Université de Montréal

Characterization of the UM171-Graft:

Dissecting the lymphoid lineage potential of the UM171-Graft.

Par

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Ce mémoire intitulé

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Présenté par

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A été évalué par un jury composé des personnes suivantes

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Résumé

De nos jours, la greffe de cellules souches hématopoïétiques (GCSH) de type allogénique est le traitement standard pour de nombreuses hémopathies malignes, et ce, en dépit des taux élevés de complications liées à la transplantation, telles que les infections, les rechutes et la maladie du greffon contre l'hôte (GVH), associées à cette procédure. Depuis les deux dernières décennies, plusieurs stratégies visant à contrôler la maladie du GVH ont émergé et incluent la déplétion partielle et la manipulation *ex vivo* des cellules T du greffon. En revanche, ces stratégies peuvent entrainer de sévères déficits immunitaires post-greffe. En comparaison avec les autres sources de cellules souches, une faible incidence de la maladie du GVH et de rechute est observée chez les patients recevant une greffe de sang de cordon ombilical (SC). En contrepartie, ce type de greffe engendre des retards dans la restauration immunitaire, rendant ainsi les patients plus susceptibles aux agents pathogènes. À l'inverse, une augmentation plus importante de la production de cellules T naïves, d'émigrants thymiques récents et de l'abondance des clonotypes des cellules T fut détectée chez les patients ayant reçu une GCSH dérivée du sang d'un seul cordon traité avec UM171, une molécule utilisée pour l'amplification *ex vivo* des CSHs dérivés de SC, relativement aux patients ayant reçu une GCSH standard. Dans ce mémoire, nous essaierons de comprendre le mécanisme moléculaire sous-jacent à la restauration immunitaire chez les patients transplantés avec un greffon de SC traité avec UM171, nous tenterons de déceler des progéniteurs lymphoïdes au sein des cellules de SC traitées avec UM171 et nous essaierons d'identifier un marqueur de surface qui pourrait être utilisé pour enrichir les progéniteurs lymphoïdes.

Nos résultats démontrent la présence de deux "clusters" lymphoïdes, PCL et LMPP, qui expriment potentiellement CD10, et sont plus nombreux dans les échantillons de SC $CD34^+$ traités avec UM171 comparativement aux contrôles. En utilisant les marqueurs de surface CD10 et CD45RA, nous avons pu identifier deux populations $CD34^+$ ($CD10^{med}CD45RA^{med/lo}$ et $CD10^{hi}CD45RA^{hi}$) qui sont multipliées dans les cultures traitées avec UM171 relativement aux contrôles. En outre, nous démontrons également que seules les cellules $CD45RA^-$ peuvent générer les deux populations $CD10^+$ *in vitro*, ce qui suggère que les cellules $CD10^{med}CD45RA^{med/lo}$ définissent une population plus primitive que les cellules $CD10^{hi}CD45RA^{hi}$. De surcroit, nous proposons que la population $CD10^{med}CD45RA^{med/lo}$ pourrait contenir des LMPPs qui se différencieront en PCLs inclus dans la population $CD10^{hi}CD45RA^{hi}$. D'un point de vue fonctionnel, nous observons un développement et une maturation des lymphocytes T nettement plus rapide pour la population $CD10^+CD34^+$ relativement à la population $CD34^+$ totale à 3 et 6 semaines, ce qui suggère que CD10 pourrait être utilisé comme marqueur de progéniteurs lymphoïdes. Par ailleurs, nous détectons également un pourcentage plus élevé de cellules ayant un phénotype ILC dans la population $CD10^+CD34^+$ comparativement à la population $CD34^+$ totale, ce qui suggère que la population $CD10^+CD34^+$ peut potentiellement contenir des progéniteurs lymphoïdes capables de produire différents types de cellules immunitaires. D'autre part, la population $CD45RA^+$ produit plus rapidement des lymphocytes T contrairement à la population $CD45RA^-$. En conséquence, nous proposons que la population $CD45RA^-$ contient des cellules primitives (ex. CSH) qui à leur tour génèrent des progéniteurs multipotents contenus dans la population $CD10^+CD34^+$ (ex. LMPP) et qui éventuellement produiront des progéniteurs à potentiel plus restreint inclus dans la population $CD45RA^+$ (ex. CLP).

En somme, ce mémoire identifie pour la première fois une population $CD10^+CD34^+$ présente dans le greffon traité avec UM171 avec un potentiel T et produisant potentiellement des ILCs. L'un des problèmes majeurs liés à la GCSH dérivée du SC est une restauration immunitaire retardée; par conséquent, optimiser l'infusion de cellules $CD34^-$ en augmentant le nombre de progéniteurs lymphoïdes pourrait considérablement aider à stimuler la restauration immunitaire chez les patients recevant une GCSH dérivée de SC.

Mots clés: UM171, Sang de Cordon, Greffe de cellules souches hématopoïétiques, Restauration immunitaire, Progéniteurs lymphoïdes, CD10, Cytométrie en flux.

Abstract

Currently, allogeneic hematopoietic stem cell transplantation is a standard treatment for many hematological malignancies, although high rates of transplant-related complications such as infections, relapse, and GVHD has constrained the implementation of HSCT. Strategies have emerged to manage GVHD and include partial T-cell depletion and *ex vivo* manipulation of donor T cells, albeit they have adverse effects on post-transplantation immune recovery and can cause profound immunodeficiency. In comparison to other stem cell sources, a lower incidence of chronic GVHD and relapse is reported in patient receiving cord blood (CB) transplant, although they also display an enhanced susceptibility to pathogens caused by an ineffective T-cell reconstitution. In contrast, we reported a greater increase in naïve T cells production, recent thymic emigrants and T cell clonotype from 3 months to 6 and 12 months in patient transplanted with a single CB graft expanded with UM171, a small molecule used for the *ex vivo* expansion of CB HSCs, as compared to counterpart patients receiving unmanipulated CB graft. In this master research, we aimed to understand the molecular mechanism underlying T-cell reconstitution in patients transplanted with UM171-expanded CB graft. We tried to identify lymphoid progenitors within the highly heterogeneous $CD34^+$ CB cells treated with UM171 using Cite-Seq, find a surface marker that can be used to enrich for early lymphoid progenitors using flow cytometry, and assess their lineage potential using artificial thymic organoids.

Our results highlight the presence of two lymphoid clusters, CLP and LMPP, expressing CD10. The LMPP cluster is about 6 fold expanded in UM171-treated cells as compared to uncultured and DMSO-treated cells. Using the marker CD10 and CD45RA we identify two $CD34^+$ populations ($CD10^{med}CD45RA^{med/lo}$ and $CD10^{hi}CD45RA^{hi}$) that are significantly expanded in $CD34^+$ CB cells cultured in the presence of UM171 in contrast to uncultured $CD34^+$ CB cells and DMSO-supplemented cultures. Furthermore, we demonstrate that only the CD45RA-negative cells can give rise to both CD10-positive subsets *in vitro*, suggesting that $CD10^{med}CD45RA^{med/lo}$ cells define a subset with a more primitive phenotype than $CD10^{hi}CD45RA^{hi}$. We propose that the $CD10^{med}CD45RA^{med/lo}$ subset could contain LMPPs that will commit to CLPs contained within the $CD10^{hi}CD45RA^{hi}$ subset. Functionally, we compared T-cell development and maturation of the $CD10^+CD34^+$ subset (including $CD10^{med}CD45RA^{med/lo}$ and $CD10^{hi}CD45RA^{hi}$) to the $CD45RA^+CD34^+$, $CD45RA^-CD34^+$ and total $CD34^+$ subsets and denote a faster T cell development and maturation at 3 and 6 weeks within the CD10-positive subset in contrast to the total $CD34^+$ subset, suggesting that CD10 can be used as a marker of lymphoid precursors in UM171 cultures. We also observe a higher percentage of ILC-like cells within the CD10-positive subset as compared to total $CD34^+$ cells suggesting that the CD10-positive subset potentially contains lymphoid precursors with multilineage capacity. Faster T cell development is observed for the $CD45RA^+$ subset whereas $CD45RA^-$ cells display the slowest T cell development, hence we propose that the $CD45RA^-$ subset contains primitive cells (*e.g.* HSC) that segregate into lineage-biased multipotent progenitors enriched in the CD10-positive subset (*e.g.* LMPP) that will give rise to lineage-restricted precursors that are $CD45RA^+$ (*e.g.* CLP).

In sum, our findings represent the first identification of a CD10-positive population within the UM171-expanded graft that has T potential and could potentially produce ILCs. Delayed immune reconstitution is a major obstacle impeding the implementation of CB HSCT; therefore optimizing infusion of $CD3^+$ donor cells by enriching for lymphoid precursors could help boost T-cell reconstitution following allogeneic HSCT.

Keywords: UM171, Cord blood, $CD34^+$ cells, Hematopoietic stem cell transplantation, lymphoid progenitors expansion, CD10, Flow cytometry.

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aGVHD: acute Graft-versus-host disease

AhR: Aryl hydrocarbon receptor

AL: Acute leukemia

ATG: Antithymocyte globulin

ATO: Artificial thymic organoids

BM: Bone marrow

CBU: Cord blood unit

CLP: Common lymphoid progenitor

cGVHD: chronic Graft-versus-host disease

CNI: Calcineurin

CMP: Common myeloid progenitor

Cite-Seq: Cellular indexing of transcriptomes and epitopes by sequencing

CSA: Cyclosporine

DLA: Dog leukocyte antigen

DLI: Donor lymphocyte infusion

DN: Double Negative

DP: Double positive

ELP: Early lymphoid progenitor

ETP: Early thymic progenitor

FACS: Flow cytometry

GI: Gastrointestinal

GVHD: Graft-versus-host disease

GVL: Graft-vs-Leukemia

HDAC: Histone deacetylase

HLA: Human leukocyte antigen

HPC: Hematopoietic progenitor cell

HSC: Hematopoietic stem cell

HSCT: Hematopoietic stem cell transplantation

HSPC: Hematopoietic stem and progenitor cell

ILC: Innate lymphoid cell

LMPP: Lymphoid-primed multipotent progenitor

LT-HSC: Long-term hematopoietic stem cell

MLP: Multipotent lymphoid progenitor

MPP: Multipotent progenitor

mRNA: messenger RNA

MTX: Methotrexate

NK: Natural Killer

OS: Overall survival

PB: Peripheral blood

PBSCs: Peripheral blood stem cells

RNA-Seq: RNA sequencing

RTE: Recent thymic emigrant

SAA: Severe aplastic anemia

SP: Single positive

SRC: Scid repopulating cell

SR1: StemRegenin-1

ST-HSC: Short-term hematopoietic stem cell

TBI: Total body irradiation

TCR: T cell receptor

TRC: Transplant-related complications

TREC: TCR excision circle

TRM: Transplantation related mortality

TSA: Trichostatin A

TSP: Thymus seeding progenitor

UCB/ CB: Umbilical cord blood

UMAP: Uniform manifold approximation and projection

VPA: Valproic acid

3D: Three-dimensional

5azaD: 5-aza-20 –deoxycytidine

À ma mère, une femme forte et indépendante qui m'a appris à ne jamais baisser les bras. Elle est ma source de réconfort et la boussole qui me permet de naviguer à travers les tempêtes. Sans toi maman, je n'existerai pas.

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Chapter 1: Introduction

1. Hematopoietic stem cell transplantation (HSCT): Origin and early advancements.

The early concept of hematopoietic stem cell transplantation (HSCT) emerged in 1949 when Jacobson and colleagues demonstrated that they could occlude the damages caused by total body irradiation (TBI) by using lead to shield the spleen of mice (Jacobson *et al*, 1949). This finding concurred with the collective efforts of the scientific community to repair the radiative damages affecting the survivors of atomic bomb explosions in Japan, and thus was greeted with enthusiasm. In essence, Jacobson's group set the first stone of the HSCT foundation (Jacobson *et al*, 1949). Grounded on this initial finding, a hypothesis arose in the scientific community: the "humoral hypothesis". The "humoral hypothesis" stated that humoral factor(s) located in the spleen/marrow of the animal help protect from TBI (Storb, 2019). Two years later, Lorenz and colleagues reported the survival of mice and guinea pigs following TBI when infused with marrow cells, thereby further expanding the HSCT foundation (Lorenz *et al*, 1951).

The "humoral hypothesis" was dismissed by the mid-1950s as several laboratories proved that it is the donor cells seeding the marrow that protect against radiation (Mathé *et al.,* 1965). These lines of evidences offered new perspectives for the treatment of malignant blood disorders and supported the emergence of HSCT. HSCT relies on: 1) Suppression of the patient's diseased marrow/ impaired immune cells using high dose chemotherapy/radiation, and 2) seeding the patient's marrow with healthy immune cells from a donor's graft (Mathé *et al.,* 1965).

In 1958, six workers of nuclear reactor accidentally exposed to TBI had a marrow transplantation performed by Mathé and colleagues, representing the first stem cell transplantation done in human. Surprisingly, and despite any knowledge on HSCT, four out of six workers survived, although the donor's cells did not permanently engraft (Watts, 2010). In 1961, Till, McCulloch and colleagues identified hematopoietic stem cells (HSCs) and demonstrated that a single cell from the bone marrow (HSC) could produce myeloerythroid colonies in the spleen and reconstitute lethally irradiated mice (Till and

McCulloch, 1961; Becker *et al.,* 1963; Siminovitch *et al*., 1963). Few years later, in 1965, Mathé's group decided to use marrows from six relatives to treat a leukemia patient following TBI (Mathé *et al.,* 1965). Interestingly, the brother's marrow engrafted and the patient went into remission before dying from graft-versus-host-disease (GVHD), a major complication of HSCT resulting from donor T cells attacking healthy cells from the host (Sweeney and Vyas, 2019).

Bortin reviewed the outcome of 200 human marrow grafts performed between 1957 and 1967 and reported that these patients died from various transplantation related mortality (TRM) causes such as GVHD, infection, graft failure or leukemia relapse, thus pinpointing the biological layers of complexity underlying HSCT (Bortin, 1970). At this point in history, albeit great advancements have been made, a major gap remained in the understanding of HSCT, histocompatibility matching and GVHD management. This might be explained by the fact that early biological studies of HSCT employed inbred mice, a model organism that does not require histocompatibility matching and demonstrates multiple biological differences with humans. As a consequence, the premature translation of mouse-based HSCT studies to humans resulted in high TRM rates. Ultimately, the recurrent failure of HSCT in humans forced immunologists to conclude that biological transfers between individuals, such as bone marrow transplant, are beyond the bounds of possibility.

Per contra, some resilient researchers such as Van Bekkum's group in the Netherlands working with primates, George Santos at Johns Hopkins working with rats and the Seattle group, led by E. Donnall Thomas and working with outbred dogs, maintained their efforts toward understanding HSCT. As a consequence of their collective efforts, TBI administration was optimized and myeloablative/immunosuppressive drugs (cyclophosphamide, antithymocyte globulin (ATG) and busulfan (BU)) were introduced and highlighted for their tumor cell killing properties, comparable to TBI (Santos, 1995). Furthermore, the Seattle group, led by E. Donnall Thomas, designed an *in vitro* histocompatibility typing for inbred dogs (Epstein *et al.,* 1968). Pivotal to the understanding of HSCT and GVHD, canine studies from 1968 demonstrated that dogs receiving a dog leukocyte antigen (DLA)-matched graft from related or unrelated donors survived significantly longer than the counterpart littermates receiving DLA-mismatched graft,

forasmuch as minor histoincompatibility led to GVHD development (Epstein *et al.,* 1968). In parallel, the efforts toward designing post transplantation drug regimens to manage GVHD increased and led to the emergence of methotrexate (MTX) and folic acid antagonist (Storb *et al.,* 1970).

With the acquired knowledge on HSCT, clinical trials resumed in 1968. A 5-month-old boy suffering from thymic alymphoplasia and agammaglobulinemia was successfully transplanted intraperitoneally with his sister's marrow and peripheral blood cells (Gatti *et al.,* 1968). The patient went into complete remission, thus representing one of the first successful HSCT in human. As a success does not come alone, in the following years, multiple patients with advanced hematological malignancies and severe aplastic anemia (SAA) successfully underwent HSCT, although they often developed transplant-related complications (TRC) such as fungal and viral infections, which drove the optimization of transfusion and management of infectious diseases (Weiden *et al*., 1979; Hansen *et al.*, 1979). Nowadays, from the thousands of allogeneic HSCT accomplished each year worldwide, more than half are for acute leukemia (AL; Henig and Zuckerman, 2014).

1.1 Hematopoietic stem cell transplantation (HSCT): Major Complications.

The emergence of chronic GVHD among long-term survivors shaded the HSCT success story, which urged the development of therapeutic approaches to control GVHD (Georges and Storb, 2016). The combination of MTX and calcineurin (CNI) inhibitors such as cyclosporine (CSA), allowed improvements in GVHD prevention (Storb *et al.*, 1986). Alternating cyclosporine and MTX is the current approach used to prevent GVHD (Lee, 2022).

Unfortunately, despite these advances, in the case of leukemia and other malignant blood diseases, the rate of disease relapse following HSCT remained high, which shed light on an important concept: graft-vs.-leukemia (GvL) which was addressed by Weiden and the Seattle group, led by E. Donnall Thomas, in 1979 (Weiden *et al.*, 1979). In GvL reaction, donor T cells, and other immune cells, eradicate residual leukemia cells surviving chemotherapy/radiotherapy (Sweeney and Vyas, 2019). This led to the introduction of donor lymphocyte infusion (DLI) as a novel treatment to control relapse (Kolb *et al.*, 1990).

DLI compensates for the immune exhaustion of donor T cells observed post-HSCT, which is an important cause of relapse (Frey and Porter, 2008). As the expected corollary of DLI is GVHD, the current treatments used to control relapse depends on multiple factors such as disease type and burden, the site of relapse and HSCT conditions and include modified second transplants, chemotherapy/immunotherapy and targeted disease therapy (Barrett and Battiwalla, 2010). Nowadays, DLI is only used in some cases of relapsed AL (Wang *et al.,* 2019).

