In-vitro Generation of potent T-lymphoid Progenitors in a feeder-cell-free DL-4 system

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ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
Ahr	Aryl hydrocarbon receptor
Bcl11b	B-cell lymphoma/leukemia protein
bHLH	Basic helix-loop-helix protein
BM	Bone marrow
BMI1	Polycomb ring finger oncogene BMI1
CB	Cord blood
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic acid
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CTLs	Cytotoxic T lymphocytes
CTP	T-lineage committed progenitors
CXCL12	C-X-C Chemokine ligand
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DL	Delta-like ligand
DN	Double negative cell
DNA	Deoxyribonucleic acid
DP	Double positive cell
E2A	E2A helix-loop-helix transcription factors
EBF	Early B-cell factor
EBV	Ebstein Barr Virus
EGF	Epidermal growth factor
ELP	Early lymphoid progenitors
ETP	Early thymic progenitor
FACS	Fluoresence activated cell sorting
FGF-7	Fibroblast growth factor 7
Flt3	Fms-related tyrosine kinase 3
FTOC	Fetal thymic organ culture
GATA3	GATA transcription factor 3
GFI1	Growth factor independent 1 transcription repressor
GH	Growth hormone
GSK	Glycogen synthase kinase
GvHD	Graft-versus-host disease
GvL	Graft-versus-leucemia
HES1	Hairy and enhancer of split
HIV	Human immunedeficiency virus
HLA	Human leukocyte antigen (HLA)
HOXB4	Homeobox protein Hox-B4
HPE	Homeostatic peripheral expansion

HSCs	Haematopoietic stem cells
HSCT	Haematopoietic stem cell transplantation
HSZT	Hämatopoetische Stammzelltransplantation
ICN	Intracellular domain of Notch
Id2	Inhibitor of DNA binding
IGFBP1	Insulin-like growth factor-binding protein 1
IGF-I	Insulin-like growth factor-I
Ikaros	Ikaros family zinc finger protein 1
IL	Interleukin
IL2Rγ	Interleukin 2 receptor gamma
IL7Rα	Interleukin 7 recetpor alpha
ILC	Innate lymphoid cells
ISP	Immature single positive cell
JAG	Serrate like ligands Jagged
LCK	Lymphocyte-specific protein tyrosine kinase
Lck	Lymphocyte-specific protein tyrosine kinase
LDA	Limiting dilution analysis
LEF	Lymphoid enhancer-binding factor
LHRH	Luteinizing hormone-releasing hormone gene
Lin-	Lineage negative
LMPP	Lymphoid-primed multipotent progenitor
lo	Low
LRF	Zinc finger and BTB domain containing 7A
LSK	$Lin^{-}Sca1^{+}c\text{-}kit^{+}$
LT-HSC	Longterm repopulating haematopoietic stem cell
mAbs	Monoclonal antibodies
MAML	Mastermind coactivator
MAPK	Mitogen activated protein kinase
MCSF	Messenger ribonucleic acid
MCSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
miRNA	MicroRNA
MLP	Multipotent early lymphoid progenitors
MLR	Mixed lymphocyte reactions
MPP	Multipotent progenitor
MS5	Murine stromal cell MS5
mTECs	Medullary thymic epithelial cells
Myb	Myeloblastosis viral oncogene homolog
NK	Natural killer
NOD/SCID/$\gamma c^{-/-}$	Non obese diabetic/SCID/common gamma chain null mouse
NSG	Non-obese-diabetic/SCID/common-gamma-chain-null mouse
Pax5	Paired box protein 5
PBMC	Peripheral blood mononuclear cell
PI3K	Phosphoinositide 3-kinase
preTCR	Pre-T cell receptor
proT	Prothymocyte

PSGL-1	P-selectin glycoprotein ligand-1
pTa	Pre-T cell receptor alpha
PU.1	Transcription factor PU.1
Rag	Recombination activation gene
RBP	Recombining binding protein suppressor of hairless
RORgamma	RAR-related orphan receptor gamma
RTE	Recent thymic emmigrant
RTOC	Rreaggregate thymus organ cultures
Runx1/CBFβ	Runt-related transcription factor 1/ Mammalian core binding factor beta complex
S17	Murine stromal cell line S17
SCF	Stem cell factor
SCID	Severe combined immune deficiency
SDF	Stromal derived factor
SOX17	SRY-related HMG-box protein 17
SP	Spleen focus forming virus proviral integration oncogene
SR1	Stemregenin 1
TACE	Tumor necrosis factor alpha converting Enzyme
TAT	HIV transactivating protein
TCF	T-cell-specific transcription factor
TCR	T cell receptor
TEC	Thymic epithelial cells
TF	Transcription factor
TPO	Thrombopoietin
TREC	T-cell receptor excision circles
TSLP	Thymic stromal lymphopoietin
TSP	Thymus seeding progenitor
TSt-4	Thymic stromal cell line TSt-4
ZAP	Zeta-chain-associated protein kinase 70

SUMMARY

Human leukocyte antigen (HLA)-mismatched haematopoietic stem cell transplantation (HSCT) represents an important therapeutic option for patients lacking suitable donors. Delayed posttransplant immune recovery constitutes one of its major complications and is most pronounced in the T cellular compartment. A novel strategy to promote *de novo* thymopoiesis from donor derived HSCs and to accelerate T cellular reconstitution in patients after HSCT consists in the adoptive transfer of *in vitro* generated T cell progenitor cells. Identification of Notch1 as the key regulator of early T-lineage development has allowed the generation of Notch ligand-based culture systems, which provide a powerful tool to generate T-lymphoid progenitors *in vitro*. The efficacy of murine T-lymphoid progenitors to promote T cell reconstitution has been well demonstrated in conventional mouse models. In consistency, *in vitro*-generated human T cell progenitors were demonstrated to promote thymic recovery in humanized mice. Yet, positive effects of *in vitro* generated human T cell precursors on peripheral T cell reconstitution have not been demonstrated. Moreover currently used Notch-based co-culture systems consist of genetically modified murine cell lines. With view to establishing a clinically applicable system, feeder-cell-free Notch-ligand culture systems for the generation of T-lymphopoietic progenitors are warranted.

During my PhD project I developed a new culture system based on the immobilized Notch ligand Delta-like-4 (DL-4).

Exposure of human $CD34^+$ cord blood cells to immobilized DL-4 enabled the *in vitro* generation of high number of T cell progenitors, which harboured the phenotype of immature early thymic progenitor cells (ETP) and prothymocytes (proT). ETP and proT cell generated during DL-4 culture upregulated essential genes involved in early T-lymphoid development (i.e. IL7Rα, PTα, RAG1 and BCL11b) and had undergone stage-specific recombination of the T cell receptor (TCR) locus in a similar way as in native human thymopoiesis. In limiting dilution analysis after secondary OP9/DL-1 co-culture, DL-4 progenitors displayed a highly increased T-lymphoid potential, which could be entirely attributed to the ETP and proT subset.

When transferred into NOD/SCID/$\gamma c^{-/-}$ mice, DL-4 primed T cell progenitors migrated to the thymus and accelerated intrathymic T cell differentiation and emergence of functional, mature and polyclonal αβ T cells in the periphery. In a co-transplantation approach, which more closely mimics a clinical setting, DL-4 progenitors and untreated $CD34^+$ cells from HLA-disparate donors

were simultaneously injected in the same recipient. This procedure allowed even more rapid and more robust T cell reconstitution. HLA-tracking of the distinct graft sources further showed, that DL-4 progenitors specifically reconstituted the T-lymphoid compartments.

This work provides further evidence for the ability of *in vitro*-generated human T cell progenitors to promote *de novo* thymopoiesis and shows for the first time, that these cells accelerate peripheral T cell reconstitution in humanized mice. The availability of the efficient feeder-cell-free DL-4 culture technique represents an important step towards the future clinical exploitation translation of *in vitro* generated T-lymphoid progenitor cells to improve posttransplant immune reconstitution.

Key words: T cell development, T-lymphoid progenitor cells, Notch1, DL-4 protein, immunereconstitution after HSCT

RESUMÉ

L'allogreffe des cellules souches hématopoïétiques (CSH) dans les situations d'incompatibilité HLA partielle représente une option thérapeutique irremplaçable pour des patients nécessitant une greffe de cellules souches hématopoïétiques, en absence d'un donneur HLA-identique. Toutefois, le retard de la restauration du système immunitaire en particulier dans du compartiment lymphocytaire après greffe est l'une des complications majeures. Une nouvelle stratégie pour promouvoir la reprise de la thymopoïèse à partir des CSH provenant du donneur et d'accélérer la reconstitution cellulaire T chez des patients après greffe de CSH consiste en le transfert adoptif des progéniteurs T générés *in vitro*. L'identification de Notch1 comme le régulateur-clé du développement lymphocytaire T a permis l'établissement de systèmes de culture à base de ligands de Notch, qui permettent la génération efficace de progéniteurs lymphoïdes T *in vitro*. L'efficacité des progeniteurs T-lymphoïdes murins pour promouvoir la reconstitution des lymphocytes T a été bien démontrée dans des modèles de greffe chez la souris. De même, des progéniteurs T-lymphopoïétiques humains générés *in vitro* et greffés aux souris humanisées favorisent la reprise de la thymopoïèse. Pourtant, aucune donnée n'a encore démontré leur capacité à donner naissance à un compartiment lymphocytaire T périphérique. De plus, les systèmes de co-culture à base de ligand de Notch actuellement utilisés consistent en des lignées stromales murines génétiquement modifiées. Afin d'établir un système cliniquement applicable, il est donc indispensable d'établir des systèmes de culture qui soutiennent la génération de progéniteurs T en absence d'un support des cellules nourricières.

Au cours de mon projet de thèse, j'ai développé un nouveau système de culture pour la génération des progéniteurs T-lymphopoïétiques humains T basé sur l'immobilisation du ligand de Notch Delta-like-4 (DL-4) sous sa forme protéique.

La culture des progéniteurs hématopoïétiques $CD34^+$ issue de sang en présence de DL-4 immobilisé permet la génération d'un grand nombre de cellules ayant un phénotype de progéniteurs thymiques précoces (early thymic progenitor: ETP) et de prothymocytes (proT). Les cellules ETP et ProT ainsi générées expriment à des niveaux élevés des gènes impliqués dans le développement lymphocytaire précoce (i.e. pTa, Rag1, IL7Ra et BCL11b). Elles montrent des signes de réarrangement du récepteur des cellules T (TCR) similaires à leurs homologues thymiques. Par des expériences de dilution limite sur une co-culture OP9/DL-1 secondaire, j'ai pu montrer que les progéniteurs générés sur DL-4 possédaient un potentiel lymphoïde T très augmenté, qui pourrait être entièrement attribué aux sous populations ETP et ProT.

Suite à leur transfert dans des souris NOD/SCID/$\gamma c^{-/-}$, les progéniteurs lymphoïde T générés par exposition a DL-4 sont capable de migrer dans le thymus, d'y poursuivre des étapes ultérieures de leur développement et d'accélérer la différentiation T intra thymique ainsi que l'émergence des lymphocytes T mature, polyclonaux et fonctionnels en périphérie. Dans une approche de co-transplantation, qui se rapproche des conditions cliniques envisagées, j'ai simultanément injecté dans le même récipient des progéniteurs générées sur DL-4 et des cellules $CD34^+$ non traitées (d'un 2ème donneur HLA-incompatible). Cette procédure a permis une reconstitution des lymphocytes T encore plus rapide et plus. Etant donné que les progéniteurs T générées sur DL-4 et les cellules $CD34^+$ non-traitées étaient issue de deux donneurs avec un HLA différent, cette expérience a permis de montrer que les progéniteurs préalablement exposés à DL-4 reconstituaient spécifiquement les compartiments lymphoïdes T alors que les autres lignées hématopoïétiques provenaient des progéniteurs $CD34^+$ non-traités.

Le travail accompli pendant mon projet de thèse renforce l'hypothèse selon laquelle les progéniteurs T générés *in vitro* promeuvent la thymopoïèse humaine dans des souris humanisées. Alors que les publications antérieures ne faisaient état que d'une thymopoïèse active, nous avons démontré pour la première fois que ces progéniteurs accélèrent également la reconstitution des cellules T périphériques chez des souris humanisées. La mise en place du système de culture cellulaire DL-4, qui soutient la génération des progéniteurs T en absence d'un support de cellules nourricières représente une étape majeure pour une future application clinique des progéniteurs lymphoïde T comme un nouveau traitement pour améliorer la reconstitution immunitaire après greffe de CSH.

Mots clés: développent des lymphocytes T, progéniteurs lymphoïdes T, Notch1, ligand de Notch DL-4 , reconstitution immunitaire après greffe des CSH

ZUSAMMENFASSUNG

Die Wiederherstellung der T-lymphozytären Immunität nach T-Zell depletierter hämatopoetischer Stammzelltransplantation (HSZT) ist ein langwieriger Prozess. Eine potentielle Strategie zur Beschleunigung der Neubildung von T-Zellen aus den transplantierten Stammzellen besteht in der Gabe von T-lymphozytären Vorläuferzellen. Die Entdeckung von Notch1 als wichtigster Regulator der frühen T-Zell-Entwicklung hat zur Etablierung Notchligand-basierter Zellkulturen geführt, mit deren Hilfe T-lymphoide Vorläuferzellen aus hämatopoetischen Stammzellen *in vitro* gebildet werden können. Das therapeutische Potential dieses Zelltyps wurde eindrucksvoll in konventionellen, syngenen und allogenen Maustransplantationsmodellen belegt, in denen nach Injektion *in vitro* generierter, muriner T-Vorläuferzellen eine Verbesserung der Neubesiedlung des Thymus sowie eine beschleunigte Wiederherstellung der T-zellulären Immunität erreicht werden konnte. Notchbasierte Co-Kultursysteme wurden ebenfalls für die *in-vitro* Herstellung humaner T-lymphoider Vorläuferzellen verwendet. Das *in-vivo* Potential humaner T Vorläuferzellen ist bislang jedoch nur lückenhaft charakterisiert: Zwar konnte gezeigt werden, dass humane T-Vorläuferzellen den hypoplastischen Thymus von immundefizienten NOD/SCID/$\gamma c^{-/-}$ Mäusen besiedeln können. Ihre Wirksamkeit, die Wiederherstellung eines funktionellen, peripheren T-Zellkompartiments zu beschleunigen, gelang bislang jedoch nicht. Darüber hinaus werden Notchliganden in derzeit verwendeten Kultursystemen von genetisch modifizierten, murinen Stromazellen präsentiert. Die Entwicklung stromazellfreier, proteinbasierter Notchligand-Kultursysteme ist daher von grosser Bedeutung für eine mögliche therapeutische Nutzung *in vitro* generierter T-Vorläuferzellen.

Durch Immobilisierung von Notchligand Delta-like 4 (DL-4) habe ich im Rahmen meines PhD Projekts ein stromazellfreies Kultursystem zur Züchtung T-zellulärer Vorläuferzellen aus humanen CD34+ Nabelschnurblutzellen etabliert. In DL-4 Kultur generierte Zellen besitzen phänotypische und molekulare Eigenschaften von frühen thymischen Vorläuferzellen (ETP) und Prothymocyten (proT). ETP und proT Zellen aus DL-4 Kulturen exprimieren wesentliche Gene der frühen T-Zellentstehung (z.B. *IL7Ra, PTa*, *RAG1* und *BCL11b*). Die entwicklungsstadium-spezifischen TCR-Rekombinationsprozesse in DL-4 Zellen erfolgen nach dem gleichen Muster wie in der nativen Thymusentstehung. Die in DL4 Kultur generierten T-Vorläuferzellen können sich in reife T-Zellen weiterentwickeln und durchlaufen die weitere T-Zelldifferenzierung innerhalb kürzerer Zeit als native $CD34^+$ hämatopoetische Vorläuferzellen.

Darüber hinaus können DL-4 generierte T-Vorläuferzellen nach Xenotransplantation den hypoplastischen Thymus von immundefizienten NOD/SCID/$\gamma c^{-/-}$ Mäusen besiedeln, intrathymische T-Zellentwicklung begünstigen und die Neubildung reifer und funktionaler T-Zellen in der Peripherie beschleunigen. Zur Simulation einer klinischen Anwendung führte ich weiterhin Co-Transplantationen mit DL-4 Vorläuferzellen und unbehandelten $CD34^+$ Zellen in gleiche Empfänger durch und konnte hiermit eine weitere Verbesserung der Immunrekonstitution erzielen. Durch Verwendung HLA-divergenter Spender in diesen Versuchen konnte ich zeigen, dass DL-4 Zellen sich vornehmlich in T-Zellen weiterentwickelten, während die restlichen Blutzellreihen von unbehandelten CD34-poitiven Zellen gebildet wurden.

Im Rahmen dieses Projekts konnte ich mit einem für die klinische Anwendung geeigneten Kulturmodell wichtige präklinische Belege für das therapeutische Potential *in vitro* generierter T-Vorläuferzellen erbringen. Diese Arbeit bildet somit eine wichtige Grundlage für eine zukünftige klinische Anwendung von T-Vorläuferzellen zur Verbesserung der T-Zell-Immunität nach HSZT.

Stichwörter: T-Zellentwicklung, T-Vorläuferzellen, Notch1, Notchligand DL-4, Immunrekonstitution nach HSZT

INTRODUCTION

1 T CELL RECONSTITUTION AFTER HSCT

Allogeneic haematopoietic stem cell transplantation (HSCT) offers an effective treatment for a broad spectrum of malignant and non-malignant disorders. In the early years of the era of bone marrow (BM) transplantation, HSCT was restricted to patients with human leukocyte antigen (HLA)-identical donors. Advances in graft handling and injection of highly purified $CD34^+$ selected grafts have enabled HSCT from partially HLA-mismatched donors to become a widely accepted therapeutic option for patients lacking an HLA-identical donor.

While these procedures allow rapid restoration of haematopoiesis, the transfer of a functional immune system is much harder to achieve. Prolonged posttransplant immune deficiency, which is most evident in the T cell compartment, is a major challenge clinicians have to deal with after HSCT. Opportunistic viral, bacterial and fungal infections are the leading cause of death in recipients of HLA-mismatched transplants most occurring within the first 100 days after HSCT. Moreover, delayed T cell recovery is associated with an increased risk of graft rejection and relapse (Parkman and Weinberg 1997; Ruggeri, Peffault de Latour et al. 2011).

After HSCT, T cell recovery occurs through two mechanisms: one thymus independent and one thymus-dependent.

Homeostatic peripheral expansion (HPE) of mature T cells transferred with the graft occurs within only 10-15 days and can partially correct numerical T cell defects. In the lymphopenic HSCT patients HPE is triggered by (i) high cytokine levels (results of reduced consumption during lymphopenia) and (ii) by interactions with antigen present in the host, which not only include pathogen but also mismatched MHC antigens of the host. The first mechanism explains, why T cells expanded in these condition have activated phenotypes, an inversed CD4/CD8 ratio and contain much less naïve T cells than normal individuals. High expansion via HPE, which continues until a particular threshold of T cell numbers is obtained, further goes along with telomere shortening and leads to higher apoptosis rates of expanded T-lymphocytes. Furthermore, the T cell repertoire generated during HPE is restricted by the limited TCR specificities contained in the graft. The absence of newly produced naïve T cells and the frequent interaction with pathogens in the host (that were not necessarily present in the donor), lead to the dominance of T cells with particular TCRs and to a gradual restriction of the

TCR repertoire. For all these reasons thymus-independent T cell recovery can indeed provide initial immune competence against (commonly shared) viral infections (e.g. EBV, CMV) but is not sufficient to sustain long term T cell recovery (for review see (Fry and Mackall 2005)).

The functional recovery of T cells with physiological properties and broad TCR-specificities relies on the thymus-dependent generation of naïve T cells. Since the immunological education of *de novo* generated T cells occurs in the recipients' thymus, emerging T cells are host-tolerant and provide a broad repertoire of naturally selected naïve T cells. *De novo* T cell development from donor haematopoietic progenitors requires several successive differentiation steps - first in the BM (to generate a lymphoid progenitor able to migrate to the thymus) and then in the thymus itself (where, after commitment to the T cell lineage, T cell precursors proceed through the complex process of thymopoiesis). Both steps are disturbed after HSCT. The generation of T cell progenitors in the BM and their delivery to the thymus is a limiting step in post-HSCT recovery (Zlotoff, Zhang et al. 2011). Furthermore, intrathymic T cell differentiation is altered for several (often interdependent) reasons: the thymic microenvironment can be damaged by the conditioning regimens, graft versus-host-disease, infectious disease or inflammatory status (Krenger, Blazar et al. 2011). Moreover age-related thymic involution contributes to a further delay of T cell reconstitution in adult patients (Parkman and Weinberg 1997; Small, Papadopoulos et al. 1999; Krenger, Blazar et al. 2011). In view of the above it becomes obvious that thymus-dependent T cell recovery is a long lasting process. The generation of thymus-derived naïve T cells requires 6-12 months in children and may take up to several years in adults, which reflects the age-dependent inefficiency of posttransplant thymic rebound (Weinberg, Annett et al. 1995; Parkman and Weinberg 1997; Ruggeri, Peffault de Latour et al. 2011). Complete restoration of the T cell compartment with a polyclonal T cell repertoire takes even longer and naïve T cells and central T memory cell counts may never reach pre-transplant levels (Komanduri, St John et al. 2007).

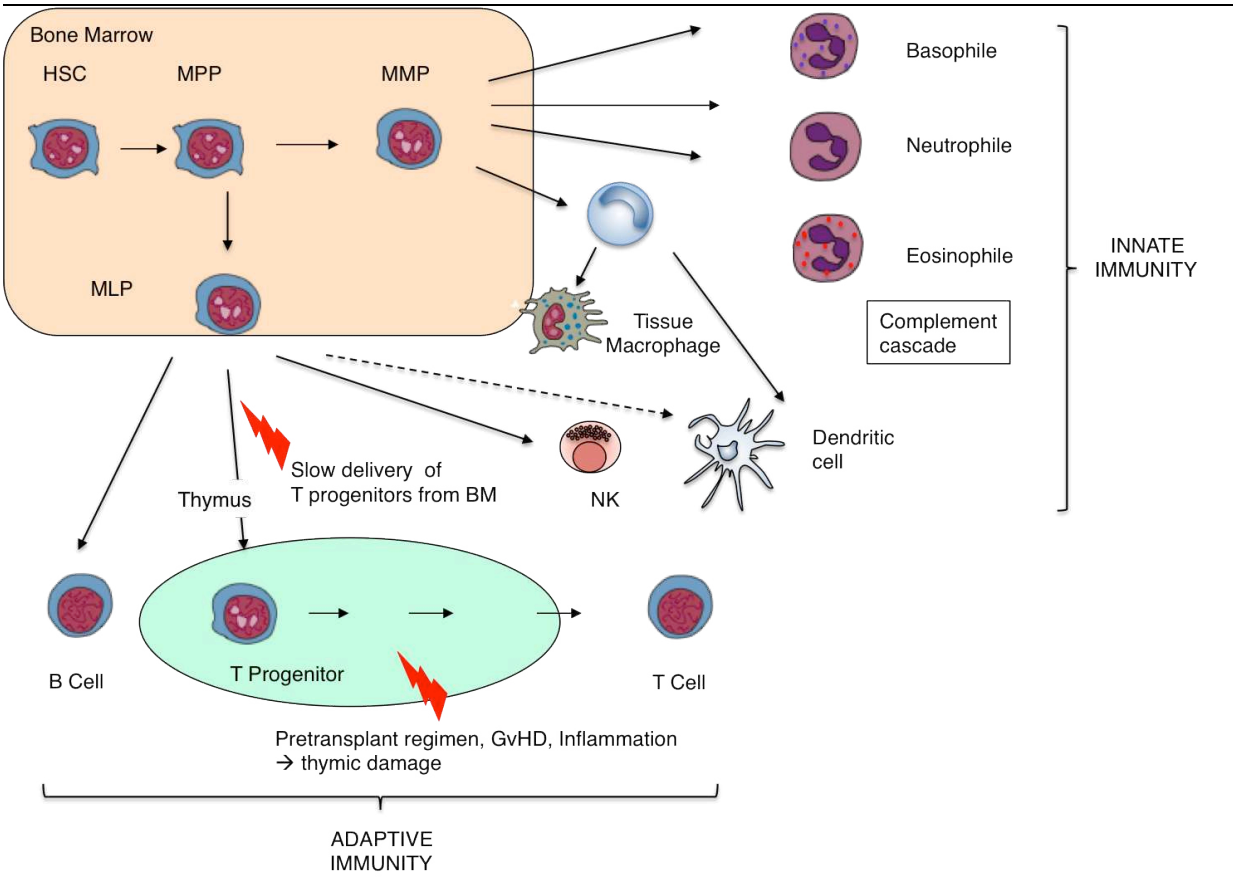

Figure 1: The innate and the adaptive immunity recover with different kinetics after HSCT

After HSCT, reconstitution of innate immunity occurs rapidly, whereas reconstitution of adaptive immunity is delayed. NK cells, monocytes, granulocytes and dendritic cells recover rapidly following HSCT. T cell regeneration is typically delayed and incomplete after HSCT because (i) T-cell progenitors have to be generated in the BM. (ii) the specialized thymic microenvironments is often perturbed due to preparative chemotoxic regimen, GvHD, inflammation. Abbreviations: HSC: Haematopoietic stem cell, MPP: Multipotent progenitor, MMP Myelomonocytic progenitor, MLP: Multipotent lymphoid progenitor, NK: Natural Killer cell. (Figure adapted from (Fry and Mackall 2005)

2 POTENTIAL STRATEGIES TO ENHANCE IMMUNE RECONSTITUTION AFTER HSCT

In order to shorten T cell reconstitution and to reduce infectious and non-infectious complications, various adoptive immunotherapeutic approaches have been evaluated. The large number of potential strategies can be roughly grouped into therapies supporting thymus-independent T cell recovery versus approaches improving *de novo* thymopoiesis.

The former includes the adoptive transfer of allodepleted donor T cells and infusion of pathogen-specific T cells and aims to transiently provide overall or antigen-specific specific T cell immunity. The later consists of hormonal or cytokine based therapies to improve the posttransplant thymic microenvironment and adoptive transfer of committed T cell progenitors.

2.1 Acceleration of Thymus independent T cell recovery

2.1.1 Allodepletion

Allodepletion consists of *ex vivo* depletion of anti-host-activated T cells that shall maintain most of the donor's T cells' immunocompetence against infectious agents. Alloreactive donor T cells can be specifically activated against major HLA-incompatible antigens by mixed lymphocyte reactions (MLRs). Activated (alloreactive) donor T cells can then be selectively killed using monoclonal antibodies (mAbs) against molecules up regulated during T cell activation (e.g. CD25, CD69, CD71 or CD137 (Fehse, Frerk et al. 2000; Fehse, Goldmann et al. 2000; Andre-Schmutz, Le Deist et al. 2002; Koh, Prentice et al. 2002; Solomon, Mielke et al. 2005; Hartwig, Nonn et al. 2006; Samarasinghe, Mancao et al. 2010)) or by photodynamic cells purging (Chen, Cui et al. 2002). A number of these approaches have been demonstrated to efficiently reduce or prevent GvHD in murine HSCT models. The feasibility and efficacy of *ex vivo* allodepletion protocols to accelerate posttransplant immune recovery has been demonstrated in a number of preclinical and clinical studies (Cavazzana-Calvo, Fromont et al. 1990; Fehse, Frerk et al. 2000; Fehse, Goldmann et al. 2000; Koh, Prentice et al. 2002; Godfrey, Krampf et al. 2004; Solomon, Mielke et al. 2005; Amrolia, Muccioli-Casadei et al. 2006). Although allodepletion protocols have been shown to markedly reduce GvHD incidence and improve post-transplant immunity in adult patients, they have been rarely used in children to date. One major restriction relates to the fact that today's protocols are not effective enough to provide the drastic degree of allodepletion that is required when used in patients with a low bodyweight. Novel depletion techniques (such as immunomagnetic depletion or photodynamic purging) have provided encouraging preclinical

results in terms of depletion efficiency. These new approaches seem to hold promise for extending the spectrum of clinical applicability to paediatric patients.

2.1.2 Injection of pathogen specific T cells

Injection of pathogen specific T cells represents another approach for shortening post-transplant immunodeficiency. Current protocols allow expanding pathogen specific cytotoxic T lymphocytes (CTLs) against CMV, EBV and adenovirus *in vitro*. The potential of these pathogen specific T cells has been in several clinical studies. CMV-specific CTLs can control CMV pneumonia that does not respond to antiviral chemotherapy (Riddell, Watanabe et al. 1992; Walter, Greenberg et al. 1995). EBV-specific CTLs have become an important therapeutic option in the treatment and prevention of post-transplant lymphoproliferative disease (Heslop, Ng et al. 1996; Rooney, Smith et al. 1998). Anti-*Aspergillus* CTLs have been developed preclinically and their ability to reduce infection-related mortality is currently tested in clinical trials (Beck, Topp et al. 2006; Tramsen, Koehl et al. 2009). More recently, new protocols avoiding long culture periods have been developed. They include sorting with (1) tetramer complexes (i.e. a specific MHC-peptide combinations). This technique is fully adapted to pathogens that induce a strong clonal immune response such as CMV (Feuchtinger, Opherk et al. 2010). (2) Secretion based sorting assays allow to specifically isolate T cells that secrete IFNγ after a short term stimulation with given antigens (Parietti, Nelson et al. 2012). To provide combined immunity against all these pathogens, trivirus-specific CTLs (against CMV, EBV and adenovirus) have been generated in a single culture and are currently tested in a clinical trial (Hamel, Blake et al. 2002; Leen, Christin et al. 2009).

HPE generates a T cells with both quantitative and qualitative deficiencies. These T cells only provide a transient T cell pool with restricted TCR specificities. The same holds true for therapies based on *in vitro* expanded mature T cells: their clinical application requires a seropositive donor for a given pathogen; the monospecificity of the generated T cells affords only limited immune protection to patients, which are often multi-infected; the activated donor T cells retain a residual risk to induce GvHD.

Ideally, approaches to improve immune recovery should not only accelerate the T cell reconstitution within the first months post-transplantation but should provide the transplanted patient with a stable, polyclonal and naïve T cell repertoire.

2.2 Acceleration of Thymus dependent T cell recovery

A promising approach consists of enhancing the development of T cells from donor-derived HSCs in the recipient's thymus and to thus accelerate the generation of donor-derived, host-tolerant T cells. As mentioned above, both the altered thymic microenvironment and the delayed delivery of T-lymphoid progenitors to the thymus contribute to prolonged T cell lymphopenia after HSCT. Therefore strategies designed to accelerate thymus dependent T cell recovery follow two different but complementary conceptions: The first consists of enhancing intrathymic T cell development and tissue repair via administration of cytokines, hormones and growth factors. The second is based on the adoptive transfer of committed T-lymphoid precursors (Figure 2).

2.2.1 Promotion of intrathymic T cell development

2.2.1.1 IL-7

IL-7 plays a key role in T cell development, survival and homeostasis in both humans and mice. It signals via a receptor complex containing the IL-7 receptor-alpha (IL-7Rα) and the common cytokine receptor-γ-chain (γc), the later being shared by other members of the IL2-receptor family (i.e. IL-2R, IL-4R, IL-7R, IL-9R, IL-15R and IL-21R). The IL–7R complex is expressed by early lymphoid progenitors in the BM, early thymic precursors and resting T cells. The IL-7R complex is expressed by early lymphoid progenitors in the bone marrow, early thymic precursors and resting T cells. The importance signalling IL-7 receptor dependent signalling in T cell development is highlighted by the phenotype of two forms of human severe combined immune deficiency (SCID): X-linked SCID, in which patients lack T and NK cells but dispose of B-cells, is characterized by deletions in the IL2RG gene, which encodes for γc (Kondo, Takeshita et al. 1993; Noguchi, Nakamura et al. 1993). In contrast defective IL7Rα expression causes T(-)B(+)NK(+) SCID, indicating that T cell deficiency (but not NK- or B-cell-deficiency) results from inactivation of IL-7-receptor alpha signalling in X-linked SCID (Puel, Ziegler et al. 1998). In preclinical studies in murine HSCT models, post-transplant administration of IL-7 enhanced both thymopoiesis and homeostatic proliferation of *de novo*-generated T cells (Fry, Christensen et al. 2001; Alpdogan, Muriglan et al. 2003). However further studies concluded, that the immune restorative capacity of IL-7 can be mainly attributed to effects on mature T cells and not to stimulation of thymopoiesis (Broers, Posthumus-van Sluijs et al. 2003; Fry, Moniuszko et al. 2003; Storek, Gillespy et al. 2003; Shultz, Lyons et al. 2005; Beq, Nugeyre et al. 2006). Although clinical data regarding the efficacy and safety of IL-

7 therapy in HSCT patients, the clinical use of IL-7 has to be considered with care, for its potential risk to aggravate GvHD: High serum IL-7 levels in HSCT patients have been linked to a greater risk of acute GVHD suggesting a role in acute GvHD induction (Dean, Fry et al. 2008). While administration of IL-7 was safe and enhanced thymopoiesis in allogeneic murine HSCT models with T cell depleted grafts, it aggravated GvHD when in combination with non-T cell-depleted grafts (Sinha, Fry et al. 2002). A study by our group showed that IL-7 administration did not result in a beneficial effect in the setting of HLA-compatible or partially compatible HSCT but did have a favourable impact on T cell development in a fully HLA-incompatible setting (Andre-Schmutz, Bonhomme et al. 2004). These results indicate that post-HSCT administration of IL-7 should be restricted to recipients of autologous or T cell-depleted grafts.

2.2.1.2 Hormones

Age-dependent changes in two distinct endocrine pathways have been linked to thymic involution and have been shown to exert opposing effects on T cell development. Gonadal steroids (the production of which increases during puberty) inhibit thymopoiesis in an age-dependent manner. By contrast, growth hormone (GH) and insulin-like growth factor-I (IGF-I) stimulate thymopoiesis, but their production declines with age. In view of the hormones' opposing effects on early T cell development, both endocrinal pathways have been modified their potential benefit in T cell reconstitution.