1.2 Hematopoietic stem cell transplantation (HSCT): Stem cell sources.

1.2.1 Peripheral Blood stem cells (PBSCs)

Donating bone marrow cells requires invasive procedures that generate relative inconvenience and discomfort for donors. Moreover, retrieving sufficient bone marrow cells to sustain permanent engraftment can be challenging, which encouraged scientists to search for alternative sources of HSCs for transplantation. As previous studies highlighted that peripheral blood (PB) contains stem and progenitor cells (Cirenza *et al.*, 1996), researchers multiplied their efforts toward using PB as an alternative stem cell source (To *et al.*, 1997). The low concentration of HSCs in unmanipulated PB was a major obstacle for its use in HSCT (Goodman and Hodgson, 1962). Nonetheless, scientific advancements made in the field of pheresis technology and the discovery of the $CD34^+$ PB cell mobilizing agent G-CSF allowed to significantly increase the $CD34^+$ cell dose in collected PB, thus favoring its use in HSCT as an effective replacement for BM. Since 1995, 60%-70% of current allogeneic transplants use PB instead of BM as stem cell source (Gratwohl *et al*., 2009).

1.2.2 Umbilical Cord Blood stem cells (CB HSCs)

Umbilical cord blood (CB), another viable graft source for HSCT, emerged in the mid-1980s. Studies conducted by Broxmeyer and colleagues revealed that, similarly to BM, human CB contains hematopoietic progenitor cells (Broxmeyer *et al*., 1989). As a result, a 6-years-old boy with Fanconi anemia underwent HSCT using CB in 1988 (Gluckman *et*

al., 1989). This first successful CB-HSCT story was the result of a multi-institutional collaboration and set the path for the numerous cases of CB transplantation that followed.

In the 5 years that followed, the acceptable engraftment rate and lower incidence of GVHD observed in pediatric patients transplanted with a CB graft harvested from a single donor helped prove the potential of using CB as a substitute to BM (Wagner *et al*., 1995). In comparison to BM grafts, slight HLA mismatches in CB graft do not cause an increase in the incidence of acute and chronic GVHD (Avery *et al.,* 2011).

Although a higher HLA disparity in CB grafts is tolerated, a donor-recipient matching at six HLA loci is the current standard for CB selection (Avery *et al*., 2011). In comparison, BM and PB grafts require at least 8-10 identical HLA loci. Interestingly, increasing the CB $CD34^+$ cell dose can partially overcome HLA-disparity and strongly correlates with engraftment and overall survival (OS) (Gluckman *et al.,* 1997; Rocha *et al*., 2009). CB also offers a potent GvL effect, albeit CB engraftment is slower as compared to counterpart stem cell sources (PBSC and BM) (Kurtzberg et *al.,* 1996; Rubinstein *et al.,* 1998; Gluckman *et al.,* 1997; Rocha *et al*., 2001; Barker *et al*., 2001).

CB can easily be obtained during childbirth, although its use in HLA-matched sibling transplantation highly depends on birth and could only be performed if the condition of a child affected by a disease requiring transplantation was known prior to the birth of the donor (American Academy of Pediatrics, 1999). Furthermore, the limiting stem cell dose per cord blood unit (CBU) restricted the implementation of this source to pediatric settings (Eapen *et al*., 2007). Currently, to circumvent the low stem cell dose, the standard procedure during CB HSCT in adults involves the transplantation of two CBUs (double CB transplantation) that are closely HLA matched to each other (Hashem and Lazarus, 2015).

1.2.3 Comparison of BM, PB and CB stem cell sources in the context of HSCT.

By dint of the advancements made in HSCT and the emergence of different stem cell sources, clinicians must appropriately select the stem cell source prior to transplantation (either BM, PB or CB). In a HLA-matched related donor HSCT setting, a meta-analysis of 11 randomized control trials comparing BM and PB grafts indicated comparable OS, TRM

and disease-free survival between the two sources (no study comparing CB, BM and PB HLA-matched related donor HSCT; Chang *et al*., 2012). On the one hand, a faster neutrophil and platelet recovery along with a lower incidence of relapse was observed for patients transplanted with a PB graft. On the other hand, a lower incidence of grade 2-4 and 3-4 acute GVHD and chronic GVHD, respectively, was reported for BM graft recipients.

For some patients, no related donor is available. In this context, clinicians can use matched unrelated donor for HSCT. Anasetti and colleagues conducted a randomized control trial to investigate the differences between PB and BM grafts in an HLA-matched unrelated setting (Anasetti *et al.*, 2012). In this study, Anasetti and colleagues observed no significant difference in 2-year OS, relapse and acute GVHD incidence between the 2 groups. Moreover, Anasetti and colleagues inferred that PB graft recipients might have a higher risk of developing chronic GVHD, although they might also have a lower risk of graft failure compared to BM graft recipients. A study conducted by the Dilloo's group challenged these findings, as they reported no significant difference in the incidence of acute and chronic GVHD among pediatric patients transplanted with either a BM or PB graft (Meisel *et al.,* 2013). In this setting, the pediatric patients received ATG for GVHD prophylaxis. Discrepancies in PB-related GVHD risk between these two studies can be accounted by the use of ATG and the patient's age.

In a large non-randomized retrospective analysis of 1525 patients with acute leukemia, Eapen and colleagues analyzed the differences between BM, PB and CB unrelated transplants (Eapen *et al.*, 2010). In terms of relapse and leukemia-free survival, no difference was observed between CB and HLA-matched BM or PB grafts (7-8/8 alleles matched). Patients receiving CB grafts had a higher rate of TRM than HLA-matched BM or PB grafts recipients. The incidence of grade 2-4 acute and chronic GVHD was lower after CB transplantation in contrast to PB graft recipients. Similarly a lower incidence of chronic GVHD was reported for CB graft recipients when compared to BM transplants, whereas no differences was observed in the incidence of acute GVHD between CB and BM transplants (Eapen *et al.*, 2010).

However, in a study conducted by Jacobson and colleagues, they reported no significance difference in 2-year OS, TRM, relapse, progression-free survival and grade 2-4 acute GVHD between CB and PB graft recipients (Jacobson *et al.*, 2012). Nonetheless, Jacobson and colleagues reported a higher rate of infections (viral and bacterial) for the double CB recipients as compared to PB transplant. Interestingly, they reported a significantly delayed T cells reconstitution at 1-6 month post-transplant in double CB recipients (vs PBSC recipients) which can account for the higher rate of TRC and indicate that CB-HSCT can result in delayed T cells reconstitution. Table **1** summarizes the key advantages and disadvantages of each stem cell source (adapted from Juric *et al.*, 2016).

Stem Cell	Advantages		Limitations	
Source	Donor	Recipient	Donor	Recipient
BM		Low risk of GvHD	Invasive HSC collection Requires hospitalization	
PBSC	No general anesthesia No hospitalization	Faster engraftment Faster immune reconstitution High GvL effect		Higher risk of GvHD
CB	Non-invasive	Low risks of GvHD and relapse Increased level of HLA-disparity is tolerated		Low number of HSC Slow immune reconstitution

Table 1. Comparing the benefits and drawbacks of hematopoietic stem cell sources pre-HSCT.
Table adapted from Juric *et al.*, 2016.
BM, Bone marrow; PBSC, Peripheral blood stem cell; CB, Cord blood; GvHD, graft-versus-host disease; HSC, hematopoietic stem cell; GvL, graft-versus-leukemia, HLA, human leukocyte antigen.

Emerging therapies and treatments for GVHD management can abrogate the increased risk of GVHD in patients receiving PB graft (Granata *et al*., 2019). Additionally, the emergence of small molecules to expand CB stem cells can help alleviate the low stem cell dose burdening the use of CB transplants (Fares *et al.*, 2014). In the same fashion, post-transplant infusion of regulatory T cells could help mitigate the slower T cells reconstitution, thus reduce susceptibility to opportunistic pathogens and help reduce relapse in CB graft recipients (Di Ianni *et al.*, 2011; Di Ianni *et al.*, 2011).

1.3 Hematopoietic stem cell transplantation (HSCT): Expanding CB HSCs.

Hematopoietic stem cells (HSCs) are essential elements of PB, BM and CB transplants. HSCs have both self-renewal and multipotency capacities and can reconstitute the entire hematopoietic system of a patient post-HSCT (Figure 1.1; adapted from Ratliff *et al.*,

2014). As such, HSCs can reconstitute both myeloid and lymphoid lineages, whereas committed progenitors such as CLPs and CMPs can only give rise to cells from one lineage (myeloid or lymphoid; Figure 1; Ratliff *et al.*, 2014). In BM, HSCs represent less than 0.01% of cells and this percentage further decreases in CB (Walasek *et al.*, 2012; Daniel *et al.*, 2016).

Due to the limiting stem cell dose in CB, several strategies focusing on the *ex vivo* expansion of HSCs have emerged to obtain adequate quantities for HSCT (≥ 2.5 × 10^7 nucleated cells/kg for partially matched CB; Eapen *et al*., 2008) and hereby exploit the advantages associated with this stem cell source. These strategies include: i) addition of several cytokines to the culture media (although the effects are transient; Zhang and Lodish, 2008), ii) over-expression of HSC-specific genes (Walasek *et al.,* 2012), iii) co-culture with stromal cells (McNiece *et al.*, 2004), iv) use of small-sized chemical agents (De Lima *et al.*, 2008; Nishino *et al.*, 2009; Peled *et al.*, 2012) and, v) use of recombinant proteins to control developmental pathways (Krosl *et al.*, 2003).

Although most of aforementioned strategies have shown promising results, clinical settings require highly sterile conditions which can easily be compromised by employing strategies such as recombinant proteins, vectors to over-express HSC genes, or co-culture with stromal cells, all requiring extensive handling. On the contrary, small molecules represent promising strategies to expand HSCs and HSPCs *ex vivo* as they are more stable than biologicals and can easily be optimized and produced in large quantities by laboratories and pharmaceutical companies, which explains the enthusiasm associated with the use of small molecule to expand HSCs and HSPCs (Fares *et al.*, 2015).

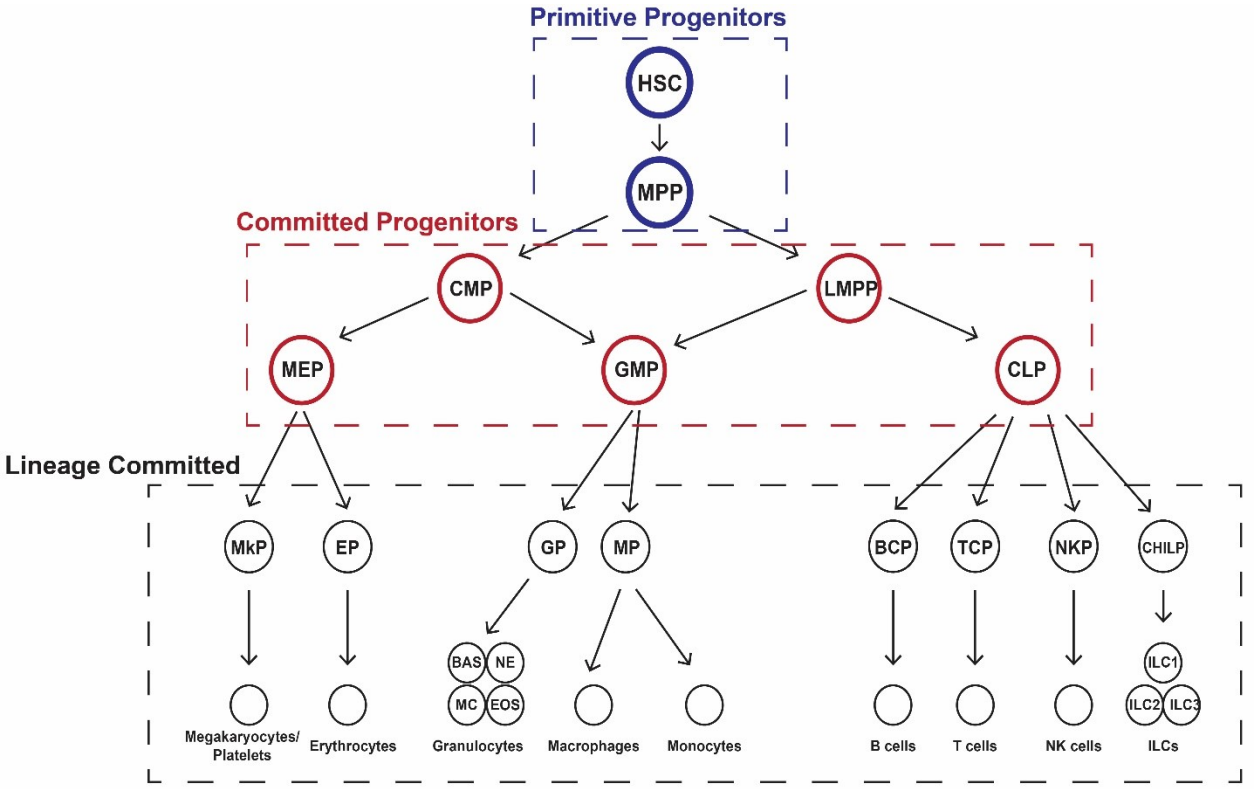


Figure 1.1 Committment during hematopoiesis.
Figure adapted from Ratliff *et al.* (2014).
HSC, hematopoietic stem cell; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor; BCP, B cell progenitor; TCP, T cell progenitor; NKP, NK cell progenitor; CHILP, common helper innate lyphoid progenitor; MP, macrophage progenitor; GP, granulocyte progenitor; EP, erythrocyte progenitor; MkP, megakaryocyte progenitor; BAS, basophils; NE, neutrophils; MC, mast cells; EOS, eosinophils; NK, natural killer; ILC, innate lymphoid cells.

1.3.1 UM171

In the last decade, a multitude of small molecules have been used to expand CB HSCs *ex vivo* such as valproic acid, trichostatin A, nicotinamide and StemRegenin-1 (SR1; Boitano *et al.*, 2010; Mahmud *et al.*, 2014; Horwitz *et al.*, 2014; Saraf *et al.*, 2015) . In 2014, our group discovered a small molecule, UM171, which promotes the expansion of CB HSCs (Fares *et al.*, 2014). We recently demonstrated that UM171 potentiates the activity of the CULLIN3-E3 ubiquitin ligase complex $CRL3^{KBTBD4}$, leading to increased proteasomal degradation of components of the LSD1/RCOR1 CoREST complex (Chagraoui *et al.*, 2021). Interestingly, UM171-induced modulation of the $CRL3^{KBTBD4}$ complex leads to the re-establishment of H3K4me2 and H3K27ac epigenetic marks which, in turn, potentially result in the upregulation of HSPCs associated genes preventing

HSC attrition during their *ex vivo* expansion (Chagraoui *et al.*, 2021). UM171 produced a 5- and 10-fold expansion of $CD34^+$ and $CD34^+CD45RA^-$ CB cells, respectively, over 12 days fed-batch cultures as compared to control conditions (Fares *et al.*, 2014; Fares *et al.*, 2015). Moreover, a ~13-fold increase in long-term reconstituting HSC (LT-HSC) frequency was reported for UM171 exposed cells compared to control or uncultured $CD34^+$ cells (Fares *et al.*, 2014; Fares *et al.*, 2015). Interestingly, the effect of UM171 on mature cell output suppression was enhanced by adding SR1, thus indicating a collaboration between the two molecules (SR1 and UM171) to expand these cells *ex vivo*. However, the greater increase in abundance of $CD34^+CD45RA^-$ cells in cultures treated with UM171 compared to SR1 suggests that, as opposed to SR1, UM171 targets phenotypically more primitive cells (Fares *et al.,* 2014). Accordingly, LT-HSC frequencies measured with UM171 treatment were not significantly altered by SR1 addition.

1.4 Hematopoietic stem cell transplantation (HSCT): the clinical application of UM171.

1.4.1 Transplantation of UM171-expanded graft.

Since its discovery, over 60 patients were successfully transplanted with a single cord blood unit (CBU) expanded with UM171 (7-days *ex vivo* expansion; Chagraoui *et al*., 2021). During this process, the CBU is thawed and $CD34^+$ cells are selected 7 days prior to infusion (at D-7) whereas the $CD34^-$ portion is cryopreserved (**Figure 1.2**; adapted from Cohen *et al.*, 2019). $CD34^+$ cells are then expanded in fed-batch culture with UM171 during 7 days. The patients undergo myeloablative or functionally myeloablative conditioning regimen 2 days prior to CB infusion (at day 5 of the expansion). Then, the patient receives the UM171-expanded graft and either on the same day, or a day later, the patient is infused with the $CD34^-$ portion of the expanded CB to reduce susceptibility to opportunistic pathogens post-HSCT (**Figure 1.2**; adapted from Cohen *et al*., 2019). In a clinical study investigating the benefits of UM171-CB transplants for 22 patients with high-risk haematological malignancies, we reported the successful transplantation of 21 patients with a single UM171-expanded CB graft with no graft failure or unexpected adverse events (Cohen *et al.*, 2019). In comparison to patients transplanted previously at

the same facility with unmanipulated CB, BM and PB grafts, patients receiving a UM171 graft had a significantly shorter median time to engraftment of 100 neutrophils per μL as compared to PB recipients. Intriguingly, we reported no significant differences in the engraftment time of 500 neutrophils per μL between BM, PB and CB graft recipients and thus, no significant difference was observed in their duration of hospital stay. A similar platelet recovery was reported for the UM171 graft recipients when compared to the patients receiving unmanipulated cord blood, whereas PBSC and BM recipients had a faster platelet recovery. Interestingly, the patients transplanted with a UM171-graft had a low incidence of grade 3-4 acute GVHD (10%) and no moderate to severe chronic GVHD (0%). A low incidence of TRM (5%) and relapse (21%) was also denoted in UM171 graft recipients at 1 year post-HSCT (Cohen *et al.*, 2019).

In consideration of a major limitation of unmanipulated CB (Eapen *et al.*, 2010), the low stem cell dose, UM171 offers promising therapeutic applications as the use of UM171 to expand CB graft can offer better HLA matching by improving accessibility to CB availability for racial and ethnic minorities (Dumont-Lagacé *et al.*, 2022). Additionally, by increasing the patient's accessibility to a CB with a better HLA matching, UM171 also allows to decrease TRM, an outcome often associated with HLA matching (Dumont-Lagacé *et al.*, 2022).

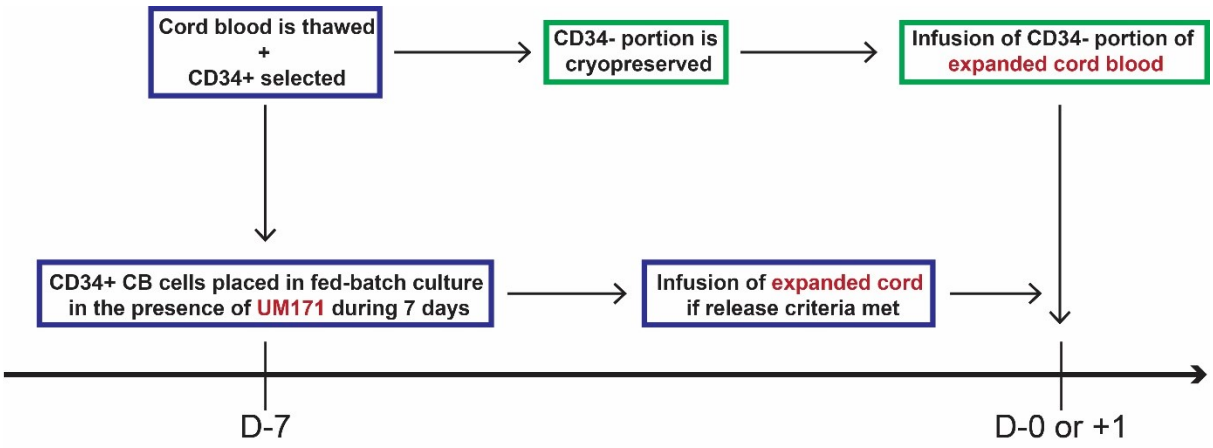


Figure 1.2. Schematic representation of the transplantation of a single UM171-expanded graft.
Figure adapted from Cohen *et al.* (2019).
Representative protocol of the expansion of a cord blood unit with UM171 during HSCT.

1.4.2 The effects of UM171 on T cell immunity during HSCT.

Impaired T cell immunity is another major problem encountered in CB-HSCT (Juric *et al.*, 2017). Delayed T cell reconstitution increases the patient's susceptibility to severe infections, which in turn increases the rate of TRM (Lucchini *et al.*, 2015; De Koning *et al.*, 2016). In the literature, lower TRM correlates with faster $CD4^+$ T cells recovery, and a lower incidence of GVHD and relapse are associated with an increased diversity of T cell populations (Yew *et al.*, 2015, Admiraal *et al.*, 2016; Gkazi *et al.*, 2018). Furthermore, limiting stem cell dose and HLA-mismatches can also delay T cell reconstitution (Van Heijst *et al.*, 2013; Yamaguchi *et al.*, 2015; Milano *et al.*, 2016). In the context of HSCT, T cell depletion is an important process that help reduce GVHD, although it also result in a significantly delayed immune reconstitution (Klein *et al.*, 2017). Immunity plays a crucial role in GvL reaction (Sweeney and Vyas, 2019). Consequently, T cell depletion can increase the rate of disease relapse due to deficient GvL effect (Hiwarkar *et al.*, 2015; Milano *et al.*, 2016). Furthermore, T cell reconstitution is essential to reduce susceptibility to opportunistic pathogens (De Koning *et al.*, 2016). More precisely, enhanced susceptibility to pathogens was reported in CB graft recipients caused by an ineffective T-cell reconstitution (Lucchini *et al.*, 2015; De Koning *et al.*, 2016). Along these lines, T-cell reconstitution is crucial to improve management of GVHD, TRM/TRC and disease relapse.

Reconstitution of the T cell repertoire revolves around two main events, (1) homeostatic expansion of peripheral T cells and (2) *de novo* thymopoïesis (Van den Brink *et al.*, 2015; Velardi *et al.*, 2021). During the first event, the recipient T cells surviving conditioning or the donor T cells from the graft undergo peripheral expansion, albeit the $CD8^+$ memory T-cell population prevalently expands resulting in defective immune response (Toubert *et al.*, 2012). During *de novo* thymopoïesis, naïve T cells are produced and educated within the recipient's thymus to complete the T-cell repertoire reconstitution (Chaudhry *et al.*, 2017; Velardi *et al.*, 2021). Lymphoid precursors originating from donor's HSCs and/or rare multipotent lymphoid progenitor (MLP) present in the graft will migrate from the BM to seed the recipient's thymus where thymus seeding progenitors (TSPs) will interact with the thymic microenvironment to regulate thymocytes development (Koch *et al.*, 2008).

The extensive manipulation of CB during the *ex vivo* expansion process with UM171 results in a lower number of infused donor T cells ($CD34^-$ infusion containing $CD3^+$ T-cells) in contrast to unmanipulated CB (Dumont-Lagacé *et al.*, 2021). We demonstrated that, despite receiving reduced T cell dose, the peripheral blood T cells count at 3, 6, and 12 months post-HSCT were similar for patients receiving a UM171-graft in comparison to a contemporary cohort of 12 patients that received unmanipulated CB graft (at the same institution with similar conditioning regimens; Dumont-Lagacé *et al.*, 2021). We also denoted an increased T cell receptor (TCR) diversity at 1 year and a more striking increase in naïve T cell and recent thymic emigrants (RTEs) at 3, 6 and 12 months among the UM171 recipients in contrast to the unmanipulated cohort. The number of unique clonotypes was similar between both cohorts at 3 months post-HSCT. At 6 and 12 months the number of unique clonotypes increases for UM171 graft recipients while it remained stable for unmanipulated graft recipients. These observations suggest that T cell reconstitution in UM171 patients is not affected by the lower T cell dose infusion (Dumont-Lagacé *et al.*, 2021). As T cell reconstitution and diversity can result from either homeostatic expansion or *de novo* thymopoïesis, we assessed the frequency of $CD34^+CD7^+$ common lymphoid progenitors (CLPs) in $CD34^+$ cells cultured in the presence/absence of UM171 (35nM; Dumont-Lagacé *et al.*, 2021). Interestingly, a 5-fold increase in CLP frequency was observed in UM171 cultures compared to DMSO-supplemented cultures and CLPs isolated from UM171 cultures were more proliferative in comparison to CLPs obtained from DMSO-supplemented cultures (Figure **1.3**; Dumont-Lagacé *et al.*, 2021). Based on RNA-sequencing analysis, we obtained an enrichment of a CLP associated gene signature for CB cells treated with UM171 compared to DMSO (Figure **1.3;** Dumont-Lagacé *et al.*, 2021). Rapid thymopoïesis recovery following HSCT has been associated with a lower cGVHD incidence and an enhanced immunity and, in turn, incidence of GVHD can affect T cell reconstitution (Yew *et al.*, 2015; Imamura *et al.*, 2003). In spite of similarities in the incidence of GVHD between both cohorts, patients from the unmanipulated cohort required further systemic immunosuppression, which might have affected T cell reconstitution (Dumont-Lagacé *et al.*, 2021). Moreover, the small sample size of both cohort also limits the interpretation of the results.

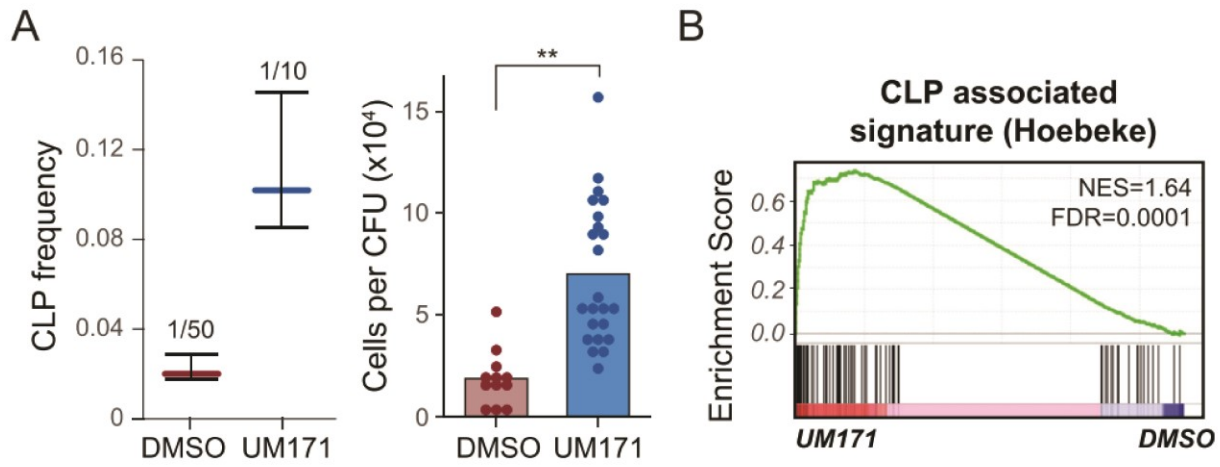


Figure 1.3. The number of CLP increases in CD34+ cord blood cells cultured in the presence of UM171.
Figure is obtained from Dumont-Lagacé *et al.* publication (2021).
CLPs; common lymphoid progenitors.
(A) CLP frequency in CD34+ cord blood cells cultured in the presence of DMSO or UM171 (35nM; left). Number of cells produced per CLP in DMSO or UM171 (right).
(B) Gene set enrichment analysis of CLP-associated genes in UM171- or DMSO- cultures. *p<0.05. **p<0.01.

Interestingly, results reported by Dumont-Lagacé and colleagues suggest that, in addition to increase the stem cell dose per CBU, UM171 could also enhance T cell reconstitution (Dumont-Lagacé *et al.*, 2021; Dumont-Lagacé *et al.*, 2022). Thus, these studies indicate that UM171 can potentially alleviate both factors limiting the implementation of CB HSCT, the low stem cell dose and the slow immune reconstitution (Table **1**; adapted from Juric *et al.,* 2016). Nonetheless, future studies should aim to decipher the molecular mechanisms underlying the observed benefits that UM171 has on immune reconstitution following HSCT.

1.5 Objectives and hypotheses of the Memoire.

We previously reported an increase in $CD34^+CD7^+$ CLP frequency in UM171 cultures as compared to DMSO-supplemented culture, thus highlighting the ability of UM171 to expand lymphoid progenitors (Dumont-Lagacé *et al.*, 2021). As this was the first study assessing the lymphoid reconstitution potential of the UM171 graft, further phenotypical and functional characterization are required to establish the effects that UM171 have on the proliferation and differentiation of lymphoid progenitors. In this study, we tried to detect the presence of lymphoid progenitors within the highly heterogeneous $CD34^+$ CB cells treated with UM171 and find surface marker that can be used to demarcate these lymphoid progenitors. Potential findings of this study can help provide insights and direction for the optimization of the $CD3^+$ T cell infusion to enhance T-cell reconstitution in patients undergoing allogeneic HSCT with UM171-treated CB transplants. Using a combination of phenotypical approaches such as cellular indexing of transcriptomes and epitopes by sequencing (Cite-Seq), flow cytometry (FACS) and a functional approach such as artificial thymic organoid (ATO) co-culture we address the following unanswered questions:

1) Can we validate the expansion of lymphoid progenitors in $CD34^+$ cord blood cells cultured *in vitro* in the presence of UM171?
2) Can we identify surface marker(s), other than CD7, that can be used to enrich for lymphoid progenitors in UM171-treated $CD34^+$ culture?
3) Which lymphoid progenitors are expanded by UM171 (MPP, LMPP or CLP)?
4) What is the functional potential of lymphoid progenitors present in UM171-treated $CD34^+$ culture (T/B/NK/ILC potential)?

The convergence of these questions lead to the emergence of the following hypotheses:

I. UM171 can be used to expand lymphoid progenitors *ex vivo*.
II. Based on literature, CD10 can be used as a marker of lymphoid progenitors in $CD34^+$ cord blood cells cultured *in vitro*.

Chapter 2 – Results

2.1 Characterization of the lymphoid potential of the UM171-Graft.

2.1.1 Cite-Seq analysis revealed the presence of lymphoid progenitors in UM171 graft that can potentially be identified phenotypically using the surface marker CD10.

To gain further insights on the lymphoid potential of the UM171 graft, we applied a cellular indexing of transcriptomes and epitopes by sequencing (Cite-seq) to visualize the changes in the transcriptome of $CD34^+$ CB cells upon exposure to UM171. To enrich for progenitors, we also sorted the UM171-treated CB cells (day 7) into a $CD86^-$, $FCER1A^-$ double negative (DN) population, thereby depleting mast cells and dendritic cells from the UM171 graft and enriching for progenitors. The resulting UM171 DN-sorted population represents 8-15% of the total UM171. This experiment entailed immunophenotyping of $CD34^+$ CB cells (Fresh, DMSO, UM171 and DN-sorted) with 58 surface markers coupled to a transcriptome analysis using a 10X genomics single-cell sequencing protocol. DMSO-supplemented cultures and unmanipulated CD34+ CB cells (Fresh) are used as controls in this setting. The experiment was designed and performed by colleagues (Chagraoui *et al.*). The collected data were processed, normalized and integrated by other members of the lab (Chagraoui *et al.*). We generated a two-dimensional embedding using the uniform manifold approximation and projection (UMAP) algorithm based on a weighted combination of RNA and protein data (wnnUMAP) providing robustness to our transcriptomic analyses. A reference-based mapping approach using the Human Cell Atlas dataset allowed to annotate each cell in our dataset. Selected marker genes from literature were used to confirm cluster annotation, thereby providing further insights on the identity of all the cell type within the wnnUMAP graph-based clustering (performed by Chagraoui J.) (Figure 2**B**). Analysis of the clusters topological localization showed striking variation between uncultured (Fresh) and cultured $CD34^+$ cord blood cells (UM171, UM171 DN-sorted and DMSO) in the first dimension (UMAP1) which can be accounted by the anticipated biological differences occurring during the *ex vivo* culturing process

(Figure 2**A**, Zhang and Lodish, 2005). We observed a great cluster overlap along UMAP1 and UMAP2 between UM171 and UM171 DN-sorted (Figure 2**A**). As anticipated, we mainly denoted clusters of $CD34^+$ progenitors in the fresh graft, which is coherent with the $CD34^+$ pre-enrichment and positive selection performed (Refer to material and methods; Figure 2**B**). Per contra, clusters of mature cells such as neutrophil, monocyte, eosinophil and dendritic cells are mostly observed in cultured $CD34^+$ cells (DMSO and UM171), which can be accounted by the culturing process allowing commitment and differentiation of HSCs and HSPs into mature cells (Figure 2**B**). Moreover, Figure 2**B** highlights the presence of common lymphoid progenitor (CLP) and lymphoid-primed multipotential progenitors (LMPP) clusters in the UM171, UM171 DN-sorted and Fresh graft, although these clusters seem depleted control in DMSO cultures (Figure 2**A** and 2**B**).

Interestingly, estimates of the fold difference of the LMPP cluster (containing predicted LMPP cells, referred to as CD34+ LMPP in Figure 2**B**) between Fresh (d0) and UM171 (d7) grafts showed that predicted LMPPs are 6 fold enriched in UM171 (d7) as compared to Fresh (d0) (Figure 2**C**). Similarly, predicted LMPPs are 6 fold enriched in UM171 (d7) as compared to DMSO (d7) (Figure 2**D**).

On the other hand, our analyses revealed an enrichment of predicted CLPs (referred to as CD34+ CLP in Figure 2**B**) in the Fresh (d0) graft when compared to the UM171 (d7) graft, which can be explained by the fact that the expansion factor was not taken into account in our analyses (Figure 2**D**). More precisely, the same number of cells were sequenced for each condition, although at day 7 the UM171 graft contains about 40 times more cells. Therefore, we expect a stronger enrichment of the CLP and LMPP clusters within the UM171 graft as compared to the uncultured cells (Fresh). Furthermore, we reported an enrichment of $CD34^+$ HSCs of ≈5 fold in the UM171 graft as compared to the DMSO graft, in agreement with previous publications (Fares *et al.*, 2014; Fares *et al.*, 2015), thereby validating the robustness of our analysis.

In terms of spatial localization, the CLP and LMPP clusters had similar topological location in the fresh (d0) and superimposed DMSO-UM171-UM171 DN sorted (d7) UMAPs and, it was at the apex of the second dimension (UMAP2) that the predicted CLP and LMPP

score was the strongest (Figure 2**E**). In accordance with the CLP and LMPP enrichment reported in figure 2**D**, CLP and LMPP localized to the non-overlapping portion of the DMSO-UM171 superimposed UMAPs, thereby further supporting the increased CLP frequency observed in $CD34^+$ CB cells exposed to UM171 (35nM) as compared to DMSO (Figure 2**D** and 2**E**, Dumont-Lagacé *et al.*, 2021).

We next performed a differential gene expression analysis across fresh, DMSO, UM171 and UM171 DN-sorted CD34+ cells (Figure 2**F**). We observed differences between DMSO and UM171 in the expression of HSC genes, which is in agreement with the expansion of HSCs reported in $CD34^+$ CB cells exposed to UM171 *in vitro* (vs DMSO; Figure 2**F**; Fares *et al.*, 2014). In addition, an increase in the expression of several lymphoid precursors-associated genes such as *CD7, MME, CD22, CCR7* and *ITGB7* is observed in the UM171 and the UM171 DN-sorted graft as compared to the DMSO graft (Figure 2**F**). Consistently, several genes expressed in the lymphoid lineages such as T cells, B cells, innate lymphoid cells (ILC) and natural killer (NK) cells seemed to be upregulated in $CD34^+$ cells cultured in the presence of UM171 in contrast to DMSO-supplemented $CD34^+$ cord blood cells (Figure 2**F**; Hagihara *et al.*, 2003; Lee *et al.*, 2011; Haddad *et al.*, 2004). As the differences observed in the expression of several HSC and lymphoid lineage genes between $CD34^+$ CB cells exposed to UM171 and DMSO-supplemented $CD34^+$ cord blood cells is not striking, further experiments are required to validate these changes.