Sex steroid ablation can delay or reverse thymic involution (Utsuyama and Hirokawa 1989). Castration performed in mice before HSCT enhanced thymic and peripheral T cell recovery, without exacerbating GvHD and maintaining Graft-versus-leukaemia (GvL) activity (Goldberg, Sutherland et al. 2005; Goldberg, Alpdogan et al. 2007; Goldberg, King et al. 2009). In a recent pilot study, LHRH-agonist goserelin was used for temporary sex-steroid blockade in a cohort of aged patients undergoing HSCT. Goserelin accelerated neutrophil and overall lymphocyte recovery and T cell recovery in particular. Improvement of T cell recovery occurred via thymus-dependent regeneration, as indicated CD4 cell counts (both total and naïve), increase of T cell receptor excision circles (TRECs) transcripts. Moreover adjuvant goserelin improved peripheral T cell function, survival and engraftment rates and did not exacerbate GvHD (Sutherland, Spyroglou et al. 2008).

Growth hormone (GH) and its proximal mediator (IGF-I) stimulate lymphopoiesis (Clark 1997). Administration of GH or IGF-I inverts age-related thymic involution and

enhances T lymphopoiesis (Clark 1997). Besides direct effects on lymphopoiesis (Huang and Terstappen 1994; Hanley, Napolitano et al. 2005), GH and IGF-I further indirectly promote thymic recovery by supporting TEC proliferation (Chu, Schmitz et al. 2008). In murine HSCT models, posttransplant IGF-I administration increased thymic cellularity and enhanced peripheral T cell reconstitution without exacerbating GvHD (Alpdogan, Muriglan et al. 2003). In HIV-infected patients GH administration augmented the volume of the thymus, improved thymic T cell output and increased peripheral CD4 cell numbers (total and naïve), emphasizing the importance of GH in T-lymphopoiesis (Napolitano, Schmidt et al. 2008). Very recently, IGF-I was successfully used to restore thymopoiesis in a child with Omenn syndrome with failed T cell reconstitution 6 months after HSCT (Ma, Shah et al. 2010; Wendorff, Koch et al. 2010).

2.2.2 Improvement of thymic tissue repair

Efficient thymopoiesis relies on the crosstalk between developing thymocytes and the non-haematopoietic stromal microenvironment. Stress induced TEC injury (e.g. by irradiation, chemotoxic drugs, GvHD) aggravates the perturbed *de-novo* thymopoiesis after HSCT. A better preservation of the thymic microenvironment should hence afford improved T cell recovery.

IL22 production by intrathymic innate lymphoid cells (ILC) was recently suggested to promote epithelial tissue repair and thereby the regeneration of the thymic microenvironment after stress induced damage and after HSCT in mice. In preclinical tests, administration of IL-22 increased early proliferation of TECs and enhanced overall thymic recovery after sublethal and lethal irradiation (Dudakov, Hanash et al. 2012).

Fibroblast growth factor 7 (FGF-7) provides cytoprotection against radiation- and chemotherapy-induced damage to TECs and improves thymic tissue repair (Farrell, Bready et al. 1998; Danilenko, Montestruque et al. 1999). FGF-7 also increases intrathymic IL-7 production (by TECs), which contributes to thymocyte survival and maturation *in vivo* (Min, Panoskaltsis-Mortari et al. 2007). In autologous primate HSCT models, FGF-7 improved the preservation of the thymic architecture and increased thymus-dependent T cell reconstitution (Seggewiss, Lore et al. 2007). In a clinical trial, FGF-7 prevented high-dose chemotherapy induced mucositis in allogeneic HSCT patients (Spielberger, Stiff et al. 2004; Stiff, Emmanouilides et al. 2006). In view of the extensive preclinical data on FGF-7's thymopoiesis-enhancing effects, administration of this molecule is a promising strategy.

2.2.3 Adoptive transfer of *in vitro*-generated T cell precursors

In contrast to all other haematopoietic lineages T cells develop at two distinct sites: First in the BM, where T-lymphoid progenitors are generated and then in the thymus itself, where T cell differentiation occurs. The generation of donor derived T-lymphoid progenitors in the recipients BM and their diminished entry to the recipients' thymus was described as a limiting step for thymus dependent T cell reconstitution after HSCT (Zlotoff, Zhang et al. 2011).

Modification of grafts by inclusion of BM derived committed progenitor cells has therefore been suggested as one strategy to circumvent the limiting step of lymphoid progenitor generation in the BM. Addition of murine BM prone lymphoid progenitors to HSC grafts has been subsequently demonstrated to transiently accelerate thymus-dependent T cell recovery and immunity against murine CMV (Arber, BitMansour et al. 2003). Importantly the inclusion of such progenitor cells in the graft never induced GvHD in mice, because donor progenitors underwent intrathymic education in the recipient. However, T-lymphoid progenitors represent a very rare cell population. The isolation of sufficient numbers is a key limitation even when working in mouse HSCT models. Given that such lymphoid progenitors occur at even lower frequencies in humans and further decline with age, their isolation from donor BM cells is not a valid option for a clinical use (Six, Bonhomme et al. 2007; De Smedt, Leclercq et al. 2011).

Speeding up thymopoiesis by injecting *in-vitro*-generated T cell precursors represents another strategy to reduce posttransplant T cell lymphopenia. During the past years, a number of seminal discoveries have improved our understanding of early steps in T cell development. The identification of Notch1 as the key regulator in early T cell development provided not only substantial novel biological insights. It also led to the establishment of cell culture systems, which now enable us to generate early T-lymphoid progenitors *in vitro*.

To further introduce this novel strategy the following chapters will provide an introduction into

(i) Early T cell development and thymopoiesis

(ii) Signalling pathways and molecular regulation of early steps in T cell development

(iii) Notch ligand culture systems for the *in vitro* generation of T cell progenitors.

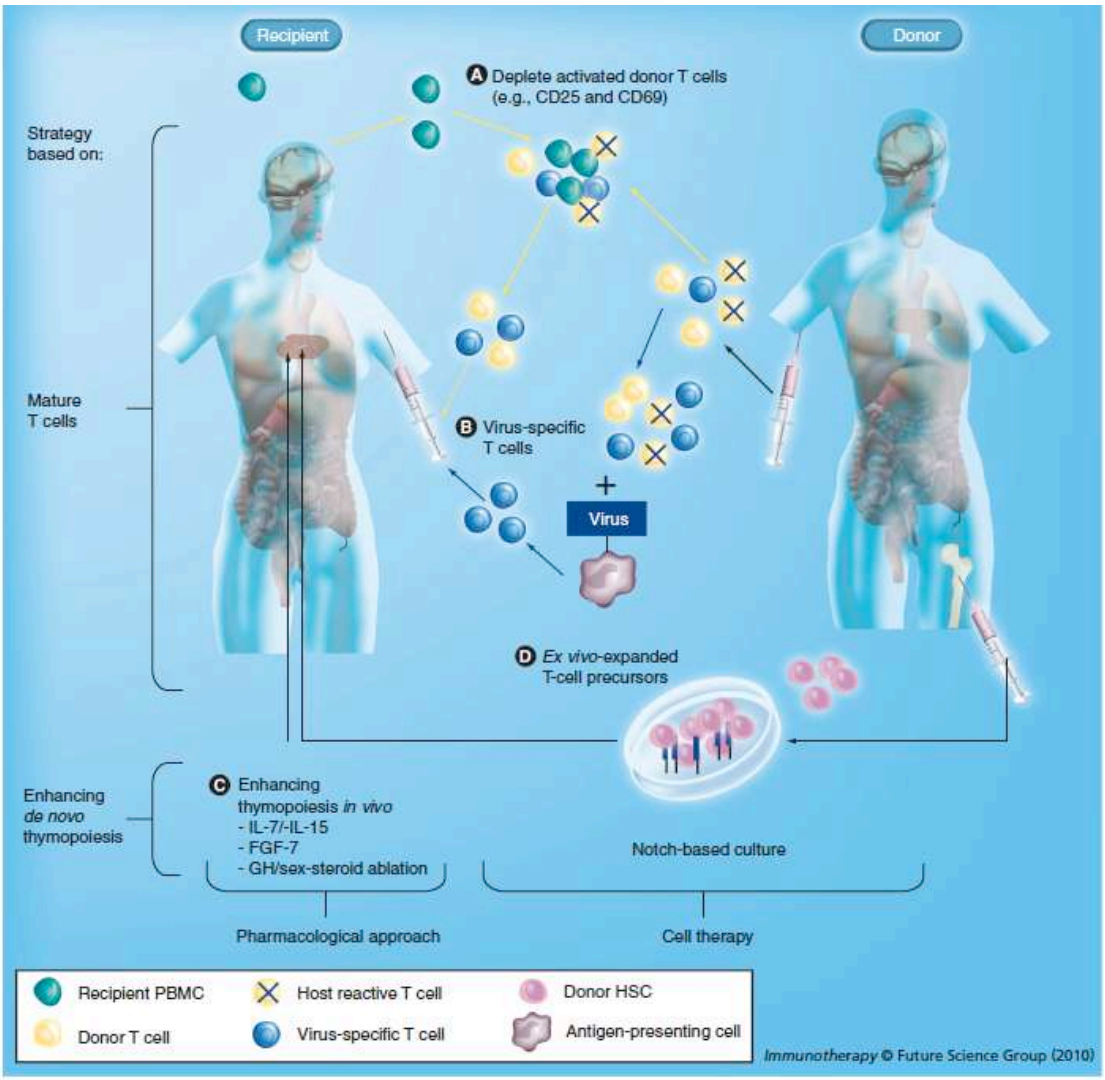

Figure 2: Strategies to enhance T cell reconstitution after T cell-depleted haematopoietic stem cell transplantation (from Reimann et al. 2010).

Four different approaches to accelerate posttransplant T cell recovery are presented. Strategies A and B are based upon injection of mature T cells. C and D aim to accelerate *de novo* thymopoiesis. **(A)** Allodepletion allows selective removal of alloreactive donor cells after activation in mixed lymphocyte reactions with either irradiated PBMC or lymphocytes from the recipient. Specific changes in surface-marker expression (CD25, CD69, CD71 and CD137) or accumulation of photosensitizers allows identification of alloreactive T cells and their subsequent removal with immunotoxins, immunomagnetic depletion or photodynamic purging. **(B)** Pathogen-specific donor T cells can be selectively expanded by exposure to antigen-presenting cells expressing different viral antigens. Strategies to promote *de novo* thymopoiesis include pharmaceutical **(C)** and cellular **(D)** approaches. **(C)** Thymopoietic agents include administration of cytokines (IL-7, IL-15) or growth factors (FGF-7) implicated in normal T cell developments and administration or ablation of hormones involved in thymopoiesis. **(D)** Notch-based cultures promote T-lineage commitment of human HSCs *in vitro*. Transfer of *in vitro*-generated T cellular precursors represents a strategy to enhance *de novo* thymopoiesis. GH: Growth hormone; HSC: Haematopoietic stem cell; PBMC: Peripheral blood mononuclear cell..

3 EARLY T CELL DEVELOPMENT AND THYMOPOIESIS

3.1 Overview on T cell development

Thymopoiesis depends on the continuous supply of T cell progenitors from the BM that reach the thymus via circulation. However, thymus settling presumably represents a very rare event and therefore the study of the precise nature of thymus seeding progenitors is very difficult.

Like all haematopoietic lineages, T cell progenitors finally derive from multipotent HSCs residing in BM. T cell differentiation from HSC involves a series of discrete differentiation steps that gradually restrict this multipotency toward more restricted T-lineage precursors. These stages can be identified through specific surface markers.

In mice HSCs are comprised within the most immature $Lin^{-}Sca1^{+}c\text{-}kit^{+}$ (LSK) cell population. Acquisition of Flt3 marks the transition towards non-renewing multipotent progenitors (MPPs), which in turn give rise to Rag1-positive early lymphoid progenitors (ELPs). Expression of IL7Rα marks the transition to the common lymphoid progenitor (CLP) that can only differentiate into T- or B-lymphocytes and natural killer (NK) cells. While CLPs were originally proposed as the thymus seeding progenitors, the presence of CLP in the blood remains controversial and subsequent investigation revealed the more advanced CLP-2 and the peripheral blood prone T-lineage committed progenitors (CTPs) could seed the thymus after intravenous injection.

A multitude of progenitors thus have the ability to contribute to T-lymphopoiesis and are candidates for thymus seeding progenitor (TSP). Given the variety of candidates and the fact that thymus settling represents a very rare event, the contributions from each population to thymic input under physiological conditions is difficult to assess.

Blood borne TSP migrate the thymus through post-capillary venules near the thymic corticomedullary junction. TSP are attracted to the thymus by adhesion molecules such as fibronectin laminin, collagen type IV, P-selectin and chemokines. In fetal murine haematopoiesis, progenitor cells expressing C-X-C chemokine receptor type 4 (CXCR4), CC chemokine receptor 7 (CCR7) and CCR9 receptors, whose ligands C-X-C Chemokine ligand 12 (CXCL12), CCL19/21 and CCL25 are expressed by the thymic stroma, enter preferentially

in the thymus (Dando, Tavian et al. 2005). Chemokines also play important role in the migration of thymocytes through the different parts of the thymus.

During further T cell differentiation, thymocytes move in a highly ordered manner through the distinct regions of the thymus. After entrance at the corticomedullary junction, the most immature thymic subsets migrate to the cortex and further on to the subcapsular region before they begin to travel back towards the medulla. At each stage, thymocytes receive distinct, sub-region specific differentiation signals from thymic stromal cells and dendritic cells (DCs). These signals govern the processes of β-selection, positive selection, and negative selection that successively shape immature thymocytes into mature T cells with a diverse array of T cell receptors (TCRs). Apart from that spatial organisation of the differentiation process, developmental steps can be phenotypically defined. The most immature stages are found in the $CD4^-/CD8^-$ double-negative (DN) compartment. During maturation, DN thymocytes develop – via the transient CD4 (in human T cell development) or CD8 (in murine T cell development) immature single positive (ISP) stage - into $CD4^+/CD8^+$ double positive (DP) cells. DP cells then further differentiate into either $CD4^+$ or $CD8^+$ single positive (SP) cells.

In murine T cell development, the DN compartment can be further divided into four differentially advanced substages (DN1-DN4) by differential surface expression of CD44 and CD25. The earliest thymocyte subset, the early thymic progenitor (ETP) is comprised within the most immature $CD44^+/CD25^-$ DN1 subset. The ETP is phenotypically defined as $LSK^-/CD44^+/CD25^-$ cells and constitutes a very rare subset (approximately 0,01% of the young mouse thymus, even less in adult mice). During the ETP stage, interaction of the immature progenitors with the thymic microenvironment induces the molecular programme for T-lymphoid engagement. While ETPs are considered as canonical T cell precursors for their efficiency in generating downstream T-lineage progeny, they retain NK and DC potential and – depending on specific ETP subsets – very little B cell and myeloid potential. Unlike most genes, which have a stable sequence in each cell, the TCRs are made up of a series of alternative gene fragments. In order to create functional TCRs, DN thymocytes use several DNA-interacting enzymes to cut and rearrange these gene fragments. The outcome of this process assures a different sequence of each TCR. Initiation of the rearrangement process occurs during the $CD44^+/CD25^+$ DN2 stage: DN2 thymocytes upregulate recombination-activation gene (Rag) 1 and 2 to induce the VDJ recombinase enzyme complex, which initiates rearrangement of the TCRβ locus. The cellular disadvantage in the rearrangement process is that it creates many non-functional TCRβ chains. To assure that only thymocytes with

functional TCR rearrangements differentiate into mature T cells, thymocytes have to undergo a first intrathymic selection process, which is called β-selection. This step occurs at the $CD44^-/CD25^+$ DN3 stage: At this stage, rearrangement of the TCRβ chain is completed. The TCRβ then pairs with the pre-T cell receptor alpha (pTα) to form the pre-T cell receptor (preTCR). If the resulting preTCR is functional, it can then interact with TCR signalling proteins and induces downstream signalling, which allows proliferation and further differentiation of the T cell precursor. Progenitors with non-functional TCRβ rearrangements can again undergo TCRβ rearrangement for several times. However all progenitors that do not finally succeed to express a productive TCRβ chain will eventually be eliminated by programmed cell death. Apart from β-selection, the DN3 stage is characterized by definitive T-lineage commitment (and loss of non-T-lineage potential) of the progenitor. PreTCR signalling further induces rearrangement of the TCRα chain and promotes downregulation of CD25. This marks the progression to the $CD44^-/CD25^-$ DN4 stage and the end of the DN stage. Thymocytes then upregulate either CD8 (in murine T-development) or CD4 (in human T-development) and thereby reach the intermediate immature single positive (ISP) stage. While developmental steps at DN stages and β-selection occur in the subcapsular regions of the thymus, thymic precursors migrate back towards the central regions afterwards, where they acquire the $CD4^+CD8^+$ double positive (DP) stage. DP cells represent more than 85% of all thymocytes and first express a mature TCRαβCD3 complex. However, most DP express TCRαβ/CD3 complexes, that cannot interact with MHC-I or II and are thus non-functional. These cells are eliminated during positive selection: DP cells are highly susceptible to apoptosis and their survival depends on on-going TCR signalling. Only DP cells with functional TCRαβ/CD3 complexes receive a survival signal and will pass the positive selection checkpoint.

Thymocytes having successfully passed positive selection migrate towards the corticomedullary junction and undergo negative selection, against self-antigens expressed by cortical stromal and DCs. Thymocytes that recognise autologous peptides receive apoptosis signals to avoid immune response against self and thus autoimmunity reactions. Depending on whether a given precursor preferentially recognizes MHC-I, or -II, it then matures into a CD8 or CD4 mature T lymphocyte. CD8 and CD4 cells then exit the thymus via the medulla into the peripheral blood.

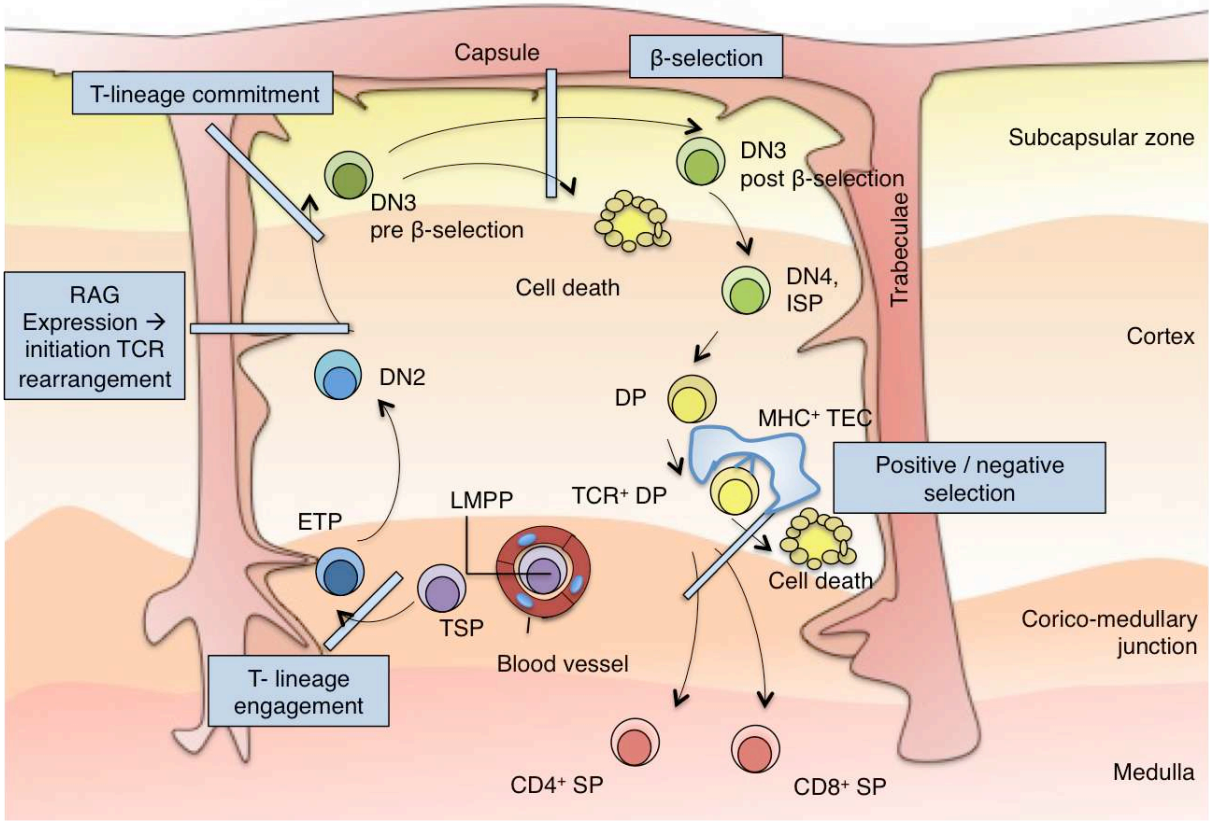

Figure 3: The topography of intrathymic T cell development (adapted from Rothenberg, Moore & Yui, 2008)

Cross-section through a murine adult thymic lobule showing the migration path of T cell precursors during development. Immigrant precursors initially enter the thymus through blood vessels near the corticomedullary junction, the early T cell precursors (ETP) subsequently migrate and differentiate from double negative (DN) to double positive (DP) to single positive (SP) stages, through the distinct microenvironments of the thymus. ETPs appear to have the options to expand in the corticomedullary junction region or to differentiate into DN2 cells that then migrate from the site of entry deep within the cortex to the outer rim of the cortex. Beta-selection occurs during the accumulation of the DN3 T cells in the extreme outer portion - the subcapsular zone - of the thymus. A directional reversal of migration back across the cortex towards the medulla occurs for the later stages of thymocyte development, when thymocytes reach the DP stage. DP cells interact with thymic epithelia cells (TECs) via TCR-MHC interaction and undergo positive and negative selection. ISP: Immature Single Positive; LMPP: lymphoid primed multipotent progenitor, NK: Natural Killer; TCR: T Cell Receptor.

3.2 Particularities of human T cell development

Much of our current knowledge about T cell development has been obtained from highly fine-tuned, genetically modified mouse models that can be diversely combined in an almost mathematical manner. In contrast human T cell development is far less well characterized and much more fragmentary. It is tempting to fill these lacunas by extrapolation from our conception of T cell development in mice, given the high degree of similarity between both species. However this approach is delusive for several reasons.

Progenitor stages traversed during different developmental stages in the BM and in the thymus display substantial differences between the two species by means of their lineage potential, their phenotype and their molecular characteristics. A very recent study shows that lifelong T cell homeostasis depends on on-going thymopoiesis in mice, but much less so in human. This impressively demonstrates that we cannot freely extrapolate our mouse-centred conception of T cell development to physiological conditions in human (den Braber, Mugwagwa et al. 2012).

Human HSC express the CD34 antigen and this marker has been useful in elucidating pathways of distinct haematopoietic lineages. HSCs, MPPs and multipotent early lymphoid progenitors (MLP) are found in the $Lin^-/CD34^+/CD38^{lo/-}$ fraction of human cord blood or BM. They can be further distinguished based on CD90 and CD45RA expression. The $CD90^+/CD45RA^-$ subset contains the pluripotent, self-renewing HSCs, MPPs are found in the $CD90^-/CD45RA^-$ subset and MLP reside in the $CD90^-/CD45RA^+$ subset. Human MLP cells have an increased potential to differentiate in lymphoid lineages and have lost their erythroid and megakaryocytic potential. However they still possess potential to generate monocytes, DCs and macrophages. A true CLP, which can only differentiate into T, B and NK cells, has not been identified in human haematopoiesis yet. Likewise the precise nature of the direct thymus seeding progenitors remains elusive. Collective findings of the past years suggest, that different progenitor population can act as MLP and thymus seeding progenitor in human. The ability of human MLP to engage towards either a T-cell or a B-cell fate varies with age. Fetal MLPs have a balanced T and B cell potential. In contrast the T-lymphoid potential of postnatal MLPs is considerably lower and further decreases during childhood. This age-dependent B over T bias of human MLP further complicates the precise phenotypical characterization of the human CLP equivalent.

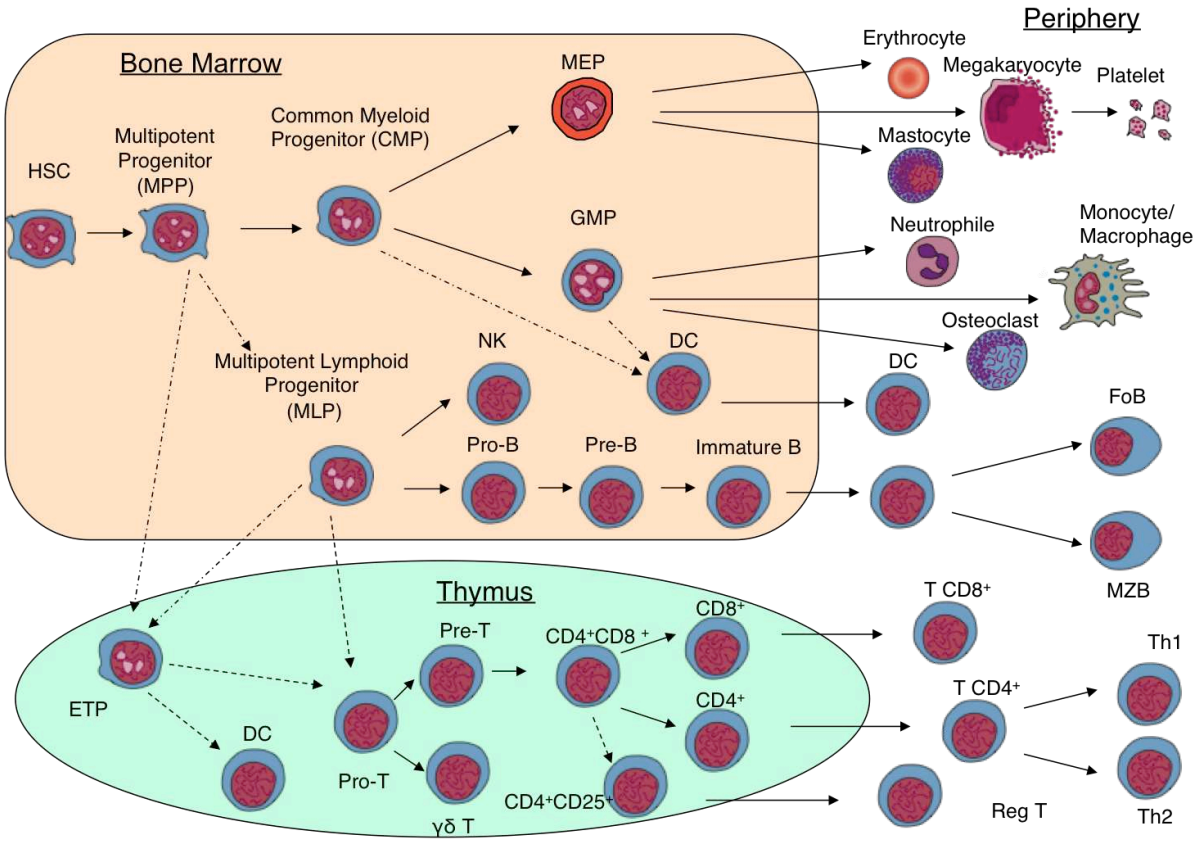

Figure 4: Schema of human haematopoietic differentiation (kindly provided by E.M. Six)

All haematopoietic cells are derived from pluripotent HSC. These cells differentiate into mature haematopoietic cells through various intermediate cell types that are defined by expression of cell surface antigens. Traditionally, it has been assumed that the first step consists in the differentiation of HSC into myeloid precursors on one side and lymphoid precursors on the other side. While myeloid precursors give rise to erythroid, megakaryocyte and mono/granulocytes, lymphoid precursors are supposed to generate T-, B- lymphocytes and NK cells as well as a proportion of DCs. HSC: Haematopoietic Stem cell, MEP: Megakaryocyte–erythroid progenitor cell, DC: dendritic cell, ETP: Early thymic progenitor, Pre-T prethymocyte Th: T-Helper Cell, GMP: granulocyte macrophage progenitor

CD7 and CD10 represent the earliest known markers expressed by the earliest recognizable T- and B-progenitors respectively. Previous attempts to better identify human CLP (and early T-lymphoid progenitors) within the $Lin^-/CD34^+/CD38^{lo/-}$ compartment have therefore made use of these two markers.

CD7 is one of the earliest T-lineage specific surface markers upregulated in T cell development (Sutherland, Rudd et al. 1984; Haynes, Martin et al. 1988). $CD34^+/CD45RA^+/CD7^+$ progenitors

are found at very low frequencies in fetal bone marrow and in CB. $CD34^+/CD45RA^+/CD7^+$ progenitors can only give rise to lymphoid lineages, but not to myeloid and erythroid cells and thereby fulfil CLP criteria. Yet, $CD34^+/CD45RA^+/CD7^+$ progenitors are clearly polarized towards the T/NK lineage as compared to their B-lymphoid potential (Allman, Karnell et al. 2001; Hao, Zhu et al. 2001; Haddad, Guardiola et al. 2004; Haddad, Guimiot et al. 2006). Haddad et al. demonstrated that $CD34^+/CD45RA^+/CD7^+$ progenitors were selectively recruited to the thymus in *ex vivo* colonization assays and therefore hypothesized that they corresponded to direct prethymic progenitors (Haddad, Guimiot et al. 2006). Interestingly, $CD34^+/CD45RA^+/CD7^+$ can only be detected in fetal bone marrow and CB, but sharply decline thereafter and cannot be found in adult BM. In postnatal thymopoiesis however, sequential upregulation of CD7 and T-lineage engagement occurs at very immature intrathymic stages only, evoking thymus seeding by a $CD7^-$ progenitor (Hao, George et al. 2008). Moreover, the human thymus contains low numbers of progenitors, that can develop into B-lymphoid, myeloid and erythroid lineages *in vitro* (Weerkamp, Baert et al. 2006). These findings evoke the existence of multipotent progenitor populations distinct from the T/NK biased $CD34^+/CD45RA^+/CD7^+$ cells that assure lymphopoiesis and especially thymopoiesis after birth.

More than 20 years ago, $Lin^-/CD10^+$ progenitors from fetal BM were first shown to be able to undergo primary steps in T cell differentiation *in vitro* and were thus proposed to correspond to human MLP (Hokland, Hokland et al. 1987). Galy et al. described $Lin^-/CD34^{hi}/CD45RA^+/CD10^+$ cells in postnatal BM, which contained a broad B, T, and NK potential but lacked non-lymphoid potential. Further studies concluded that bone marrow $CD34^+CD10^+CD19^-$ cells were relatively B-cell committed, since they harboured partial DJ_h rearrangements and displayed B-lineage specific gene expression profile (Hokland, Hokland et al. 1987; Galy, Verma et al. 1993; Galy, Travis et al. 1995; Davi, Faili et al. 1997; Dworzak, Fritsch et al. 1998; Rossi, Yokota et al. 2003). In a more recent report by our group the $CD34^+/CD10^+$ subset was further separated by depletion of $CD24^+$ cells. The $CD34^+/CD10^+/CD24^-$ fraction from CB and adult BM was highly enriched for common lymphoid potential (B, T, NK) but still retained residual myeloid potential. In contrast expression of CD24 went along with loss of T cell potential and marked early B cell commitment. Importantly, the multi-lymphoid $CD34^+CD10^+CD24^-$ cells were detected in peripheral blood and at immature thymic developmental stages, suggesting their potential role as a thymic precursor throughout life. Interestingly this study also demonstrated a sharp

decrease of the $CD34^+/CD10^+/CD24^-$ population during the first years of age, which went along with a decrease of T-lymphoid potential (Six, Bonhomme et al. 2007).

Collectively these findings suggest that different progenitor population might act as MLP and thymus seeding progenitor in human. The precise contribution of a given progenitor to T- and B-cell development and to thymopoiesis is unknown but appears to depend of age and is most evident between fetal and postnatal stages.

Although the precise nature of the earliest thymic precursor in human T cell development remains elusive, intrathymic $CD34^+/CD45RA^+/CD7^+$ progenitors are currently accepted to represent the equivalent of the ETP stage. Like in mice, human ETP are comprised within the CD4/CD8 DN compartment at very low frequencies. Early human T cell development is marked by further upregulation of CD7, IL-7Rα, CXCR4 and CD5. Along with that CD10 is downregulated. The emerging $CD34^+/CD45RA^+/CD7^+/CD5^+$ subset has been named proT stage. Progenitors at the proT stage are T-lineage polarized but still remain at least in vitro B, DC and at a very low degree myeloid potential (Weerkamp, Baert et al. 2006).

Expression of CD1a marks transition to the downstream preT stage and definitive T-lineage commitment. This process goes along with initiation of TCR rearrangements within the TCR-δ, TCR-γ, and TCR-β loci that will determine the developmental outcome (Dik, Pike-Overzet et al. 2005): progenitors with in-frame TCR-δ and TCR-γ rearrangements will further develop into $CD3^+$/ TCR-$\gamma\delta^+$ T cells, whereas a TCR-β chain will pair with the pre-Tα to form the pre-TCR complex. At the preT stage and the following CD4 ISP stage, precursors undergo β-selection to than become $CD4^+/CD8^+$ DP thymocytes (Figure 5).

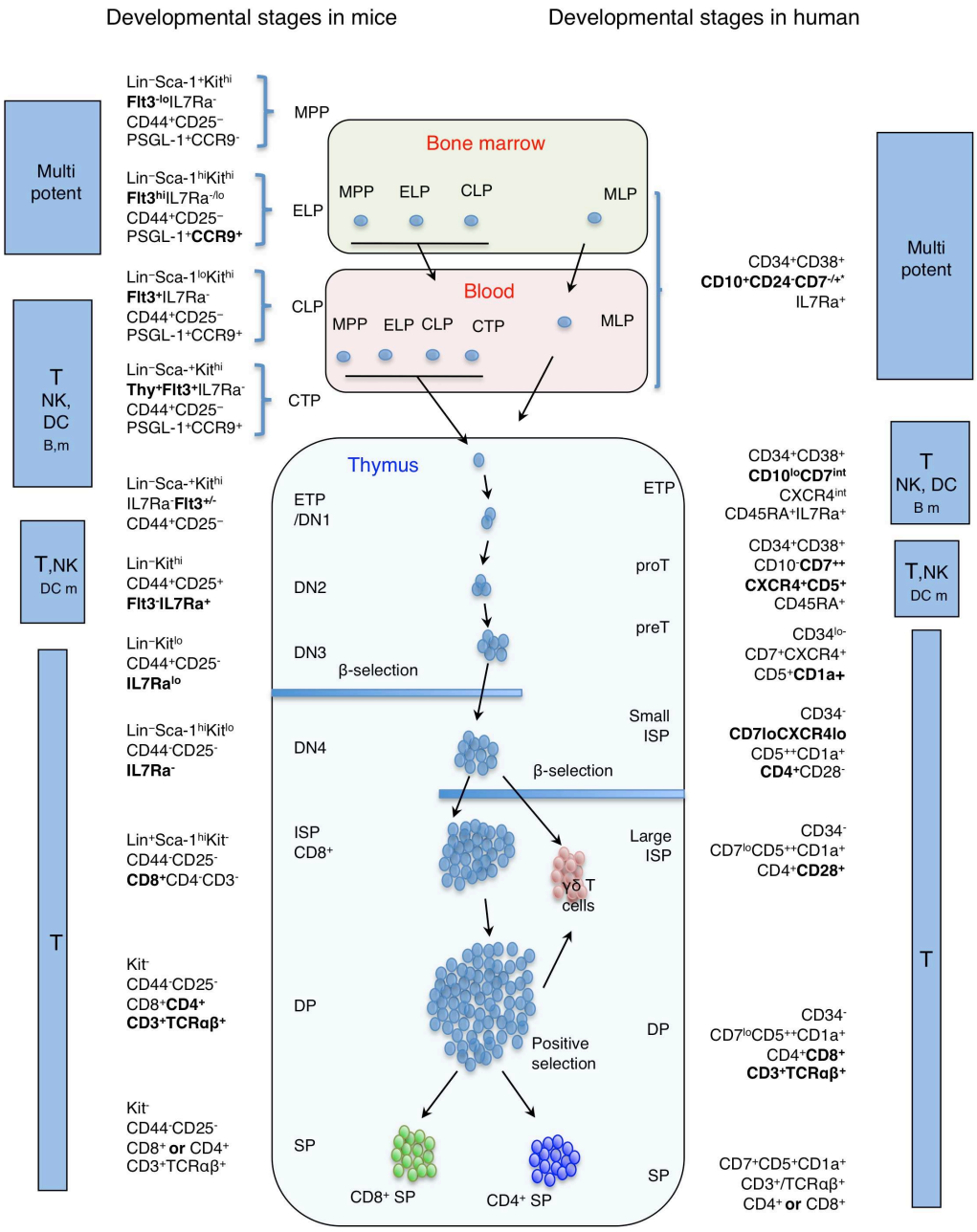

Figure 5: Comparison of known stages of murine and human T cell development

Figure 5: Comparison of known stages of murine (left part) and human (right part) T cell development. Lineage commitment in mice occurs at the transition from the DN2 to the DN3 stage and at the transition from the proT to the preT stage in human. *Phenotype of MLP cells in human BM differs depending on ontogenic stage. In fetal BM and CB, $Lin^-/CD34^+/CD38^-$ MLP express CD45RA and CD7, in adult BM they do not express CD7 and CD45RA but are $CD10^+/CD24^-$

Abbreviations: MPP: multipotent progenitor, MLP: multipotent early lymphoid progenitor, ELP: early lymphoid progenitor, CLP: common lymphoid progenitor, CTP: circulating T cell progenitors, DN: CD4/CD8 double negative, ETP: early thymic progenitor, proT: prothymocyte, preT: pre-thymocyte, ISP: immature single positive, DP: double positive, SP: single positive, Lin: Lineage negative.

4 REGULATION OF EARLY STEPS IN T CELL DEVELOPMENT

Early T cell development depends on the interaction of the thymic microenvironment with the "thymic migrants". Thymic epithelial cells provide critical signals to the immature lymphoid progenitors to guide them through the processes of T cell engagement, commitment and β-selection. Notch1 is the essential factor for the instruction of T-lineage engagement and interplays with other signalling pathways (IL-7, Wnt and CXCR4) to orchestrate T cell differentiation until β-selection.

4.1 Signalling pathways in early T cell development

4.1.1 Notch1/DL-4 signalling

The most important trigger for a T cell engagement is the activation of Notch1 signalling induced by Delta-like Notch ligands expressed on thymic stroma.

Notch proteins are essential regulators of a broad spectrum of cell fate decisions and differentiation processes during fetal and postnatal development and at several stages of haematopoiesis. Originally identified in Drosophila melanogaster, they are highly conserved among different species. Mammals express 4 Notch receptors (Notch 1-4), which can be activated by 5 different Notch ligands. These ligands can be subdivided into 2 classes: the Serrate like ligands Jagged1 and -2 and the Delta-like ligands -1, -3 and -4 (DL-1, DL-3, DL-4). Both receptors and ligands are transmembrane proteins with variable numbers of epidermal growth factor (EGF)-like domains. Upon interaction with its ligand the intracellular portion of the Notch receptor undergoes a series of proteolytic cleavages that liberate the intracellular domain of Notch (ICN). The cleaved ICN translocates to the nucleus, where it heterodimerizes with the transcription factor (TF) Recombining Binding Protein suppressor of hairless-k (RBP-Jk). RBP-Jk is thereby converted from a transcriptional repressor into a transcriptional activator. Further recruitment of the Mastermind like co-activator (Maml) then initiates transcription of Notch target genes. The structure of the RBP-Jk/ICN/Maml complex was originally conceived as a monomer bound to DNA, but recent studies show, that it can also bind as a dimer or in cooperation with E2A, a member of E-protein family of basic helix–loop–helix (bHLH) proteins (Figure 6).

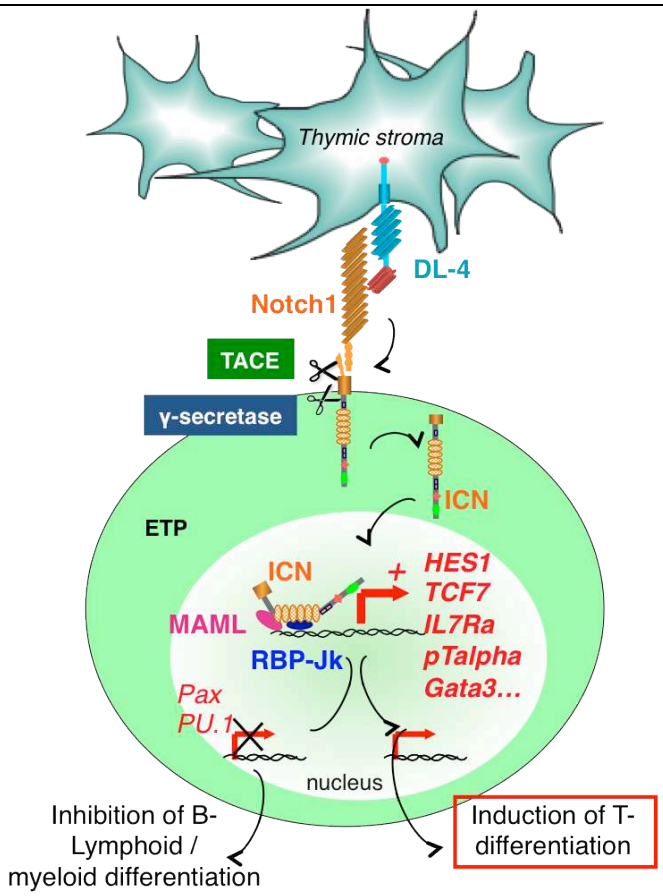

Figure 6: Proteolytic cascade induced by Notch1/DL-4 interaction in the thymus (kindly provided by Emmanuelle Six)

Thymic stromal cells and early thymic progenitors interact via DL-4/Notch1. After ligand-receptor binding 2 successive proteolytic cleavage steps are induced: the metalloprotease TACE first liberates the extracellular Notch1 domain bound to DL-4. In a successive step the γ-secretase/presenilin complex cleaves the intracellular Notch-domain (ICN), which is then translocated to the nucleus. The ICN then associates with the TFs RBP-Jk and Mastermind-like proteins (Maml), recruits further activators and initiates transcription of essential genes for early T-lymphoid engagement and commitment. This complex silences genes involved in B-lymphopoiesis and myelopoiesis.

In the thymus, the monomeric form of Notch1 targets the hairy enhancer of split (Hes) TFs, cell cycle proteins and a number of essential genes for early T cell commitment, namely CD25, Gata3, IL7Rα, TCF1 and pTα (Garcia-Peydro, de Yebenes et al. 2006; Rothenberg, Moore et al. 2008; Weber, Chi et al. 2011). Notch1 further autoregulates its own transcription by cooperating with E2A. This positive autoregulatory feedback-loop is important for the instauration and maintenance of the Notch1-signal during early thymopoiesis (Yashiro-Ohtani, He et al. 2009). Notch receptors can be further modulated through extracellular and

intracellular modifications. For example, glycosylation of the extracellular domain of Notch1 and Notch2 by Fringe glycosyltransferases modifies their affinity for the different ligands. In this, Lunatic Fringe strengthens DL-mediated but reduces Jagged1-mediated Notch1 signalling. In contrast, for the case of Notch2 it potentiates both DL-mediated and Jagged-1-mediated signalling (Van de Walle, De Smet et al. 2011).

Notch1 and Notch2 exert opposing roles at distinct stages in haematopoiesis and T cell development. While Notch-2 signalling contributes to the HSC maintenance in the BM, Notch1 is a key factor for T-cell differentiation. These differences can be partially explained by (i) the distinct expression pattern of Notch2 and Notch1 in the BM and the thymus, (ii) the preferential activation of Notch1 and Notch2 by different ligands and (iii) by an organ specific expression profile for the distinct Notch ligands. For instance, Jagged-1 is primarily expressed at the surface of stromal cells in the BM and the fetal liver. Jagged-1 preferentially activates Notch-2, which in turn confers important signals for the maintenance of HSC in the BM (Varnum-Finney, Halasz et al. 2010). In contrast, Notch1 is not activated in HSC from the BM: first because neither DL-1 nor DL-4 ligands (its principal ligands) are expressed in the BM, second because BM progenitors express only low levels of Notch1 (Ohishi, Varnum-Finney et al. 2000) and third because Leukaemia/lymphoma-Related Factor (LRF) represses Notch1 activation in the BM (Maeda, Merghoub et al. 2007). Since Notch1 signalling can repress early stages of early B-cell development, absence of Notch1 signalling is important to assure early B-lymphoid development in the BM.

TECS highly express DL-4 and (to a lesser extent) DL-1. The Notch1/DL-4 interaction is essential for the organ-specific induction of T cell development in the thymus. The instructive organ-specific role of Notch1 to implement a T over a B-cell fate has been demonstrated by several studies modifying Notch1-activation at distinct haematopoietic stages. Absence of Notch-1 or its inhibition blocks early intrathymic T cell differentiation and leads to ectopic intrathymic B-cell development. In consistency, ectopic Notch-1 activation in BM HSC inhibits B-cell development and induces aberrant T cell development in the BM (Allman, Karnell et al. 2001; Maeda, Merghoub et al. 2007).

Historically it was proposed, that DL-1 and DL-4 represented two independent Notch1 activators in early T cell development. The fact that, DL-1 expressing BM stromal cells (OP9-DL1) allowed completion of T cell differentiation from HSC *in vitro* while simultaneously inhibiting B-cell and myeloid development made DL-1 the first candidate to determine T cell

lineage specification (Schmitt and Zuniga-Pflucker 2002). However, this initial hypothesis was later on attenuated by the following findings: DL-1 knockout mice have normal *in vivo* T cell development. Although expressed in the thymus, DL-1 is mainly found on thymic blood vessels and only at low levels in thymic epithelial cells (Hozumi, Negishi et al. 2004; Koch, Fiorini et al. 2008; Van de Walle, De Smet et al. 2011). Unlike DL-1, DL-4 is highly expressed by thymic epithelial cells of the cortex, which is the supposed anatomical site for T cell commitment. Conditional deletion of DL-4 on thymic epithelial cells leads to a complete block of T cell development *in vivo* (Koch, Fiorini et al. 2008). In contrast, ectopic expression of DL4 induces extrathymic T cell development (Yan, Sarmiento et al. 2001; Dorsch, Zheng et al. 2002). Subsequent studies furthermore demonstrated that DL-4 binds Notch1 with the highest affinity and is its most potent activator (Besseyrias, Fiorini et al. 2007; Mohtashami, Shah et al. 2010; Van de Walle, De Smet et al. 2011). These findings constituted the dominant role of DL-4 as the essential and only non-redundant Notch ligand in T cell development (Koch, Fiorini et al. 2008), (Mohtashami, Shah et al. 2010), (Besseyrias, Fiorini et al. 2007), (Hozumi, Mailhos et al. 2008).

Early in T cell development Notch1-signalling is sharply activated and subsequently provides important signals for the differentiation, commitment and proliferation of T-lymphoid progenitors (Yashiro-Ohtani, He et al. 2009; Ma, Wei et al. 2012). This includes upregulation of an array of genes that are essential for early T-lymphoid differentiation. Such genes are *Hes1*, *TCF1* and *GATA3* and a number of cytokine receptors. These genes provide survival signals for T-progenitors before and after β-selection (e.g. *IL7Rα* and *IL2Rα*), induce the rearrangement of the TCR locus (e.g. *RAG1* and *RAG2*) or constitute the preTCR and TCR complex (e.g. *PTα*, *CD3-δ, -γ* and *-ε*). The precise role of these genes throughout T cell differentiation will be further detailed in the chapter "Launching the T-developmental programme – a molecular view". Also see figure 8.

In both mice and human, Notch1-signalling remains active beyond the ETP stage and increases throughout all T cell developmental stages until the β-selection. Thereafter Notch1 is rapidly downregulated by pre-TCR signalling. During β-selection Notch1 is differentially regulated in humans and mice. While the requirement for Notch at the β-selection checkpoint is absolute in mice, human preT cells can – albeit less efficiently- further proceed towards that DP stage in the absence of Notch (Magri, Yatim et al. 2009; Taghon, Van de Walle et al. 2009). Moreover, non-physiologically high Notch activation inhibits TCR-αβ differentiation and skews differentiation towards the γδ lineage in human (Van de Walle, De Smet et al. 2009).

4.1.2 IL-7 receptor signalling

IL-7 plays a key role in the development, survival and homeostasis of human and murine T cells. In mice, IL-7 is also involved in B-cell development. In human MLPs, IL7Rα is expressed at very low levels. In contrast murine CLP are IL7Rα negative. When thymic seeding progenitors encounter first Notch1 activation, IL7Rα is rapidly upregulated. Signalling via the IL-7 receptor promotes survival and proliferation of early thymic progenitors (in mice). IL-7Rα expression decreases from the DN2 stage on and reaches lowest levels at the DP stage. Successfully selected CD4 and CD8 SP cells re-upregulate IL7Rα again. The temporal attenuation of IL-7 receptor signalling is essential for definitive T cell commitment and for the passage through β- and positive selection. Several *in vitro* T cell differentiation models with human and murine progenitors have provided evidence for this. For instance, high IL-7 concentrations in OP9/DL-1 cultures block T cell development at pre-β-selection stages and lead to the expansion of non-committed DN2/DN3 or proT progenitors (Zakrzewski, Kochman et al. 2006; Ikawa, Hirose et al. 2010; Six, Benjelloun et al. 2011). In consistency, the regular expression of *LCK*, *PTα* and *BCL11b*, which are essential for T-lineage commitment at the DN3 stage, depends on low *in vitro* IL-7 concentrations. Of these factors, Bcl11b represses *IL7Rα* transcription and might therefore account for the attenuation of the IL-7 receptor signal at downstream developmental stages (Di Santo 2010; Ikawa, Hirose et al. 2010). On a mechanistic level, silencing of IL-7R signalling during β- and positive selection might be important to assure that only thymocytes with productive preTCRs/TCRs survive this checkpoint. The downstream pathway of the IL-7R, the preTCR and the TCR/CD3 complex involves the signal transducer PI3K, which confers prosurvival signals to thymocytes. On-going signalling via the IL-7R might therefore reduce the susceptibility of thymocytes to undergo apoptosis and would favour the generation of T cells with impaired TCRs (Vicente, Swainson et al. 2011).

In a similar way, IL-7R signalling strength changes in early human thymopoiesis (Garcia-Peydro, de Yebenes et al. 2006; Gonzalez-Garcia, Garcia-Peydro et al. 2009). Intriguingly, IL7Rα decreases less drastically; it remains expressed at low mRNA and protein levels even in DP cells. However, IL-7R-mediated signalling in human DP does not induce the PI3K pathway (as it is observed in SP thymocytes that proliferate in response to IL7 signalling) and thereby should not provide a non-physiological survival advantage to DP cells during

positive selection (Swainson, Kinet et al. 2007; Vicente, Swainson et al. 2011).

4.1.3 Wnt

Wnt signalling is required for thymocyte development at pre-β selection stages and during transition from the DP to the SP stage (Rothenberg, Moore et al. 2008). Besides regulating T cell development it also involved in HSC maintenance (Luis, Weerkamp et al. 2009).

Wnt proteins are secreted glycoproteins that regulate cell-cell interactions in various cell types and species. The central player in the Wnt-signalling cascade is the cytoplasmic protein β-catenin. In the absence of Wnt signalling, β- catenin is continuously phosphorylated by glycogen synthase kinase (GSK) 3β and subsequently degraded. Binding of Wnt to its receptor complex prevents this phosphorylation. The stabilized β-catenin then translocates to the nucleus and activates TFs of the Tcf/Lef group. Several lines of evidence support the role of Wnt in early thymopoiesis: Wnt proteins are expressed by thymic epithelial cells and Wnt signalling is highly active in early thymic progenitors (Weerkamp, Baert et al. 2006). Tcf-1, a main target of Wnt in thymocytes is the essential TF for the initiation of T-lineage specification and differentiation programme (Weber, Chi et al. 2011). In consistency, Wnt1 and Wnt4 deficient mice display proliferative defects at the DN2 and DN3 (Mulroy, McMahon et al. 2002) and disruption of Wnt signalling in early T progenitors by Lck-Cre induced deletion of β-catenin blocks T cell differentiation at the β-selection (Xu, Banerjee et al. 2003; Janas, Varano et al. 2010). Stabilization of β-catenin in DN thymocytes allows passage through β-selection even in the absence of a functional preTCR (Xu, Banerjee et al. 2003). Wnt also promotes proliferation and survival of DP cells and the final differentiation of mature SP cells. In thymopoiesis Wnt does not only affect T cell progenitors. It also promotes the development of TECs by activating the TF FoxN1 (Balciunaite, Keller et al. 2002).

It has to be mentioned that there is some inconsistency among studies of Wnt function in T cell development. Most of them used different genetically modified mice, in which Wnt-signalling was either turned on or off. These dichotomic models do not well depict the complex function of Wnt signalling in T cell development, which depends on different Wnt activation levels and on the crosstalk with other pathways. Canonical Wnt signalling regulates haematopoiesis in a dosage-dependent manner. Mild Wnt-activity enhances HSC maintenance, whereas intermediate levels support T cell differentiation (Luis, Naber et al. 2011). During

these different stages Wnt appears to act in synergy with (i) Notch (e.g. in upregulation and activation of TCF-1, a key player in T cell commitment) to induce proliferation and instruct cell fate decisions (Weerkamp, van Dongen et al. 2006), with (ii) CXCR4 during β-selection and with (iii) TCR-signals at DP stages of T cell development.

4.1.4 CXCR4-SDF1α

Interaction of SDF1α with its receptor CXCR4 confers important chemotactic stimuli to human and murine haematopoietic progenitors (Aiuti, Webb et al. 1997). SDF1α-CXCR4 interaction was proposed to be involved in the initial migratory phases of immature thymocytes (Annunziato, Romagnani et al. 2001).

In mice, SDF1α is expressed by TECs in the thymic cortex and regulates the migration of DN (Boehm and Bleul 2006). In contrast to other chemokines, SDF1α not only acts as a chemoattractant but also is required for early progenitor proliferation and thymocyte survival during β-selection. Lck-Cre induced CXCR4 deletion in DN2 thymocytes results in increased apoptosis of DN3 cell and inhibits passage through β-selection (Trampont, Tosello-Trampont et al. 2009). Consistent with this, pharmacological inhibition of CXCR4-signalling inhibits the differentiation of DN3 cells into DP cells *in vitro* and *in vivo*. CXCR4 acts via the PI3K and the Mitogen activated protein kinase (MAPK) signal transducers, which are also activated by preTCR downstream cascade. CXCR4 therefore functions as a co-stimulator to sustain the preTCR-signal during β-selection (Janas and Turner 2010).

Although not described in such detail, CXCR4 seems to play a dual role in human thymopoiesis too. In the human thymus, SDF1α is expressed at the corticomedullary junction and at subcapsular regions and thus at subregions, where thymus migration and β-selection occur (Annunziato, Romagnani et al. 2001; Hernandez-Lopez, Varas et al. 2002). SDF1α-CXCR4 interaction was proposed to be involved in the initial migratory phases of immature thymocytes (Annunziato, Romagnani et al. 2001). Likewise CXCR4 signalling promotes the proliferation and further differentiation of immature $CD34^+$ human thymocytes. While low in most immature $CD34^+/CD10^+/CD7^{int}$ ETP stage, CXCR4 levels increase at the proT stage and remain high throughout all DN stages until the CD4ISP stage. In FTOC cultures seeded with $CD34^+$ thymic progenitors, SDF1α deprivation or CXCR4 inhibition drastically reduced

proliferation and induced a developmental arrest before β-selection. In contrast, stimulation with SDF1α particularly promoted the expansion of $C34^+$ progenitors and simultaneously improved the generation of CD4 ISP cells. Interestingly, CD4 ISP themselves responded with much less proliferation to SDF1α treatment, which evidences that human thymocytes depend on CXCR4 signalling before and during but not after β-selection. Consistent with this stage-specific role, CXCR4 expression levels declined upon differentiation into DP cells. Combination of SDF1α and IL-7 further increased the proliferation of $CD34^+$ progenitors in FTOC. In line with this, CXCR4 and IL7R both confer downstream signals via PI3K. With what has been described above (see chapter 4.1.2), it would be interesting to know, whether the synergy of CXCR4 and IL-7 only affects the proliferation of progenitors before β-selection or also the β-selection process itself. However, this issue was not addressed by this study (Hernandez-Lopez, Varas et al. 2002) (Figure 7).

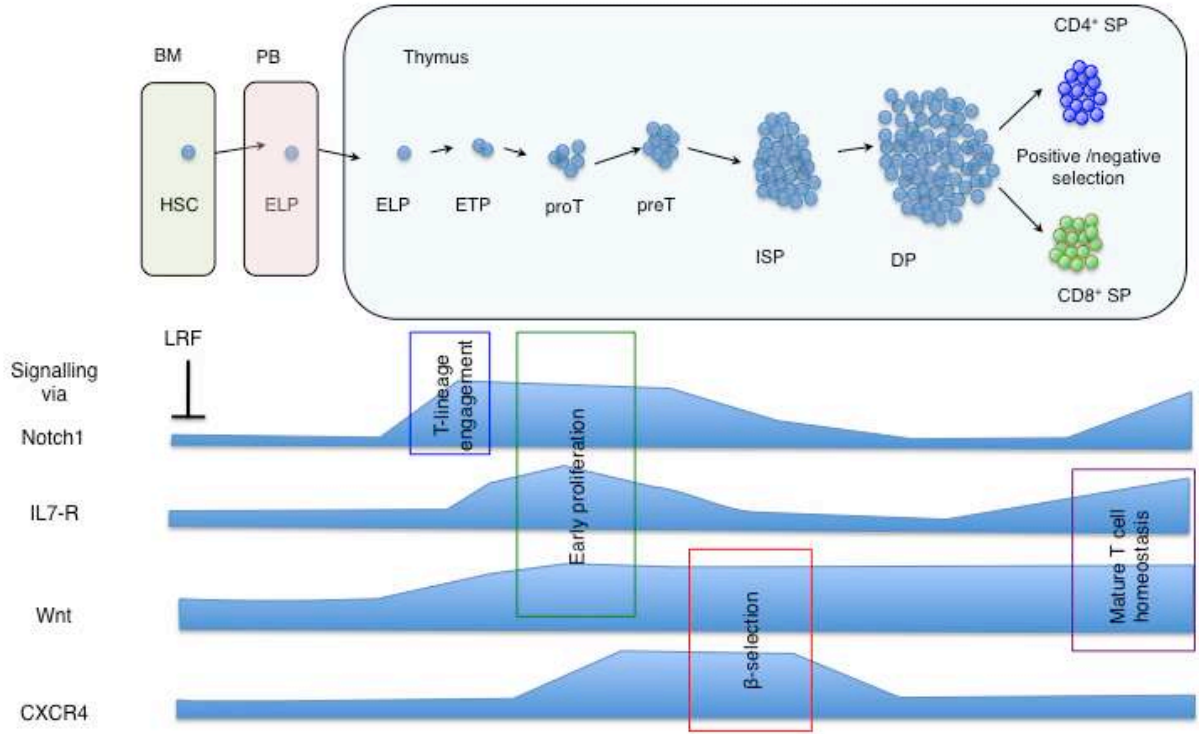

Figure 7: Proposed interaction of Notch1, IL-7, Wnt- and CXCR4 signalling during early thymopoiesis

In the BM, low levels of Wnt-signalling are required for the HSC maintenance. In contrast, Notch1 signalling is not activated due to (i) repression by LRF and (ii) absence of suitable ligands. Progenitors entering the thymus encounter first Notch1 activation. This activates the T-lymphoid differentiation programme, which includes upregulation of IL7Rα and Tcf1 expression. Combined signalling via IL-7R, Wnt and CXCR4 induces proliferation of immature thymocytes before the pre-β-selection checkpoint. During β-selection IL-7R signalling has to be silenced, while Notch1, Wnt and CXCR4 remain active and sustain the preTCR-signal. After successful β-selection Notch1 and CXCR-4 are downregulated, while Wnt-signalling remains active at this stage. Once cells have passed towards the SP stage, signalling via IL-7 becomes again important for the homeostasis of the mature T cells.

4.2 Launching the T-developmental programme – a molecular view

T cell engagement of HSC depends on the stage specific combination of distinct transcription factors (TFs) and the variation of their activity. At present, a consensual concept distinguishes two activation steps, in which (i) a multipotent lymphoid progenitor is primed towards a - still reversible – T-lymphoid progenitor and (ii) then becomes a committed T cell precursor. This implies restriction of the lineage potential and engagement towards a program supporting terminal differentiation (Rothenberg, Moore et al. 2008).

Although Notch1 is the master regulator for the induction of the T cell development, it appears unlikely that it is the only trigger for the induction of the T cell program: (i) Notch1 is a widely used signalling trigger in haematopoietic and non-haematological development. (ii) Genes upregulated in response to Notch (i.e. *TCF1, pTα, GATA3, CD3γ, -δ, -ε, IL2Rα, IL7Rα, Bcl11b*) are activated at distinct stages of T cell development (i.e. DN1, DN2, DN3 stage) and not all of them contain CSL/RBP-Jk/Mastermind binding sites. This implies that they are not direct Notch1 targets (Rothenberg 2012). (iii) Some canonical Notch1 targets are important for efficient induction of T cell development but are partially redundant (Wendorff, Koch et al. 2010). Therefore implementation of the T-lineage programme depends on the activation of cooperative-signalling pathways that act in synergy with Notch1 to induce a highly complex transcriptional program.

Priming and engagement towards the T cell program in mice is conferred by the subsequent activation of three main TFs: TCF1, GATA3 and Bcl11b. These three TFs are not expressed at prethymic stages, presumably due to repression of Notch1 in the BM. Within the earliest intrathymic progenitor stages expression levels of TCF1, GATA3 and Bcl11b sharply increase and are sustained at varying levels during further T cell development. TCF1 and GATA3 mark the transition from the prethymic precursor towards the T cell primed progenitor. Activation of Bcl11b leads to definitive T-lineage commitment (Rothenberg 2012). Contexts explained in this part of the introduction are the results of studies in mice.

4.2.1 TCF-1

In the past years TCF-1 has emerged as the principal downstream target of Notch1 that initiates T cell development (Weber, Chi et al. 2011). TCF-1 is one of the first genes expressed at the ETP stage. Ectopic overexpression of TCF-1 in HSC is sufficient to activate the T cell

identity program *in vitro* and *in vivo* even in the absence of Notch1 signalling. Among all previously studied TFs activated by Notch, TCF-1 was the first protein demonstrated to do so. TCF-1 activates transcription of *GATA3, BCL11b, IL2RA, CD3ε, IL7Rα* and some further genes that are essential for T-lineage engagement. Via positive autoregulation it further sustains its own transcription (Weber, Chi et al. 2011). TCF-1 further controls TFs that act in synergy with Notch1 to shape the T-lineage program *(*e.g. Helix-loop-helix protein, E2A and Id2) (Germar, Dose et al. 2011). Intriguingly, TCF-1 was originally described as a key target of β-catenin during canonical Wnt-signalling. Its additional role as a Notch target provides further evidence for the presumed cooperation of Notch1 and Wnt in T-lymphoid engagement. In this context, one might assume the following synergy: Notch1 increases the synthesis of TCF-1 protein, which is then activated by the sustained Wnt-signalling during further T cell differentiation. This model would be quite in harmony with the above-mentioned finding that Wnt acts in a dose dependent manner during thymopoiesis.

4.2.2 GATA-3

GATA-3 is another key regulator of T cell development. It contains highly conserved zinc finger domains to specifically bind to the consensus GATA motifs in promoter regions of several genes (Orkin 1992). Overexpression of GATA-3 enhances early differentiation of immature thymocytes and promotes the transition towards the DP stage. The activity of GATA-3 at such distinct stages can be partially explained by different occupancy patterns of GATA-3 at distinct developmental stages. When first upregulated in DN1 cells, GATA-3 mainly occupies stem/progenitor specific genes. Cooperation with Notch1 and E2A at later stages leads to a switch towards T cell identity genes such as *PTα* (Zhang, Mortazavi et al. 2012), (Hozumi, Negishi et al. 2008). GATA-3 is not only regulated through transcriptional control but also at posttranscriptional levels. PI3K-Akt-mTOR dependent pathways enhance translation efficacy of GATA-3 mRNA and MAP kinase dependent mechanisms can stabilize GATA-3 protein and thereby fine-tune its levels (Ciofani and Zuniga-Pflucker 2005; Yamashita, Shinnakasu et al. 2005; Cook and Miller 2010). Both, PI3K and MAP kinase are signal transducers in the downstream cascade of CXCR4 and of the preTCR. This further highlights the role of GATA-3 during β-selection (Janas and Turner 2010). Given the multiple points of intervention of GATA-3 in the control of the T-lineage program, its regulation must be highly precise.

4.2.3 Bcl11b

While TCF-1 and GATA-3 are essential for the initiation of the T cell program, definitive commitment depends on Bcl11b. Bcl11b is a bifunctional C2H2 zinc finger TF, that was first discovered as a suppressor of T cell lymphomas and as a nuclear receptor of COUP-TF (Liu, Li et al. 2010). Within haematopoiesis, Bcl11b is restricted to T-lineage cells (and at low levels NK-cells). In early T cell development Bcl1b especially intervenes during β-selection (Wakabayashi, Watanabe et al. 2003; Inoue, Kanefuji et al. 2006; Kastner, Chan et al. 2010). The dramatic upsurge of *BCL11b* expression at the DN2/DN3 stage coincides with the time frame when T-lineage progenitors become definitely committed to a T cell fate and loose their NK-lineage potential (Wakabayashi, Watanabe et al. 2003; Inoue, Kanefuji et al. 2006; Kastner, Chan et al. 2010). Three recent reports revealed, that in the absence of BCL11b activation, T cell progenitors failed to definitely commit, while their proliferation and survival were not affected (Ikawa, Hirose et al. 2010; Li, Leid et al. 2010; Li, Burke et al. 2010). In consistency, deletion of Bcl11b prolonged the time frame in which T cell progenitors retained multipotent lineage potential (Ikawa, Hirose et al. 2010). Apart from directing T-lineage commitment, Bcl11b is involved in maintaining T-lineage restriction: Loss of Bcl11b in already T-committed DN3 cells abrogated the T-lineage transcription programme (i.e. silencing of TCF1, GATA3 expression) but boosted the expression of NK-lineage genes, which eventually lead to the emergence of cell that resembled to normal NK-cells (Li, Burke et al. 2010).

TCF-1, GATA-3 and Bcl11b are thus key TFs during early T cell development and commitment and orchestrate the specific expression of numerous genes involved in this process. This occurs in two subsequent waves: Upregulation of TCF-1 and GATA-3 at earliest progenitor (DN1) levels prepares the activation of the T-lineage identity genes and the downregulation of multiple ‘stemness/non-T lineage’ associated genes. This leads to an increased expression of IL2Rα, IL7Rα, CD3δ CD3ε and induction of Rag1. The increase of Bcl11b levels at the DN3 stage coincides with T-lineage commitment and activates genes required for the assembly of the preTCR-complex (i.e. pTα, ζ-chain-associated protein kinase (ZAP) 70) (Heng and Painter 2008) (Figure 8).