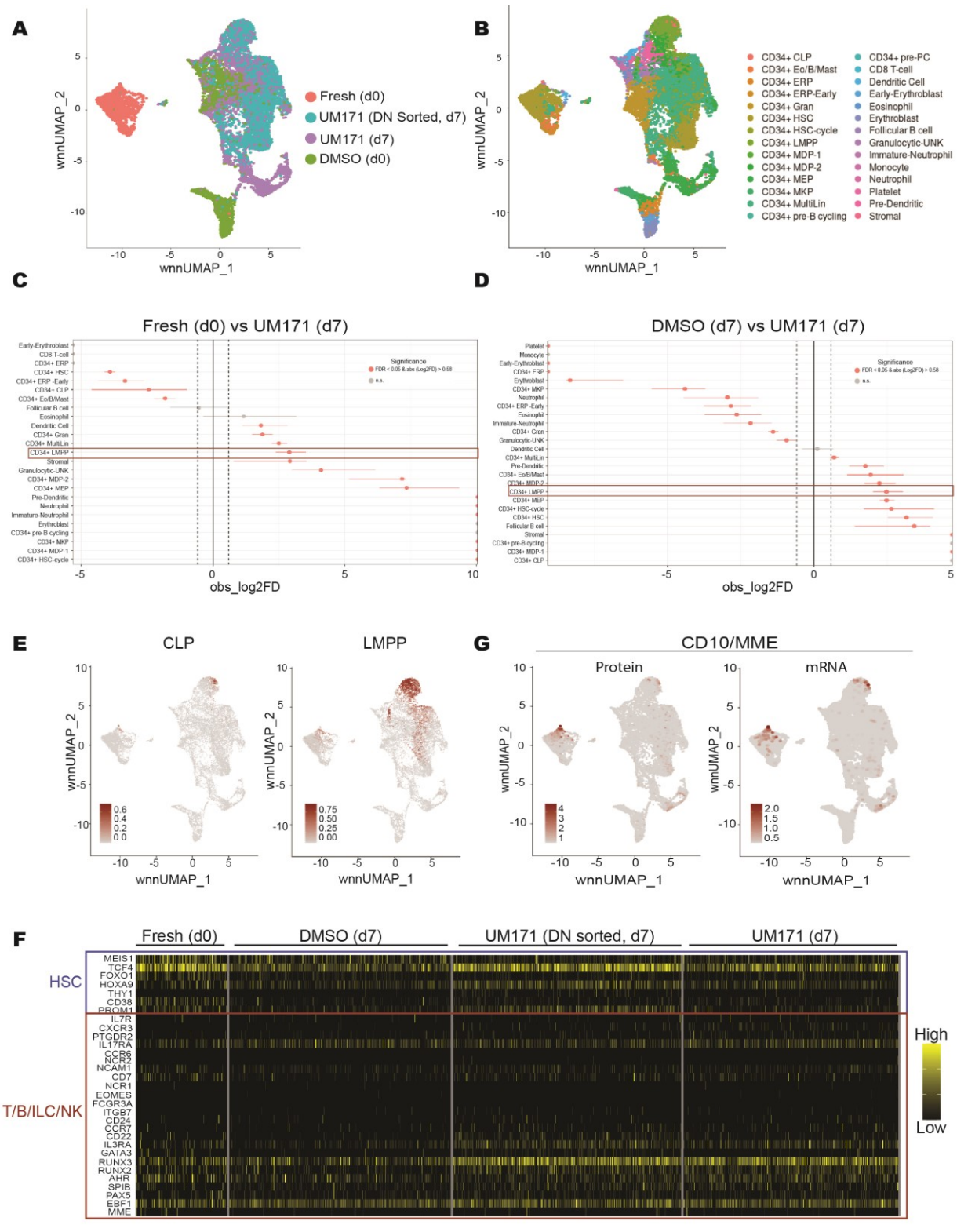
In early publications, CD7 was defined as one of the earliest T-cell surface marker in T-cell ontogeny (Sutherland *et al.*, 1984; Haynes *et al.*, 1988; Haynes *et al.*, 1989) thence heralding the subsequent identification of $CD7^+$ lymphoid-restricted progenitors in CB (Hao *et al.*, 2001; Hoebeke *et al.*, 2007). Accordingly, Dumont-Lagacé and colleagues used the surface marker CD7 to isolate CLP in $CD34^+$ CB cells cultured in the presence of UM171 (Dumont-Lagacé *et al.*, 2021). It has been demonstrated that in CB, $CD34^+CD38^-CD7^+$ coexpress CD10 (Hao *et al.*, 2001).

In contrast, Yoshida and colleagues demonstrated that within LT-HSCs ($CD34^+CD38^-$) the $CD10^+CD7^-$ and $CD10^+CD7^+$ lymphoid precursor subsets have distinct progenitor capacity (Yoshida *et al.*, 2006). More precisely, they reported that T cells represented more than

70% of total human CD45$^+$ cells in CD10$^+$CD7$^+$ recipients, whereas for the CD10$^+$CD7$^-$ recipients, T cells represented less than 30% of total human CD45$^+$ cells at 8 weeks post-transplantation in newborn NOD-scid/IL2rgKO. Hao and colleagues provided further insights by identifying a rare subpopulation of thymocyte progenitors lacking the expression of the surface marker CD7 (CD34$^+$Lin$^-$CD7$^-$; Hao *et al*., 2008). In fact, they proposed to use CD7 expression in human CD34$^+$Lin$^-$ thymocytes to track commitment stages where (1) primitive CD7$^-$ progenitors with lympho-myeloid-erythroid potential will commit to (2) CD7^{intermediate}CD10$^+$ progenitors with multilymphoid potential (B/NK/T) and eventually yield (3) CD7^{hi} progenitors with restricted T/NK potential (Hao *et al.*, 2008). Consistently, Six and colleagues reported a B, T and NK potential of a CD34$^+$CD10$^+$ (Lin$^-$) progenitor population found in CB and BM (Six *et al.*, 2007). In sum, these studies highlight the multilymphoid potential of CD10$^+$CD7$^{-/int}$ CLPs in contrast to the T-lineage biased potential of CD7^{hi} CLPs, thereby indicating that CD10 might be more efficient in segregating early lymphoid progenitors with multilymphoid potential than CD7.

Interestingly, in a previous pilot single-cell RNA sequencing experiment performed by Chagraoui and colleagues, they reported the presence of multiple lymphoid clusters in the UM171 DN-sorted graft (Figure **S1**, Chagraoui J., unpublished). In this setting, the DN (FCER1A$^-$, CD86$^-$) subset represented 15% of the total UM171 graft and the lymphoid clusters represented 25% of the DN thus, approximately 3.75% of the total UM171 graft (Figure **S1**, Chagraoui J., unpublished). Furthermore, they denoted that the expression of *MME*, *CCR7* and *KIAA0087* was specific to lymphoid clusters, which further validates the use of CD10 as a marker of lymphoid progenitors in CD34$^+$ CB cells exposed to UM171 (Figure **S1**, Chagraoui J., unpublished). Other genes involved during lymphopoïesis include *CD22, FLT3, ITGB7 and BLNK/BCL6* (Wagner *et al.*, 1996; Tarlinton *et al.*, 1997; Duy *et al.*, 2010; Zriwil *et al.*, 2018; Zhang *et al.*, 2022; Figure **S1**). We selected genes producing surface proteins and tried to design the optimal panel composed of lymphoid-associated surface markers by FACS. As the expression of CD22, FLT3, ITGB7 and CCR7 was not constant in cultured CD34$^+$ cells exposed to UM171 we restricted our analysis to the CD10 surface marker. Ergo, we hypothesized that we can use the surface marker CD10 to enrich for lymphoid progenitors with multilymphoid potential in CD34$^+$ CB cells cultured in the presence of UM171 (35nM).

We next determined whether the mRNA and protein expression of CD10 was coherent with the spatial localization of the CLP and LMPP clusters (Figure 2**E** and 2**G**). CD10 protein and mRNA levels had highly similar topological location, thus confirming the robustness of our transcriptome profiling (Figure 2**G**). Furthermore, the strongest expression of CD10 protein and mRNA was observed at the apex of the first and second dimension (UMAP1 and UMAP2) of the UM171 UMAP (Figure 2**G**). As shown in panel e, the spatial localization of CD10 (mRNA and protein) along the second dimension of the UM171 and UM171 DN-sorted UMAPs concurs with the CLP and LMPP clusters, hence validating the use of CD10 as a phenotypic marker of lymphoid progenitors. However, not all LMPPs and CLPs express CD10 which suggests that CD10 alone is not sufficient to isolate all LMPPs and CLPs contained within UM171 cultures.



(Figure 2 Continues on next page)

Figure 2. Cite-Seq analysis revealed the presence of lymphoid progenitors in cells exposed to UM171. Lymphoid progenitors (CLP and LMPP) can be identified phenotypically using the surface marker CD10.
Cite-Seq experiment was designed by Chagraoui J. Raw data were acquired, processed and integrated by Chagraoui J and Lehnertz B. and the resulting data were analyzed by Maalaoui H.
(A) UMAP visualization of uncultured CD34+ cord blood cells (Fresh) or cultured *ex vivo* in presence of UM171 (35nM), DN sorted and DMSO. UM171 DN sorted; CD34+ cord blood cells were sorted into CD86-, FCER1- double negative population after 7 days of culture in the presence of UM171.
(B) UMAP visualization of annotated populations in CD34+ cord blood cells (Fresh) or cultured *ex vivo* in presence of UM171, DN sorted and DMSO.
(C) and (D) ScProportion test showing the log2 fold change of annotated populations between uncultured (fresh) and UM171-treated DN sorted CD34+ cord blood cells (c) and between DMSO-supplemented and UM171-treated DN sorted CD34+ cord blood cells (d).
(E) UMAP representing common lymphoid progenitors (CLP) and lymphoid-primed multipotential progenitors (LMPP) predicted score in overlapping fresh CD34+ CB cells, CD34+ cells DMSO-supplemented and exposed to UM171 (including DN sorted) after integration.
(F) Heatmap showing differentially expressed genes accross conditions (Fresh, UM171, UM171 DN sorted and DMSO-supplemented CD34+ cells).
(G) UMAP displaying the feature plot of the CD10 antibody and MME (CD10) RNA levels of the overlapping UM171-DMSO wnnUMAPs displayed in a) at a single cell resolution.

2.1.2 UM171 does not induce CD10 expression on CD34$^+$ CB cells.

Cite-Seq analysis indicated that CD10 is strongly expressed in lymphoid progenitors, albeit whether the expression is simply the result of an upregulation of *MME* in CD34$^+$ CB cells induced by UM171, needs to be further investigated. As a first line of evidence, we analyzed the transcriptome of UM171-treated CD34$^+$ CB cells obtained via RNA-Seq experiment performed previously by colleagues (Fares *et al.*, 2014). A few years ago, we reported that the expression of EPCR, a LT-HSC marker, was rapidly induced at the mRNA and protein level in CD34$^+$ CB cells treated with UM171, at 12 and 24 hours respectively (Fares *et al.*, 2014; Fares *et al.*, 2017). Conversely, we reported only a slight increase in EPCR expression in CD34$^+$ CB cells exposed to other small molecules such as SR1, which was likely due to HSCs expansion (Fares *et al.*, 2014; Fares *et al.*, 2017). Figure 3 illustrates the changes in mRNA levels of *MME* (CD10) and *PROCR* (EPCR: positive control) over 72 hours in CD34$^+$ CB cells cultured in the presence of UM171 or DMSO. We observed a rapid up-regulation of the *PROCR* level in CD34$^+$ cells exposed to UM171 as compared to the DMSO-supplemented CD34$^+$ cell cultures (Figure 3**A**). Strikingly, at only 12 hours, the *PROCR* mRNA level almost doubled in CD34$^+$ cells treated with UM171 (vs DMSO-supplemented) validating the strong transcriptional induction of *PROCR* caused by UM171, an epigenetic modulator, as previously reported (Figure 3**A**). On the contrary, even after 72 hours, we observed negligible fluctuation in the *MME* levels between DMSO-supplemented and UM171-treated CD34$^+$ CB cells, likely due to systematic variation in large-scale RNA sequencing (Figure 3**B;** Costa-Silva *et al.*, 2017) thus, an increase in CD10 level must be associated with an increase in the number of

cells that express CD10. Therefore, RNA sequencing indicates that CD10 is not a marker induced by UM171 *in vitro*, yet further functional and transplantation assays are required to confirm that CD10 can be used as a phenotypic marker of lymphoid progenitors.

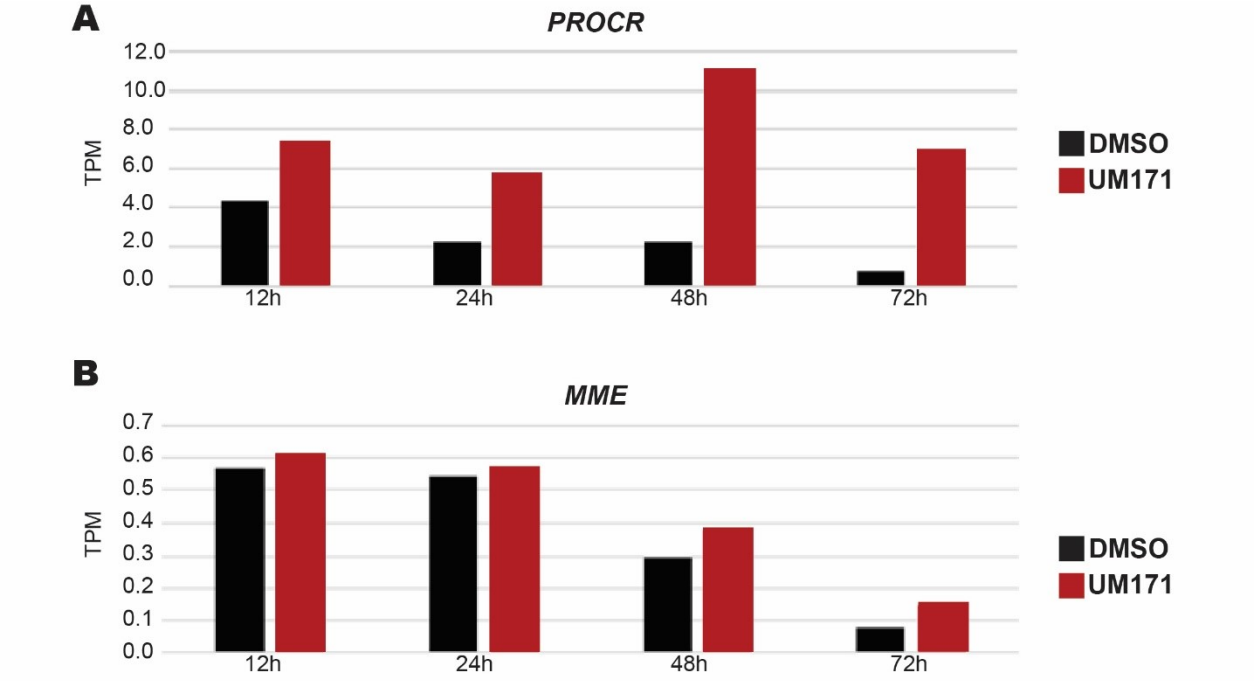


Figure 3. UM171 does not induce CD10 expression on CD34+ CB cells.
Data were obtained from the RNA-Seq experiment performed by colleagues and partially analyzed by HMa (Fares *et al.*, 2014). CD34+ cord blood (CB) cells were cultured during 72 hours in the presence of DMSO or UM171 (35nM).
(A) Expression of *PROCR* (EPCR) in transcript per million (TPM). Comparison of *PROCR* transcript level between DMSO-Supplemented CD34+ cord blood cells and UM171-treated (35nM) CD34+ cord blood cells at 12, 24, 48 and 72 hours. *PROCR* was used as a control to illustrate the pattern associated with an UM171-induced mRNA upregulation *in vitro*.
(B) Expression of *MME* (CD10) in transcript per million (TPM). Comparison of *MME* transcript level between DMSO-Supplemented CD34+ cord blood cells and UM171 treated CD34+ cord blood cells at 12, 24, 48 and 72 hours.

2.1.3 Two CD10-positive subsets are expanded in CD34^{+} cord blood cells cultured in the presence of UM171.

As our Cite-Seq data suggested that CD10 strongly associates with the CLP and LMPP clusters that are depleted in DMSO cultures (vs UM171), we assessed whether we could recapitulate the expansion of the CD10-positive subsets *in vitro*. To do so, we expanded CD34^{+} CB cells in the presence of either DMSO or UM171 (35 nM) during 7 days and we analyzed the samples for the presence of CD10-positive cells by FACS (Figure 4**A**). Intriguingly, we denoted the presence of two distinct CD10-positive subsets that differed in the mean fluorescence intensity for the surface markers CD10 and the lineage commitment marker CD45RA across all conditions (CD10^{med}CD45RA$^{med/lo}$ and

$CD10^{hi}CD45RA^{hi}$; Figure 4**B**-**D**). Furthermore, to enrich for CD10-expressing progenitors, we gated on $CD34^{hi}$ cells (Figure 4**B**-**D**). We reported the highest percentage of $CD10^{med}CD45RA^{med/lo}$ subset in the UM171-treated CB cells (vs Fresh and DMSO cultures) (Figure 4**B** and 4**C**). Moreover, a higher percentage of the $CD10^{hi}CD45RA^{hi}$ subset was also observed in the UM171 cultures in comparison to the DMSO-supplemented cultures (Figure 4**B**).