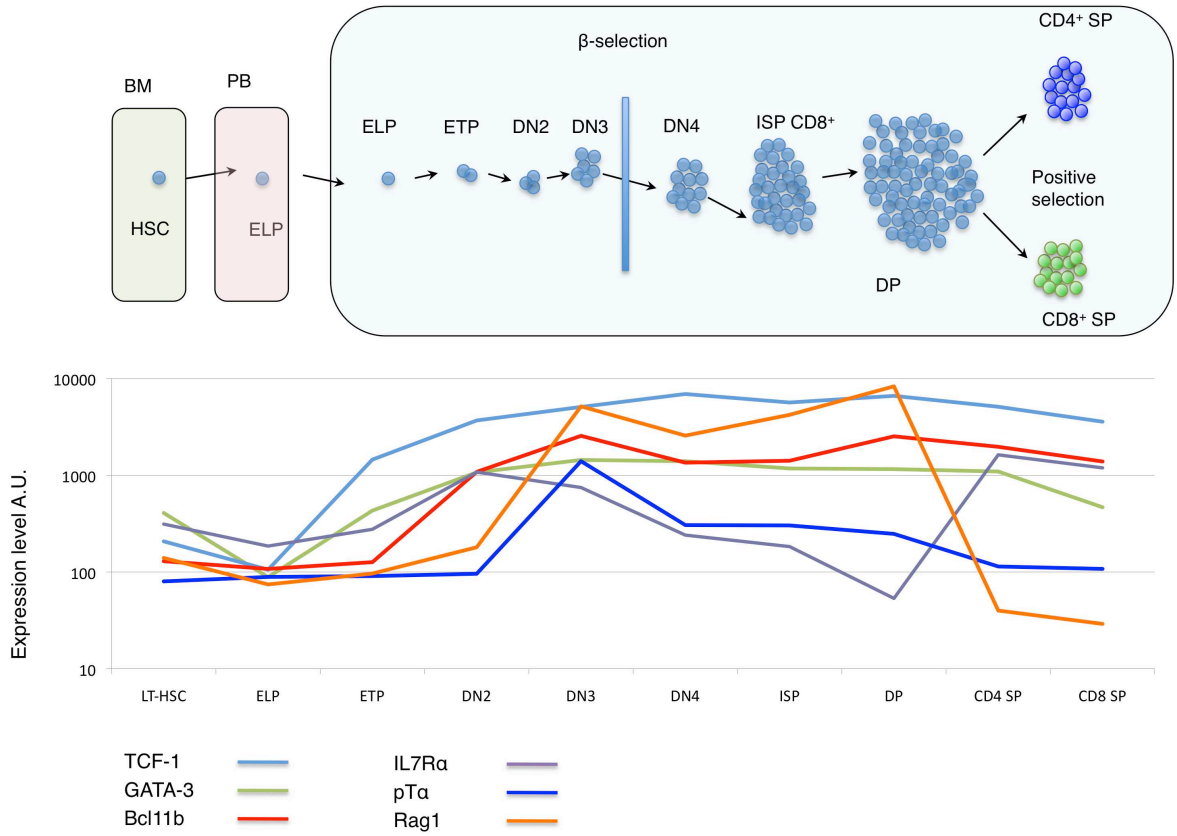

Figure 8: Regulatory gene expression during distinct stages of T-lymphoid differentiation

Curves show the kinetic of increase and decrease of the indicated genes at distinct stages during murine T cell development. Graphs were generated after analysis of publicly available Microarray data on the gene expression data from the Immunological Genome Project (Immgen, http://www.imgen.org). A.U. Arbitraty Units.

5 IN VITRO SYSTEMS TO RECAPITULATE T CELL DEVELOPMENT

5.1 FTOC and RTOC

First attempts to reconstruct T-lymphopoiesis in the early 1980s were based on fetal thymus organ cultures (FTOC), in which thymic lobes explanted from 14-15 day old mouse embryos were depleted of endogenous thymocytes and subsequently seeded with defined haematopoietic progenitors. FTOC support the entire program of T cell development, including positive and negative selection (Jenkinson, Franchi et al. 1982). First attempts to extend the FTOC system to human T cell development consisted of human fetal thymus fragments that were injected with $CD34^+$ progenitors (Baum, Weissman et al. 1992). Given the ethical concern of this method, xenogenic FTOC system based on the fetal thymus of immune deficient mice were subsequently developed (Plum, De Smedt et al. 1994).

The three dimensional architecture of the original FTOC system depicts the *in vivo* migration process quite well but its complexity hampers to study the contribution of specific thymic compartments. Reaggregate thymic organ culture (RTOC), in which distinct stroma and epithelial of the thymus are individually recombined, allow to better determine the contribution of each cell type to T cell development (Anderson, Jenkinson et al. 1993). Nevertheless, when working with human progenitors, the reconstitution efficiency of xenogenic FTOC and RTOC remains low. They depend on many variables (such as the difficult manipulation of the thymic explants) and not necessarily on the T cell potential of a given progenitor. This makes it challenging to further exploit these systems for the evaluation of the T-lymphoid potential of a given human progenitor cell.

5.2 Feeder cell based approaches

To create less demanding and more standardized culture conditions, a number of cell lines have been tested for their potential to support T-lymphopoiesis *in vitro*. The BM derived murine stromal cell lines S17 and the Macrophage colony-stimulating factor (MCSF) deficient OP9 turned out to be very efficient to support B-lymphopoiesis from HSC. However, induction of T-lymphopoiesis was much harder to achieve. First promising results were obtained with thymic stromal cell lines. The thymic fibroblastic cell line TSt-4 established from fetal thymus of C57BL/6 mice and the θ35 thymic stromal cell line from BALB/cJ mice supported the

differentiation of DN murine thymocytes into DP cells (Watanabe, Mazda et al. 1992; Montecino-Rodriguez, Johnson et al. 1996). Unfortunately, these cell lines only allowed induction of T cell differentiation with low efficacy and did not sufficiently silence the developmental program for non-T lineages (Montecino-Rodriguez, Johnson et al. 1996; Wada, Masuda et al. 2008). The difficulties encountered during the development of such cell lines raised the concern, that T cell differentiation required complex molecular and cellular interactions that could not be provided by a stromal cell line.

During the late 1990s, several studies gathered mounting evidence for the importance of Notch1 in early T-lymphopoiesis (Pui, Allman et al. 1999; Radtke, Wilson et al. 1999; Wolfer, Bakker et al. 2001). This successively led to the development of Notch ligand expressing cell lines.

5.3 Feeder cell based Notch ligand cultures

Jaleco et al. developed a first Notch based culture system, in which S17 stromal cells were genetically modified to express Notch ligand DL-1 (S17/DL-1 cells). When seeded with human $CD34^+$ CB haematopoietic progenitors, S17/DL-1 cells promoted the generation of T/NK precursors and blocked B-cell development. They further allowed the generation of low numbers of $CD4^+/CD8^+$DP cells (Jaleco, Neves et al. 2001).

Zuniga-Pflucker et al. showed that OP9 cells do not express Notch ligands and thereby explained their incapacity to support *in vitro* T cell development. After retroviral transfection with DL-1, OP9/DL-1 cells induced the normal T cell differentiation programme in murine fetal liver and BM LSK cells and supported murine T-lymphopoiesis from the early DN stage, through the DP stage until functional single-positive $CD8^+/TCR\alpha\beta^+$ T cells (Schmitt and Zuniga-Pflucker 2002; Awong, Herer et al. 2011). The OP9/DL-1 system also induces T cell development in human BM and CB derived HSC and recapitulate the essential steps of human T cell differentiation from the $CD34^+/CD38^-$ progenitor to the $TCR\alpha\beta^+$ DP stage (De Smedt, Hoebeke et al. 2004; La Motte-Mohs, Herer et al. 2005).

Given its high efficacy the OP9/DL1 cells became the most commonly used system for *in vitro* T cell differentiation. However, the discovery that DL-4 is the essential and most potent Notch ligand in thymopoiesis (Hozumi, Negishi et al. 2004; Besseyrias, Fiorini et al. 2007; Hozumi, Mailhos et al. 2008; Koch, Fiorini et al. 2008) prompted revisions on the original

OP9/DL-1 system and favoured the further development of OP9/DL-4 cells. DL-4. OP9/DL-4 cells induce higher Notch1 signalling activity in progenitor cells than OP9/DL-1 cells. Thereby they are more efficient in supporting T cell development and in inhibiting B-lymphopoiesis / myelopoiesis (Mohtashami, Shah et al. 2010). Although OP9/DL-4 have been increasingly used in the past years, at present the OP9/DL-1 co-culture still remains the mostly used culture system for T cell differentiation.

To further mimic the thymic niche a number of DL-1 or DL-4 expressing thymic cell lines such as the thymic stromal cell line Tst4 (Tst4/DL-1 and Tst/DL-4) and the thymic epithelial cell lines TEC84 (TEC/DL-1) have been established (Meek, Cloosen et al. 2009) (Beaudette-Zlatanova, Knight et al. 2011). Tst4/DL-1, Tst/DL-4 and TEC/DL-1 cells all promote *in vitro* T cell development, but are not more efficient than OP9/DL-1 or OP9/DL-4 cells.

5.4 Feeder cell free Notch ligand cultures

In feeder cell based Notch ligand cultures the isolated role of Notch1 activation in T cell development is obscured by additional factors that can be secreted by the stroma cells themselves (e.g. SDF1α secretion by OP9 cells). Moreover, these culture systems consist of genetically modified murine cell lines and are thereby not eligible for clinical use. In order to overcome this obstacle, recent studies have aimed at developing fully molecularized Notch ligand culture by making use of purified, plate bound Notch ligands.

5.5 DL-1

In a series of studies that spanned the past decade, the Bernstein group has developed a DL-1 based culture system to expand HSCs *in vitro*.

One technical obstacle to be solved during this process was to find DL-1 forms that activate Notch signalling. The first constructs these researchers developed consisted of the extracellular (ec) domain of the human DL-1. The soluble form of this DL-1 protein could bind to Notch but did not induce Notch signalling. Activation of Notch could only be obtained when DL-1 was immobilized to the plastic surface of the culture dish. This was achieved when the

DL-1 ec domain was fused to the Fc-fragment of human IgG (Varnum-Finney, Wu et al. 2000).

At the time, the Bernstein group originally conceived this DL-1 culture (the late 1990s), the precise role of Notch1 in T cell development was not yet elucidated. These researchers started from the observation that constitutive Notch activation in HSC immortalized pluripotent haematopoietic progenitors and therefore sought to use DL-1 for the expansion of HSC (Varnum-Finney, Xu et al. 2000).

Culture of murine or human haematopoietic progenitors with plate bound DL-1 allowed multilog-fold expansion of haematopoietic progenitors that displayed short-term myeloid and lymphoid repopulation potential *in vivo* (Varnum-Finney, Brashem-Stein et al. 2003; Dallas, Varnum-Finney et al. 2007). Although no direct evidence was provided, these scientists further postulated that long-term repopulation HSCs (LT-HSCs) were concomitantly expanded in DL-1 cultures. DL-1 culture also induced early T cell development in murine LSK and human $CD34^+/CD38^-$ CB cells and allowed *in vitro* expansion of T/NK lymphoid progenitors (Ohishi, Varnum-Finney et al. 2002; Dallas, Varnum-Finney et al. 2007). Originally, a dual role for the DL-1/Notch1 interaction was proposed to explain the apparently conflicting findings, in which DL1 sustained HSC maintenance on one hand and on the induction of differentiation on the other. A recent work of the same group suggests that effects of DL-1 on HSC maintenance are conferred via Notch2, while DL-1/Notch1 interaction accounts for effects on T cell differentiation (Varnum-Finney, Halasz et al. 2010). Unlike OP9/DL-1 cells, immobilized DL-1 did not support T cell differentiation development beyond the DN2 in mice / the proT stage in humans. Moreover it favoured the generation of NK cells. This was explained by a lack of Wnt-signalling in these reduced culture conditions (Aoyama, Delaney et al. 2007).

5.6 DL-4

In a similar approach, a number of previous studies explored the potential of DL-4 fusion proteins to induce T cell development in haematopoietic progenitors. Lefort et al. introduced a two-phase culture system, in which $CD34^+$ CB cells were first expanded on MS5 stromal cells and then transferred to DL-4 coated culture wells. Immobilized DL-4 induced immunophenotypic and molecular changes consistent with T lineage engagement and silenced B cell transcripts. These early changes mediated commitment and acceleration of T cell development *in vitro* (including completion of the TCR rearrangement in secondary OP9-DL1

co-culture) (Lefort, Benne et al. 2006). As observed in the DL-1 system, DL-4 culture supported T cell differentiation only to pre-β-selection stages and led to emergence of NK-like cells upon prolonged exposure (Haraguchi, Suzuki et al. 2009). Absence of signals activating Wnt and CXCR-4 dependent pathways were proposed to account for this observation, as a murine DL-4 system supplemented with Wnt activators and murine CXCR4 ligand CXCL12 allowed to overcome this block at a pre-β-selection stage (Janas and Turner 2010).

6 *POTENTIAL OF IN VITRO GENERATED T CELL PROGENITORS TO PROMOTE POST-TRANSPLANT T CELL RECONSTITUTION*

Notch ligand culture systems provided a biotechnological tool for the *in vitro* generation of T-lymphoid progenitor and thereby prompted their further clinical exploitation to accelerate T cell reconstitution after HSCT. As described above, the OP9/DL-1 cells support the entire T cell differentiation process *in vitro.* A major challenge therefore consisted in establishing culture conditions allowing the specific expansion of early progenitors, which (i) are still capable to seed the thymus and (ii) have not undergone positive or negative selection *in vitro* and thus at extrathymic sites.

The van den Brink group increased IL-7 concentrations in OP9/DL-1 cultures, to block T cell development before β-selection. Thereby they succeeded to generate high numbers of DN2/DN3 cells from murine LSK cells. Co-transfer of *in vitro* generated murine T-lymphoid progenitors with unmanipulated LSK cells into lethally irradiated allogeneic recipients promoted *de novo* thymopoiesis, accelerated the emergence of host-tolerant, functional $CD4^+$ and $CD8^+$ T cells and thereby afforded improved anti-infective and anti-tumour immunity (Zakrzewski, Kochman et al. 2006). Murine T cell progenitors accelerated T cell reconstitution independently of MHC disparities, never caused GvHD and were even applicable in a setting without stem cell rescue (Zakrzewski, Suh et al. 2008). This work provided the proof of concept for the potential of *in vitro* expanded T cell precursors and prompted the extension of this approach to human cells.

In 2009, Awong et al. made use of the OP9/DL-1 system to generate high numbers of $CD7^+/CD5^+$ proT cells from human $CD34^+$ CB cells. When adoptively transferred into newborn immunodeficient mice, *in vitro* generated proT cells were able to repopulate the thymus and to enhance thymic reconstitution. In this study, administration of human IL-7 was

required for the thymic engraftment of the proT cells *in vivo* (Awong, Herer et al. 2009).

Meek et al. used the Tst4/DL-1 andTst4/DL-4 co-culture to generate T cell progenitors (with the same phenotype as mentioned above) from G-CSF mobilized $CD34^+$ cells. These cells reconstituted the thymus of immunodeficient mice and further differentiated into mature $TCR\alpha\beta^+$ thymocytes. In this study, the Tst4/DL-4 cultured human progenitor cells were incapable of reconstituting non-T-lineages (Meek, Cloosen et al.).

Although these new findings represented a remarkable progress towards the further clinical exploitation of *in vitro* generated T-lymphoid progenitors, a number of issues had not been addressed by these studies.

1) None of them showed, that *in vitro* expanded human T-progenitors could give rise to mature and functional $CD4^+$ or $CD8^+$ T-lymphocyte in the periphery. Although this might reflect non-physiological conditions of T cell development in xenotransplantation models, this issue needs to be further addressed.
2) Current expansion protocols are much less efficient for the generation of human T-progenitors from BM-derived HSCs.
3) The use of genetically modified murine cells, expressing Notch ligands is a considerable safety concern for a further clinical use.

The Bernstein group addressed the latest of these three issues and succeeded to develop a fully molecularized Notch ligand culture. DL-1 expanded murine LSK cells enhanced thymic engraftment and accelerated T cell reconstitution in a murine allotransplantation model (Dallas, Varnum-Finney et al. 2007). Consistent with this, DL-1 cultured $CD34^+/38^{-/lo}$ CB progenitors displayed an increased capacity to repopulate the BM and the thymus of NSG mice but did not accelerate the emergence of peripheral T cells (Ohishi, Varnum-Finney et al. 2002). In 2010, these researchers succeeded in transferring this procedure into a clinical protocol: In this study DL-1 progenitors enhanced short-term bone marrow engraftment and accelerated neutrophil recovery in myeloablated patients undergoing double-unit cord blood transplantation (Delaney, Heimfeld et al. 2010). However, DL-1 expanded progenitors did not improve posttransplant T cell recovery (I.Bernstein, personal communication to Isabelle André-Schmutz).

OBJECTIVE OF PHD PROJECT

The objective of my PhD project was to make use of immobilized DL-4 to establish a feeder-cell free Notch ligand culture for the *in vitro* generation of human T-lymphoid progenitors and to test their potential to accelerate T cell recovery.

Immobilized DL-4 had been previously described to be sufficient for launching the T cell developmental program in human $CD34^+$ CB cells *in vitro*. However, the capacity of DL-4 to drive immature haematopoietic progenitors towards a T cell fate and to generate potent human T-lymphoid progenitors at clinically exploitable yields needed to be further explored. Moreover, this proposed protocol depended on a two-phase culture system, in which feeder $CD34^+$ CB cells were first expanded on MS5 stromal cells before being transferred to immobilized DL-4 ligand (Lefort, Benne et al. 2006).

We further simplified this approach and bypassed the requirement of the MS5 pre-amplification stage to generate T-lymphoid progenitors from $CD34^+$ CB cells *in vitro*. In a first step, I characterized *in vitro* properties of T-lymphoid progenitors from DL-4 cultures. In a next step, I adoptively transferred DL-4 progenitors into NSG mice to explore their potential to promote T cell recovery. I showed that highest numbers of early T-lymphoid progenitors and highest *in vitro* T-lymphoid potential were found after 7 days of culture and that T-lymphoid progenitors yielded at that time point promoted thymic and peripheral T cell recovery in two different HSCT approaches in NSG mice (Article "Human T-lymphoid progenitors generated in a feeder-cell-free DL-4 culture system promote T cell reconstitution in NOD/SCID/$\gamma c^{-/-}$ mice").

When studying the capacity of immobilized DL-4 to induce the T-lineage programme I realized that this approach was efficient to induce changes consistent with early lineage engagement. However, it failed to support final commitment and T cell differentiation beyond the β-selection checkpoint. This was in contrast to what can be observed in OP9/DL-1 co-cultures. I hypothesized that this particularity of the DL-4 system was due to the absence of additional signals that are supplied by feeder-cell based cultures but not in the completely molecularized DL-4 system. To further explore these differences and to propose mechanisms that might be at cause, we performed comparative analysis of distinct progenitor populations isolated from DL-4 culture, OP9/DL-1 co-culture or native thymocytes (supplemental results part 1: "Comparison of *in vitro* generated T-lymphoid progenitors with native T-lymphoid progenitor populations").

The DL-4 system explained in part 1 and 2 was set up for the generation of T-lymphoid progenitors from CB derived HSCs. Although CB transplants have become a valid graft alternative, postnatal HSCs from BM or mPB still constitute the major graft source for HSCT. In terms of a further clinical application of the DL-4 culture, its adaption to such HSC sources would be of high importance. Unfortunately, initial experiments revealed that the DL-4 system was much less efficient for generating T-lymphoid progenitors from postnatal HSCs. I therefore sought of reasons that might account for the little propensity of postnatal HSCs to generate T-lymphoid progenitors in the DL-4 culture (supplemental results part 2 ”extension of the DL-4 system to postnatal HSCs”).

RESULTS

1 HUMAN T-LYMPHOID PROGENITORS GENERATED IN A FEEDER-CELL-FREE DL-4 CULTURE SYSTEM PROMOTE T CELL RECONSTITUTION IN NOD/SCID/γC MICE (PUBLISHED RESULTS)

1.1 Exposure to immobilized DL-4 induces phenotypical changes consistent with early T-lymphoid engagement and allows *in vitro* generation of early T-lymphoid progenitors

CB derived $CD34^+$ progenitors exposed to immobilized DL-4, expressed cell surface CD7 within 3 days of culture and thus acquired a $CD34^+/CD45^+/CD7^+$ ETP-like phenotype. This population, constituted the predominant population after 7 days. During further culture CD7 levels increased and CD34 levels declined. This led to the emergence of a $CD34^-/CD7^{++}$ proT1 population, from which $CD5^+$ proT2 cells and low numbers of $CD1a^{int}$ preT cells emerged within 14 days. Even upon prolonged culture, DL-4 progenitors never expressed CD4, CD8, cell surface CD3 and TCRαβ indicating a developmental block at the preT stage. In contrast, an emerging downstream subset of the proT1 population expressed the NK-lineage markers NKP46 and CD56. Control cultures with Fc-fragments did not support the generation of CD7 positive progenitors. In quantitative terms, a 7-day DL-4 culture yielded 5.0 x 10^4 ETP-cells from an input 2 x 10^4 $CD34^+$ cells (comprising only 180 ETP cells) corresponding to a 280-fold increase of this population.

1.2 DL-4 progenitors display molecular characteristics of early T–lymphoid progenitors

To confirm that acquisition of the ETP and proT1 phenotype went along with T-lineage engagement we studied the expression of early T lineage genes in these populations. In contrast to CD34+/CD7- cells *PTα*, *RAG1*, *IL7Rα* and *BCL11B* transcripts were first detectable in ETP cells and further increased in proT1 cells. In contrast, *SPI-1* and *PAX5* transcription levels were downregulated in ETP and proT1 cells.

We further analysed TCR rearrangement events at different times of cultures: After 7 days when ETP cell were predominant we did not observe any TCR rearrangement events. Non-T cell specific Dδ2-Dδ3 and Vδ2-Dδ3 rearrangements were first observed between day 7 and day 10 and coincided with the proliferation of proT-1 cells. T-lineage specific

rearrangements within the TCR-δ (Dδ2-Jδ1), TCR-γ locus (Vγ9 and Vγ11) were first observed after 14 days of culture and paralleled with increasing levels of proT2 cells. Incomplete rearrangements in the TCR-β (Dβ1-Jβ1, Dβ 1-Jβ2) locus appeared after prolonged DL-4 culture and coincided with apparition of preT cells.

1.3 DL-4-primed ETP and proT1 cells have high T-lymphoid potential

To demonstrate that DL-4 culture conferred an increased *in vitro* T-lymphoid potential I performed Limiting dilution analysis (LDA) with DL-4 cells in secondary OP9/DL1 co-cultures. DL-4 progenitors retrieved from day 7 cultures displayed a 25 fold higher T-cell progenitor frequencies than untreated CB cells. Secondary OP9/DL1 co-cultures with sorted DL-4 progenitor population revealed that the increase of T-lymphoid potential could be entirely attributed to the ETP and proT1 subsets. Moreover, DL-4 ETP and proT1 cells accomplished final T cell differentiation within shorter time than the more immature $CD34^+/CD7^-$ progenitors. Finally, $CD7^+$ DL-4 progenitors were capable of reconstituting the thymus of NSG mice.

1.4 DL-4 progenitors seed the thymus, accelerate thymic reconstitution and give rise to mature, circulating T-lymphocytes *in vivo*

The *in vivo* T-lymphoid potential of DL-4 cells was assessed in two different xenotransplantation models: (i) in irradiated adult NSG and (ii) in newborn non-irradiated NSG mice. Control conditions consisted of uncultured $CD34^+$ CB cells and of progenitors from Control-Fc cultures. Analysis of thymic engraftment and peripheral T cell reconstitution were performed at 8 weeks (adult NSG) and 4 weeks (neonate NSG) posttransplant respectively. I found active thymopoiesis 20 of 22 DL-4 progenitor recipients vs. 13 of 20 recipients of uncultured CD34 CB cells. None of the recipients of Fc-control cultured cells showed thymic reconstitution. DL-4 recipients displayed more advanced thymopoiesis than NSG injected with untreated $CD34^+$ progenitors. I furthermore performed a kinetic analysis of thymic engraftment in newborn NSG. DL-4 recipients showed first signs of thymic engraftment and active thymopoiesis after as little as 1 week posttransplant. In contrast thymopoiesis in $CD34^+$ progenitors it only occurred at 3 weeks posttransplant.

Moreover, I detected mature peripheral T cell in about a third of DL-4 recipients, but only in one recipient of untreated $CD34^+$ cells. Finally, DL-4 reconstituted thymi displayed a polyclonal TCRβ VJ recombination pattern.

1.5 Co-transplantation of DL-4 progenitors and untreated $CD34^+$ cells promotes thymopoiesis and accelerates peripheral T cell reconstitution

To mimic HSCT settings, resembling more closely to clinical conditions, I performed co-transplantations with DL-4 primed progenitors and untreated $CD34^+$ cells. Co-transplantation considerably enhanced thymic engraftment, accelerated intrathymic T cell differentiation and further improved peripheral T cell reconstitution. Peripheral T cells derived from spleens of co-transplanted recipients were able to secrete IFN-γ upon polyclonal stimulation.

Since I used DL-4 progenitors and $CD34^+$ from HLA-disparate donors in the co-injections, I could track the specific contribution of each cell types to the distinct haematopoietic lineages. DL-4 progenitors preferentially reconstituted the peripheral T cellular compartment (78-83 %), while the vast majority (>97%) of non-T-lineage cells were progeny of the untreated $CD34^+$ cell fraction.

Human T-lymphoid progenitors generated in a feeder-cell-free DL-4 Culture system promote T cell reconstitution in NOD/SCID/γc Mice

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TRANSLATIONAL AND CLINICAL RESEARCH

Human T-Lymphoid Progenitors Generated in a Feeder-Cell-Free Delta-Like-4 Culture System Promote T-Cell Reconstitution in NOD/SCID/$\gamma c^{-/-}$ Mice

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Key Words. Hematopoietic stem cell transplantation • Immunotherapy • T-lymphoid precursor cells • DLL4 protein • Notch1

ABSTRACT

Slow T-cell reconstitution is a major clinical concern after transplantation of cord blood (CB)-derived hematopoietic stem cells. Adoptive transfer of in vitro-generated T-cell progenitors has emerged as a promising strategy for promoting de novo thymopoiesis and thus accelerating T-cell reconstitution. Here, we describe the development of a new culture system based on the immobilized Notch ligand Delta-like-4 (DL-4). Culture of human $CD34^+$ CB cells in this new DL-4 system enabled the in vitro generation of large amounts of T-cell progenitor cells that (a) displayed the phenotypic and molecular signatures of early thymic progenitors and (b) had high T lymphopoietic potential. When transferred into NOD/SCID/$\gamma c^{-/-}$ (NSG) mice, DL-4 primed T-cell progenitors migrated to the thymus and developed into functional, mature, polyclonal αβ T cells that subsequently left the thymus and accelerated T-cell reconstitution. T-cell reconstitution was even faster and more robust when ex vivo-manipulated and nonmanipulated CB samples were simultaneously injected into NSG mice (i.e., a situation reminiscent of the double CB transplant setting). This work provides further evidence of the ability of in vitro-generated human T-cell progenitors to accelerate T-cell reconstitution and also introduces a feeder-cell-free culture technique with the potential for rapid, safe transfer to a clinical setting. STEM CELLS *2012;30:1771–1780*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Unrelated cord blood transplantation (UCBT) has become an important therapeutic procedure for patients lacking human leukocyte antigen (HLA)-compatible donors [1]. Although originally conceived for hematopoietic stem cell transplantation (HSCT) in children, the use of double cord blood (CB) units circumvents the limitation of low cell doses and makes double UCBT (dUCBT) a valid option in adults. However, serious infections constitute the leading nonmalignant cause of post-transplant morbidity and mortality and are undoubtedly related to delayed T-cell immune reconstitution and impaired de novo thymopoiesis [2–5]. Donor-derived T cells generated in the recipients thymus first appear 9 months after UCBT [1]. Complete restoration of the T-cell compartment with a polyclonal T-cell repertoire takes even longer and may never reach pretransplant levels [3, 6].

After HSCT, de novo T-cell generation from donor hematopoietic progenitors is disturbed at several successive steps. First, the generation of T-cell progenitors in the bone marrow (BM) and their delivery to the thymus have been shown to represent a limiting step in post-HSCT recovery [7]. Furthermore, conditioning regimens, graft versus-host-disease, infectious disease, and inflammatory status damage the thymic stroma and thereby alter intrathymic T-cell differentiation [8].

A promising approach for accelerating de novo T-cell development consists in bypassing T-cell precursor delivery from the BM by adoptively transferring in vitro-generated T-lymphoid progenitors. The latter should be capable of

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immediately seeding the thymus and then rapidly generating a wave of donor-derived, polyclonal, host-tolerant T cells.

Notch-1 signaling plays a major role for priming human stem cells (HSCs) toward the T-cell lineage and thus for early thymopoiesis [9–11]. Notch-based culture systems, such as delta-like-1 (DL-1)-expressing BM stromal cells (OP9/DL-1 cells), allow in vitro generation of large numbers of human T-cell progenitors that have been demonstrated to promote de novo thymopoiesis in humanized mice [12–14]. This working hypothesis was further supported by observations in conventional mouse models: when transferred into allogeneic recipients, murine T-cell progenitors generated in OP9/DL-1 coculture accelerated de novo thymopoiesis and emergence of host-tolerant, functional T cells that afforded protection against infectious agents and residual tumor cells when transferred into an allogeneic HSCT model [15, 16].

With a view to establish a clinically applicable system, feeder-cell-free Notch-ligand culture systems for the generation of T-lymphopoietic progenitors are warranted. Replacement of the OP9/DL-1 cells with the Fc-fusion-protein DL-1/Fc enabled high-grade expansion of hematopoietic progenitors in vitro. These progenitors were found to enhance bone marrow engraftment and myeloid reconstitution in xenotransplantation models and in a recent phase I clinical trial [17, 18]. Unfortunately, this procedure does not appear to have an influence on the kinetics of T-cell reconstitution.

Recent studies have shown that (a) DL-4 is the essential Notch 1 activator in the T-engagement of hematopoietic stem cells [19–21] and (b) immobilized DL-4 induces phenotypic changes reminiscent of early T-cell development in $CD34^+$ CB cells having been expanded in an MS-5 culture system [22]. By extending this approach, we found that immobilized DL-4 supported the in vitro generation of T-cell progenitor cells from $CD34^+$ CB cells in a feeder-cell-free culture system. We further demonstrated that DL-4 generated T-cell progenitors displayed the phenotypic and molecular signatures of very immature thymic precursors. Upon transfer in NOD/SCID/$\gamma c^{-/-}$ (NSG) mice, these progenitors were capable of migrating to the thymus and differentiating into mature polyclonal T cells. This work provides a basis for rapidly translating our ex vivo culture system into clinical procedures for accelerating immune reconstitution after partially HLA-mismatched, allogeneic HSCT.

Materials and Methods

Human Cells and Tissues

CB samples not eligible for banking were used, following the provision of informed consent by the child's mother.

Human thymus tissue samples were obtained from children undergoing heart surgery. The samples were collected as previously described [23] and used in accordance with the French legislation and ethical guidelines.

Cell Preparation

$CD34^+$ hematopoietic progenitors were magnetically sorted from human CB and thymus, as previously described [23]. For quantitative polymerase chain reaction (PCR) experiments, $CD34^+$ cells were further sorted on an ARIA II system (BD Biosciences, San José, CA) to exclude residual $CD3^+$, $CD56^+$, and $CD19^+$ cells.

Generation of the Delta-4 Fc Fusion Protein and a Control IgG2b Fc-Fragment

The Delta-4 Fc fusion protein construct was generated by cloning cDNA encoding the soluble domain of hDL-4 (aa1-526) (kindly provided by Adrian Harris) into the pFuse-hFC1 vector (InvivoGen, San Diego, CA, http://www.invivogen.com/) containing the coding region for the human IgG2bFc fragment. The DL-4-Fc fusion construct (or the pFuse-hFc1 fragment alone) was then subcloned into an MSCV-IRES-PuroR retroviral vector and transduced into HEK293T cells. The DL-4-Fc fusion protein and the control IgG2b Fc-fragment were produced and purified by PX'Therapeutics (Grenoble, France, http://www.px-therapeutics.com/).

Exposure of $CD34^+$ Progenitor Cells to Notch Ligand DL-4

$CD34^+$ CB cells were cultured in 24-well plates previously coated with DL-4 (5 μg/ml) or control-Fc (5 μg/ml) for 24 hours at 4°C, blocked with bovine serum albumin 2% in phosphate-buffered saline (PBS) for 1 hour at 37°C, and washed with PBS. Cultures were initiated at a concentration of 2×10^4 cells per well in X-VIVO 20 medium (BioWhittaker, Walkersville, MD), supplemented with 20% defined fetal calf serum (Hyclone, Thermo Fisher Scientific, Illkirch, France) and the recombinant human cytokines interleukin-7 (IL-7), Flt3-ligand (Flt-3), stem cell factor, and thrombopoietin (all at 100 ng/ml and all purchased from either R&D Systems [Minneapolis, MN, www.rndsystems.com] or PeproTech Inc. [Rocky Hill, NJ, www.peprotech.com]). If the culture lasted longer than 1 week, cells were transferred into freshly coated wells at a maximum concentration of 1×10^5 cells per well. After 7 days of culture on DL-4, cultured cells were sorted by fluorescence-activated cell sorting (FACS) to exclude $CD34^-/CD7^-$ myeloid cells from subsequent analyses. The cells in the resulting fraction are referred to hereafter as "DL-4 cells."

The OP9/DL-1 In Vitro T-Cell Differentiation Assay

The T-lymphoid potential of native $CD34^+$ CB cells and T-cell progenitors generated by exposure to DL-4 was assessed via limiting dilution assays (LDAs) in OP9/DL-1 cocultures, as previously described [24].

Quantitative, Real-Time PCRs

Total RNA was isolated with the RNeasy Micro Kit (Qiagen, Courtaboeuf, France, http://www.qiagen.com) and then reverse-transcribed using Multiscribe reverse transcriptase and oligo(dT) primers with the high-capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, http://www.lifetechnologies.com). Diluted cDNA was used as a template for quantitative, real-time PCR reactions in TaqMan gene expression assays for Pre-T-cell Receptor alpha (*PTα*), interleukin-7 receptor alpha (*IL7Rα*), recombination activating gene 1 (*RAG1*), B-cell lymphoma/leukemia 11B (*BCL11B*), spleen focus forming virus proviral integration oncogene 1 (*SPI-1*), and paired box protein Pax-5 (*PAX5*) (Applied Biosystems, US, http://appliedbiosystems.com). The PCRs were performed on an ABI Prism 7900 system (Applied Biosystems, US, http://appliedbiosystems.com), according to the manufacturer's instructions. All transcript levels were normalized against the results for human *glyceraldehyde-3-phosphate dehydrogenase*.

T-Cell Receptor Recombination Analysis

T-cell receptor (TCR) recombination analysis of in vitro-generated DL-4 cells and native human thymocytes was performed as previously described [25]. The TCR-*β*-rearrangement repertory of human T cells recovered from the thymuses of transplanted NOD/SCID/$\gamma c^{-/-}$ mice (referred to here as "NSG" mice) was measured using the ImmunTraCkeR assay (ImmunID Technologies, Grenoble France, http://www.immunid.com/), as previously described [26].

Flow Cytometry Analysis and Cell Sorting

Monoclonal antibodies against CD1a (HI149), CD3 (SK7), CD4 (SK3), CD5 (UCHT2), CD7 (M-T701), CD8 (RPA-T8), CD16 (Leu11c), CD34 (8G12), CD45, CD45RA (HI100), CD56 (My31), CXCR4 (12G5), and 7-aminoactinomycin D (7AAD) were obtained from BD Biosciences. Monoclonal antibodies against TCRαβ (IP26A) and TCRγδ (IMMU510) were obtained from Beckman Coulter (Brea, CA, https://www.beckmancoulter.

com). Cell suspensions were stained and analyzed on an 8-color FACSCanto II cytometer (BD Biosciences, http://www.bdbiosciences.com). The data were analyzed using FlowJo software (Treestar, Ashland, OR, http://www.flowjo.com/) after gating on viable, 7AAD-negative cells. Absolute cell numbers were determined using Caltag counting beads (Invitrogen, Camarillo, CA, www.invitrogen.com). Cell subsets were sorted on an ARIA II system.

Adoptive Transfer of In Vitro-Generated T-Cell Progenitors into NSG Mice

The NSG mice (obtained from The Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) were housed in a pathogen-free facility. DL-4 cells (5×10^5) were injected intrahepatically into newborn, nonirradiated NSG mice or intravenously into adult (4-week old), 3Gy-irradiated NSG mice. Control mice were injected with either 1.5×10^5 noncultured $CD34^+$ cells or 1×10^6 cells cultured on immobilized control-Fc molecules. Regardless of the type of cells injected (i.e., DL-4 cells, control-Fc cells, or noncultured $CD34^+$ cells), each animal received a total of 1.5×10^5 $CD34^+/CD7^-$ cells.

Transplanted mice were subcutaneously injected with 5 mg of recombinant human IL-7 (kindly provided by Cytheris, Issy-les-Moulineaux, France, http://www.cytheris.com/) every 7 days. Nonirradiated newborn NSG mice were sacrificed at 1, 2, 3, or 4 weeks and 3 Gy-irradiated adult NSG mice at 8 weeks post-transplant. Cells were harvested from the femur, spleen, thymus, and peripheral blood and analyzed by flow cytometry, as described above. All experiments and procedures were performed in compliance with the French Ministry of Agriculture's regulations on animal experiments.

Cytokine Production

Peripheral blood mononuclear cells from spleens were obtained after mechanic disruption and red blood cell lysis. Splenocytes were cultured in the presence of 100 U/ml IL-2 and 5 ng/ml IL-7 for 7 days. The cells were then stimulated with phorbol myristate acetate (PMA) (50 ng/ml), ionomycin (1 mg/ml), and Golgi-Stop reagent for 3 hours. Intracellular IFN-γ was measured using standard flow cytometry procedures.

Statistical Analysis

Nonparametric Mann-Whitney *U* tests were performed using Prism 4 software (GraphPad Software Inc., LA Jolla, CA, http://www.graphpad.com).

RESULTS

In *Vitro* Exposure of CB $CD34^+$ Cells to a DL-4 Fusion Protein Induces Phenotypic Changes that are Consistent with Early T-Cell Development

Purified $CD34^+$ CB cells cultured with DL-4-Fc fusion protein (DL-4) began to express CD7 within 3 days (Fig. 1A, upper panel). This expression paralleled the upregulation of CD45RA (Supporting Information Fig. S1, middle panel). CD7 expression continued to increase until day 7 and was correlated with a decrease in CD34 expression and the emergence of a $CD34^-/CD7^{++}$ population. A T-cell progenitor subset expressing CD5 (Fig. 1A medium panel), intracellular CD3epsilon (Fig. 1B upper panel), and CXCR4 (Supporting Information Fig. S1 lower panel) emerged from within the $CD34^-/CD7^{++}$ population between days 7 and 10. By day 14, the $CD34^-/CD7^{++}/CD5^+$ population had started to express low levels of CD1a (Fig. 1B, upper panel). In human postnatal thymocytes, the early thymic progenitor (ETP) ($CD34^+/CD45RA^+/CD7^+$), proT1 ($CD7^{++}/CD5^-$), proT2 ($CD7^{++}/CD5^+$), and preT stages ($CD7^{++}/CD5^+CD1a^+$) mark successive T-cell developmental stages before beta selection [12]. Since we observed the characteristic expression of these antigens in DL-4 culture, our $CD34^+/CD45RA^+/CD7^+$, $CD7^{++}/CD5^-$, $CD7^{++}/CD5^+$, and $CD7^{++}/CD5^+/CD1a^+$ subsets will be referred to hereafter as ETP, proT1, proT2, and preT cells.

The lack of any CD4, CD8, surface CD3, or TCR expression in DL-4 cultures indicated that a T-cell development was blocked at this stage. A subset of the $CD34^-/CD7^{++}$-population was found to coexpress NKP46 and CD56 at day14, indicating differentiation toward a natural killer (NK) lineage. Phenotypically, the NK- and the T-lineage-engaged populations could be clearly distinguished from each other by mutually exclusive expression of NK-precursor markers (i.e., NKP46 and CD56) and T-precursor markers (i.e., CD5 and intracellular CD3) (Fig. 1B, lower panel). In line with this differential marker expression, the NK-precursor population did not express CXCR4 (Fig. S1B, lower line).

$CD34^-/CD7^-$ cells had a myeloid phenotype and were excluded by FACS from all subsequent analyses. The remaining DL-4 fraction thus contained $CD34^+/CD7^-$, ETP, and proT1 cells. In contrast, $CD34^+$ CB cells exposed to the control IgG2b Fc-fragment ("control-Fc cells") never gave rise to $CD7^+$ T-cell progenitors (Fig. 1A, lower panel). The vast majority of control-Fc cells had a myeloid phenotype (data not shown) and only a small proportion was $CD34^+$.

In quantitative terms, 2×10^4 $CD34^+$ cells (containing only 170 ETP cells) gave rise to an average of 5.0×10^4 ETP-cells after 7 days of culture (Table 1, third row). This count did not change thereafter, whereas the mean number of proT1 and proT2 cells increased from 5.6×10^4 on day 7 to 4.1×10^5 after 14 days of DL-4 culture (data not shown).

DL-4 Cells Display the Molecular Characteristics of Early T-Cell Progenitors

To confirm that acquisition of the ETP and proT1 phenotype had opened (and were actively transcribing) a T-cell differentiation program after 7 days of DL-4 culture, we studied the cells' expression of genes involved in early T-cell development and compared them with the $CD34^+/CD7^-$ subset. The latter did not express the *PTα*, *IL7Rα*, *RAG1*, and *BCL11B* genes (data not shown). In contrast, all these genes were initially expressed in ETP cells and substantially upregulated in proT1 cells. Accordingly, *SPI-1* was downregulated in the proT1 subset and *PAX5* was silenced in all subsets after DL-4 exposure (Fig. 2B). Although we did not detect any TCR rearrangements after 7 days of DL-4 culture (Supporting Information Table S1), longer culture times did induce these events in a similar pattern as seen in human thymopoiesis (Supporting Information Table S1).

DL-4-Primed ETP and proT1 Cells Have High T-Lymphoid Potential

As demonstrated above, the DL-4 cells' phenotypic and molecular characteristics were consistent with early T-cell engagement. In order to quantify the cells' in vitro T-lymphoid potential, serial dilutions obtained after 0, 7, and 14 days of cultures were placed in secondary OP9/DL-1 cocultures and analyzed in an LDA (Table 1). A cell able to complete T-cell differentiation in vitro is referred to hereafter as a "T-cell precursor." Noncultured $CD34^+$ cells displayed a T-cell precursor frequency of 1 in 349 (Table 1). Seven days of exposure to DL-4 induced a 29-fold increase in the T-cell precursor frequency (i.e., to 1 in 12). Although high numbers of proT1 and proT2 cells were generated after, a considerably lower T-cell precursor frequency was found after 14 days of culture (1 in 92); this correlated with the high number of NK-like cells observed after prolonged culture. Likewise, exposure to DL-4 produced a multilog-fold increase of T-cell precursors, whereas 58 T-cell precursors were found in 2×10^4 noncultured $CD34^+$ CB cells, 13.4×10^3 T-cell precursors were generated during a 7-day DL-4 culture (corresponding to a 230-fold increase [Table 1, third row]).

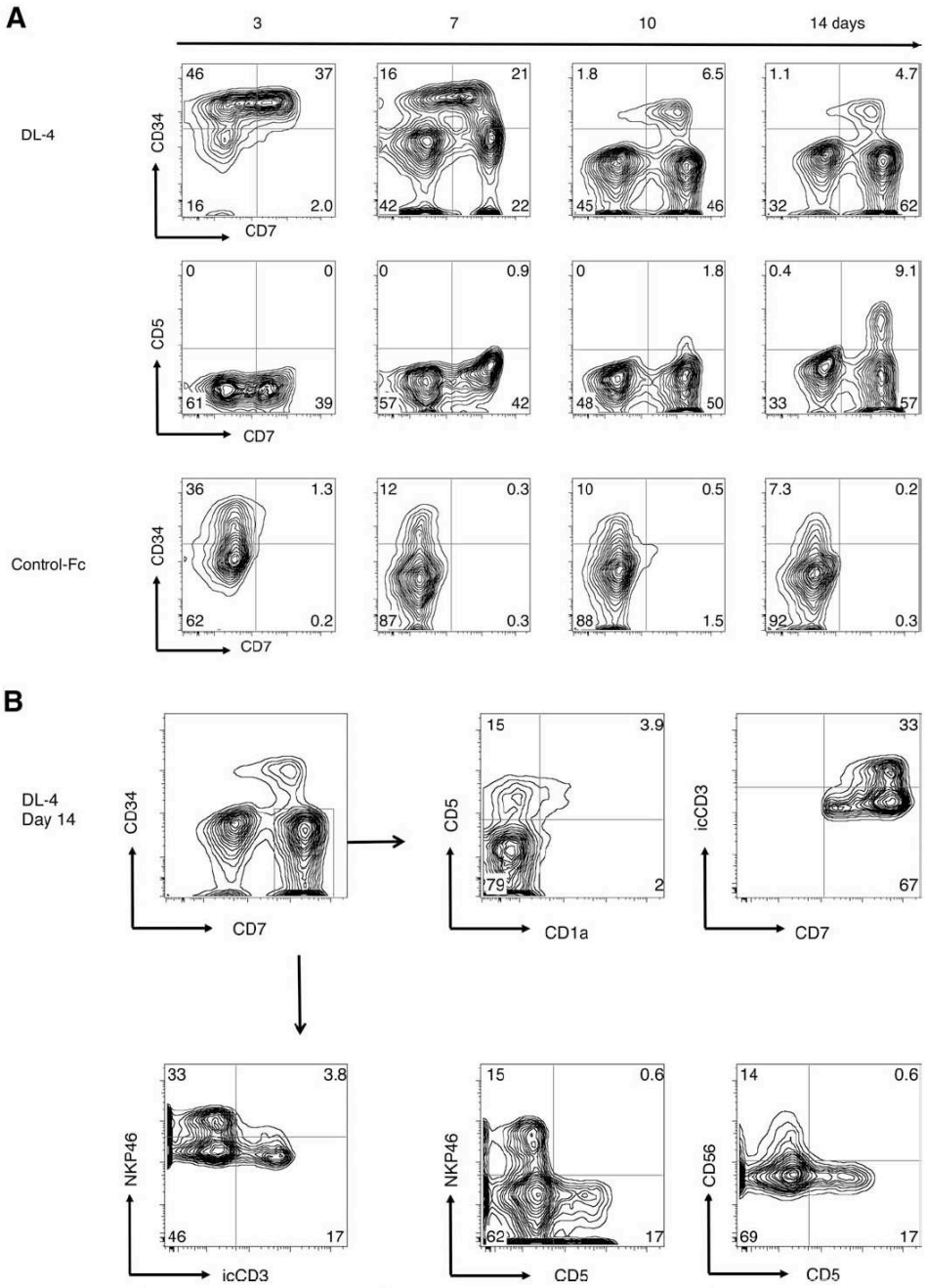

Figure 1. Emergence of $CD7^+$ cells after exposure of $CD34^+$ cells to immobilized delta4. (**A**): $CD34^+$ cord blood cells were plated into dishes precoated with either DL-4 (upper lines) or control-Fc (lower line) and cultured for 14 days. Cultured cells were analyzed by flow cytometry for surface expression of CD34, CD7, and CD5 (as early markers of T-cell commitment) at the indicated time points. (**B**): Flow cytometry analysis of DL-4 cells from a 14-day culture. $CD34^-/CD7^{++}$ cells were analyzed for surface expression of CD5, CD1a, NKP46, CD56, and intracellular expression of CD3epsilon. Abbreviation: DL-4, delta-like-4.

To identify the cell subset responsible for the marked increase in T-cell precursor frequency, serial dilutions of the distinct $CD34^+/CD7^-$, ETP, and proT1 DL-4 cell subsets from day 7 cultures were expanded in OP9/DL-1 cocultures, as described above. The $CD34^+/CD7^-$ cell population displayed much the T-lymphoid potential as untreated $CD34^+$ cells (1 in 248). In contrast, ETPs and proT1 cells displayed much higher T-cell precursor frequencies (1 in 14.9 and 1 in 7.5, respectively) (Supporting Information Table S2). When injected into 4-week-old sublethally irradiated NSG mice, ETP and proT1 DL4 cells were able to reconstitute the thymus in three out of four mice as compared to none of the mice (0/2) injected with $CD34^+/CD7^-$ DL-4 cells (Supporting Information Fig. S2A). Interestingly, ETP and pro-T-cells

Table 1. Exposure to DL-4 increases the T-cell precursor frequency of $CD34^+$ cells

DL-4 culture time in days	0	7	14
T cell precursor frequency[-1a]	349 [319–379]	12 [9.5–15.6]	92 [83–100]
$CD34^+$ or DL-4 cell numbers[b]	2×10^4	1.5×10^5 [$1.0–1.7 \times 10^5$]	7.0×10^5 [$5.8–8.7 \times 10^5$]
ETP cell numbers[b]	170 [160–180]	5.0×10^4 [$3.3–6.7 \times 10^4$]	5.3×10^4 [$4.8–5.8 \times 10^4$]
Absolute T cell precursor numbers[c]	58	13.4×10^3	7.5×10^3

[a]T-cell precursor: a cell able to generate $CD4^+/CD8^+$ or $CD3^+/TCR\gamma\delta^+$ T cells in an OP9/DL-1 coculture. The precursor frequency is the median [interquartile range] from three independent experiments. The quoted numbers of DL-4 cells were generated from 2×10^4 $CD34^+$ cord blood (CB) cells.
[b]The number of DL-4 cells generated from 2×10^4 $CD34^+$ CB cells.
[c]The number of $CD34^+$ or DL-4 cells (second row) divided by the corresponding T-cell precursor frequency (first row).
Abbreviations: DL-4, delta-like-4; ETP, early thymic progenitor.

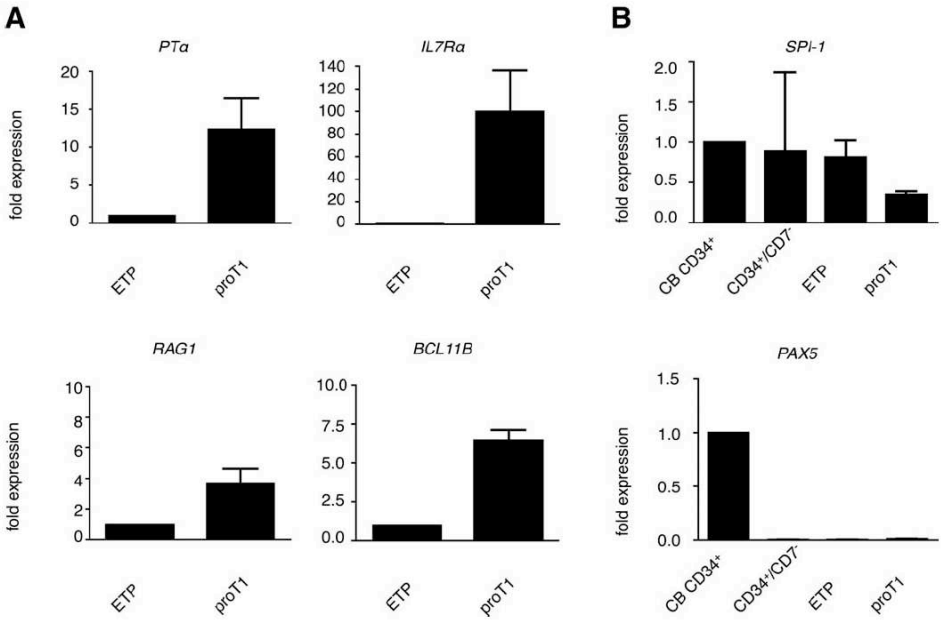

Figure 2. The gene expression profile of delta-like-4 (DL-4) cell subsets. (**A**): TaqMan polymerase chain reaction gene expression analysis in $CD34^+/CD7^-$, ETP, and proT1 DL-4 subsets generated after 7 days of culture. Transcript levels for the indicated genes were normalized against human GAPDH expression. Expression of T-cell developmental genes within the $CD34^+/CD7^-$ subset was either not detected (*PTα*) or negligible (*IL7Rα, RAG1*, and *BCL11B*). Levels found in the proT1 population were compared with those in the ETP subset. The columns represent the median-fold expression values obtained in three independent experiments. Bars indicate the interquartile range. (**B**): Transcript levels for the indicated genes were normalized against human *GAPDH* expression. Gene expression levels found in DL-4-primed cell populations were compared with the initial expression levels of *SPI-1* and *PAX5* in noncultured $CD34^+$ cells. The columns represent median-fold expression values obtained in three independent experiments. Bars indicate the interquartile range. Abbreviations: BCL11B, B-cell lymphoma/leukemia 11B; ETP, early thymic progenitor; IL7Rα, interleukin 7 receptor alpha; PTα, pre-T-cell receptor alpha; RAG1, recombination activating gene 1; SPI-1, spleen focus forming virus proviral integration oncogene 1, PAX5, paired box protein 5; GAPDH, glyceraldehyde-3-phosphatase dehydrogenase.

retained the ability to produce B and myeloid cells (data not shown). This finding formally demonstrates that the increased T-lymphoid potential of DL-4 cells is correlated with their CD7 expression. Furthermore, mature $CD4^+/CD8^+$ double-positive cells and $\gamma\delta TCR^+/CD3^+$ T cells emerged from ETPs and proT1 cells within 2 weeks, whereas an additional week was necessary to produce mature T cells from $CD34^+CD7^-$ DL-4 cells or uncultured $CD34^+$ cells (Table 2). These results demonstrate that the ETP and proT1 progenitor subsets correspond to a more advanced T-cell developmental stage than the $CD34^+/CD7^-$ subset.

DL-4 Progenitors Seed the Thymus and Give Rise to mature, Circulating T-Lymphocytes In Vivo

The T-cell potential of day-7 DL-4 cells was further investigated in two different xenotransplantation models. Four-week-old, sublethally irradiated NSG mice and newborn, nonirradiated NSG mice were injected (intravenously and intrahepatically, respectively) with either day-7 DL-4 cells (after exclusion of myeloid cells by FACS) or untreated $CD34^+$ cells. Analyses were performed at 8 weeks or 4 weeks post-transplant, respectively.

In the adult cohort, we found thymic engraftment in 10 of the 12 mice injected with DL-4 cells, six of the seven mice injected with noncultured $CD34^+$ cell, and none of the mice injected with cells from control-Fc cultures. In the nonirradiated newborn transplantation model, thymic engraftment was observed in all 10 DL-4 recipients but only seven of the 12 recipients of noncultured $CD34^+$ cells. Intrathymic T-cell development was more advanced in recipients of DL-4-cells, as suggested by higher percentages of mature $CD3^+/TCR\alpha\beta^+$

Table 2. The kinetics of T-cell differentiation for noncultured $CD34^+$ cells and progenitor subsets retrieved from a DL-4 culture

Days of OP9/DL-1 coculture	Phenotype %	Noncultured $CD34^+$	DL-4 progenitor subset		
			$CD34^+/CD7^-$	ETP	proT1
7	CD7/CD5	6.5	0.9	36.6	81.6
	CD5/CD1a	0.7	0.3	8.1	51.5
14	CD7/CD5	61.4	25.9	95	97
	CD5/CD1a	4.2	5.7	38	31
	TCRγδ/CD3	0	0	0.35	0.9
	CD4/CD8	0	0	3.3	3.8
21	CD7/CD5	86	60	96	96
	CD5/CD1a	15	32	50	68
	TCRγδ/CD3	0	0.95	1.5	7.8
	CD4/CD8	5.0	7.8	27	62

DL-4, delta-like-4; ETP, early thymic progenitor.

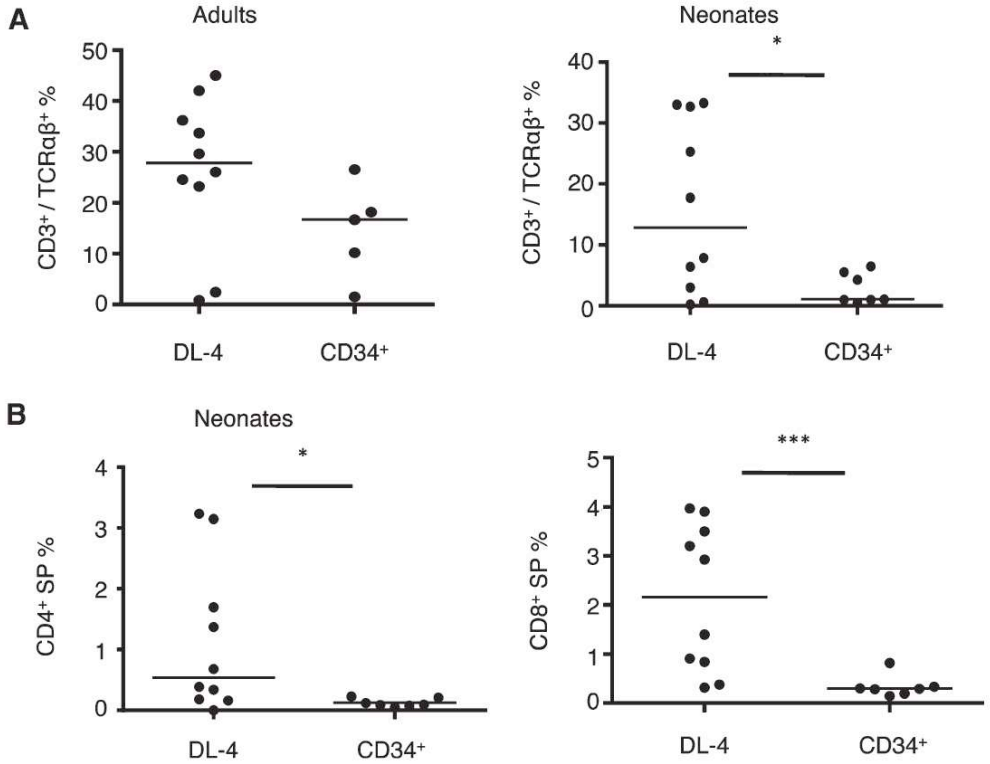

Figure 3. DL-4 cells can reconstitute the thymus in NOD/SCID/$\gamma c^{-/-}$ (NSG) mice. Human cord blood (CB) $CD34^+$ cells were exposed to DL-4 in culture for 7 days. The DL-4-primed cells were sorted by flow cytometry, as described in Materials and Methods. Four-week-old irradiated NSG mice or nonirradiated newborn NSG mice were transplanted with 1.5×10^5 noncultured $CD34^+$ CB cells (adult: $n = 7$, newborn: $n = 12$) or 5×10^5 sorted DL-4 cells (adult $n = 12$, newborn: $n = 10$) in three independent experiments. (**A**): Mice were sacrificed after 8 weeks and human $CD45^+$ thymocytes were quantified by flow cytometry in each of the three groups. Positive thymic engraftment was defined as $>2.5 \times 10^3$ $hCD45^+$ cells per thymus. Mice with positive thymic engraftment (adult: 10/12 in DL-4 vs. 6/7 in $CD34^+$ group; newborn: 10/10 in DL-4 vs. 7/12 in $CD34^+$ group) were further analyzed for CD4, CD8, CD3, and TCRαβ expression by gating on $hCD45^+/7AAD^-$ cells. The figure shows the percentage of $CD3^+/TCR\alpha\beta^+$ cells present in individual adult (left) and newborn (right) mice. Each point indicates a percentage observed in an individual mouse. Bars represent median values. *, $p < .05$. Data were combined from three independent experiments. In one of the untreated $CD34^+$ recipients, $CD3^+/TCR\alpha\beta^+$ could not be analyzed due to technical reasons. (**B**): Percentage of $CD4^+$ single-positive (SP) cells and $CD8^+$ SP cells found in the thymus of individual newborn NSG mice. Horizontal bars represent median values, *, $p < .05$; ***, $p < .001$. Abbreviation: DL-4, delta-like-4.

cells, CD4 single-positive (SP) cells, and CD8 SP cells (Fig. 3A, 3B and Supporting Information Fig. S2B). In nonirradiated newborn NSG mice, the low degree of BM chimerism (2%) contrasted with high thymic engraftment (38%) and suggested BM-independent repopulation of the thymus by DL-4 cells.

In the cohort of irradiated adult mice, mature T cells were detected in the spleen of four of the 12 DL-4 recipients but in only one of the seven noncultured $CD34^+$ recipients. Furthermore, circulating mature T cells were found in the peripheral blood of three DL-4 recipients and none of the noncultured

Table 3. Kinetics of thymic reconstitution after transfer of DL-4 cells or untreated $CD34^+$ cells in nonirradiated newborn NOD/SCID/$\gamma c^{-/-}$ mice

	Days post-transplant	7	14	21	28
DL-4	Human $CD45^+$ cells[a]	3/3	5/6	6/6	10/10
	T-cell differentiation[b]	1/3[c]	4/6	5/6	10/10
$CD34^+$	Human $CD45^+$ cells	0/2	0/6	3/6	7/12
	T-cell differentiation	0/2	0/6	2/6	7/12

[a]Proportion of mice with human intrathymic $CD45^+$ cells.
[b]Defined by the presence of intrathymic human $CD7^{++}/CD5^+/CD1a^+$ preT cells, Supporting Information Fig. S2C for descriptive phenotype.
[c]Early T-cell development at day 7 defined by presence of intrathymic human $CD7^{++}/CD5^+$ proT2 cells, Supporting Information Fig. S2C for descriptive phenotype.
Abbreviation: DL-4, delta-like-4.

$CD34^+$ cell recipients. These results demonstrate the DL-4 cells' ability to give rise to circulating mature T cells in vivo within 2 months. In both adult and newborn recipients, most of the thymic $CD3^+$ T cells belonged to the αβ lineage (Supporting Information Fig. S2B) and displayed a polyclonal TCRβ VJ recombination pattern (Supporting Information Fig. S3).

DL-4 Progenitors Seed the Thymus Faster than Untreated $CD34^+$ Cells

To test the kinetic of thymic human engraftment and T-cell differentiation in vivo, we performed serial analysis in a large cohort of NSG neonates ($n = 29$) transplanted with DL-4 progenitors or untreated $CD34^+$ cells. As depicted in Table 3, first signs of thymic engraftment with human cells could be detected in DL-4 recipients as soon as day 7 post-transplant. The majority of DL-4 recipients exhibited a robust ongoing intrathymic T-cell differentiation at day 14 (presence of intrathymic $CD7^{++}/CD5^+/CD1a^+$ preT cells and in some case $CD4^+/CD3^+$ cells) (Supporting Information Fig. S2C). In contrast, thymopoiesis did not occur before day 21 post-transplant in recipients of untreated $CD34^+$ cells (Table 3).

Cotransplantation of DL-4 Progenitors and Untreated $CD34^+$ Cells Promotes Thymopoiesis and Accelerates Peripheral T-Cell Reconstitution

As described above, DL-4 cells were able to seed the thymus and give rise to mature circulating T cells when transferred into two different NSG mouse models of transplantation. Moreover, we found that T-cell reconstitution was faster in mice injected with DL-4 progenitors than in animals having received untreated $CD34^+$ cell recipients. However, in the experiments presented above, mice received either sorted DL-4 cells or noncultured $CD34^+$ cells. This transplantation condition differs markedly from a clinical CB transplantation setting, in which in vitro-generated, DL-4 cells would be injected in addition to untreated $CD34^+$ cells. To further explore the in vivo T-lymphoid potential of DL-4 progenitors in a setting that more closely resembled clinical conditions, we cotransplanted 1.5×10^5 untreated $CD34^+$ cells and 5×10^5 DL-4 progenitors from non-HLA-matched donors into 4-week-old NSG mice. Eight weeks after transplantation, we found thymic engraftment in all mice coinjected with DL-4/$CD34^+$ cells. In the latter animals, levels of thymic engraftment were higher (Fig. 4A) and intrathymic T-cell development was more advanced (Fig. 4B) than in animals injected with untreated $CD34^+$ cells. Two months after transplantation, we found mature T cells in the spleen of four of the 6 DL-4/$CD34^+$ recipients, but only in four of the 12 DL-4 cell recipients and one of the seven mice injected with untreated $CD34^+$ cells (Supporting Information Fig. S4A). Total intrasplenic T-cell counts (median [interquartile range]) in DL-4/$CD34^+$ recipients were considerably higher than in DL-4 recipients (8.4×10^4 [$5.8 \times 10^4 - 4.4 \times 10^5$] vs. 0.4×10^4 [$0.4–5.4 \times 10^4$], respectively) and reached levels similar to those usually obtained at 12 weeks post-transplantation in $CD34^+$ cell recipients (8.5×10^4 [0.3–18.4]). We observed T cells in the peripheral blood of four of the six mice coinjected with DL-4/$CD34^+$ cells. Upon polyclonal in vitro stimulation, T cells derived from spleens of DL-4/$CD34^+$ reconstituted recipients were able to produced IFN-γ (Fig. 4D).

Four recipients received DL-4 cells from an HLA-$A2^-$ donor and untreated $CD34^+$ cells from an HLA-$A2^+$ donor. In this system, the engrafting cells' expression (or not) of HLA-A2 identified them as being derived from DL-4 progenitors or untreated $CD34^+$ cells. At the time of analysis, the vast majority (>97%) of engrafted human $CD45^+$ cells in the BM, spleen, and peripheral blood were progeny of the untreated $CD34^+$ cell fraction (Fig. 4C and Supporting Information Fig. S4B). In contrast, 89% of splenic T cells and 78% of peripheral blood T cells were found to progeny of the DL-4 cell fraction (Fig. 4C and Supporting Information Fig. S4B). Taken as a whole, these data show that the DL-4 cells' potential for promoting T-cell reconstitution is enhanced when they are cotransplanted with untreated $CD34^+$ cells.

Discussion

In this study, we showed that brief exposure to immobilized DL-4 specifically induces a T-cell differentiation program in CB-derived $CD34^+$ cells and thus enables the efficient generation of early T-cell progenitors in a feeder-cell-free culture system. There was strong evidence to suggest that DL-4 cells are indeed early T-cell progenitors, in view of their immunophenotype, gene expression pattern, TCR rearrangement pattern, and, most importantly, ability to accelerate T-cell differentiation both in vitro and in vivo. Strikingly, DL-4 cells adoptively transferred into NSG mice were able to repopulate the thymus, accelerate de novo thymopoiesis, give rise to mature peripheral T cells, and significantly quicken T-cell reconstitution.

In human thymopoiesis, ETP cells are the most immature T-cell-engaged population and account for less than 0.5% of the immature double-negative compartment [27–30]. We showed that 7 days of DL-4 culture particularly enabled the enrichment of this rare cell type. ETP and proT1 cells generated after 7-day culture did not express CD1a, had not undergone TCR rearrangements, and retained in vivo non-T-lymphoid potential, defining them as T-cell-engaged progenitors but not fully committed precursors. This is consistent with previous findings demonstrating that early stages of T-cell differentiation harbor plasticity for other lineages when placed in appropriate conditions [31]. Acquisition of the ETP and proT1 phenotypes was correlated with higher levels of the direct Notch targets *IL7Rα* and *PTα* [32–35] as well as of *RAG1*, which is essential for correct expression of a pre-TCR. Likewise, we found upregulation of BCL11B, which is involved in the survival and proliferation of T-cell precursors prior to β-selection [36–39]. The TCR rearrangement events found after long culture times were correlated with the appearance of proT1, proT2, and preT cells. These three cells populations had much the same TCR patterns as we (unpublished data) and others have observed for native thymopoiesis [32].

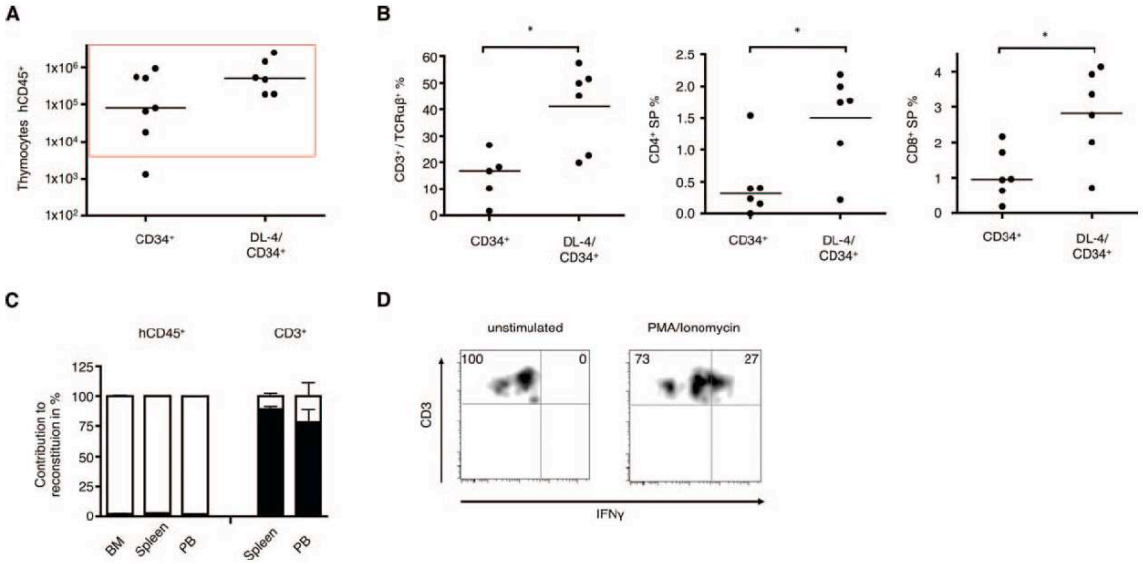

Figure 4. Cotransplantation of DL-4-primed progenitors and untreated $CD34^+$ cells promotes thymopoiesis and accelerates peripheral T-cell reconstitution. (**A**): Four-week-old, sublethally irradiated NSG mice were coinjected with 5×10^5 DL-4 cells (corresponding to the progeny of 7×10^4 $CD34^+$ cells) and 1.5×10^5 noncultured $CD34^+$ cells. Mice were sacrificed after 8 weeks and human $CD45^+$ thymocytes were quantified by flow cytometry. Positive thymic engraftment was defined as $>2.5 \times 10^3$ $hCD45^+$ cells per thymus (specimens within a red rectangle). Thymic reconstitution after cotransplantation was compared with that seen in mice injected with noncultured $CD34^+$ cells alone. Horizontal bars indicate median values. (**B**): Analysis of intrathymic T-cell development in mice injected with noncultured $CD34^+$ cells ($n = 7$, three independent experiments) or DL-4/$CD34^+$ cells ($n = 6$, two independent experiments). The figure shows the percentage of $CD3^+/TCR\alpha\beta^+$ (left), $CD4^+$ SP (middle), and $CD8^+$ SP (right) cells found in individual NSG mice, with the median percentage indicated by horizontal bars. *, $p < .05$. In one of the untreated $CD34^+$ recipients, $CD3^+/TCR\alpha\beta^+$ could not be analyzed due to technical reasons. (**C**): Four mice were coinjected with HLA-$A2^-$ DL-4 cells and HLA-$A2^+$ noncultured $CD34^+$ cells. The relative contribution of the DL-4 cells and $CD34^+$ cells to either $CD45^+$ hematopoietic or $CD3^+$ T-cell reconstitution was determined by quantifying HLA-$A2^-$ cells (black bars) and HLA-$A2^+$ cells (white bars) within a given population. Data are represented as the mean percentage ± SD. (**D**): Splenic T cells detected in cotransplanted mice were cultured with IL-2 and IL-7 for 7 days, then stimulated with PMA (50 ng/ml) and ionomycin (1 mg/ml) for 3 hours and assayed for intracytoplasmic IFN-γ expression. Abbreviations: BM, bone marrow; DL-4, delta-like-4; SP, single positive; PB, peripheral blood; PMA, phorbol mystirate acetate.