Interestingly, an assessment of the absolute count indicated a significant enrichment of both subsets in $CD34^{+}$ cells upon exposure to UM171 (Figure 4**D**). More precisely, we observed a 2-fold net expansion of the $CD10^{hi}CD45RA^{hi}$ in the UM171-supplemented cultures as compared to Fresh $CD34^{+}$ cultures ($p=0.0022$) as well as a 6-fold and 7-fold net expansion of the $CD10^{med}CD45RA^{med/lo}$ in the UM171-treated cultures in comparison to Fresh ($p=0.0028$) and DMSO-supplemented cultures ($p=0.0025$), respectively (Figure 4**D**). Overall, the aforementioned results highlighted that UM171 significantly expands CD10-positive subsets in $CD34^{+}$ CB cells. Therefore, based on the expression of the surface marker CD45RA, we propose that the $CD10^{med}CD45RA^{med/lo}$ subset could contain LMPPs and, as commitment lineage specification progresses, CD45RA level will increase resulting in the LMPPs producing $CD45RA^{+}$ CLPs comprised within the $CD10^{hi}CD45RA^{hi}$ subset (Ueda *et al.*, 2000; Majeti *et al.*, 2007; Haddad *et al.*, 2004).

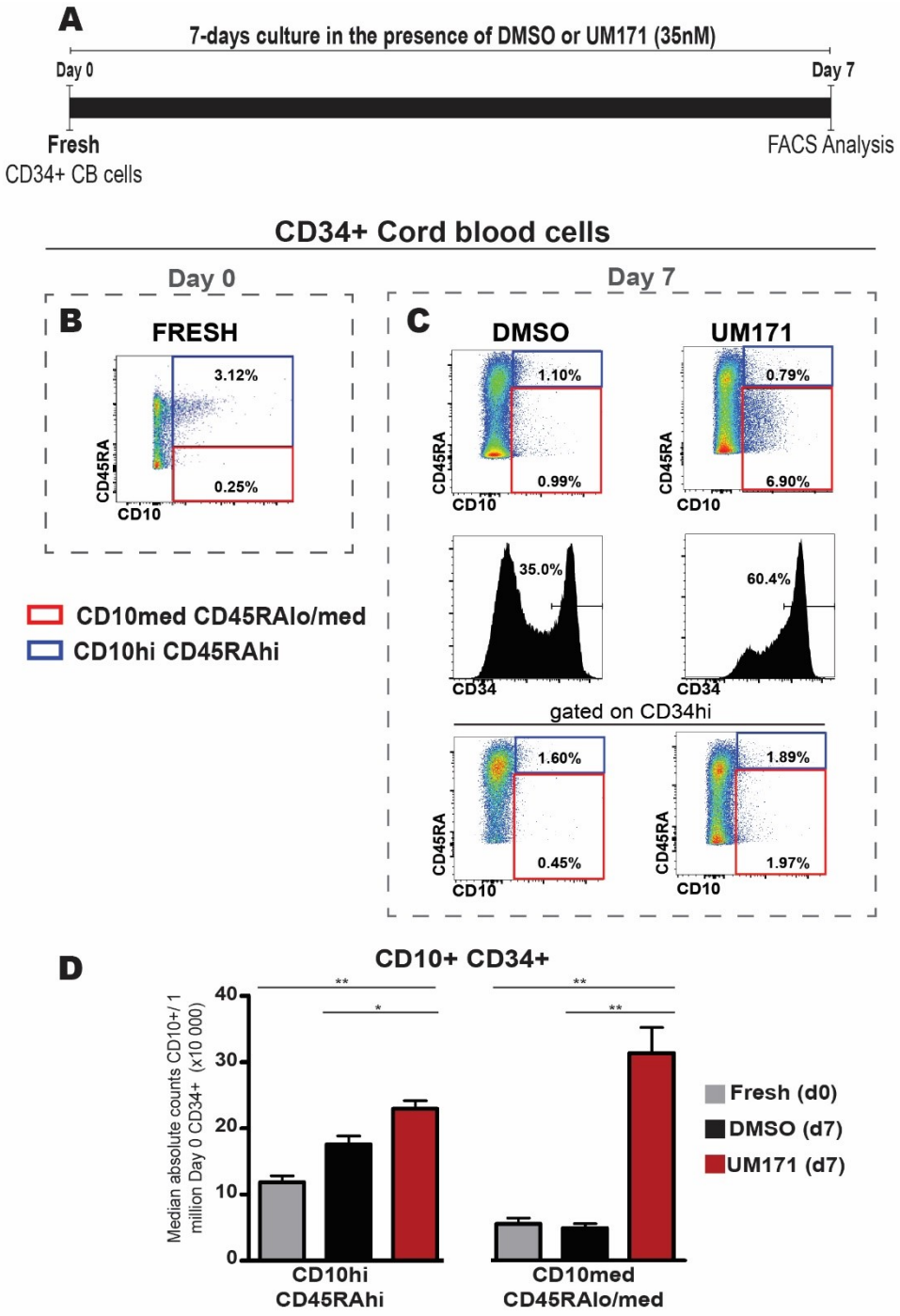


Figure 4. CD10-positive subsets are expanded in CD34+ cord blood cells cultured in the presence of UM171.

(A) Experimental outline of the 7 days expansion of CD34+ Cord blood (CB) cells.

(B) Representative FACS profile of CD10, CD45RA and CD34 surface expression in uncultured CD34+ CB cells (day 0).

(C) Representative FACS profile of CD10, CD45RA and CD34 surface expression in CD34+ CB cells culture in DMSO-supplemented media (left panel) or UM171-treatment (right panel) during 7 days.

(D) Median absolute count of CD10hi,CD45RAhi and CD10med,CD45RAlo\med subsets for one million of day 0 CD34+ CB cells in fresh uncultured, DMSO-supplemented and UM171-treated CD34+ CB cells. Representative of at least 3 independent experiments. *$p \leq 0.05$. **$p \leq 0.005$. Data were analyzed using the non-parametric-Mann-Whitney test comparing UM171 (d7) vs Fresh (d0) and UM171 (d7) vs DMSO (d7).

2.1.4 Only CD45RA-negative cells can give rise to the CD10^{med}CD45RA$^{med/lo}$ subset.

Several studies have used CD45RA as a marker of lineage commitment and therefore we would expect that the CD10^{med}CD45RA$^{med/lo}$ subset differentiates into lineage-committed CD10^{hi}CD45RA^{hi} lymphoid progenitor cells *in vitro* (Ueda *et al.*, 2000; Haddad *et al.*, 2004; Majeti *et al.*, 2007). To gain further insights on whether the CD10^{med}CD45RA$^{med/lo}$ subset originates from CD45RA-positive lineage-committed cells that loose CD45RA expression *in vitro* or if the CD10^{med}CD45RA$^{med/lo}$ progenitors will give rise to the CD10^{hi}CD45RA^{hi} subset, we decided to sort CD34^{+} CB cells (fresh, d0) into the CD45RA^{+}CD34^{+} and CD45RA^{-}CD34^{+} populations by FACS and then to culture each population in the presence of DMSO or UM171 (35nM) for 7 days (Figure 5). FACS analysis indicated that only the CD45RA^{-}CD34^{+} population can yield both CD10-positive subsets (CD10^{med}CD45RA$^{med/lo}$ and CD10^{hi}CD45RA^{hi}) whereas the CD45RA^{+}CD34^{+} can only generate the CD10^{hi}CD45RA^{hi} subset thus, indicating that CD10^{med}CD45RA$^{med/lo}$ defines a subset with a more primitive phenotype (Figure 5). Interestingly, a higher percentage of the CD10^{med} subset is observed in the UM171-treated cultures (vs DMSO-supplemented cultures) which recapitulates the UM171-induced expansion of the CD10^{med} subset mentioned earlier.

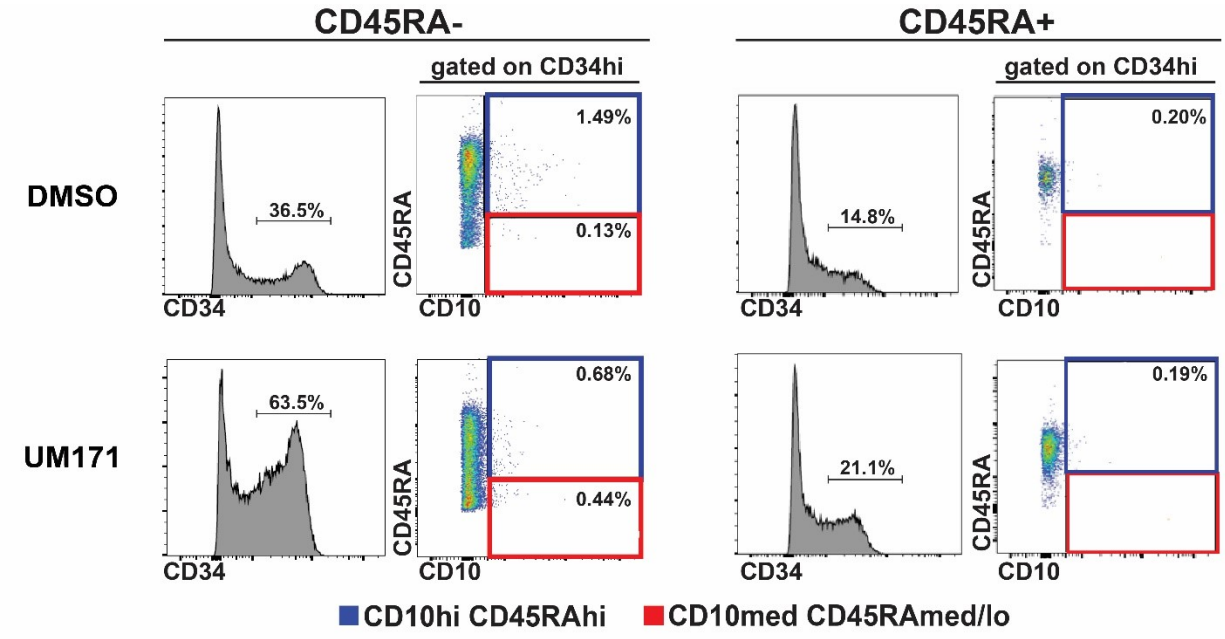


Figure 5. Only CD45RA-negative cells can give rise to the CD10med,CD45RAmed/lo subset. Representative FACS profile of CD10, CD34 and CD45RA surface expression in CD34+ cord blood cells exposed to UM171 (35nM) or DMSO during 7 days in vitro. Representative of at least 3 independent experiments.

2.1.5 CD10-positive subset displays faster T cell differentiation kinetic than Total CD34$^+$ subset.

We next assessed whether CD10 marked early lymphoid progenitors or if CD10 was abnormally expressed in the non-lymphoid populations *in vitro*. To decipher the lymphoid potential of the UM171 graft, we used an artificial thymic organoid (ATO) to generate a three-dimensional (3D) aggregate of HSPCs combined to a standardized stromal cell line expressing Notch ligand (Delta-like ligand 4 [DLL4]; Figure 6**A**). We expanded fresh CD34$^+$ CB cells in the presence of UM171 (35nM) during 7 days, followed by a FACS sorting into different subsets and a co-culture with MS5-hDLL4 in ATO during 6 weeks as described by Seet *et al.* (2017; Figure 6**A**). This approach relies on a serum-free condition and thus, help reduce the discrepancies in reproducibility caused by a serum-containing medium as observed in the monolayer systems (Seet *et al.*, 2017; Montel-Hagen *et al*, 2020). Furthermore, functional perturbation experiments suggested great variations in the molecular mechanisms underlying early T cell development between mice and humans, thereby depreciating the extrapolation of the insights on T cell development obtained via the mouse models to the humans (Taghon *et al.*, 2009; Van de Walle *et al.*, 2009; Ha *et al.*, 2017). Nonetheless, Notta and colleagues reported the presence of two distinct compartments diverging on the acquisition of CD45RA: 1) the CD38$^{-/low}$CD45RA$^-$ compartment that entails HSCs, MPPs and megakaryocyte-erythroid progenitors and, 2) the CD38$^{-/low}$CD45RA$^+$ compartment that comprises the hematopoietic progenitor cell (HPC) population encompassing lymphoid-primed precursors and the monocyte/dendritic cell lineages (Notta *et al.*, 2011; Notta *et al.*, 2015). In mice, studies reported that CD10 (Galy *et al.*, 1995; Doulatov *et al.*, 2010) can be used to identify the CD45RA$^+$ HPC populations enriched in lymphoid potential. Thus, following 7-days culture of CD34$^+$ CB cells in the presence of UM171, we decided to sort the UM171-treated CD34$^+$ CB cells into 4 subsets: 1) CD10$^+$CD34$^+$ (containing both the CD10^{med} and CD10^{hi} described earlier), 2) CD45RA$^-$CD34$^+$, 3) CD45RA$^+$CD34$^+$ and 4) Total CD34$^+$ to compare T cell differentiation and maturation kinetic. Additionally, we included the uncultured CD10-positive subset (Fresh, CD34$^+$CD10$^+$) to analyze whether UM171 has an effect on the lymphoid potential of our CD10-positive subset. Accordingly, we would expect to observe a faster T-cell development within the CD45RA$^+$CD34$^+$ (refer to as CD45RA$^+$)

compartment as compared to the CD45RA$^-$CD34$^+$ (refer to as CD45RA$^-$) compartment in UM171-treated CB cells. Moreover, our approach was used to further validate whether the CD45RA$^+$CD34$^+$ compartment is enriched in lymphoid progenitors by comparing its lymphoid potential to the Total CD34$^+$ subset. Lastly, this experiment allowed us to determine whether our CD10-positive subset corresponds to a population containing lineage-restricted lymphoid precursors (CD45RA$^+$) or a population containing precursors with multi-lineage potential (CD45RA$^-$). One should bear in mind that the interpretation we propose here is limited since the results are obtained from a single pilot experiment and that no cell count were acquired. A robust interpretation of this experiments would require additional replicate with cell count and the appropriate statistical analysis.

At 3 weeks of co-culture in ATO, the UM171-treated CD10-positive subset still had lymphoid-myeloid proliferative potential, albeit a higher percentage of non-myeloid cells was observed (73% vs 23% respectively, Figure 6**B**) which was consistent with the T-lineage-biased ATO microenvironment in which Notch signalling promotes T cell differentiation and maturation of thymocyte precursors (Taghon *et al.*, 2009). In terms of T cell differentiation, at 3 weeks, half of the non-myeloid cells within the UM171-treated CD10-positive subset were double positive (CD4$^+$CD8$^+$; Figure 6**B**). Interestingly, at 3 weeks, we reported almost exclusively non-myeloid cells within the CD45RA$^+$ subset (86% vs 9% myeloid cells) and the opposite scenario was observed for the CD45RA$^-$ subset where the majority of cells were myeloid (80% vs 15% non-myeloid cells; Figure 6**B**). Consistently, more than half of the non-myeloid cells were CD4 and CD8 double positive within the CD45RA$^+$ subset whereas half of the cells were negative for CD4 and CD8 within the CD45RA$^-$ subset (Figure 6**B**). In comparison, a skewing toward myeloid lineage was observed in the Total CD34$^+$ subset (60% vs 32% Non-myeloid cells) and, similar to the CD45RA$^-$ subset, half of the non-myeloid cells were double negative for the markers CD4 and CD8, at 3 weeks (Figure 6**B**). Therefore, our data suggest that, at 3 weeks, the lymphoid potential is enriched in the CD45RA$^+$ subset as compared to the Total CD34$^+$ subset. In contrast, the CD45RA$^-$ subset maintained a myeloid-lymphoid potential at 3 weeks (Figure 6**B**). The CD10-positive subset recapitulated the lymphoid phenotype observed in the CD45RA$^+$ subset where the majority of cells were non-myeloid (73% vs 23% myeloid) and half of the cells were double positive for the markers CD4 and

CD8, albeit the myeloid potential was still present which contrast to the CD45RA$^+$ subset lacking myeloid cells at 3 weeks, suggesting a lymphoid-skewed multipotential of the CD10-positive lymphoid progenitors (Figure 6**B**). As we reported previously (Fares *et al.*, 2014), UM171 expands CD34$^+$CD45RA$^-$ HSCs *in vitro* thus, the multilineage potential observed within the CD45RA$^-$ subset potentially pertains to enriched HSCs expanded by UM171 as compared to the CD45RA$^+$ and total CD34$^+$ subsets. Similar T cell differentiation kinetic was observed among the CD10-positive subsets whether UM171 was present or not at 3 weeks (Figure 6**B**). Although similar at 3 weeks, both CD10-positive subsets (UM171 and Fresh) showed a higher percentage of CD4$^+$CD8$^+$ DP and a lower percentage of CD4$^-$CD8$^-$ DN thereby validating that CD10 can be used to enrich for T-cell precursors, as mentioned in the literature. A striking increase in T cell differentiation was observed for the CD45RA$^+$ subset in contrast to the CB CD34$^+$CD3$^-$ HSPCs differentiation reported by Seet and colleagues (Seet *et al.*, 2017), confirming that CD45RA efficiently demarcates the T-lineage committed precursors.

At 6 weeks, the differences in T-cell differentiation across the subsets recapitulated the tendency observed at 3 weeks (Figure 6**B**). More precisely, a myeloid potential, yet reduced, was observed for the Total CD34$^+$ and CD45RA$^-$ subsets whereas the CD10-positive and CD45RA$^+$ subsets were lacking myeloid populations (Figure 6**B**). Consistently, almost all of the non-myeloid cells were CD4$^+$CD8$^+$ DP for the CD45RA$^+$ and CD10-positive subsets suggesting a faster T-cell differentiation than their counterpart subsets still containing double negative lymphoid cells (CD45RA$^-$ and total CD34$^+$; Figure 6**B**). Although similar, the CD45RA$^-$ subset had a greater myeloid potential than the Total CD34$^+$ subset thus confirming the presence of immature progenitors within the CD45RA$^-$ subset. At 6 weeks, we observed great similarities between the Total CD34$^+$ subset and the CD34$^+$CD3$^-$ HSPCs (reported by Seet *et al.*, 2017) in terms of T-cell differentiation. Interestingly, in regards to the CD10-positive subsets, two distinct developmental stages were observed within the uncultured CD10-positive cells (fresh) that were lacking in the CD10-positive subset treated with UM171, a CD4$^-$CD8$^-$ T cell population (double negative, representing 6% of the total non-myeloid cells) and a CD8$^+$ T cell population (single positive, representing 6% of the total non-myeloid cells) (Figure 6**B**). Whether this discrepancy was due to a higher heterogeneity of immature and mature lymphoid

progenitors present within the fresh CD10-positive subset (vs UM171-treated CD10-positive subset) needs to be confirmed. Based on the aforementioned results, we propose that the CD45RA^{-} subset contains primitive cells (*e.g.* HSCs) that segregate into lineage-biased multipotent progenitors enriched in the CD10-positive subset (*e.g.* LMPP) and, as cell lineage specification and commitment progress, these lineage-biased multipotent progenitors will give rise to lineage-restricted precursors that are CD45RA^{+} (*e.g.* CLP). This suggested mechanism is based on data obtained from a single pilot experiment including percentages rather than absolute counts therefore, additional replicates with cell count as well as the appropriate statistical analysis are required to validate the latter proposition.

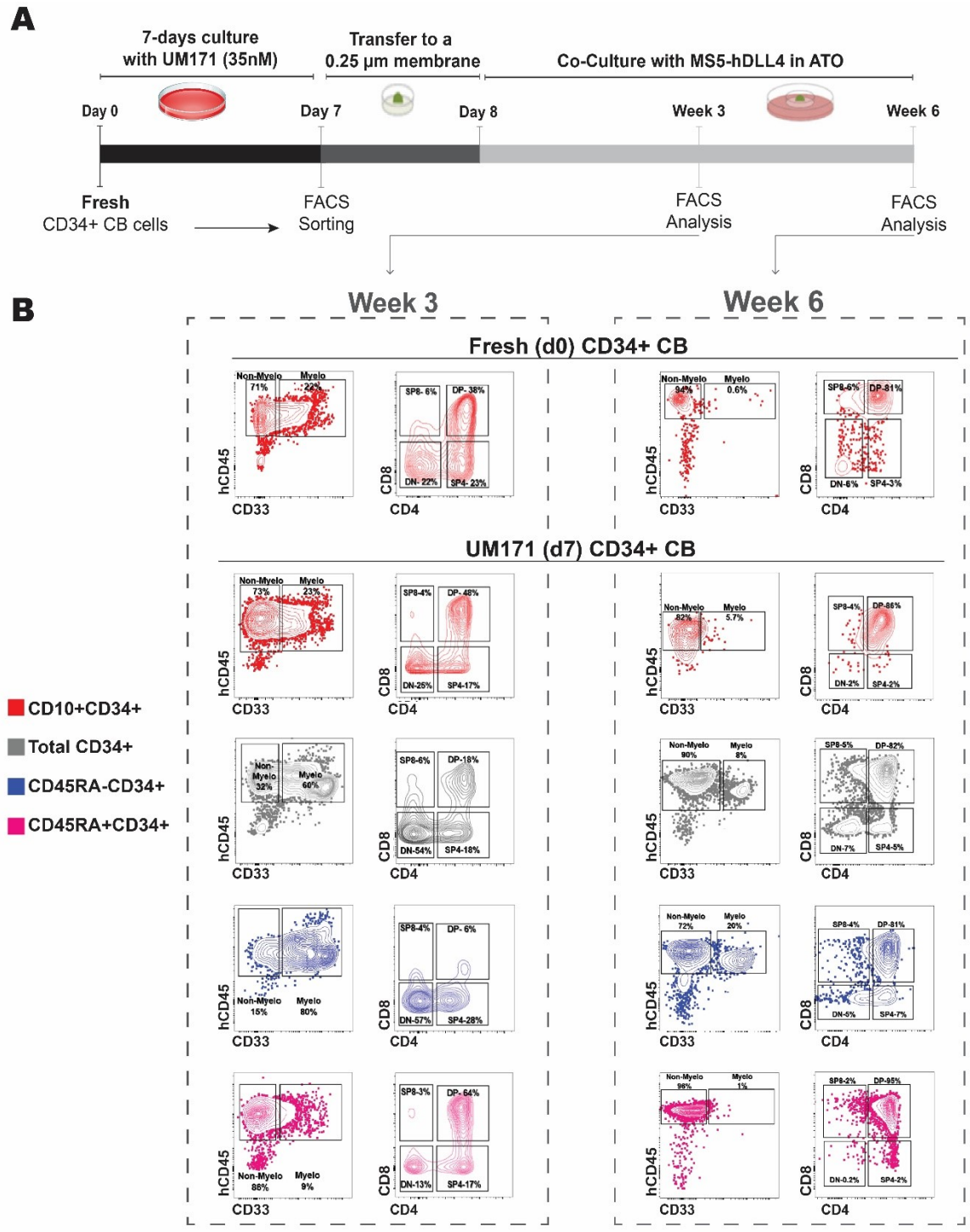


Figure 6. CD10-positive subset displays greater T cell differentiation potential than Total CD34+ subset.

(A) Experimental outline of the artificial thymic organoid (ATO) protocol.

(B) Contour plot showing CD33, hCD45, CD8 and CD4 surface expression in CD10+, total CD34+, CD45RA- and CD45RA+ subsets uncultured (Fresh) or cultured in presence of UM171 (35nM) during 7 days. FACS profile were obtained at 3 and 6 weeks co-culture in ATO. CD8 and CD4 surface expression are presented after gating on CD33 negative cells (non-myeloid cells). SP8/4; single positive CD8+/CD4+ t cells, DN; double negative t cells (CD8-CD4-) and DP; double positive t cells (CD8+CD4+). Representative of a pilot experiment designed and initiated by Chagraoui J.

2.1.6 CD10-positive subset displays greater thymopoïesis potential than Total CD34$^+$ subset.

We next analyzed whether maturation of T cells also occured within the different subsets at 6 weeks (Figure 7). We observed mature recombined T cells across all conditions, suggesting that CD10 alone is not sufficient to demarcate lymphoid precursors (Figure 6 and 7). Consistent with the kinetic of T-cell differentiation observed for the different subsets (Figure 6), maturation was greater for the CD45RA$^+$ subset, followed closely by the CD10-positive subset, the Total CD34$^+$ and the lower percentage of CD3$^+$TCR-αβ$^+$ T cells was reported for the CD45RA$^-$ subset, overall recapitulating the T-cell differentiation kinetic observed earlier (Figure 6 and 7). Intriguingly, the percentage of CD3$^+$TCR-αβ$^+$ T in CB CD34$^+$CD3$^-$ HSPCs reported by Seet and colleagues at 6 weeks is similar to the percentage of CD3$^+$TCR-αβ$^+$ T observed in the CD45RA$^-$ subset (Seet *et al.*, 2017). In sum, the CD34$^+$CD3$^-$ subset had a comparable percentage of T-cell differentiation to the CD10-positive subset and the Total CD34$^+$ subset at 3 and 6 weeks respectively and a similar percentage of CD3$^+$TCR-αβ$^+$ T to the CD45RA$^-$ subset at 6 weeks, suggesting a high degree of heterogeneity of progenitors within the CD34$^+$CD3$^-$ subset in contrast to our subsets sorted using CD10 and CD45RA surface markers (Seet *et al.*, 2017).

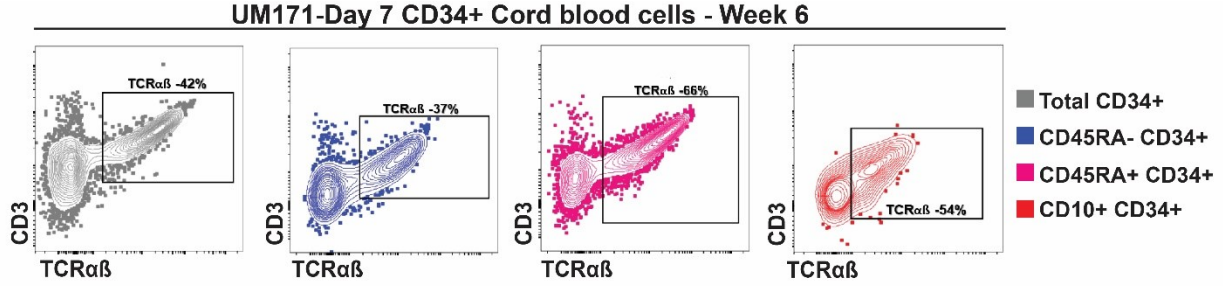


Figure 7. CD10-positive subset displays greater thymopoiesis potential than Total CD34+ subset. Contour plot showing CD3, TCRαß surface expression in Total CD34+, CD45RA-CD34+, CD45RA+CD34+ and CD10+CD34+ derived progenies at 6 weeks co-culture in ATO. Subsets were cultured in the presence of UM171 during 7 days prior to co-culture in ATO. CD3 and TCRαß surface expression are presented after gating on CD33 negative cells (non-myeloid cells). Representative of a pilot experiment. Experimental outline is show in figure 6 panel a.

2.1.7 UM171 enhances lymphopoïesis in CD10-positive CD34+ CB cells.

Lavaert and colleagues recently reported the presence of an early $CD10^+$ TSP1 expressing high level of stem and progenitor genes (Lavaert *et al.*, 2020). They proposed that the level of stem and progenitor genes decreases as $CD10^+$ TSPs differentiate into $CD7^+$ ETPs displaying IL-7R signalling, promoted by strong level of Notch activation (Lavaert *et al.*, 2020). However, they did not detect ILC or NK precursors using the BM and thymus datasets and further inferred that it was unlikely that either TSP subsets ($CD10^+$ TSP1 or $CD7^+$ TSP2) represents ILC-NK precursors (Lavaert *et al.*, 2020). On the other hand, Canque and colleagues identified two populations of early lymphoid progenitors (ELPs) populations differing based on CD127 (IL7R) expression ($CD127^+$ and $CD127^-$ ELPs) where solely the $CD127^-$ ELPs has NK/ILC and T potential (vs $CD127^+$ that only has marginal T potential; Alhaj Hussen *et al.*, 2017). Intriguingly, Lavaert *et al.* proposed that the TSP2 subset could be a subset of the $CD127^-$ ELP population described by Canque and colleagues (Alhaj Hussen *et al.*, 2017; Lavaert *et al.*, 2020).

Intriguingly, using ATO, we obtained a higher percentage of a potential ILC-like population ($IL7R^+CD7^+Lin^-$) in the CD10-positive subset in comparison to the Total $CD34^+$, $CD45RA^-$ and $CD45RA^+$ subsets (Figure 8). In addition, we denoted the presence of $ITGB7^+ILR7^+$ populations in the $CD45RA^-$ and the $CD45RA^+$ subsets (Figure 8). Interestingly, β_7 integrins (including ITGB7) have been highlighted in the literature for their role in lymphocyte homing and localization in the gut (Berlin *et al.*, 1993; Cepek *et al.*, 1994; Wagner *et al.*, 1996; Kunkel *et al.*, 2000 and Salmi *et al.*, 2005). Therefore, these ITGB7 expressing cells (Lin^-) could potentially be common helper-like innate lymphoid cell progenitors (CHILPs) described previously as $ITGB7^+IL7R^+Lin^-$, which is coherent with the higher percentage of these potential ILC progenitors observed within the most primitive subset ($CD45RA^-$; Klose *et al.*, 2014). Furthermore, this potential population of CHILPs were depleted within the CD10-positive subset displaying the higher percentage of ILC-like cells which potentially suggests that the CD10-positive subset do not contain CHILPs-like cells. Intriguingly, Ghaedi and colleagues identified $IL7R^+$ LMPPs that could give rise to ILCs (ILC2) via CLP-independent pathways in transplantation assays (Ghaedi *et al.*,

2016). Interestingly, we denoted a smaller percentage of ILC-like cells and a prominent ITGB7^{+}IL7R^{+} CHILP population within the CD45RA^{+} subset in contrast to the CD10-positive subset. This might indicate that the CD10-positive subset contains LMPP that can give rise to ILCs and T cells or contains ILC-producing LMPPs (via CLP-independent pathways) and T-cell producing CLPs (LMPP-containing CD10^{med} and CLP-containing CD10^{hi}), whereas the CD45RA^{+} subset might contain CLPs that commit to ILC-producing CHILPs.

Furthermore, as the CD10-positive subset has T potential and could produce ILCs, this subset could originates from CD127^{-} ELPs (Alhaj Hussen *et al.*, 2017) and potentially represents another CD10^{+} TSP subset than the one identified by Lavaert *et al.* (2020). Of note, one should cautiously envision this proposition as the ILC potential and the presence of CHILPs should be validated and the lack of replicate and transplantation assay could bias our interpretation.

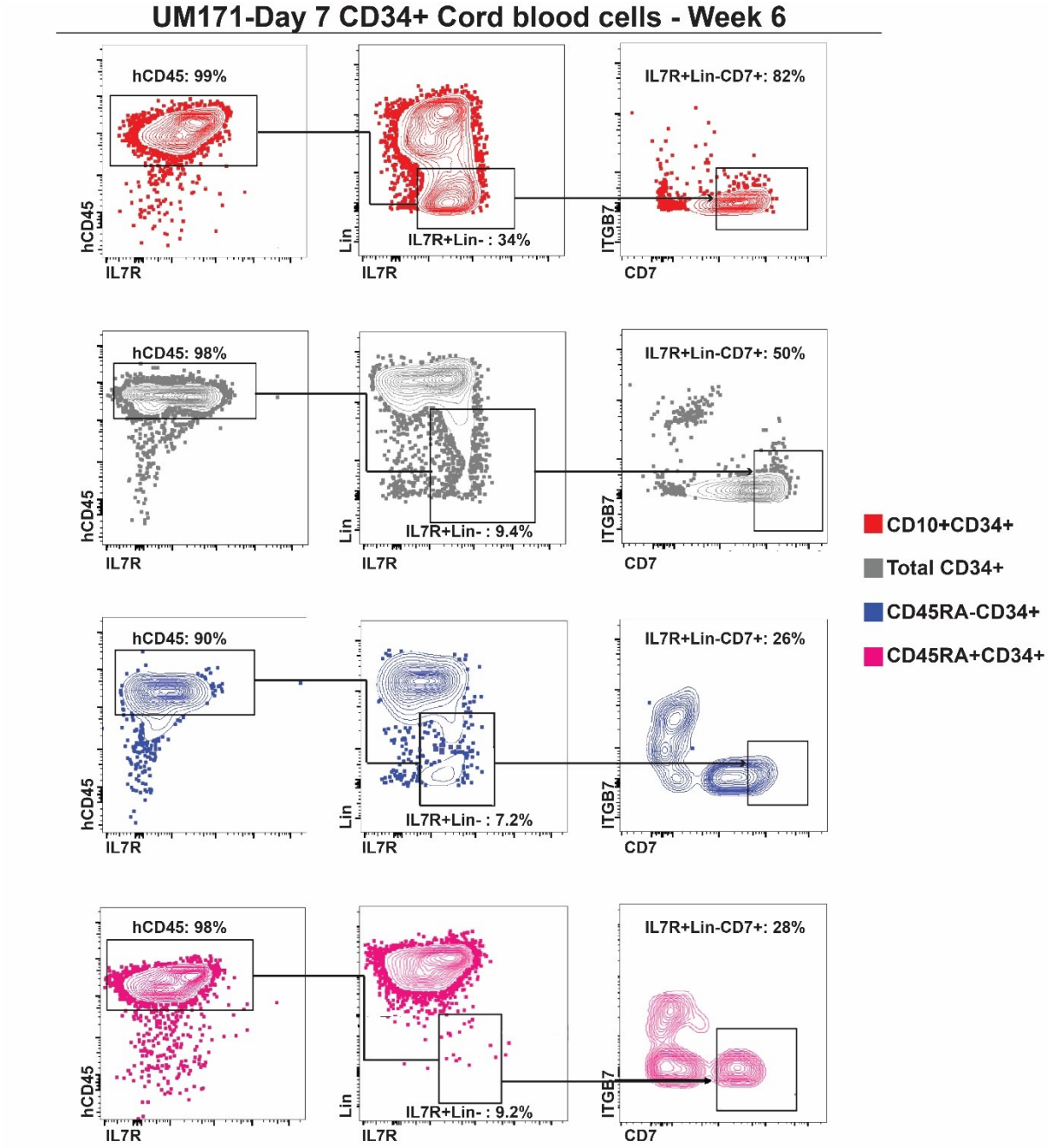


Figure 8. UM171 enhances lymphopoiesis in CD10-positive CD34+ CB cells.
Contour plot showing hCD45, Lin, ILR7 and CD7 surface expression in CD10+CD34+, Total CD34+, CD45RA-CD34+ and CD45RA+CD34+ subsets at 6 weeks co-culture in ATO. Subsets were cultured in the presence of UM171 during 7 days prior to co-culture in ATO. Representative of a pilot experiment. Experimental outline is shown in figure 6 panel a.