Induction of CD7 expression within the ETP and proT1-like DL-4 progenitor cells correlated with greater in vitro and in vivo T-cell potential. Furthermore, ETP and proT1 cells sorted from day 7 DL-4 cultures were able to complete in vitro T-cell differentiation (in secondary OP9/DL-1 coculture) to a higher extent than $CD34^+/CD7^-$ DL-4 cells or untreated CB $CD34^+$ cells did. This observation provides further functional evidence of an advanced T-cell development stage. Taken as a whole, these in vitro data clearly show that the DL-4 culture described herein recapitulate the early T-cell development stages of native thymopoiesis (as has already been described in OP9/DL-1 cocultures [12, 13]).

At the pre-β-selection stages, the T-cell differentiation of DL-4-progenitors followed the same differentiation pattern as reported in the literature for OP9/DL-1 cocultures [12, 33] and in our own unpublished observations. In contrast to OP9-DL-1 cocultures (which support the generation of mature T cells in vitro), the T-cell differentiation induced by DL-4 is blocked at the preT cell stage. This agrees with previous demonstrations in which signals other than Notch1 activation (i.e., stimulation of CXCR4 by SDF1a and activation of the Wnt pathway) are required to successfully pass through the β-selection checkpoint [34, 35]. These signals are partially secreted/expressed by OP9/DL-1 cells [40, 41]. Stimulation of CXCR4 and Wnt signaling in a murine DL-4 culture was found to partially overcome the pre-β selection block [34].

This particularity of immobilized Notch-ligand culture systems probably also explains the emergence of an NK-like population in the DL-4 culture. T and NK cells share early, Notch-dependent differentiation steps [42]. Although the $CD7^{++}$ progenitors observed in both OP9/DL-1 coculture and native thymopoiesis have T/NK potential in vitro, the emergence of a substantial NK-subset is found in neither OP9/DL-1 nor the normal thymus, possibly due to the presence of further biological signals [12, 13, 41, 42]. Given the absence of such signals (i.e., Wnt signaling and CXCR4 signaling) in DL-4 culture, we consider that the NK cells emerged from the $CD7^{++}$ bipotent T/NK progenitor via a biased differentiation pathway. The occurrence of NK cells in feeder-cell-free Notch-ligand cultures has been reported and was attributed to a lack of Wnt signaling [17, 41, 42]. In our opinion, the occurrence of biased NK cells after prolonged DL-4 culture (i.e., ≥10 days) provides a further argument for using day 7 DL-4 cells culture in transplantation experiments.

Long-term DL-1 culture (i.e., for more than 2 weeks) enables the relatively specific in vitro expansion of $CD34^+$ CB cells [20]. This contrasts markedly with DL-4 culture in which $CD34^+$ expression decreases after 1 week. ETP, ProT1, and NK-biased $CD56^+$ cells also appear in DL-1 cultures, albeit at lower frequencies and at later culture time points. These findings indicate that DL-1 and DL-4 differ in their ability to induce the generation of immature hematopoietic cells and T-cell-engaged cells, respectively. Recent findings provide a partial biological explanation for this difference. It was recently found that the DL-1-dependent expansion of $CD34^+$ hematopoietic progenitors was mediated by Notch-2 [43]. In contrast, T-cell development is strictly dependent on Notch-1 signaling [19, 20]. Furthermore, DL-4 was unambiguously found to be the essential Notch1 ligand in thymopoiesis and more potent than DL-1 for inducing the T-cell differentiation program [22, 44, 45]. These arguments sustained our choice to use DL-4 and a short culture period for this protocol.

The ability of adoptively transferred, in vitro-generated murine T-cell precursors to promote T-cell reconstitution in an allotransplant model has already been unambiguously demonstrated [15, 16]. Likewise, human T-cell progenitors generated from CB cells or mobilized HSCs on feeder cells expressing Notch ligands can transiently promote and accelerate thymopoiesis in humanized mice [12, 13]. When human $CD34^+$ cells were expanded on plate-bound DL-1, only the injection of very high cell numbers was capable of achieving thymic engraftment in NSG mice [17]. However, none of these studies described positive effects on peripheral T-cell reconstitution [12, 13, 17].

Our present results demonstrate that DL-4-primed cells displayed greater thymopoietic potential after adoptive transfer into NSG mice. Intrathymic T-cell differentiation was faster in DL-4 injected mice than in mice injected with noncultured $CD34^+$ cells. Importantly, T cells derived from injected, DL-4-exposed T-cell progenitors displayed a conventional and polyclonal CD4, CD8, and TCRαβ profile.

T-cell progenitors generated during DL-4 culture accelerated thymopoiesis to the same extent as OP9-DL-1-generated T-cell progenitors did (data not shown). Furthermore, we detected a higher degree of peripheral T-cell reconstitution in DL-4 recipients 2 months after transplantation; this constitutes a truly novel feature of the DL-4 expansion. Thymopoiesis and peripheral T-cell reconstitution were still further improved when DL-4 cells were coinjected with untreated $CD34^+$ cells. The DL-4 cells had selectively repopulated the T-cell compartment by 2 months post-transplantation, whereas the untreated $CD34^+$ cells had almost exclusively reconstituted the other hematopoietic compartments. The clearly improved T-cell recovery after cotransplantation highlights the synergy between the DL-4 primed unit and the untreated CB unit. We presume that (a) DL-4 T-cell progenitors instantly provide a wave of thymopoiesis and T-cell regeneration and (b) long-term T-cell development is sustained by the untreated CB unit, which can sustain T-cell progenitors generation in the recipient's bone marrow.

Just 1 week of DL-4 culture generated enough T-cell progenitors to envisage their adoptive transfer in vivo. In contrast to OP9-DL-1 system, the proposed DL-4 system does not contain genetically modified murine cells and thus circumvents a considerable safety risk. The use of magnetically sorted $CD34^+$ cells (as routinely performed in haploidentical human transplantation settings), with no enrichment of the more immature subsets (i.e., $CD34^+/CD38^-$) resolves a further obstacle to human application. The cotransplantation design mimics HLA-mismatched dUCBT quite closely and suggest a potential clinical application. In such a situation, DL-4 culture could be initiated with a single CB unit during 7 days, with infusion of the culture product at the same time as (or shortly after) transplantation of the untreated CB unit. In this straightforward instance, the use of DL-4 T-cell progenitors would advantageously bypass the need for de novo T-cell progenitor generation in the patient's BM; this would quicken immune reconstitution after HSCT and should reduce post-transplantation morbidity and mortality due to infections.

In addition to this direct therapeutic approach, other diseases characterized by impairments of early intrathymic T-cell development and alterations in thymic structure could potentially be treated with DL-4-primed progenitors. The latter are capable of inducing thymopoiesis even in the highly disturbed thymic microenvironment of irradiated, immunodeficient mice. In severe combined immunodeficiencies, the absence of a physiological interaction between thymic progenitors and the thymic stroma means that thymopoiesis is still disturbed after HSCT [46, 47]. Likewise, thymopoiesis is affected in chronic inflammatory disorders, HIV infection, and in the aftermath of cytotoxic, pretransplantation conditioning [8, 48–50]. Given the DL-4 primed progenitors' potential to bypass the initial stages in thymic T-cell differentiation, one can legitimately hypothesize that patients affected by these conditions would benefit from treatment with in vitro-generated progenitors. The presence of a great number of functional T-cell progenitors could provide a physiological stimulus to the altered thymic stroma and thereby help regenerate thymic function and organization.

Conclusions

This study first described a feeder-cell-free Notch-ligand culture system specifically for the rapid generation of human T-cell progenitors in vitro. Moreover, we provide the first evidence that human in vitro-generated T-cell progenitors can further differentiate into mature, polyclonal, functional peripheral T-cells in the perturbed thymic microenvironment of the NSG mouse. Coinjection of DL-4-primed and untreated $CD34^+$ CB cells further improved T-cell recovery under experimental conditions that quite closely mimic the clinical setting of dUCBT. This provides a strong argument for the therapeutic potential of DL-4 cells.

Acknowledgments

We thank Julien Rouiller, Alexandrine Garrigue for technical assistance, Olivier Pellé for cell sorting, Nicolas Montcuquet for NK staining, and Kamel Hadboun for animal care. Recombinant human IL-7 was a kind gift of Cytheris, Issy-les-Moulineaux, France. This work was supported by grants to M.C.C. and I.A.S. from DHOS/INCA/INSERM, the Fondation pour la Recherche Médicale (FRM), Cell-PID (Advanced Cell-based Therapies for the Treatment of Primary Immunodeficiency, FP7-HEALTH-2010), and a Regenerative Therapy ERC Advanced Grant (FP7 2011). C.R. was funded by postdoctoral scholarships from the Deutsche Forschungsgemeinschaft (DFG) and the FRM. A.S. received a doctoral scholarship from the Citta della Speranza.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Supplemental Results

Figure S1A

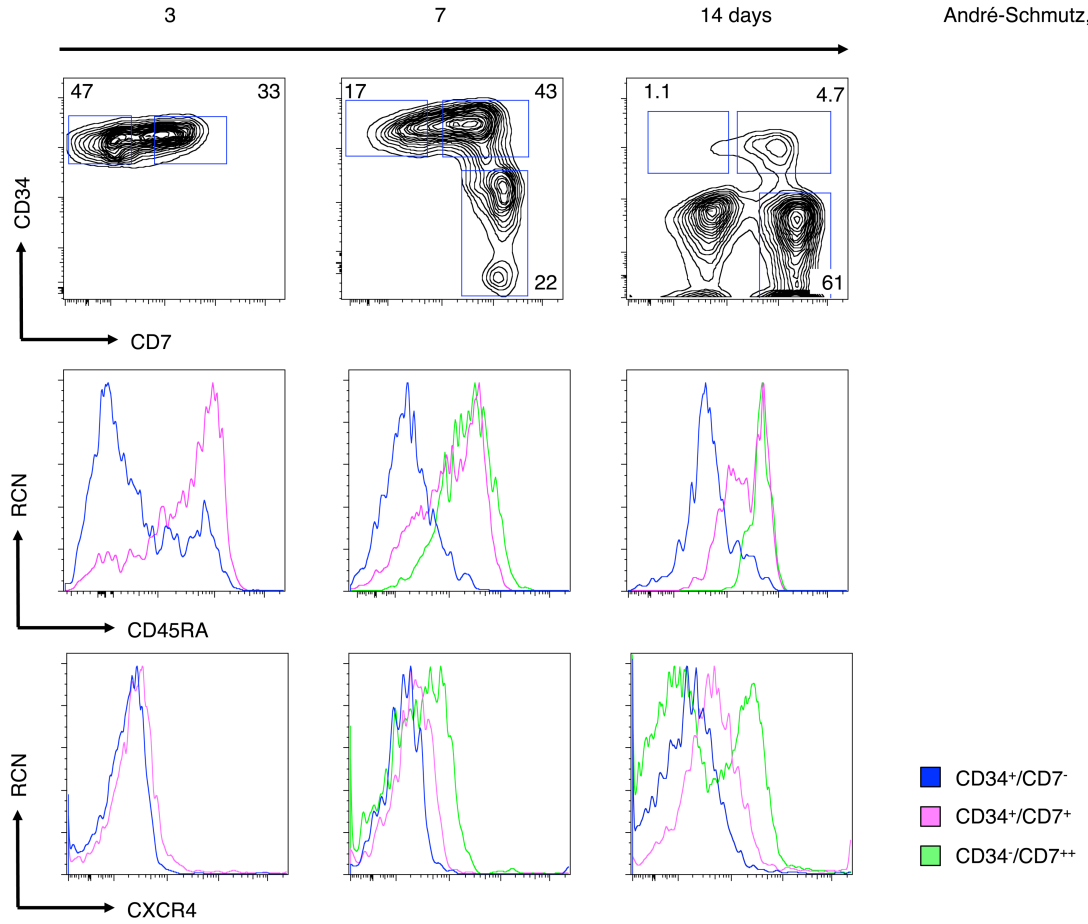


Figure S1A: DL-4 cells upregulate CD45RA and CXCR4 during culture A: Phenotypic analysis of CD34 and CD7 expression by DL-4 cells harvested after 3, 7 and 14 days of culture (upper line). The surface expression of CD45RA (middle line) and CXCR4 (lower line) was tested within the $CD34^{+}/CD7^{-}$, $CD34^{+}/CD7^{+}$ (ETP) and $CD34^{-}/CD7^{++}$ (proT) subsets. RCN: relative cell number.

Figure S1B

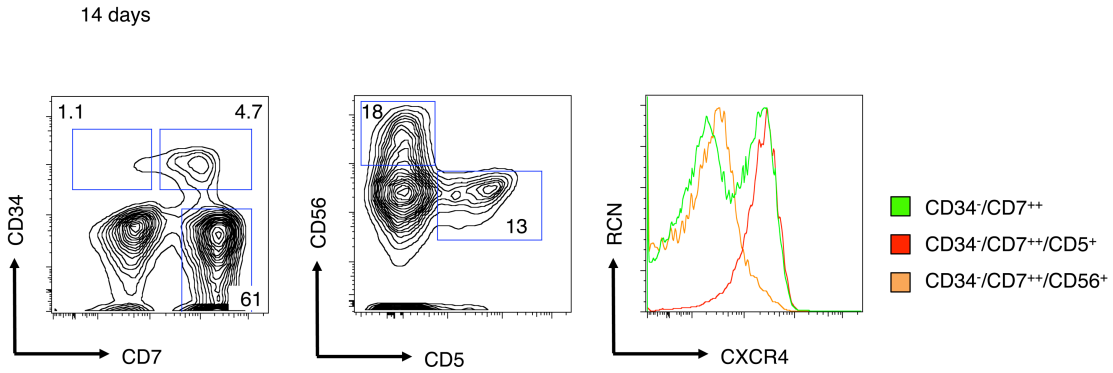


Figure S1B: DL-4 cells upregulate CD45RA and CXCR4 during culture B: Flow cytometric analysis of surface CXCR4 expression, as measured by gating on $CD34^{-}/CD7^{++}$, $CD34^{-}/CD7^{++}/CD5^{+}$ and $CD34^{-}/CD7^{++}/CD56^{+}$ subsets.

Figure S2A

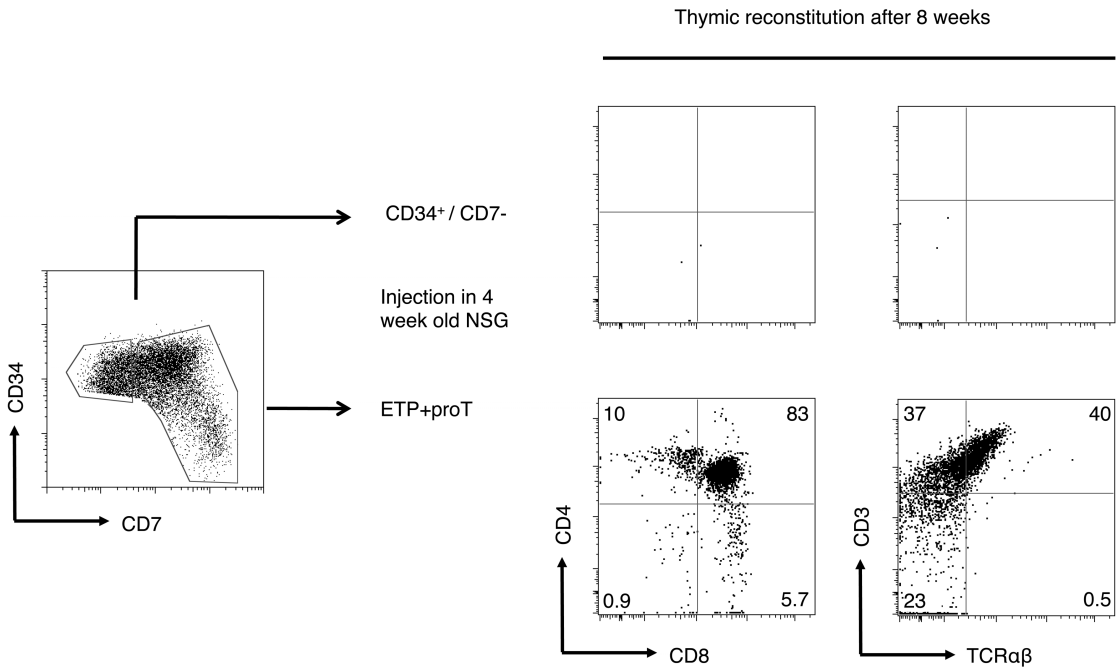


Figure S2B

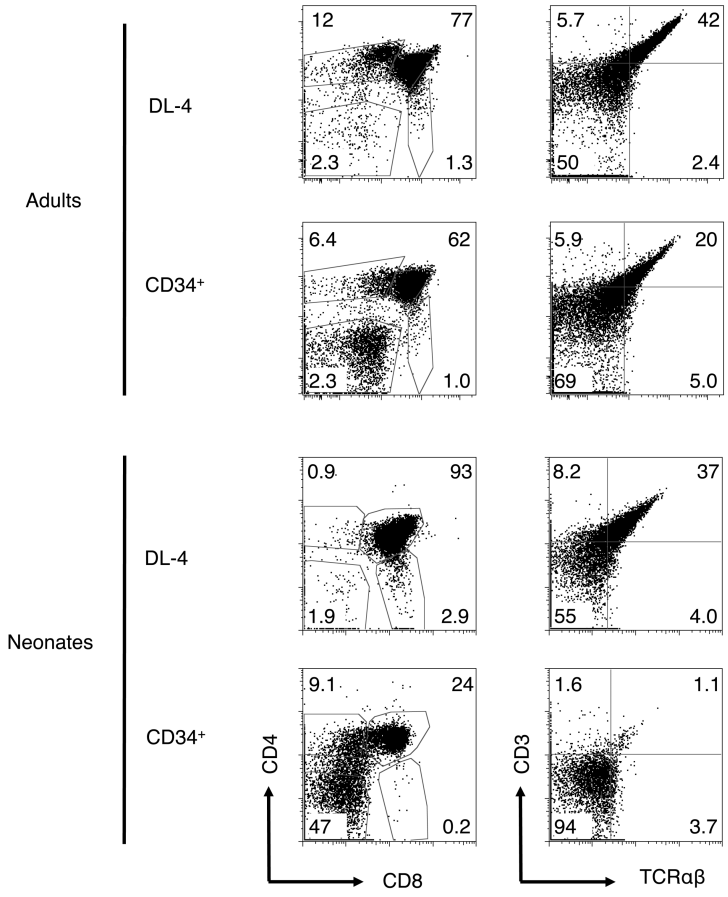


Figure S2: DL-4 cells can reconstitute the thymus when transferred into irradiated adult NOD/SCID/$\gamma c^{-/-}$ (NSG) mice and into non-irradiated, new-born NSG mice and accelerates thymopoiesis *in vivo* A: CD34+/CD7− and ETP /proT1 cells sorted from a 7-day DL-4 culture were transplanted into 4 week old irradiated NSG. Thymic reconstitution was assessed after 8 weeks by flow cytometry. CD4, CD8, CD3 and TCRαβ expression was studied within $hCD45^{+}/7AAD^{-}$ cells. B: Irradiated adult NSG mice (upper group) and non-irradiated newborn NSG mice (lower group) were injected with 5×10^5 sorted DL-4 progenitors or 1.5×10^5 non-cultured $CD34^{+}$ cells. Thymic reconstitution was assessed 8 weeks (in adult recipients) or 4 weeks (in new-born recipients) after transplantation. The figure shows the flow cytometry analysis for thymic reconstitution as described above. C: A total of 29 non-irradiated newborn NSG were transplanted with 5×10^5 sorted DL-4 progenitors or 1.5×10^5 non-cultured CD34+ cells in two independent transplantation series. Results of the thymic reconstitution kinetics in DL-4 versus untreated $CD34^{+}$ cells are described in Table 4. Phenotypic images of representative thymi recovered from DL-4 recipients at 7 and 14 days post-transplant are shown.

Figure S3

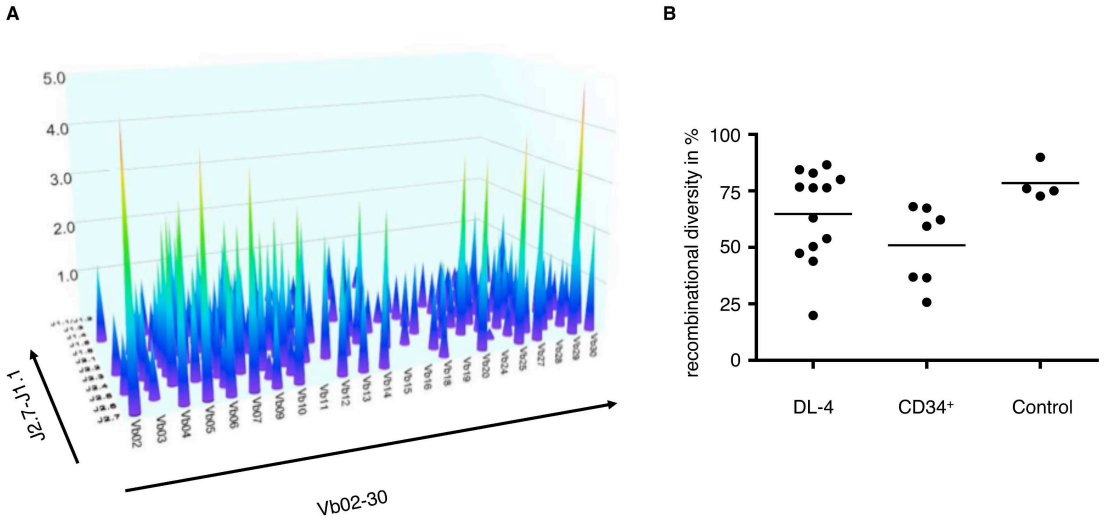


Figure S3: DL-4 cells give rise to polyclonal thymocytes in recipient mice TCRβ VJ rearrangement patterns were analysed for a number of reconstituted thymuses from adult and newborn NSG recipients. A) The TCRβ VJ recombination pattern in a representative thymus reconstituted with DL-4 cells. The image represents the result of an ImmunTraCkeR® Multiplex PCR that detects all recombination events between the TCRβ V and TCRβ J loci. B) The diagram shows the combinatorial diversity of the TCRβ VJ rearrangements in reconstituted thymuses from mice injected with DL-4-cells (left column), non-cultured $CD34^+$ cells (middle column) and control T lymphocytes from healthy donors (right column). Each dot indicates the combinatorial diversity of an individual sample. Bars indicate median values.

Figure S4

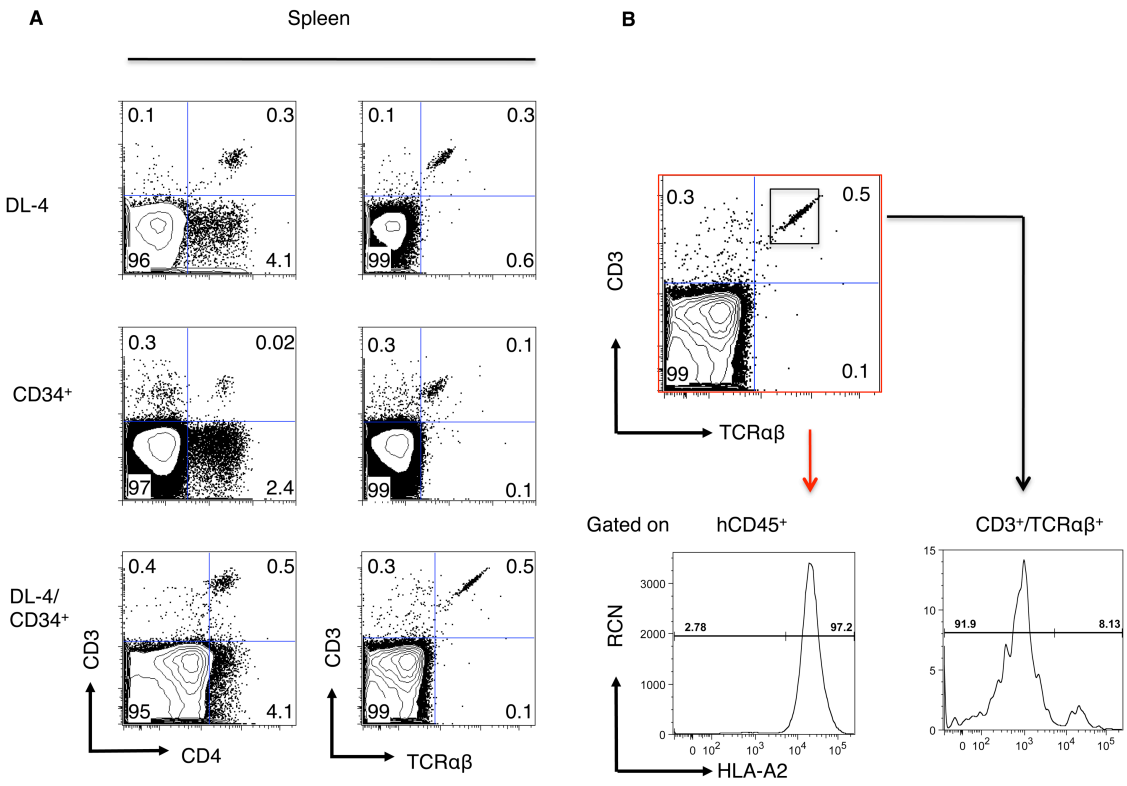


Figure S4: DL-4 cells give rise to peripheral T-cells in recipient mice and specifically reconstitute the T-cell compartment A) Mature T-cells were found in the spleen of 4 out of 12 DL-4 cell recipients, 1 out of 7 non-cultured $CD34^+$ cell recipient and 4 out of 6 mice co-transplanted with DL-4 and $CD34^+$ cells. The Figure shows a flow cytometry analysis of CD3, CD4 and TCRαβ expression in a representative spleen from each type of transplantation. B) Mice were co-injected with $HLA\text{-}A2^-$ DL-4 cells and $HLA\text{-}A2^+$ untreated $CD34^+$ CB cells. The upper panel shows an experiment gated on $hCD45^+$ spleen cells. HLA-A2 expression was examined by gating on the entire $hCD45^+$ spleen cell population (left histogram) and on the splenic $CD3^+/TCR\alpha\beta^+$ T-cells in particular (right histogram). The flow cytometry analysis pictured is representative of three different mice.

	Culture time in days				
TCR rearrangement event	0	7	10	14	18
Dδ2-Dδ3 and Vδ2-Dδ3	-	-	+	+	+
Dδ2-Jδ1	-	-	+	+	+
Vd1-Jd1	-	-	-	+	+
Vγ9 and Vγ11	-	-	-	+	+
Dβ1-Jβ	-	-	-	+	+
Vβ-Jβ2	-	-	-	-	+

Table S1: Kinetics of TCR rearrangement events during DL-4 culture

Progenitor subset[a]	**T cell precursor frequency^{-1}** [b]
CD34$^+$/CD7$^-$	**248** [191-305]
ETP	**14.9** [12.6-17.3]
proT1	**7.5** [7.1-7.9]

Table S2: The increase in T-cell potential after culture with DL-4 correlates with a T-lymphoid precursor phenotype

[a] Sorted CD34$^+$/CD7$^-$, ETP (CD34$^+$/CD7$^+$) and proT1 (CD34$^-$/CD7^{++}) cells from a 7-day DL-4 culture.

[b] T cell precursor: a cell able to generate CD4$^+$/CD8$^+$ or CD3$^+$/TCRγδ$^+$ T-cells in an OP9/DL-1 co-culture. The precursor frequency is reported as the median [interquartile range] value from three independent experiments.

UNPUBLISHED RESULTS

2 COMPARISON OF DL-4 PROGENITORS WITH NATIVE THYMIC PROGENITORS

Supplemental Material and Methods

Material and methods not explained in this chapter are detailed in the corresponding section of the article: "Human T-lymphoid progenitors generated in a feeder-cell-free DL-4 culture system promote T-cell reconstitution in NOD/SCID/$\gamma c^{-/-}$ mice" (page 62).

Cell samples

Cord blood samples were harvested on delivery of full-term, healthy pregnancies at the Intercommunal Hospital (Creteil, France) and the Saint-Vincent de Paul Hospital (Paris, France) after obtaining written, informed consent of the mothers according to French legislation and ethical guidelines. Human thymic tissue was obtained from children aged from 1 d to 10 years of age undergoing cardiac surgery. Experimental procedures with human thymic fragments were approved by the human research Ethics Committees at the Oswaldo Cruz Foundation and the Necker Children's Hospital (Paris, France).

OP9/DL-1 Co-culture

OP9/DL-1 cells were generated by Emmanuelle Six from our working group. In brief, OP9 obtained from the ATCC (CRL-2749, LGC Promochem, Molsheim, France) were transduced with a defective retrovirus containing the human Notch ligand Delta1 and GFP separated by an IRES. T lymphoid potential was assayed by plating around 5000 candidate progenitors per well of a 24-well plate previously coated with OP9-Delta1 stromal cells (using 20,000 cells the day before the co-culture). OP9/DL-1co-culture was performed in α-MEM Medium (Invitrogen, Cergy-Pontoise, France) freshly reconstituted with MilliQ Plus water (18 MΩ) and supplemented with 20% Defined Fetal Bovine Serum (Hyclone, Thermo Fisher Scientific, Illkrich, France) and the following cytokines, 2 ng/ml IL-7, 5 ng/ml FL and 10 ng/ml SCF (R&D Systems, Minneapolis, MN, USA). The stroma was replaced weekly.

Flow cytometry and cell sorting

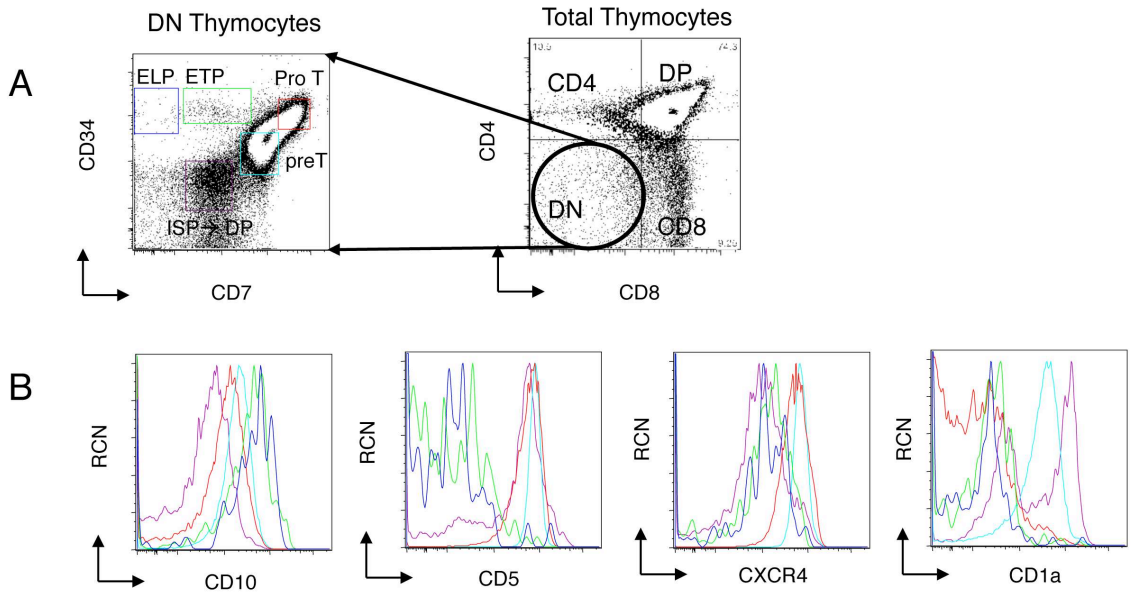
T cell progenitors generated in DL-4 or OP9/DL-1 culture or native thymocyte suspensions were incubated with monoclonal PE-, FITC-, APC-, PECY7-, V450 Horizon-, APC-H7- or Amcyan- conjugated antibodies. Antibodies to the following human antigens were used: CD3ε, CD4, CD8a, CD7, CD5, CD1a, CD45, CD56, NKp46, NKG2D and CXCR4. All

antibodies were purchased from BD Biosciences or Beckman Coulter. Cells were stained with antibodies for 15 minutes at 4°C before being washed. For intranuclear Bcl11b staining, cells were fixed/permeabilized using eBioscience fixation/permeabilization set. $5x10^5$ cells were fixed in fixation buffer at 4°C for 30 minutes and were labelled with an Ab to Bcl11b (Bethyl laboratories, Montgomery, USA) or with a control isotype for 30 minutes at 4°C. For fluorescent detection, a secondary anti-rabbit IgG-Alexa647 antibody was added and incubated for 20 minutes at 4°C. Data acquisition was performed using a FACSCanto II (BD Biosciences). Analysis was performed using Diva® and FlowJo® software.