Chapter 3 – Discussion

3.1 HSCT

3.1.1 Immune reconstitution post-HSCT.

Currently, allogeneic hematopoietic stem cell transplantation (HSCT) is the first line of treatment for various malignant and non-malignant hematopoietic disorders (Storb *et al.*, 2019). Over the past decades, a high rate of transplant-related complications (TRC) has constrained the implementation of HSCT. TRC, including infections, relapse, toxicities due to the conditioning regimen and GvHD (acute and chronic) often erode the outcome of the patient post-HSCT (Ringdén *et al.*, 2012; Ghimire *et al.*, 2017). Early contributors of transplant-related mortalities (TRM) are infection, acute GVHD and relapse whereas late contributors of TRM include chronic GVHD and organs malfunction (Ghimire *et al.*, 2017). Demarcation of GVHD from GVL effects has been challenging as they share similar underlying mechanisms grounded on alloreactivity (Negrin *et al.*, 2015). Nonetheless, many strategies have emerged to manage GVHD such as T-cell depletion from the graft (via $CD34^+$ cell selection; Klein *et al.*, 2017) and the use of post-transplant immunosuppression (Bacigalupo *et al.*, 2015) for patients transplanted with an HLA-mismatch donor graft. Recently, strategies such as partial T-cell depletion (of specific T-cell subsets; Robinson *et al.*, 2016) and *ex vivo* manipulation of donor T cells (Sengsayadeth *et al.*, 2016) were developed. Although these strategies can prevent TRM, they also have adverse effect on the post-transplantation immune recovery and a profound immunodeficiency often arise as the expected corollary of these approaches (Storek *et al.*, 2008).

Innate and adaptive reconstitution are pivotal determinants of the patient outcome following HSCT (Ogonek *et al.*, 2016). More precisely, tissue repair and the control of infection rely on the early innate immunity whereas the immune response to microbial and viral pathogens is contingent on the restoration of the adaptive immunity. GVL effects depend on the recovery of the immune functions and, in turn, autoimmune-like dysregulations increase the patient susceptibility to chronic GVHD development (Ogonek *et al.*, 2016). In comparison to innate immunity recovery occurring within weeks to few months following allogeneic HSCT, the restoration of a functional T-cell compartment can

take as long as 2 years (Storek *et al.*, 2008; Bosch *et al.*, 2012) thereby increasing the susceptibility of the patient to opportunistic pathogens and lessening the graft-versus-tumor immune responses post-HSCT (Clave *et al.*, 2013). In the peripheral blood, a normal naïve T cells count is usually observed within three to six months post-transplantation (Mehta *et al.*, 2016) whereas it can take 6-12 months to obtain a $CD4^+$ cell counts sustaining protective immunity (Storek *et al.*, 2008; Bosch *et al.*, 2012). TCR excision circles (TRECs) count, used as an indicator of the production of thymic derived naïve T cells, remains low for the 3-6 first months post-HSCT (Toubert *et al.*, 2012). Interestingly, the early formation of the $CD4^+$ T-cell compartment positively associates with the overall survival (OS) and the viremia clearance post-HSCT (Admiraal *et al.*, 2017; De Koning *et al.*, 2018; Haddad *et al.*, 2018). Furthermore, several studies also proposed a role of the innate lymphoid cells (ILCs) in GVHD (Munneke *et al.*, 2014; Bruce *et al.*, 2017; Dudakov *et al.*, 2017, Komanduri *et al.*, 2017). ILC belong to a class of effector cells involved in the early immune response against pathogens by stimulating tissues repair and they originate from a CHILP precursor (Satoh-Takayama *et al.*, 2008; Monticelli *et al.*, 2011). Munneke and colleagues analyzed the level of circulating ILC1s in PB and reported that patients that developed more severe GVHD displayed fewer circulating ILC1s in PB in contrast to healthy controls (Munneke *et al.*, 2014). In addition, Bruce and colleagues showed that intravenous infusion of donor-derived ILC2 cells in mice could alleviate the severity aGVHD by migrating to the gastrointestinal (GI) tract and producing Th2 cytokines while reducing Th1 and Th17 inflammatory cells (Bruce *et al.*, 2017). Intriguingly, using a murine GVHD experimental model, Dudakov and colleagues demonstrated that, following allo-HSCT, mice with GVHD had reduced level of intrathymic ILC3s and IL-22 and displayed a defective recovery of thymopoïesis (ILC3s produce IL-22; Dudakov *et al.*, 2017). They showed that $IL\text{-}22^{-/-}$ mice displayed more severe thymic injuries associated with GVHD post-HSCT whereas the exogenous administration of IL-22 following transplantation associated with an improved thymopoïesis and thymus-derived peripheral T cells development (Dudakov *et al.*, 2017, Komanduri *et al.*, 2017). Overall, these studies suggest that ILCs are also involved in GVHD and, to some extent, in T cell reconstitution.

Following HSCT, two distinct pathways contribute to T-cell reconstitution. The first pathway occurs early post-HSCT and consist of the peripheral expansion of either the

recipient T cells persisting through conditioning or the donor T cells from the graft (Velardi *et al.*, 2021). During this early post-transplantation period, a skewing of the TCR repertoire, due to the prevalent expansion of the $CD8^+$ memory T-cell population (cytokine-dependent), results in an inadequate immune response (Toubert *et al.*, 2012). The second pathway occurs after the recovery of the recipient thymus from conditioning-induced injuries and is essential to complete the T-cell repertoire reconstitution (Velardi *et al.*, 2021). This latter process relies on the *de novo* production and education of naïve T cells in the thymus of the recipient (Chaudhry *et al.*, 2017). Lymphoid precursors are generated in the bone marrow (BM) from the donor's HSCs and/or rare MLPs present in the graft, and seed the recipient thymus where the crosstalk between the thymus seeding progenitors (TSPs) and the thymic microenvironment regulates the development of thymocytes (Koch *et al.*, 2008).

A plethora of factors also affect T-cell reconstitution such as infection (De Koning *et al.*, 2018), relapse of the underlying disease and GVHD (Clave *et al.*, 2013). The age of the HCST recipient is also important as it strongly associates with thymic involution and the maintenance of thymic architecture, which is an important factor of *de novo* T cell generation (Van den Brink *et al.*, 2015; Krenger *et al.*, 2011; Chaudhry *et al.*, 2017). Importantly, pre-transplant parameters such as conditioning regimen, use of ATG, HLA mismatch and the graft source can also affect T-cell reconstitution (Ogonek *et al.*, 2016). In comparison to other stem cell sources used in HSCT, patients transplanted with CB display lower incidence of chronic GVHD and relapse (Ballen *et al.*, 2013; Rocha *et al.*, 2004; Laughlin *et al.*, 2004). However, delayed engraftment in CB graft recipient results in higher rates of infections (Ballen *et al.*, 2016). In addition, an enhanced susceptibility to pathogens was reported in CB graft recipient caused by an ineffective T-cell reconstitution (Lucchini *et al.*, 2015; De Koning *et al.*, 2016).

3.1.2 T-cell reconstitution in UM171-graft recipient post-HSCT.

In contrast, in a 22-patient phase I/II clinical trial, we reported a rapid and durable neutrophil engraftment translating into a low rate of TRM at 1 year for the patients transplanted with a single CB graft expanded with UM171, a small molecule used for the *ex vivo* expansion of CB HSCs (Fares *et al.*, 2014; Cohen *et al.*, 2019). In addition, a low

incidence of acute GVHD (10%) was observed for the patients receiving a UM171-expanded graft (no occurrence of acute GVHD; Cohen *et al.*, 2019) hence favouring thymus recovery. Intriguingly, in terms of T-cell reconstitution, we reported that, despite receiving a 2 times lower $CD3^+$ T cell dose, the patients transplanted with a UM171-expanded graft had similar count of T cells in peripheral blood at 3, 6 and 12 months post-HSCT than the patients receiving unmanipulated CB graft (transplanted previously in the same institution with comparable conditioning regimens). Following HSCT, the UM171-graft recipients also displayed a greater increase in naïve T cells production, recent thymic emigrants (RTEs) and T cell clonotype from 3 months to 6 and 12 months than the counterpart cohort (unmanipulated), overall suggesting *de novo* thymopoïesis. In line with *de novo* thymopoïesis, we demonstrated a 5-fold increase in the frequency of CLP that are 3 times more proliferative as compared to the CLPs obtained from DMSO-supplemented cultures (Dumont-Lagacé *et al.*, 2021). Expansion of CB transplant with UM171 allows to: 1) obtain adequate stem cell dose and, 2) increase HLA-matching level by promoting accessibility to smaller HLA-match CBU and CB availability for racial and ethnic minorities, thereby alleviating two factors negatively associated with T-cell reconstitution (De Koning *et al.*, 2016; Dumont-Lagacé *et al.*, 2022).

In this study, we aimed to understand the molecular mechanism underlying T-cell reconstitution in patients transplanted with UM171-expanded CB graft. We tried to identify lymphoid precursors within the highly heterogeneous $CD34^+$ CB cells treated with UM171 and find a surface marker that can be used to enrich for early lymphoid progenitors. This study can provide insights and direction for the optimization of the $CD3^+$ T cell infusion to enhance T-cell reconstitution in patients undergoing allogeneic HSCT with UM171-treated CB transplants.

3.2 Characterization of lymphoid progenitors in the UM171 graft.

3.2.1 The UM171 graft contains lymphoid progenitors that can potentially be identified phenotypically using the surface marker CD10.

To gain insights on the lymphoid potential, we used Cite-Seq to analyze changes in the transcriptome of $CD34^+$ CB cells exposed to UM171 (35nM, 7 days) and depleted of dendritic and mast cells (UM171 DN-sorted which is $CD86^-FCER1A^-$) as compared to Fresh $CD34^+$ and DMSO exposed cells. The results highlighted the presence of two closely related lymphoid cluster, CLP and LMPP, with overlapping spatial localization. Interestingly, the predicted LMPPs were 6 times more numerous in the UM171 graft as compared to the Fresh and DMSO-supplemented graft. Zheng and colleagues used ATAC-seq to unravel the chromatin dynamics of the transcriptional programs of CB progenitor cells (Zheng *et al.*, 2018). Interestingly, they reported a consistent accessibility of primed lymphoid genes across LMPP and CLP progenitors. In contrast, a striking decrease in the accessibility of lymphoid-primed genes was observed during the LMPP to GMP transition, thus indicating a high degree of similarity between the transcriptome of LMPP and CLP progenitors (Zheng *et al.*, 2018), which is coherent with the overlapping spatial localization of predicted LMPP and CLP along the UMAPs (at the apex along the second dimension, UMAP2). Furthermore, higher CD10 (protein and mRNA) levels were observed at the overlapping spatial localization reported for LMPP and CLP, suggesting that this marker could be used to demarcate lymphoid progenitors from a highly heterogeneous $CD34^+$ CB cells population. However, not all predicted CLPs and LMPPs express CD10 thus suggesting that CD10 alone is not sufficient to enrich for all lymphoid progenitors within the UM171 cultures. Moreover, we denoted a stronger lymphoid transcriptional signature in the UM171 graft as compared to the DMSO graft, which suggests that UM171 exposure could enhance the lymphoid potential of cultured $CD34^+$ cord blood cells.

As we reported previously, UM171 can induce the expression of surface marker such as EPCR, a marker of LT-HSCs (Fares *et al.*, 2017). Therefore, using the RNA-Seq data (obtained previously; Fares *et al.*, 2017), we demonstrated that *MME* (encoding CD10) is

not subjected to induction by the small molecule UM171 since *MME* level remained roughly unchanged even after cell division.

3.2.2 Two CD10-positive subsets are expanded in CD34+ cord blood cells cultured in the presence of UM171.

We observed the presence of two CD10-positive subsets differing based on the expression of the surface marker CD45RA and CD10 ($CD10^{med}CD45RA^{med/lo}$ and $CD10^{hi}CD45RA^{hi}$), in $CD34^+$ CB cells. We reported a 2-fold expansion of the $CD10^{hi}CD45RA^{hi}$ subset in the UM171-treated culture as compared to uncultured $CD34^+$ cells (Fresh). The most striking expansion was reported for the $CD10^{med}CD45RA^{med/lo}$ which had a 6-fold and 7-fold expansion in the UM171 cultures as compared to uncultured and DMSO-supplemented $CD34^+$ cells, respectively. Furthermore, we demonstrated that only the CD45RA-negative cells can give rise to both CD10-positive subsets *in vitro*, suggesting that $CD10^{med}CD45RA^{med/lo}$ defines a subset with a more primitive phenotype than $CD10^{hi}CD45RA^{hi}$.

During human hematopoiesis, the acquisition of the CD45RA delineates a pivotal developmental transition (Fritsch *et al.*, 1993). Along these lines, using single-cell sequencing, Velten and colleagues proposed that the expression of the surface marker CD45RA correlates with a transcriptional priming toward the lymphoid/myeloid development, as predicted by the STEMNET algorithm (Velten *et al.*, 2017). In contrast, publications have reported that common myeloid progenitors (CMPs) are lacking the surface marker CD45RA (Manz *et al.*, 2002; Chao *et al.*, 2008). In addition, prior studies reported that $CD34^+CD10^-$ cells display lymphoid-myeloid potential and that the myeloid potential is progressively lost as the cells acquire the expression of CD10, thereby highlighting the presence of distinct lymphoid progenitors in the heterogeneous $CD34^+$ CB population (Galy *et al.*, 1995; Berardi *et al.*, 1997; Ichii *et al.*, 2010). Overall, these publications support the idea that $CD10^{med}CD45RA^{med/lo}$ defines a subset of progenitors with multilineage potential whereas the $CD10^{hi}CD45RA^{hi}$ subset potentially contains lymphoid-restricted progenitors.

Intriguingly Karamitros and colleagues also denoted the presence of two LMPP populations differing based on the expression of CD10 and CD45RA by flow cytometry,

and analyzed their lineage potential using clonal *in vitro* assays (Karamitros *et al.*, 2018). The LMPP subset with a high CD10 and CD45RA (hi) expression lacked myeloid lineage potential whereas the LMPP subset with an intermediate CD10 and CD45RA (med) expression showed lymphoid-myeloid potential. As a result, they proposed that the $CD10^{hi}CD45RA^{hi}$ cells are LMPP transitioning into MLP (*i.e.* intermediate precursors; Karamitros *et al.*, 2018), insights that could be translated to the two CD10-positive subsets that we identified.

During thymopoïesis, BM-derived precursors (including LMPPs and CLPs; Saran *et al.*, 2010) settle the thymus where they enter the early T-lineage progenitor (ETP) pool (Allman *et al.*, 2003). Subsequently, ETP differentiates into the T-cell lineage in response to Notch signalling and cytokines from the thymic stroma (Petrie *et al.*, 2007). However, a cascade of various receptor-ligand interactions is crucial to regulate the entry of BM-derived precursors into the thymus (Scimone *et al.*, 2006). CCR7 has been suggested to mediate thymus seeding by BM-derived progenitors (Ueno *et al.*, 2002; Ueno *et al.*, 2004). Moreover, impaired CCR7 function has been associated with a defective negative selection due to the modifications of the thymic architecture in mice (Misslitz *et al.*, 2004; Davalos-Misslitz *et al.*, 2007; Krueger *et al.*, 2010). Interestingly, CCR7 was expressed in our $CD10^{hi}CD45RA^{hi}$ subset (data not shown) and in the UM171 DN-sorted graft (Figure 2**F**; Figure S**1**), suggesting that this subset has thymic settling potential, as it is expected for CLPs.

3.2.3 CD10-positive subset displays faster T-cell differentiation and maturation than Total CD34+ subset.

More then a decade ago, Haddad and colleagues inferred that the $CD34^{+}CD45RA^{hi}CD7^{+}$ HPCs lymphoid potential was skewed toward the T/NK lineages and that the $CD34^{+}CD45RA^{hi}Lin^{-}CD10^{+}$ HPC dominantly displayed a B-cell potential when co-cultured with OP9 cells (Haddad *et al.*, 2004). In contrast, using newborn NOD-scid/IL2rgKO transplant assays, Yoshida and colleagues challenged these results by demonstrating that $CD10^{+}CD34^{+}CD38^{-}CD7^{-}$ cells are able to repopulate the recipient thymus in addition to producing $CD19^{+}$ B cells and $HLA\text{-}DR^{+}CD33^{+}$ (myeloid cells) in peripheral blood, BM and the spleen of the murine recipients, thereby suggesting that $CD10^{+}$ progenitors have

more than a unilineage potential (Yoshida *et al.*, 2006). Six *et al.* helped clarify the contentious surrounding CD10 by splitting the $CD34^+CD10^+$ population into two progenitor subsets differing in the expression of CD24, where the $CD10^+CD24^+$ population contains the B cell-committed precursors described by Haddad and colleagues (Haddad *et al.*, 2004; Six *et al.*, 2007). Additionally, they shown that the $CD34^+CD10^+$ population of immature thymocytes had B/T and NK potential, and also displayed myeloid potential, yet greatly reduced (Six *et al.*, 2007).

As such, to determine the lymphoid potential of our CD10-positive population, we used the ATO approach to perform a T-cell differentiation kinetic over 6 weeks. We observed a faster T-cell differentiation and maturation for the CD10-positive subsets as compared to the Total $CD34^+$ subset at 3 and 6 weeks of co-culture in ATO (Figure 7**B**). Furthermore, when comparing all subsets, the greater T-cell differentiation and maturation was observed for the $CD45RA^+$ subset whereas the $CD45RA^-$ subset had the lowest T-cell differentiation and maturation. Alhaj Hussen and colleagues denoted distinct lymphoid differentiation pathways all stemming from a common $CD34^{hi}CD45RA^+$ HPCs (Alhaj Hussen *et al.*, 2017), which could explain the faster T-cell differentiation kinetic that we observed for the $CD45RA^+$ subset. Furthermore, we reported that UM171 expands HSPCs *in vitro* that are $CD34^+CD45RA^-$, which can account for the slower T-cell differentiation kinetic of the $CD45RA^-$ subset (Fares *et al.*, 2014).