For cell sorting of human thymic progenitor populations, thymocyte suspensions were stained with CD34, CD7, CD5, CD1a, CD3 and CD4, incubated for 15 minutes at 4°C before being washed. Sorting was performed using a FACS Aria II (BD Biosciences).

2.1 Phenotypical comparison of DL-4 cells with native thymic progenitors

In the human thymus, most immature subsets are found within the $CD34^+$ subset, which comprises around 1% of all thymocytes. After enrichment for $CD34^+$ cells by magnetic bead isolation, these progenitors were further phenotypically analysed using CD34 and CD7 as first differentiation markers. Consistent with previous reports, I found that the majority of $CD34^+$ thymocytes expressed high CD7 levels (Haddad, Guimiot et al. 2006; Hao, George et al. 2008). The $CD7^+$ population could be further divided into a $CD34^+/CD7^{hi}$ and a $CD34^{lo}/CD7^+$ population. A rare population (<1%) of the $CD34^+$ cells expressed intermediate or no detectable levels of CD7. Both the $CD34^+/CD7^-$ and the $CD34^+/CD7^{int}$ population expressed cell surface CD10. Increasing expression levels of CD7 within the $CD34^+/CD7^{int}$ population went along with downregulation of CD10. Progenitors at the $CD34^+/CD7^{hi}$ stage did no longer express CD10 but had become positive for cell surface CD5. CD1a was first expressed in the $CD34^{lo}/CD7^+$ population. Due to non-stringent sorting condition around 10% of post-sort thymocytes did not express CD34. These cells expressed only low levels of CD7. The $CD34^-/CD7^{lo}$ population expressed CD4 and a continuum of CD3 and CD8, indicating, that this subset comprised $CD4^+$ISP and $CD4^+/CD8^+$ DP cells. The stage specific expression of CD10, CD5, CD1a, CD4, CD3 and CD8 within the CD34 and CD7 expressing subsets allowed to phenotypically define these progenitors as ELP ($CD34^+/CD7^-$), ETP ($CD34^+/CD7^{int}$), proT ($CD34^+/CD7^{hi}$), preT ($CD34^{lo}/CD7^+$) and a continuum of ISP and DP cells (referred to as ISP→DP thereafter) ($CD34^-/CD7^{lo}$) cells (Figure 9).



C

Population	CD10	CD5	CD1a	CXCR4
$CD34^+/CD7^-$ (ELP)	+	-	-	- / low
$CD34^+/CD7$int	+	-	-	lo
CD34+/CD7hi (proT)	-	+	-	+
CD34lo/CD7+ (preT)	-	+	+	+
CD34-/CD7lo (ISP→ DP)	-	+	+	low / -

Figure 9: Phenotypical characterization of native thymic progenitor stages

A) Flow cytometric expression pattern of CD34 and CD7 expression in CD34 enriched thymocytes, which allows their sub-fraction into ELP, ETP, proT, preT and ISP→DP cells (left FACS-plot). In total thymocyte suspensions, $CD34^+$ thymocytes are found within the $CD4^-/CD8^-$ DN subset (encircled population in right dot-plot). B) Flow cytometric histograms for the cell surface expression of CD10, CD5, CXCR4 and CD1a within ELP (blue), ETP (green), proT (red), preT (blue) and ISP→DP (purple) cells. C) Comparative phenotypical description of ELP, ETP, proT, preT and ISP→DP cells.

After DL-4 culture, 6 distinct progenitor populations emerged: The $CD34^+/CD7^-$, the $CD34^+/CD7^+$, the $CD34^-/CD7^{++}/CD5^-$, the $CD34^-/CD7^{++}/CD5^+$, the $CD7^{++}/CD5^+/CD1a^+$ and the $CD34^-/CD7^{++}/CD56^+$ subsets, to which we referred to as immature $CD34^+/CD7^-$, ETP, proT1, proT2, preT and NK cells.

In DL-4 progenitors, CD34 was expressed at the ETP stage only but was sharply downregulated upon passage towards the proT1 stage. In contrast thymic proT cells highly

expressed CD34 and its downregulation only occurred at the preT stage. Moreover, the distinction between $CD5^-$ proT1 and a $CD5^+$ proT2 stage was only evident in DL-4 progenitors. In the native thymus, virtually all proT cells expressed high levels of CD5 (Figure 10A).

Given these phenotypical differences, I wanted to provide further evidence for the stage specific T-lymphoid phenotype of DL-4 progenitors and analysed them for intranuclear expression of Bcl11b and cell surface expression of CXCR4. In both DL-4 and native thymocytes, Bcl11b was first detected at the proT stage. Unlike thymic proT cells, which all expressed Bcl11b, DL-4 proT1 cells could be divided in a $Bcl11b^-$ and a $Bcl11b^+$ population. DL-4 proT2-cells were all $Bcl11b^+$ and DL-4 NK cells were all $Bcl11b^-$. In native thymopoiesis Bcl11b expression was maintained during the following preT and ISP→DP stage (Figure 10B left panel).

CXCR4 was upregulated at the proT stage in both DL-4 and native thymic progenitors. As observed for Bcl11b, DL-4 proT1 cells displayed a two-peak CXCR4 expression profile. Within the more advanced DL-4 populations, CXCR4 was only expressed in proT2 cells but not in NK cells. In sorted thymic progenitors, CXCR4 expression levels remained high at the proT and preT stage and declined at the ISP→DP stage (Figure 10 B right panel).

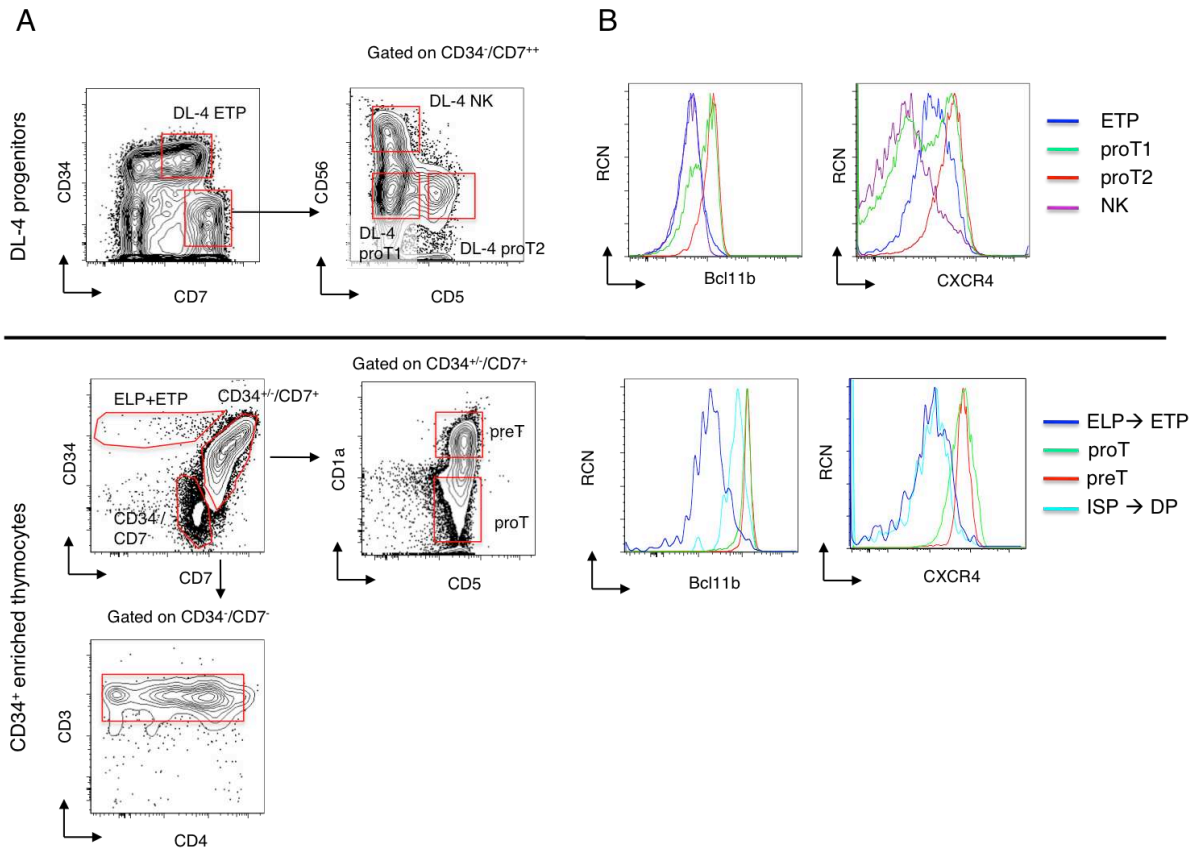


Figure 10: Expression of Bcl11b and CXCR4 in DL-4 progenitors and native thymic progenitor subsets

A: Upper panel: $CD34^+$ CB cells were cultured with DL-4 during 14 days. Based on different CD34, CD7, CD5 and CD56 expression, DL-4 ETP, proT1, proT2 and NK subsets were defined. Lower panel: Based on the distinct expression of CD34, CD7, CD5 and CD1a, CD3 and CD4, CD34 enriched human thymocytes were defined as ELP, ETP, proT, preT and ISP→ DP cells. Since the ELP and ETP fraction represented a very rare population, these two cell types were combined within one gate for further analysis and are referred to as ELP→ETP cell hereafter.

B: Intranuclear Bcl11b expression and cell surface expression of CXCR4 within DL-4 ETP (blue), proT1 (green), proT2 (red) and NK (purple) subsets (upper panel) and within native thymic ELP→ ETP (blue), proT (green), preT (red) and ISP→ DP (cyan blue) subsets (lower panel).

2.2 Native thymic progenitors and DL-4 progenitors have a similar molecular signature

ELP, ETP, proT, preT and ISP→DP populations were sorted for further molecular analysis. Since ELP and ETP fractions represented two very rare populations (less than 0,5% respectively) I combined these two progenitor types within one sorting gate to increase cellular yields (referred to ELP→ ETP thereafter). Gene expression and TCR-rearrangement analysis within the distinct thymic progenitor subsets were performed as described above (page 61-62).

2.2.1 T-lineage gene expression in native thymic progenitors and DL-4 progenitors follows similar kinetics

In native thymic progenitor populations, T-lineage specific genes *IL7Ra, PTa*, *RAG1* and *BCL11b* were all upregulated by a 2-5 fold in the proT stage as compared to the ELP/ETP stage. The further expression of these genes followed two different patterns: *IL7Ra* and *PTa* levels were highest at the preT stage and declined upon transition towards the ISP→DP stage; *RAG1* and *BCL11b* transcripts levels further increased throughout the proT, preT and ISP→ DP stage. *SPI*1 and *PAX*5 (which are involved in myeloid and B-lymphoid development) were abruptly downregulated at the proT stage (Figure 11).

In DL-4 progenitors, *IL7Ra, PTa*, *RAG1* and *BCL11B* transcripts were absent (or present at very low levels) in the immature $CD34^{+}/CD7^{-}$ subset. *IL7Ra, PTa*, *RAG1* and *BCL11B* genes were initially expressed in DL-4 ETP cells and further upregulated in proT-1 cells.

Expression levels for all analysed T-lineage genes increased between the ETP and the proT stage in both DL-4 progenitors and in native thymic progenitors (Figure 12). The relative increase of *PTa*, *RAG1* and *BCL11B* expression (numbers above the columns in Figure 12) at the proT stage was quite similar in DL-4 progenitors and in native thymocytes. In contrast, the relative increase of *IL7Ra* levels in DL-4 proT1 cells was higher than in thymic proT cells (Figure 12).

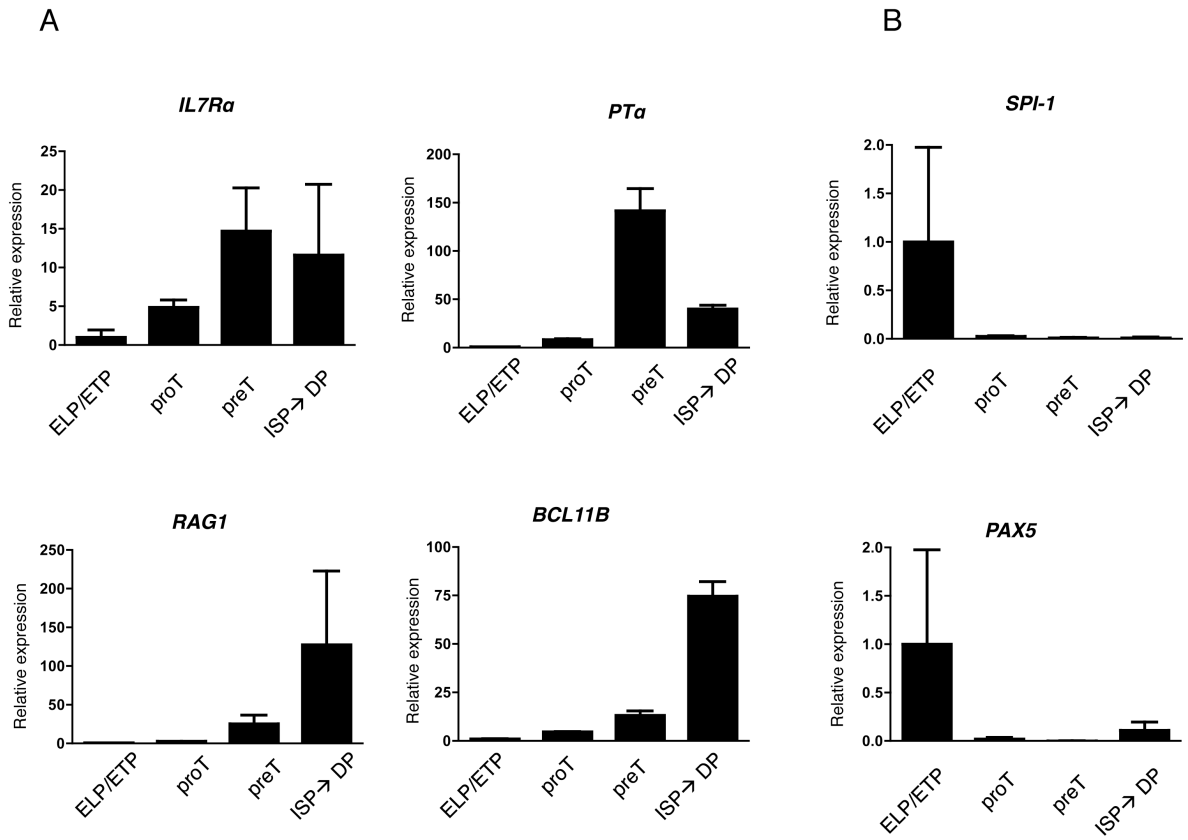


Figure 11: Gene expression profile of native T-lymphoid progenitor populations

Taqman analysis for expression of *IL7Rα*, *pTα*, *Rag1* and *Bcl11b* in native thymic ELP→ ETP, proT, preT and ISP→DP cells sorted from $CD34^+$ enriched native human thymocytes. Transcript levels for the indicated genes were normalized to human *GAPDH* expression. Gene expression levels in proT, preT and ISP→DP cells were compared to expression in the ELP→ETP subset. Data are representative for 3 independent experiments. Columns display relative expression values of a representative PCR. Error bars indicate RQ_{min} and RQ_{max} values.

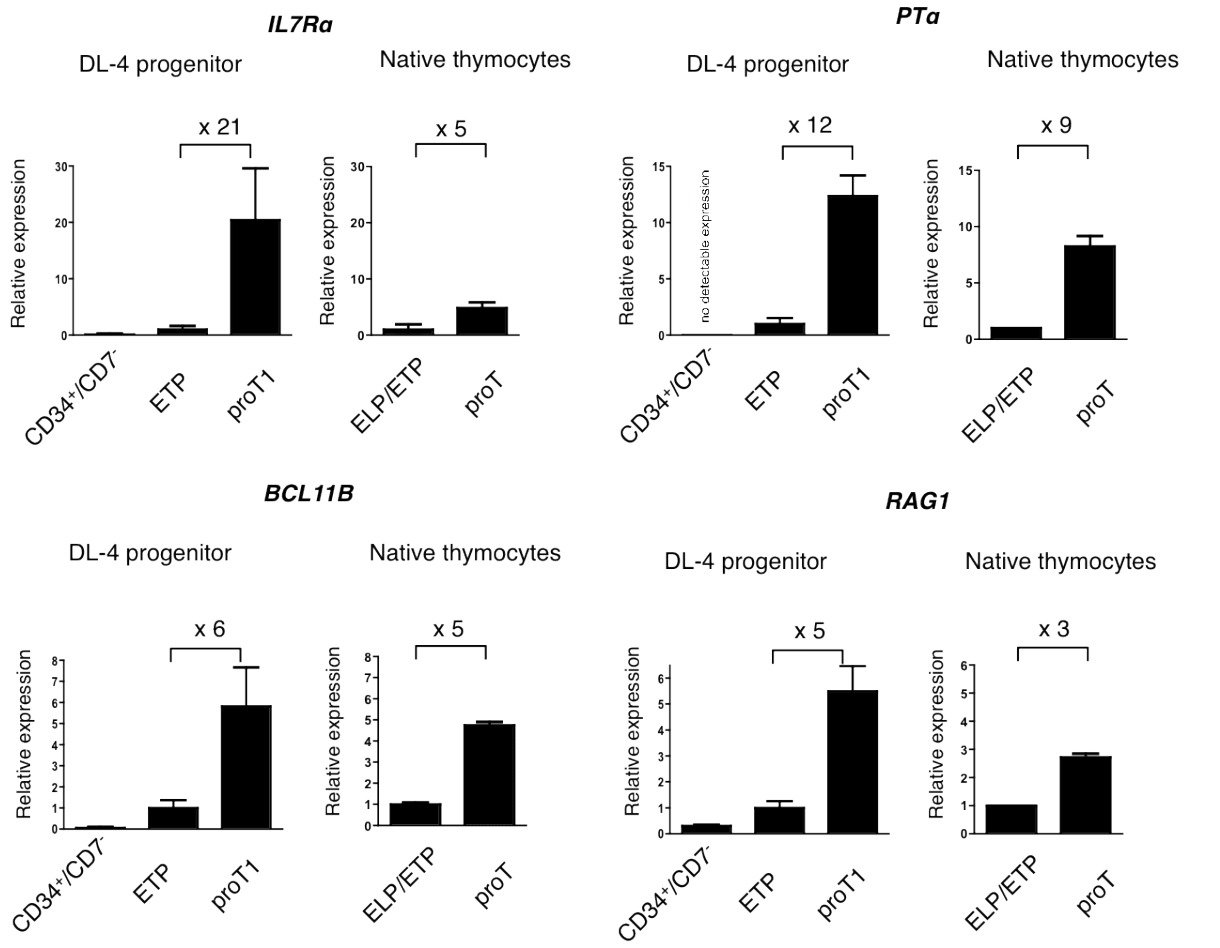


Figure 12: Comparison of gene-expression between DL-4 progenitors and native thymic T-lymphoid progenitors

Taqman analysis for *IL7Rα*, *pTα*, *Rag1* and *Bcl11b* gene expression in sorted DL-4 progenitors ($CD34^+/CD7^-$, ETP, proT1) from a day-7 culture and ELP/ETP and proT populations from native thymocytes. Transcript levels for each gene were normalized to human *GAPDH* expression. Expression levels for each gene were calibrated to the ETP in DL-4 progenitors and to the ELP→ETP population in thymic progenitors. Numbers above the columns indicate the increase in gene expression at the transition from the ETP to proT1 stage in DL-4 stage or the transition from the ELP→ ETP to the proT stage in thymic progenitors. Shown columns display relative expression values of one representative PCR. Error bars indicate RQ_{min} and RQ_{max} values.

2.2.2 Stage specific TCR rearrangements occur at the same stages in DL-4 progenitors and in native thymocytes

In DL-4 progenitors, TCR rearrangements only occurred after prolonged culture times (more than 1week). To identify the specific stages where these events first occurred, DL-4 ETP, proT1, proT2 and NK cells were analysed for TCR rearrangement. No TCR rearrangements were detected in DL-4 ETP cells (apart from very low levels of Dδ2-Dδ3 rearrangements). Incomplete (D-J) rearrangements within the δ-, γ- and β- locus first appeared within the proT1 stage and increased upon passage to the proT2 stage. Vβ-Jβ1 rearrangements indicating the presence of a completed rearrangement of the TCR-β chain, were first found in proT2 cells. Apart from very faint levels of Dδ2-Dδ3 and Vδ2-Dδ3 rearrangements, TCR recombination did not occur in DL-4 NK cells.

In a similar way ELP→ETP, proT, preT and ISP→DP cells from $CD34^+$ enriched thymocytes were analysed for TCR-recombination (Table 2.1 right column). The stage specific onset of the distinct TCR rearrangement events observed in DL-4 progenitors followed the same pattern as in native thymic ETP, proT and preT cells.

TCR locus	Rearrangement event	DL-4 subpopulation at day 14				First apparition in native thymopoiesis
		ETP	proT1	proT2	NK	
δ	Dδ2-Dδ3	-/lo	+	+	-/lo	ETP
	Dδ2-Jδ1	-	+	+	-	proT
	Vδ2-Dδ3	-	+	+	-/lo	proT
	Vδ1-Jδ1	-	-	+	-	proT→preT
γ	Vγ10	-	faint	+	-	proT
	Vγ9	-	faint	+	-	proT
	Vγ11	-	-	-	-	preT
β	Dβ1-Jβ1	-	+	+	-	proT
	Dβ1-Jβ2	-	+	+	-	proT
	Dβ2-Jβ1	-	-	+	-	proT
	Vβ-Jβ1	-	-	faint	-	proT→ preT
	Vβ2-Jβ2	-	-	-	-	preT

Table 2.1 Comparison of TCR-rearrangement pattern between DL-4 and native T-lymphoid progenitors

TCR rearrangement events within the TCRδ, TCRγ and TCRβ locus were analysed in indicated progenitor populations (ETP, proT1, proT2 and NK) from day-14 DL-4 cultures and in their thymic counterparts (thymic subpopulations sorted as described in figure 2.3). The column to the right indicates the stages, at which given TCR- rearrangement event first occurred in native thymopoiesis.

3 APPLICATION OF THE DL-4 CULTURE FOR BM DERIVED CD34$^+$ CELLS

Supplemental Material and Methods

Material and methods not explained in this chapter are detailed in the corresponding section of the article: "Human T-lymphoid progenitors generated in a feeder-cell-free DL-4 culture system promote T-cell reconstitution in NOD/SCID/γc$^{-/-}$ mice" (page 62).

Cell samples and isolation

Mononuclear cells from BM corresponded to the unused, healthy residues of allogeneic, haematopoietic stem cell harvests. CD34$^+$ cells were isolated using an indirect CD34 micro bead kit and VarioMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. For sorting of Lin$^-$/CD34$^+$/CD38$^-$ progenitors, cells were stained with CD34, CD38, 7AAD and a lineage (Lin) cocktail containing antibodies against CD3, CD56 and CD19. Cells were sorted on a FACS ARIA (BD Biosciences, San José, CA, USA) after exclusion of 7AAD positive cells.

DL-4 culture of BM cells

DL-4 coating was similarly performed as described above (page 62). Cultures were initiated with 2 x 10^4 CD34$^+$ BM cells in X-VIVO 20 medium (BioWhittaker, Walkersville, MD), supplemented with 20% defined fetal calf serum (Hyclone, Thermo Fisher Scientific, Illkirch, France) and the recombinant human cytokines interleukin-7 (IL-7), Flt3-ligand (Flt-3), stem cell factor (SCF) and thrombopoietin (TPO) (all at 100 ng/ml and all purchased from either R&D Systems (Minneapolis, MN) or PeproTech Inc. (Rocky Hill, NJ)). If the culture lasted longer than one week, cells were collected, resuspended in fresh medium and transferred into freshly coated wells at a maximum concentration of 1 x 10^5 cells/well.

3.1 DL-4 induces T cell development in postnatal HSC but with considerably lower efficiency

DL-4 culture of BM derived CD34$^+$ cells induced phenotypical changes consistent with early T cell development as well. After 3 days of culture I could first observe CD34$^+$/CD7$^+$ ETP cells. However, ETP appeared at much lower frequencies (5-10% in BM vs. 30-50% in CB cells) in BM DL-4 cultures and expressed considerably lower CD7 levels than CB derived ETP. Upon further DL-4 culture with BM cell, ETP differentiated into proT1 cells. Unlike in CB progenitors, transition from the ETP towards the proT1 stage occurred in an abrupt manner and did not pass by stages with intermediate CD34. ProT1 cells subsequently gave rise to CD7^{++}/CD5$^+$ proT2 and CD7^{++}/CD56$^+$ NK-like progenitors. As in CB, T cell differentiation in DL-4 cultures with BM cells remained blocked before β-selection (Figure 13).

In quantitative terms, T-progenitor yields from BM DL-4 culture were a 1-2 log-fold lower than from cultures with CB (Figure 15). This was most evident during the first week of culture, when ETP cells were predominantly generated in CB cultures. An input of 2 x 10^4 BM CD34$^+$ only yielded 0,5-5,0 x 10^4 ETP cells (mean 2,5 10^4), which was a 1-2 log fold lower yield compared to DL-4 cultures with equal numbers of CB cells. Prolonged culture allowed generation of up to 10^5 CD34$^-$/CD7^{++} proT1 and NK progenitors from an input of 2 x 10^4 BM CD34$^+$ cells, but again this yield was around a log-fold lower than in CB cultures (Figure 14).

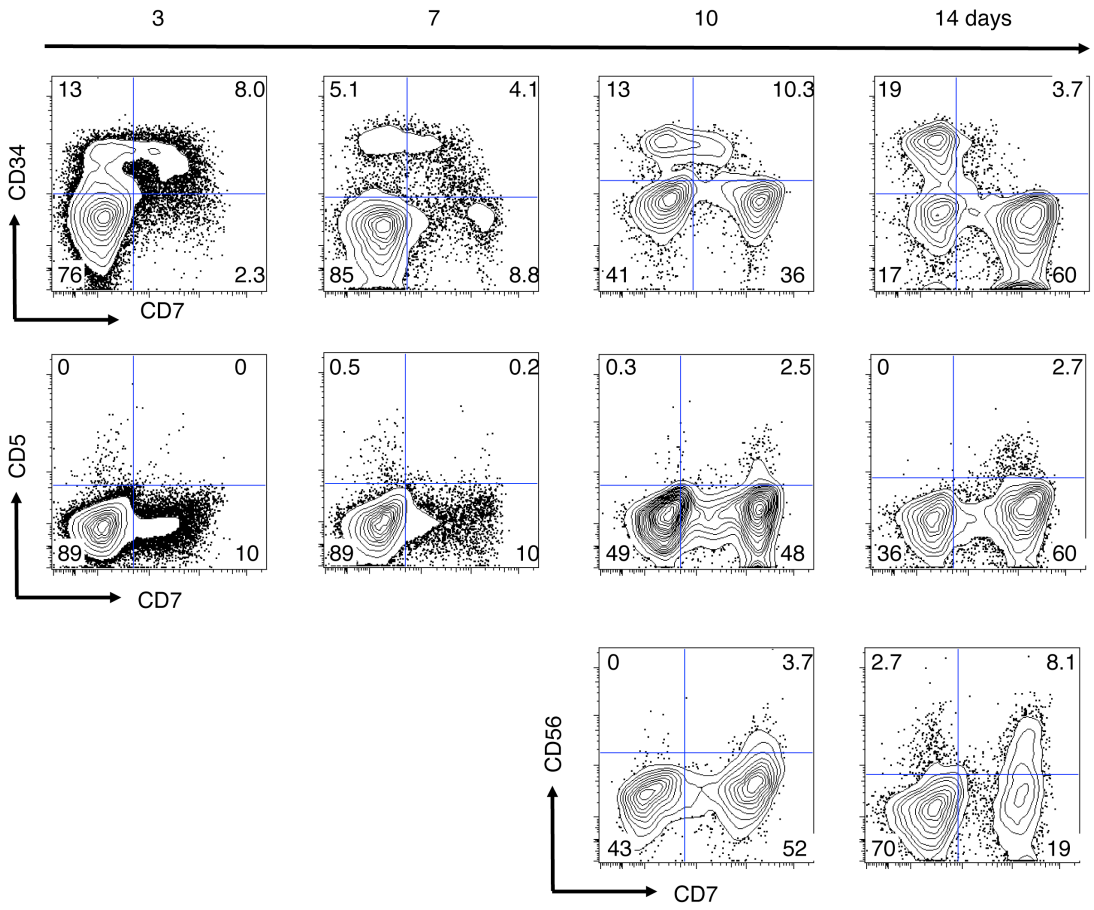


Figure 13: DL-4 induced generation of T-lymphoid progenitors from BM CD34$^+$

BM derived CD34$^+$ progenitors were cultured in DL-4 coated wells for 14 days. Expanded cells were analysed for surface expression of CD34, CD7 and CD5 and CD56 by flow-cytometry (after exclusion of death cells by 7AAD uptake) at indicated time-points.

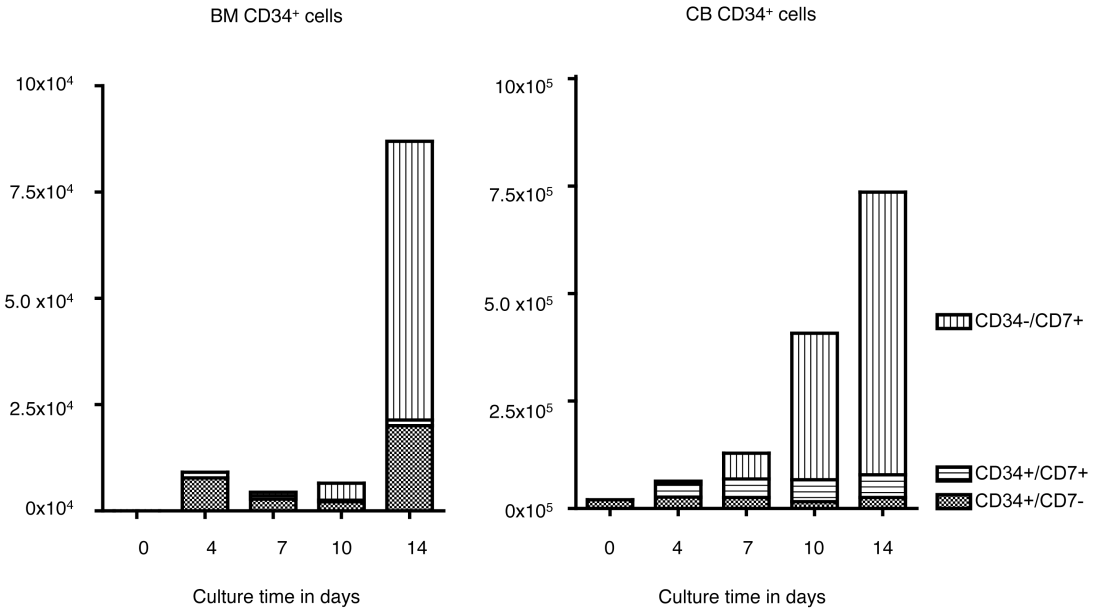


Figure 14: Comparison of T-lymphoid progenitor yields in DL-4 cultures with CD34⁺ cells from BM and CB

The yield of $CD34^+/CD7^-$, $CD34^+/CD7^+$ and $CD34^-/CD7^{++}$ DL-4 cells (see figure key) obtained after culture of $2x10^4$ BM $CD34^+$ progenitors (left diagram) or $2x10^4$ CB $CD34^+$ progenitors (right diagram) at indicated time points. The data are the mean of three independent experiments.

3.2 The $CD34^-/CD7^-$ DL-4 subset represents a transient myeloid population

Despite the low yield of T-lymphoid progenitors, we observed a 5-fold expansion of total cell numbers when culturing BM $CD34^+$ cells. However, the vast majority of these cells were $CD34^-/CD7^-$. We likewise observed this $CD34^-/CD7^-$ myeloid population in DL-4 cultures with CB cells, but in the later case it comprised a considerably lower fraction of the cellular yield.

Further phenotypical analysis of the $CD34^-/CD7^-$ cells showed that this population further co-expressed CD13, CD11b and CD14 and thereby had differentiated towards a myeloid lineage (Figure 15). Interestingly, highest levels (up to 95% of all cells) of $CD34^-/CD7^-$ myeloid cells were generated within the first week. Upon prolonged DL-4 culture (> 10 days), $CD34^-/CD7^-$ cells successively increased in granularity (higher SSC-levels), decreased in size, augmented 7AAD uptake and finally disappeared spontaneously.

When subjecting the $CD34^-/CD7^-$ population to methylcellulose assays, we found that it did not maintain any potential to generated CFU-colonies. Likewise the $CD34^-/CD7^-$ did not

retain any potential to give rise to T-lineage cells when placed in secondary OP9/DL-1 co-cultures.

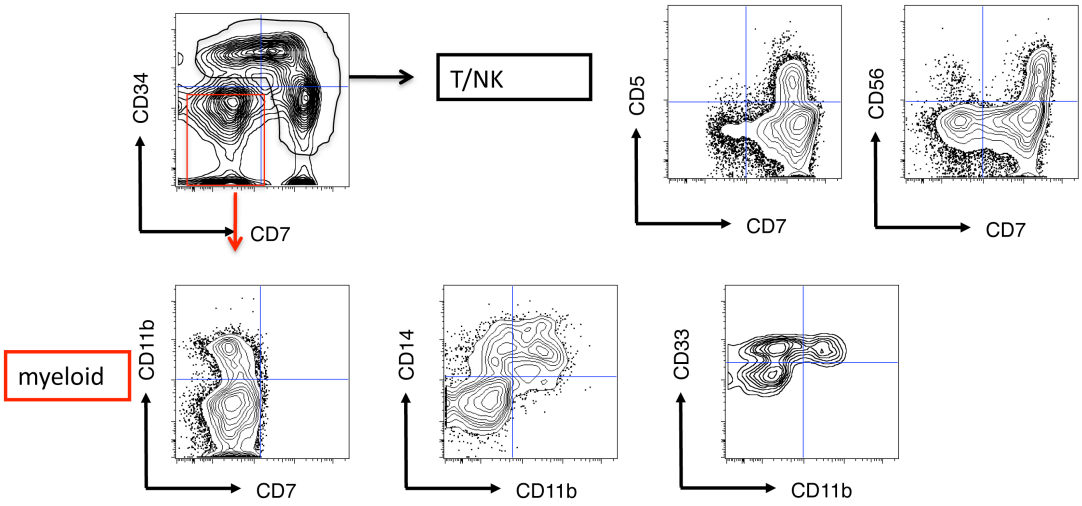


Figure 15: The CD34⁻/CD7⁻ subset emerging in DL-4 culture displays a myeloid phenotype

Upper panel, left graph: Flow cytometric analysis of DL-4 progenitors generated from CD34⁺ CB cells after 10 days of culture. The dotplot in the upper left shows differential expression of CD34 and CD7. Two principle populations can be distinguished: One subset comprising CD34⁺/CD7⁻, CD34⁺/CD7⁺ and CD34⁻/CD7⁺⁺ cells (encircled in black) and another CD34⁻/CD7⁻ population (framed in red). Upper panel, two right-hand graphs: Phenotypical analysis for CD5 and CD56 expression within the first-mentioned (encircled in black) DL-4 population. Lower panel shows phenotypical analysis for CD11b, CD14 and CD33 expression within the CD34⁻/CD7⁻ population (framed red in the upper panel).