Intriguingly, at 6 weeks, we denoted the presence of a DN and a SP8 population in the uncultured CD10-positive subset that are lacking in the UM171-treated CD10-positive subset (otherwise, both CD10-positive subsets were similar). This variation can potentially indicate a greater heterogeneity of immature and mature lymphoid precursors in the uncultured CD10-positive subset (vs UM171 CD10-positive subset), thus suggesting that CD10 more efficiently segregates immature from mature lymphoid precursors within $CD34^+$ cells treated with UM171. The kinetic of T cell differentiation and maturation that we reported for the CD10-positive subset matches what has been observed previously by Six and colleagues who denoted the presence of $CD4^+CD8^+$ DP after 3 weeks of culture of $CD10^+$ progenitors from CB (Six *et al.*, 2007). Along these lines, we propose that the $CD45RA^-$ subset contains primitive cells (*e.g.* HSCs) that segregates into lineage-biased

multipotent progenitors enriched in the CD10-positive subset (*e.g.* LMPP, $CD10^{med}CD45RA^{med}$). As cell lineage specification and commitment progress, these lineage-biased multipotent progenitors will give rise to lineage-restricted precursors that are $CD45RA^{+}CD34^{+}$ and potentially $CD10^{hi}CD45RA^{hi}$ (*e.g.* CLP).

3.2.4 CD10-positive subset displays greater T potential and produce higher percentage of ILC-like cells than Total CD34+ subset.

Using single cell sequencing approaches, Lavaert and colleagues recently identified two distinct thymus seeding progenitor (TSP) subsets differing in the expression of several markers including CD10 and CD7 (Lavaert *et al.*, 2020). They also reported that the expression of the chemokine receptors CCR7 and CCR9 peaks in the $CD10^{+}$ TSPs. They inferred that early $CD10^{+}$ TSPs drastically loose *MME* (CD10) expression as they differentiate into $CD7^{+}$ ETP, a transition induced by strong Notch signalling. In addition, this transition is marked by an increase in the expression of immature thymocyte markers including CD7, CD34 CD62L and IL7R (Lavaert *et al.*, 2020).

On the other hand, Alhaj Hussen and colleagues denoted the presence of two distinct lymphoid-restricted subsets along which lymphoid cells differentiate; these subsets differ in IL7R (CD127) expression and T cell potential (refer to as $CD127^{-}$ and $CD127^{+}$ early lymphoid progenitors (ELPs); Alhaj Hussen *et al.*, 2017). They observed a marginal T-cell potential for the $CD127^{+}$ ELPs whereas both ELP subsets have NK and ILC potential. In agreement with the literature, both ELPs subsets are found within the $CD45RA^{+}$ ($CD115^{-}$ $CD116^{-}CD123^{-}$) compartment originating from a multipotent precursor. Lavaert *et al* proposed that the $CD7^{+}$ TSP2 subset could originate from the $CD127^{-}$ ELP population (Lavaert *et al.*, 2020). When we investigated the ILC potential, we denoted a higher percentage of cells displaying an ILC phenotype ($CD127^{+}CD7^{+}Lin^{-}$) in our CD10-positive subset (vs Total $CD34^{+}$ and $CD45RA^{+}$ subsets). This observation suggests that our CD10-positive population has T potential and could potentially produce ILCs, thereby could originates from the $CD127^{-}$ ELPs described by Alhaj Hussen *et al*. However, this contradicts the findings of Lavaert and colleagues since they could not detect ILC potential in the $CD10^{+}$ TSP subset, to which our CD10-positive subset is the most similar (in the expression of the surface markers CD10, CCR7 and CD34). This discrepancy could be

explained either by: 1) our identification of a novel $CD10^+$ TSP subset displaying T potential and that can produce ILC-like cells originating from a $CD127^-$ ELP or, 2) the lack of additional surface markers and functional assays that mitigate our interpretation.

Furthermore, ITGB7 has been highlighted in the literature for its role in lymphocyte homing and localization in the gut (Berlin *et al.*, 1993; Cepek *et al.*, 1994; Wagner *et al.*, 1996; Kunkel *et al.*, 2000 and Salmi *et al.*, 2005). We also denoted the presence of $ITGB7^+ILR7^+Lin^-$ expressing cells which could be common helper-like innate lymphoid cell progenitors (CHILPs) described previously as $ITGB7^+IL7R^+Lin^-$ (Klose *et al.*, 2014). A higher percentage of CHILP-like cells was observed within the most primitive subset ($CD45RA^-$) whereas this potential population of CHILPs was depleted within the CD10-positive subset, displaying the higher percentage of ILC-like cells. Furthermore, we reported a smaller percentage of ILC-like cells and a higher percentage of potential $ITGB7^+IL7R^+$ CHILPs within the $CD45RA^+$ subset in contrast to the CD10-positive subset. The absence of CHILP-like cells within the CD10-positive subset could indicate that ILC-like cells were not produced by CHILPs within the CD10-positive subset. Interestingly, Ghaedi and colleagues identified $IL7R^+$ LMPPs that could give rise to ILCs (ILC2) via CLP-independent pathways in transplantation assays (Ghaedi *et al.*, 2016). Accordingly, we propose that the CD10-positive subset either (1) contains LMPP that can give rise to ILCs and T cells or (2) contains ILC-producing LMPPs (via CLP-independent pathways) and T-cell producing CLPs (LMPPs that are $CD10^{med}$ and CLPs that are $CD10^{hi}$). In contrast, we propose that the $CD45RA^+$ subset might contain CLPs that need to commit to CHILPs to generate ILCs thus, resulting in a slower production of ILC-like cells.

3.3 Conclusion

In sum, our findings represent the first identification of a CD10-positive population within the UM171-expanded graft that has T potential and could potentially produce ILCs. This population is significantly expanded in $CD34^+$ CB cells treated with UM171 (vs uncultured or DMSO-supplemented $CD34^+$ CB cells). We proposed that this CD10, CCR7-expressing population might be enriched in LMPPs and CLPs, that can further undergo Notch-dependent differentiation into lymphoid-restricted $CD7^+$ CLPs, identified previously by Dumont-Lagacé *et al*. (2021).

The addition of other surface markers will be required to differentiate myelo-lymphoid multipotential progenitors from lymphoid precursors within our highly heterogeneous CD10-posivite population. Moreover, transplantation assays are essential to decipher the actual lineage potential of our CD10-positive population since it is unclear how our *in vitro* ATO assay relates to *in vivo* differentiation. Additional functional assays are also required to determine whether the T and ILC cells identified phenotypically retain their function.

Delayed immune reconstitution is a major obstacle impeding the implementation of partially HLA-mismatched HSCT by increasing the rate of relapse in patients and enhancing their susceptibility to pathogens. Therefore, optimizing the $CD3^+$ donor infusion by enriching for lymphoid progenitors could help improve immune reconstitution in patients transplanted with UM171-expanded CB graft. This study represents the first step to disentangle the complex molecular mechanisms underlying T-cell reconstitution in UM171-expanded CB graft recipients and could help provide insights and direction for future strategies aiming to boost T-cell reconstitution following allogeneic HSCT.

3.4 Author Contributions

Chagraoui J. designed, performed and processed the data of the Cite-seq experiments. Lehnertz B. help processed the Cite-Seq data. The author analyzed the processed Cite-Seq data. The author designed and troubleshoot antibody panels to assess the different lymphoid markers identified previously in the UM171-DN sorted graft. The author performed all experiments assessing the expansion of CD10-positive population between UM171, DMSO and fresh cultures. Chagraoui J. designed the ATO experiment, thawed and expanded cord blood cells, performed flow cytometry sort and generated the organoids, the experiment was brought to completion by the author. The author designed, troubleshoot and validated all antibody panels generated for the analysis of the ATOs at 3 and 6 weeks and performed all analyses. Boivin I., Durand A., Garcias D. and the author performed $CD34^{+}$ cord blood cell purification. G.A. Gosselin and A. Bellemare-Pelletier provided technical support with flow cytometry sorts. The author generated all the figures (except Figure 1.3 and S1) and wrote the memoire. Bordeleau M. E. and Chagraoui J. provided critical reviewing and suggestions for the memoire. Sauvageau G. and Chagraoui J. provided project coordination and helped design all the experiments.

Chapter 4 - Material & Methods

Isolation of human CD34$^+$ HSPCs

The study was conducted in accordance with the Research Ethics Boards of Université de Montréal and Charles LeMoyne Hospital (Greenfield Park, QC, Canada) approval. Umbilical cord blood units were collected from consenting mothers at the Charles LeMoyne Hospital (Greenfield Park, QC, Canada). Umbilical cord blood units were cryopreserved for a maximum of 4 years before use. The EasySep™ positive selection kit (StemCell Technologies Cat # 18056) was used to isolate Human CD34$^+$ cord blood cells.

CD34$^+$ Primary Cell culture

Human CD34$^+$ cord blood cells were cultured in HSC expansion media composed of StemSpan SFEM (StemCell Technologies) supplemented with 100 ng/ml stem cell factor (SCF, R&D Systems), 100 ng/ml FMS-like trysine kinase 3 ligand (FLT3, R&D Systems), 50 ng/ml thrombopoietin (TPO, R&D Systems), 100 ng/ml interleukin 6 (IL-6, R&D Systems) and 10 mg/ml low-density lipoproteins (StemCell Technologies). Only the umbilical cord blood units that had a CD34+ purity and cell viability above 90% and 95% respectively were used in the experiments and randomly assigned to the conditions (fresh, DMSO-supplemented or UM171 treatment).

Flow Cytometry and Antibodies

For the identification of surface markers to isolate lymphoid precursors, mouse anti-human antibodies were employed to detect CD34 (APC, BD Biosciences), CD45RA (PE, BD Biosciences) and CD10 (BV786, BD Biosciences). A Canto II cytometer and BD LSRFortessa cytometer (BD Biosciences) were used for flow cytometry acquisition. Flow cytometry acquisition were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Cell count were analyzed using GraphPad Prism software and the figures were edited using Adobe Illustrator 2021.

To analyze cells obtained in artificial thymic organic (ATO) co-culture, mouse anti-human antibodies were used to detect hCD45 (Pacific Blue, BioLegend), CD33 (APC, BD Biosciences) CD8a (PE-Cy7, BioLegend), CD4 (Pacific Blue, BioLegend) CD3 (APC-Cy7, BD Biosciences), TCR αβ (PE, BioLegend), IL7R (PE, BD Biosciences), ITGB7 (BV711,

BD Biosciences) and CD7 (APC, BD Biosciences). The lineage negative (Lin-) mouse anti-human antibodies included CD33 (PE-Cy7, BioLegend), CD3 (PE-Cy7, BD Biosciences), CD8a (PE-Cy7, BioLegend), CD4 (PE-Cy7, BD Biosciences), CD14 (PE-Cy7, BD Biosciences) and CD56 (PE-Cy7, BD Biosciences). All flow cytometry acquisitions were obtained using BD LSRFortessa cytometer (BD Biosciences) and analyses performed using FlowJo software (Tree Star, Ashland, OR, USA) and presented using Adobe Illustrator 2021.

Artificial thymic organoid (ATO) cultures

MS5-hDLL4 cells were harvested at ≈70% confluence using trypsinization and resuspended in ATO culture medium. Fresh serum free ATO culture medium was made every week and consisted of RPMI 1640 (Corning), 4% B27 supplement (ThermoFisher Scientific), 1% penicillin/streptomycin (ThermoFisher Scientific), 1% Glutamax (ThermoFisher Scientific), PBS-reconstituted 30 µM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich), 5 ng/ml rhFLT3L and 5 ng/ml rhIL-7 (R&D Systems). MS5-hDLL4 cells were combined with the different subsets of $CD34^+$ cord blood cells ($CD45RA^-$, $CD45RA^+$ and $CD10^+$) in a 20:1 ratio (MS5-hDLL4 : $CD34^+$ CB cells) per ATO. 5000 sorted $CD34^+$ CB cells were pipetted per ATO, and 3 ATO were made per conditions. MS5-hDLL4 and $CD34^+$ CB cells were combined in a 1.5 ml Eppendorf tube and centrifuged at 300 g for 5 min (4°C, using a centrifuge equipped with swinging buckets), supernatants were discarded cautiously and brief vortexing was used to resuspend the cell pellet. We then place a 0.25 µm Millicell transwell insert (EMD Millipore) in a 12-well plate filled with 0.200 µL of ATO medium, representative of a single ATO. Five µL of Centrifuged MS5-hDLL4/$CD34^+$ CB cells was carefully pipetted and deposited centered onto the cell insert to form a drop. ATO medium was halve changed every 2-3 days and replaced with fresh medium during 6 weeks. At 3 and 6 weeks, ATO cells were harvested using a FACS buffer composed of PBS, 0.5% bovine serum albumin and 2mM EDTA (ThermoFisher). The FACS buffer was pipetted directly onto the ATO using a P1000 pipet and exerted mechanical force was used to dissociate the cells from the ATO followed by their passage through a 50 µm nylon strainer. This protocol is described by Seet *et al* (2017).

RNA-sequencing

$CD34^+$ cord blood cells cultured in the presence of UM171 during 7 days were preserved at -80°C in TRIzol Reagent (Thermo Fisher Scientific Cat # 15596026). In accordance with TrueSeq Protocols (Illumina) cDNA libraries were constructed. Sequencing was done using an Illumina HiSeq 2000 instrument. The Casava pipeline (Illumina) and Refseq release 63 were used for mapping and gene expression quantification as described previously (Fares *et al.*, 2014).

Cite-Seq

Experiment was performed by colleagues (Chagraoui J. *et al*, 2021; unpublished). In brief, this experiment included 58 different ADT antibodies to mark for all major lineages (HSPCs, lymphoid and myeloid lineages). Furthermore, four hashtags (HTO) were used to tag and distinguish the 4 conditions: 1) fresh $CD34^+$ cord blood cells (uncultured; day 0), 2) DMSO-supplemented $CD34^+$ CB cells (day 7), 3) UM171-treated $CD34^+$ CB cells (day 7) and, 4) UM171 DN-sorted $CD34^+$ CB cells (day 7). The UM171 DN-sorted condition was obtained by culturing fresh $CD34^+$ CB cells in the presence of UM171 during 7 days, and by sorting the expanded cells using flow cytometry to obtain a $CD86^-$, $FCER1A^-$ double negative (DN) population. The UM171 DN-sorted is depleted in mast ($FCER1A^+$) and dendritic ($CD86^+$) cells, thereby enriched in $CD34^+$ progenitors. For each conditions 8000 cells were sequenced using 10X Genomics single cell sequencing. This experiment was performed in accordance with the protocol described by Buus and colleagues (Buus *et al.*, 2021). Final Seurat objects were analyzed using R (version 4.0) and the Seurat scripts were generated by the Satija lab (Hao *et al.*, 2021).

Statistical analysis

Statistical analysis were performed using GraphPad Prism software using the Mann-Whitney test to compare the fold change between UM171 cultures and each of the two control (DMSO and Fresh), separately. Proliferation of cord blood cells (lymphoid cells, HSCs, etc.) do not follow a normal distribution and therefore requires the use of a non-parametric test such as the Mann-Whitney test. Error bars represents medians. Significant p values are < 0.05.

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Appendix

Supplemental Figure

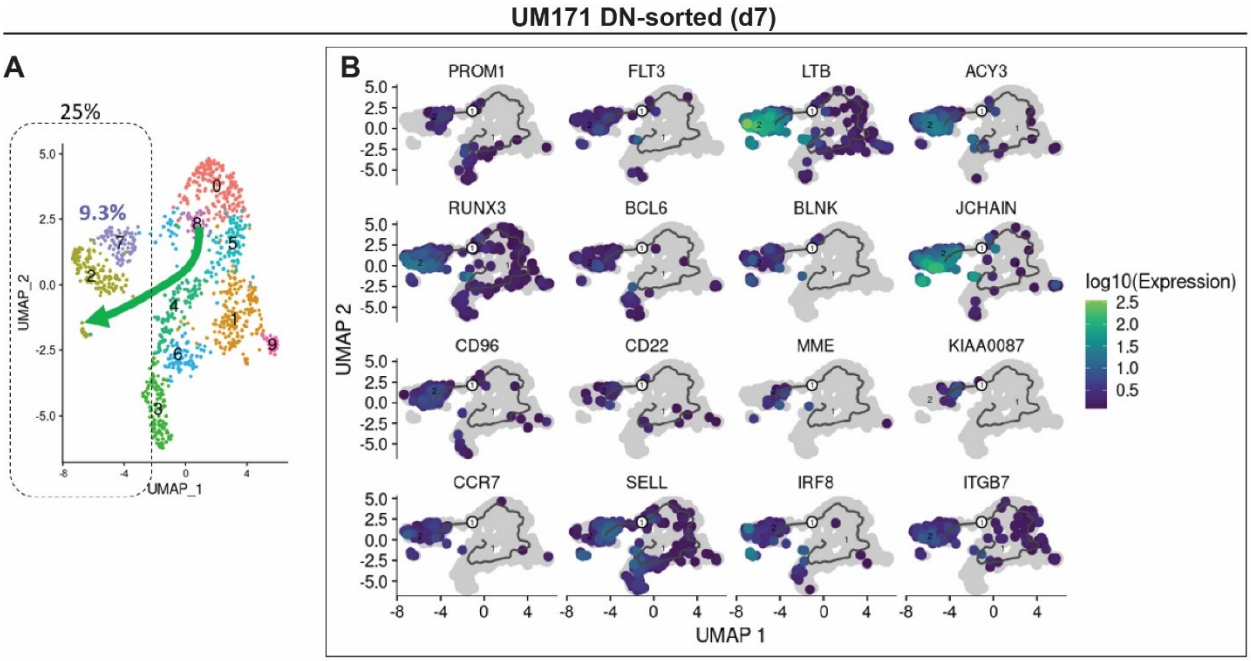

Figure S1. *MME* is expressed in the lymphoid cluster 7 of the UM171 DN-sorted graft.
Figure is a courtesy of Chagraoui J. Not published.
(A) Representative UMAP showing the clusters distribution of CD34+ cord blood cells cultured in the presence of UM171 (35nM) during 7 days. The total UM171 was sorted into a CD86- FCER1A- subset to deplete dendritic and mast cells. The DN subset represents 15% of the total UM171 graft. Cluster 2 and 7 have a lymphoid identity and represents 25% of the UM171 DN-sorted subset and 3.75% of the total UM171 graft.
(B) Representative UMAP showing the RNA level of lymphoid genes expressed in lymphoid clusters shown in a.