3.3 Sorting of CD34⁺/CD38^{lo} cells

Activation of Notch1 was previously described to inhibit engagement towards myeloid lineages in HSC. Therefore, I was surprised to find such a high rate of myelopoiesis DL-4 cultures. To explain this conflicting result, on might argue that Notch1-activation indeed inhibits the differentiation of immature HSCs into common myeloid progenitors (CMPs) (via upregulation of GATA-2) (de Pooter, Schmitt et al. 2006) but does not suppress the expansion of CMPs. Within the CD34+ cells I had used in the previous experiments, HSCs and MLPs, reside within the immature Lin⁻/CD34⁺/CD38⁻ compartment. In contrast, CMP are found in CD34⁺/CD38⁺ subset.

I therefore decided to remove CMP populations from the CD34+ bulk population by sorting Lin$^-$/CD34$^+$/CD38$^-$ cells to find out whether (i) myeloid differentiation was related to Notch-independent expansion of CMPs cells, (ii) whether lower levels of decreased myelopoiesis would favour T-lymphopoiesis.

I first isolated Lin$^-$/CD34$^+$/CD38$^-$ cells from CD34$^+$ CB cells and submitted them into DL-4 culture. This sorting step resulted in complete absence of CD34$^-$/CD7$^-$ myeloid progenitors after 3 days and reduced apparition of myelopoiesis after 7 days (Figure 16). I could further reduce the onset of myelopoeisis at day 7, when I transferred progenitors into freshly coated DL-4 plates at day 3 (Figure 17). This finding suggests that Notch signal in DL-4 culture is sufficient to inhibit myeloid differentiation from HSC at early time points but is not sufficiently sustained during prolonged culture (e.g. due to consumption or degradation). When using CD34$^+$/CD38$^-$ sorted BM marrow cells, myelopoiesis was likewise abolished at 3 days of culture and appeared at low levels after 7 days. Although the use of Lin$^-$/CD34$^+$/CD38$^-$ cells considerably reduced the emergence of myeloid cells, the efficacy for the generation of T-lymphoid progenitors was not improved (Figure 18).

In conclusion, myeloid and T-lymphoid cells in DL-4 cultures derive from different progenitor populations from within the CD34$^+$ bulk population. The proliferation of the myeloid cells appears to occur independently from Notch1. However, myelopoiesis does not seem to alter the efficacy of the DL-4 system to induce T cell development. Therefore the high degree of myelopoiesis observed in DL-4 cultures with BM cells is unlikely to account for the low yield of T-lymphoid progenitors. It appears more likely that it reflects intrinsically reduced T-lineage potential of HSCs from adult BM.

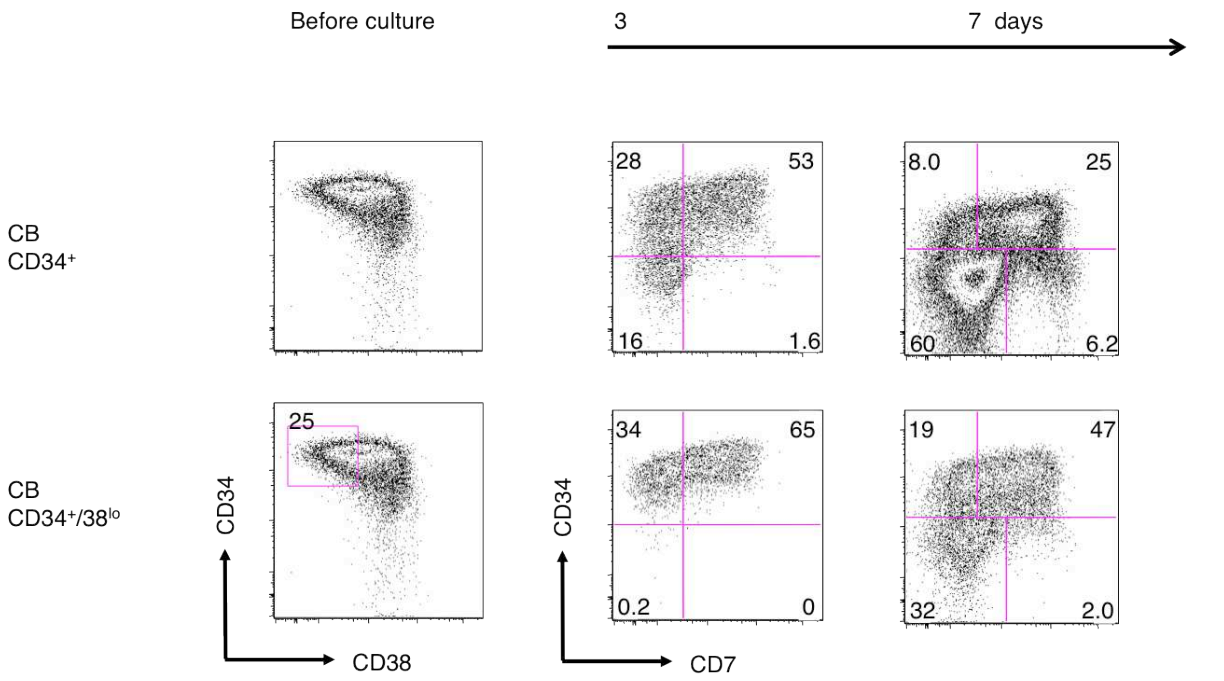


Figure 16: Impact of sorting the immature CD34$^+$/CD38^{lo} CB subset

CD34$^+$/CD38^{lo} cells were FACS-sorted from immunomagnatically purified CD34 CB cells after exclusion of CD3, CD19 and CD56. The left dotplot in the lower row shows the CD34$^+$/CD38^{lo} sorting gate.

DL-4 cultures were initiated with either CB CD34$^+$ bulk cells (upper row) or sorted CD34$^+$/CD38^{lo} progenitors (lower row) and cultured during 2 weeks. Cells were transferred to freshly coated wells after 7 days. Flow cytometry for CD34 and CD7 expression was performed at day 7 and 14 (two flow cytometric images at the left).

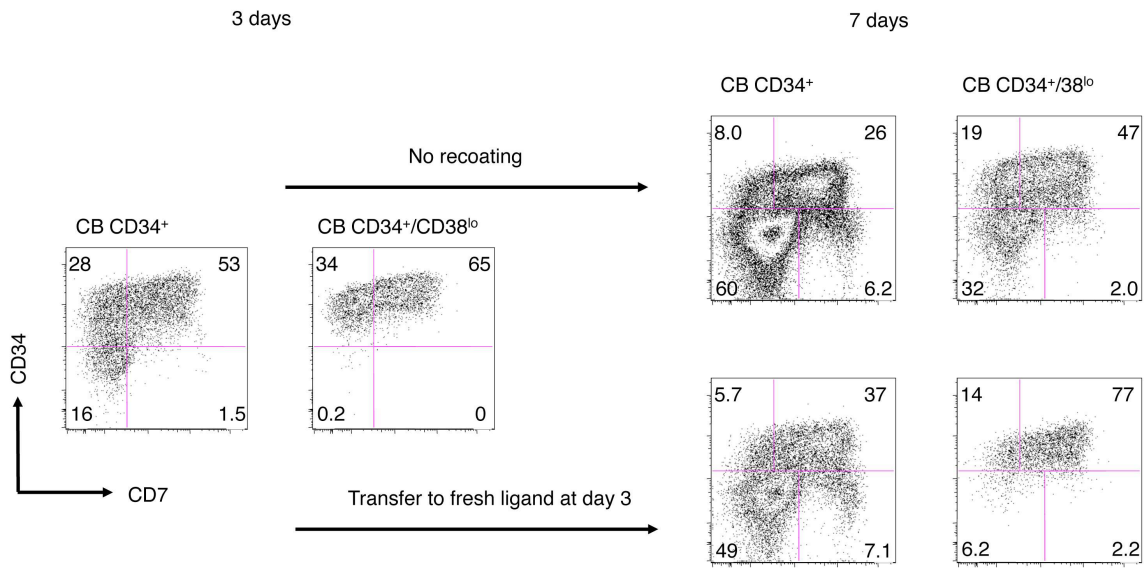


Figure 17: Impact of early renewal of DL-4 ligand

CB CD34$^+$ bulk cells or sorted CD34$^+$/CD38^{lo} cells were cultured with DL-4 during 3 days and analysed by flowcytometry for CD34 and CD7 expression (two flow cytometric images at the left). After 3 days, DL-4 progenitors either remained in the same culture well (upper panel) or were transferred into a freshly coated DL-4 culture well (lower panel). Phenotypical analysis for CD34 and CD7 expression were again performed at day 7 of culture.

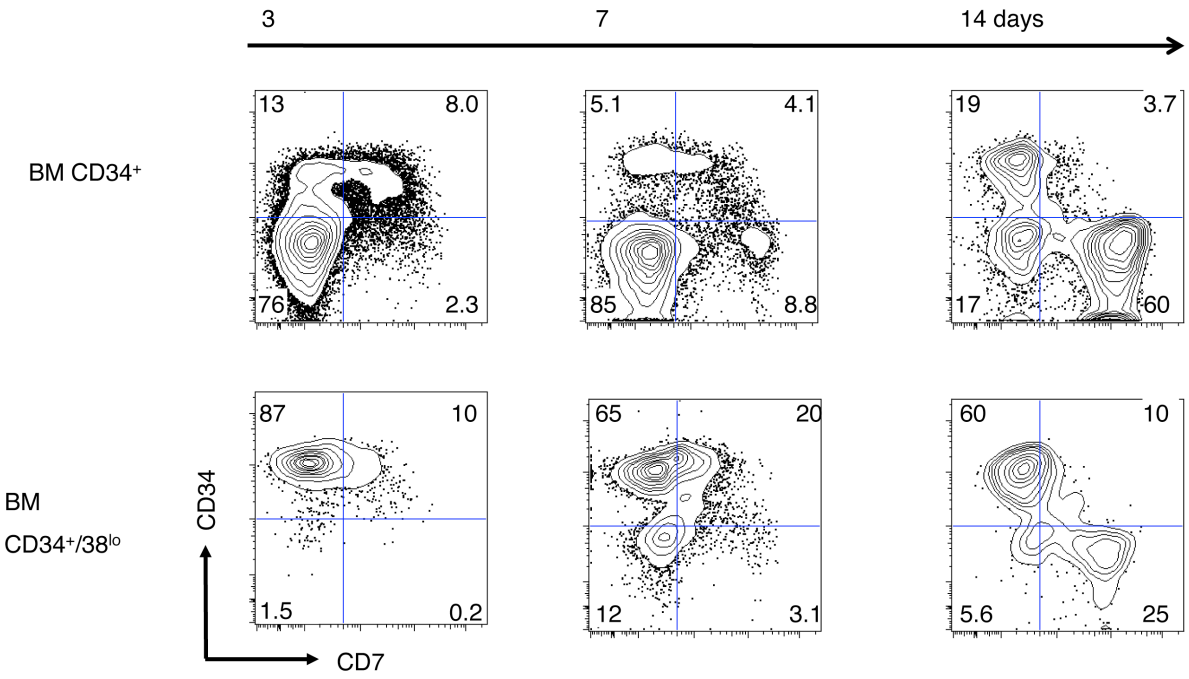


Figure 18: Impact of sorting the immature CD34^{+}/CD38^{lo} BM cells

CD34^{+}/CD38^{lo} cells were FACS-sorted from immunomagnatically purified CD34 BM cells after exclusion of CD3, CD19 and CD56.

DL-4 cultures were initiated with either BM CD34^{+} bulk cells (upper row) or sorted CD34^{+}/CD38^{lo} progenitors (lower row) and cultured during 2 weeks. Cells were transferred to freshly coated wells after 7 days. Flow cytometry analysis for CD34 and CD7 expression was performed on day 3,7, and 14.

DISCUSSION

Complementation of HSC grafts with T-lymphoid progenitors represents a promising strategy to enhance T cell recovery after allogeneic HSC. Given the scarcity of such cells, their isolation from native HSC grafts does not represent a valid option for therapeutic use. At present a number of feeder cell based culture systems have been demonstrated to efficiently promote *in vitro* generation of human T-lymphoid progenitors. However none of these protocols fulfils the strict clinical safety standards.

During my PhD project I have made use of immobilized Notch-ligand DL-4 to develop a novel feeder-cell-free culture system for the generation of T-lymphoid progenitors. Culture of $CD34^+$ CB cells in presence of DL-4 allowed the *in vitro* generation of high numbers of progenitors, that share many features with native ETPs. It induced T cell differentiation at pre-β-selection stages, but did not sustain it beyond this checkpoint. Although DL-4 culture likewise induced T-lymphoid engagement in $CD34^+$ BM cells, T-lymphoid progenitor generation was very little efficient. Finally, I could show that *in vitro* generated T-lymphoid progenitors harbour increased *in vivo* thymopoietic potential. These promising results and the fact, that the DL-4 culture might be rapidly transferred into a protocol fulfilling clinical standards, opens the possibility to use DL-4 progenitors as a complementary adoptive immunotherapy to improve posttransplant T cell recovery after allogeneic HSCT.

1 CHARACTERIZATION OF IN VITRO ETP –POTENTIAL

Early human T cell development is marked by the subsequential upregulation of CD7 on $Lin^{-/}CD34^+/CD38$- progenitor cells. In the fetal bone marrow $CD34^+/CD45RA^+/CD7^+$ progenitors have been identified as thymus colonizing cells. $CD34^+/CD45RA^+/CD7^+$ progenitors represent a major subpopulation of fetal $CD34^+$ progenitors (up to 40% at 20 weeks of gestation) and were proposed to sustain fetal thymopoiesis. From the last third of gestation on, $CD34^+/CD45RA^+/CD7^+$ progenitors numbers largely decline: in CB only 8% of $Lin^-/CD34^+/CD38^-$ progenitors and 1% of the entire $CD34^+$ progenitor population express CD7. In adult BM $CD34^+/CD45RA^+/CD7^+$ can no longer be detected. After birth, $Lin^-/CD34^+/CD10^+/CD24^-$ multipotent lymphoid progenitors represent the most advanced extrathymic progenitors with T cell potential. Likewise, $Lin^-/CD34^+/CD10^+/CD24^-$ ELP cells are the most immature intrathymic progenitor in postnatal thymopoiesis. After arrival in the thymus, they upregulate CD7 and thereby proceed towards the ETP ($CD34^+/CD7^{int}$) stage. This marks their early T-lineage engagement.

Therefore, ELPs and ETPs are the most immature T cell-engaged population in the postnatal thymus. They account for less than 0.5% of the immature DN compartment, which itself comprises only 3-5 % of all thymocytes. If assuming a total thymic cellularity of 2,0 -5,0 x 10^{10} cells, an adult human thymus does only contains 0,3-1,25 x 10^{6} ETP progenitors.

We showed that short DL-4 culture particularly promoted the generation of the rare ETP progenitors. ETPs neither expressed CD5 nor CD1a, had not started TCR recombination and retained non T-lymphoid *in vivo* potential. They are therefore engaged but not committed T-lineage progenitors. First expression of CD7 in ETPs and its further increase in proT1 cells correlated with the increased expression of early T-lineage genes *IL7Rα*, *pTα*, *RAG1* and *Bcll11b.* Moreover we found that induction of CD7 expression correlated with a higher *in vitro* T cell precursor frequency. This observation is consistent with the notion that the DL-4 induced T-lineage specification of the CD7 expressing cells occurs early in DL-4 culture. Furthermore, we found that ETP and proT1 cells from DL-4 cultures were able to complete *in vitro* T cell differentiation (in secondary OP9/DL-1 co-culture) within shorter time than $CD34^{+}/CD7^{-}$ or untreated CB cells and therefore correspond to more advanced differentiation stages.

Taken as a whole these *in vitro* data indicate that the DL-4 culture described herein recapitulates early steps of T cell development of native thymopoiesis and that the generated progenitors have properties of early thymic progenitors.

2 DL-4 PROGENITORS CORRESPOND TO ETP BY MEANS OF THEIR IN-VIVO THYMOPOEITIC POTENTIAL

We have provided a number of arguments that ETP and proT1 DL-4 progenitors are able to directly migrate to the thymus and to sustain *in vivo* thymopoiesis. When injected into newborn non–irradiated mice, DL-4 progenitors could repopulate the thymus within one week only. This step occurred independently of prior bone marrow engraftment. Moreover, we could attribute the thymus seeding capacity to the CD7 expressing DL-4 progenitors. Similar findings have been reported for $CD7^{+}$ T-lymphoid progenitors generated in OP9/DL1 or in Tst4/DL-4 co-culture (Awong, Herer et al. 2009; Meek, Cloosen et al. 2009). In using the newborn non-irradiated NSG model we quite closely mimicked a physiological steady state.

Given that the DL-4 progenitor cells injected into mice contained ETP and proT1 cells, both these population might theoretically contribute to the repopulation of the NSG thymus. It is difficult to estimate the specific *in vivo* thymus seeding potential of the ETP and proT1

fraction, because very few studies with human cells have addressed this issue. To my knowledge, the thymus seeding potential of native human ETP and proT cells (isolated from human thymus) has never been assessed in xenotransplantation models. $CD34^{+}/CD7^{++}$ progenitors obtained from OP9/DL-1 co-cultures are able to migrate towards the thymus, but this population likewise represents a continuum of ETP and proT cells (Awong, Herer et al. 2009). In mice, only canonical $ckit^{hi}$ DN1a and DN1b cells (and thus the analogous of human ETPs) can home towards the thymus homing after intravenous injection in non-irradiated recipients (Porritt, Rumfelt et al. 2004). For this reason I think that the true thymic seeding potential is comprised within the ETP population.

It is likewise assumable that more advanced T progenitor populations still retain *in vivo* thymic seeding potential, especially if injected in recipients that have received pre-transplant conditioning (i.e. by irradiation). Although unable to engraft the thymus on non-irradiated recipients, OP9/DL1 generated DN2 and DN3 progenitors indeed reconstitute the thymus of lethally irradiated recipients (Zakrzewski, Kochman et al. 2006). Interestingly, *in vitro* generated $CD7^{++}/CD5^{+}$ proT cells can efficiently reconstitute FTOCs and express high levels of genes associated with thymus homing (i.e. CCR, PSGL-1 and α2-, α4- and α5- integrin) (Awong, Herer et al. 2009)). However, these finding are only surrogate parameters and do not precisely depict migration into the non-irradiated thymus *in-vivo* and do not provide a proof for the true thymopoietic potential of these cells. The only way to elucidate the *in vivo* thymopoietic potential of the distinct *in vitro* generated human progenitors, would be to inject sorted ETP and proT cells in different recipients. This would not only be of scientific interest but could also have practical consequences: in this study we performed DL-4 cultures during 7 days to obtain optimal ETP yields. If proT1 and proT2 would still harbour *in vivo* thymus seeding activity, this would imply extension of culture times (e.g. for 10 days).

3 COMPARISON OF THE DL-4 AND THE OP9/DL1 CULTURE FOR THEIR EFFICIENCY TO GENERATE EARLY LYMPHOID PROGENITORS

In vivo thymopoiesis is a complex process, integrating distinct signalling pathways. For a long time it was assumed that - *in vitro* - this specific microenvironment could only be mimicked by the 3-dimensional architecture as it is provided by FTOC cultures. Identification of Notch1 as the central player in early T cell development and the availability Notch ligand cultures such as OP9/DL-1 cells, helped to further dissect the complexity of the

microenvironment required for T cell differentiation. The DL-4 culture, introduced in this work, solely relies on the DL-4 signal (and a cytokine cocktail consisting of SCF, Flt3L, TPO and IL-7) and thereby represents a further simplification of the OP9/DL-1 system. It is therefore of interest to compare the efficacy of the DL-4 culture with that of OP9/DL-1 cells.

In HSC-OP9/DL-1 co-cultures, the progression through the early steps of lymphoid development follows a similar phenotypical expression pattern as we have observed in DL-4 cultures: during the first week of OP9/DL-1 co-culture $CD34^+$ cells rapidly acquire CD7 as the earliest T lineage marker. $CD5^+$ proT and $CD1a^+$ preT cells first emerge during the 2^{nd} week of culture. After 1 week, ETP cells are the dominating cell population in both HSC-OP9/DL-1 co-cultures and DL-4 cultures and OP9/DL-1 generated ETPs likewise display an increased *in vitro* T-lymphoid potential (Awong, Herer et al. 2009). It is difficult to directly compare the increase in T-lymphoid potential described in previous studies with OP9/DL-1 co-cultures to ours, since they used (i) different starting populations ($CD34^+/CD38^{-/lo}$ CB vs. unsorted $CD34^+$ cells), (ii) different culture times (14 days vs. 12 days vs. 7 days) and (iii) different readouts for Limiting dilution analysis (LDA) experiments ($CD7^{++}/CD1a^+$ vs. $CD3^+/TCR\gamma\delta^+$, $CD4^+/CD8^+$ mature T cells). The Zuniga-Pflucker group co-cultured 10^4 $CD34^+/CD38^{-/lo}$ CB cells (which are comprised in 1-2x10^5 $CD34^+$ cells) on OP9/DL1 during 12 days. They found a 20-fold proliferation index, a $CD7^+$ cell frequency of 85% and a T-precursor frequency of 2^{-1}. Based on these parameters, a 12-day OP9/DL-1 co-culture with an input of 10^4 Lin-$CD34^+CD38^{-/lo}$ CB cells yielded 8.5x10^4 T cell progenitors (Awong, Herer et al. 2009). In our experiments, a 7-day exposure to DL-4 of an equivalent number of $CD34^+$ CB (i.e. 10^5 $CD34^+$ progenitors) allowed the generation of 7,5 x 10^5 DL-4 cells with a T-precursor frequency of 12^{-1} and therefore yielded a total of 6,7x10^4 functional T cell progenitors. The T cell progenitor production, we obtained after only 7 days of DL-4 culture time is therefore very close to what Awong et al. found after 12 days of HSC-OP9/DL1 culture. Furthermore, this study with OP9/DL-1 cultures defined presence of $CD7^{++}/CD1a^+$ preT cell as the positive readout of the LDA experiments and for the calculation of the T-cell progenitor frequency. This readout is thus much less stringent than ours, in which we only considered progenitors able to generate $CD3^+/TCR\gamma\delta^+$ or $CD4^+/CD8^+$ T cells as T-cell precursors. Therefore, this comparison even underestimates the T-lymphoid potential of DL-4 progenitors. We have ourselves performed 2 LDAs with progenitors from day 7 HSC-OP9/DL1 co-cultures and have applied our more stringent readouts to them (data not shown). In these tests, T cell precursor frequencies were between 21^{-1} and 46^{-1} and thus quite similar to those of DL-4 progenitors (12^{-1} after 7 days of culture).

The Zuniga-Pflucker group has likewise tested the *in vivo* potential of OP9/DL1 derived T-progenitors and found that they could reconstitute the NSG thymus. However, this study did not characterize the kinetics of thymic recovery in detail (Awong, Herer et al. 2009). Based on the comparison with these findings and on our own experiments HSC-OP9/DL1 cultures we conclude that the DL-4 and the OP9/DL1 system have an equivalent potential to produce functional ETPs in short cultures.

4 PROLONGED DL-4 CULTURE DOES NOT SUSTAIN T-LINEAGE DIFFERENTIATIONS BEYOND B-SELECTION

Unlike the OP9/DL-1 system, which recapitulates the entire process of T cell differentiation, the DL-4 culture less efficiently supports the transition from the proT to the preT stage and does not sustain passage through β-selection. These differences evoke the requirement of environmental factors that are provided by OP9/DL-1 cells but not by the entirely molecularized DL-4 system. In my view, four signals are of particular interest in this context: the Notch-signal itself, IL-7, CXCR-4 and Wnt.

Notch

In murine T cell development, a first proliferation burst occurs between the DN2 and DN3 stage. These rapidly expanding cell highly depend on Notch1-signalling (Rothenberg, Moore et al. 2008; Awong, Herer et al. 2009; Rothenberg 2012). In human HSC-OP9/DL-1 cultures, Notch1 mRNA expression in progenitors increases with time and reaches highest levels in preT cells (Awong, Herer et al. 2009). This suggests that high Notch1 activity is also required at these stages *in vitro*. In OP9/DL-1 or OP9/DL-4 cultures, the OP9 cells continuously renew DL-1 or DL-4 and thereby provide a constantly high Notch signal. In contrast, the DL-4 system consists of a single coating step with DL-4-Fc fusion protein and thereby provides a much less well-sustained Notch signal. In our experiments we found that early DL-4 renewal improved T cell differentiation and reduced emergence of myeloid cells. This indirectly suggests that the availability of DL-4 subsequently decreases during culture (e.g. due to degradation of the DL-4 protein, endocytosis, neutralization by Fc-Receptors expressing macrophages or consumption of DL-4). With this in view, the Notch signal provided in the DL-4 system might be sufficient to promote the T-lymphoid programme at very early stages. However, the emergence of high numbers of more advanced (and still Notch-

dependent) stages (i.e. proT, preT) might require higher DL-4 quantities than a simple coating step may provide.

IL7

As described above, progenitors express high IL-7Rα levels during T cell engagement but rapidly downregulated it before β -selection. In a previously described murine DL-4 culture, T cell differentiation remained blocked at the murine DN2 stage (the equivalent of the human proT stage) when using high IL-7 concentration (10ng/ml). However upon diminution of IL-7 concentrations (to 1 ng/ml), progenitors could proceed to DN3 stages (Ikawa, Hirose et al. 2010). Similar observation have been made in OP9/DL-1 cultures: high IL-7 concentrations (30-100 ng/ml) arrest T cell differentiation before β selection (in both murine and human), which can only be unblocked by reduced IL-7 concentration (2ng/ml) (De Smedt, Hoebeke et al. 2004; Zakrzewski, Kochman et al. 2006; Awong, Herer et al. 2009; Six, Benjelloun et al. 2011). In the present study, we have used very high IL-7 concentration (100 ng/ml) to favour proliferation of early thymic progenitors. However, even low IL-7 concentration (2ng/ml) did not unblock the pre-β-selection arrest of human T cell differentiation in our DL-4 system (data not shown).

CXCR4

CXCR4 signalling has been demonstrated to be important for passage through β-selection in both murine and human thymopoiesis. This led us to analyse the expression of this chemokine receptor in DL-4 progenitors and in native thymic progenitors. In both cases, we found a stage specific increase of CXCR4 expression at the proT and preT stage. While SDF1α – the CXCR4 ligand - is absent in the DL-4 system, Janas et al. showed that OP9/DL1 cells secrete SDF1α (Janas, Varano et al. 2010). These researchers also described a murine DL-4 system for the *in vitro* differentiation of murine DN thymocytes. Consistent with our findings, this DL-4 culture only sustained T cell development until the DN3 stage. However, addition of SDF1α in this system unblocked T cell differentiation, allowed passage through β-selection and generation of DP thymocytes *in vitro* (Janas, Varano et al. 2010). Missing SDF1α dependent activation of CXCR4 expressing proT cells might therefore be a reason for the developmental arrest in the DL-4 culture system. Absence of SDF1α might deprive the $CXCR4^{+}$ proT cells of an essential activator and could thereby account for the arrest of T cell differentiation before β selection.

Wnt

Although combined signalling via Notch1 and CXCR4 (i.e. DL4 and SDF1α) was demonstrated to unblock T cell differentiation, this combination still supports the generation of DP thymocytes less efficiently than OP9/DL-1 co-cultures. Further improvement of DP cell generation (to a similar extent as in OP9/DL1 co-cultures) could be achieved by inhibition of GSK3β with Chir99021 and thus by activation of Wnt-signalling (Janas, Varano et al. 2010).

In summary, these findings suggest that lack of CXCR4- and Wnt-signalling in DL-4 cultures underlies the developmental arrest at β selection but does not provide a direct explanation for the less efficient generation of proT and preT progenitors. However, one might argue that the importance of these signals (i.e. SDF1α, Wnt) is not tightly confined to the single step of β-selection but that they are already required at the proT or preT stage. Our findings that CXCR-4 levels increase at the proT stage indirectly support this assumption. Nevertheless, the precise role of CXCR-4 (and Wnt) signalling at these immature stages remains elusive. Given the progenitor stage specific dependency on Notch1, IL-7, CXCR-4 and Wnt signalling, it might be necessary to combine these signals differently at distinct points of culture to further develop the DL-4 approach into a culture system that recapitulates the entire T-lymphoid developmental program *in vitro*.

Beyond the four signals discussed in this section, stroma cells might confer further – so far unknown - signals that interplay in the regulation of T-cell differentiation at the proT and preT stage. Deeper comparative gene expression analysis of ETP, proT and preT progenitors from DL-4 cultures, OP-9/DL-1 co-cultures and from native thymi (e.g. with microarray analysis) could identify novel signalling pathways that are differently activated in these progenitors. Another approach might consist in performing micro dissections of distinct thymic subregions (i.e. from the corticomedullary junction, subcortex and the cortex). Screening these samples for distinctly expressed genes (by microarray analysis) or for cell surface markers might allow to identify new candidate molecules that are essential for the crosstalk thymic stroma and the differentiating thymocyte.

5 PROLONGED DL-4 CULTURE FAVOURS THE EMERGENCE OF A NK-BIASED POPULATION

Absence of additional proliferation and differentiation signals, which are provided in feeder-cell cultures, likewise provide an explanation for the emergence of the NK-like population in the DL-4 culture. T and NK cells share early, Notch-dependent differentiation

steps (Haraguchi, Suzuki et al. 2009) and in DL-4 cultures NK and $CD5^+$ proT2 cells emerge from a common $CD7^{++}$ progenitor. Although OP9/DL1 generated $CD7^{++}$ proT cells also harbour T/NK potential *in vitro*, abundant emergence of NK-cell does not occur in OP9/DL-1 co-cultures (Aoyama, Delaney et al. 2007; Awong, Herer et al. 2009; Haraguchi, Suzuki et al. 2009; Meek, Cloosen et al. 2009). Since T cell differentiation is blocked at the preT stage in DL-4 culture, I consider that the high degree of NK cell generation represents a salvage differentiation pathway of this bipotent T/NK progenitor. Absence of Bcll11b has been recently demonstrated to favour NK over T cell differentiation of bipotent T/NK progenitors. However, DL-4 cells express Bcl11b at mRNA and protein levels. The occurrence of NK cells in other feeder cell free Notch ligand cultures has been reported and was attributed to a lack of Wnt signalling (Ohishi, Varnum-Finney et al. 2002; Aoyama, Delaney et al. 2007; Haraguchi, Suzuki et al. 2009). One way to inhibit NK-cell differentiation in the DL-4 culture might therefore consist in providing Wnt signals (e.g. by GSK-inhibitors or recombinant Wnt protein). Whether this might in turn increase proT cell generation remains likewise to be tested. In the DL-4 culture, biased NK-cells only appeared after prolonged DL-4 culture (i.e. ≥10 days). From a practical point of view, this provides a further argument for using day 7 DL-4 cells culture in transplantation experiments.

6 *WHY IS THE DL-4 SYSTEM LESS EFFICIENT FOR THE IN VITRO GENERATION OF T-LYMPHOID PROGENITORS FROM ADULT HSCS?*

Although CB transplants have become a valid graft alternative, postnatal HSCs from BM or mPB still constitute the major graft source for HSCT. Extension of the DL-4 system to postnatal HSCs would therefore be of great importance for a future clinical exploitation. Unfortunately, the current DL-4 culture is much less efficient for generating T-lymphoid progenitors from postnatal BM derived HSCs. Moreover the ETP-population, which constituted a major population in CB cultures, is virtually not generated in DL-4 cultures with BM.

This is not entirely related to a specific incapacity of the DL-4 culture. Even in the OP9/DL-1 system, the generation of T-lymphoid progenitors from adult $CD34^+$ BM cells is much less efficient than from CB cells. The low efficacy of both culture systems therefore most likely reflects cell intrinsic property of BM progenitor cells. Three principal causes might explain the reduced *in vitro* T-lymphoid potential of postnatal $CD34^+$ cells. (i) They contain less genuine HSC and lymphoid progenitors. (ii) They have an intrinsically reduced capacity to

engage towards the T-lineage and to produce T-cells. (iii) They have a low proliferation capacity during the T –cell differentiation process.

(i) In human haematopoiesis, LT-HSCs are enriched within the $Lin^-/CD34^+/CD38^-$ fraction of the heterogeneous $CD34^+$ cell population. $Lin^-/CD34^+/CD38^-$ cells are less frequent in adult BM (8% of $CD34^+$ cells) than in CB (25% of $CD34^+$ cells). Frequency of $CD34^+CD10^+CD24^-$ human MLP also declines with age (Six, Bonhomme et al. 2007).

(ii) Apart from their differing frequencies, both human $Lin^-/CD34^+/CD38^-$ cells and $CD34^+/CD10^+/CD24^-$ MLP from adult BM display a lower cell intrinsic T-lymphoid potential (i.e. a lower T-cell precursor frequency) and a lower proliferative activity than CB cells. Moreover, there is an age dependent dominance of B- over T-lymphoid potential of human MLPs (Six, Bonhomme et al. 2007; De Smedt, Leclercq et al. 2011). Partially overlapping observations have been made in mice: both, the T-lymphoid potential (*in vitro* and *in vivo*) of LSK cells and the number of LSK $Flt3^+$ MPPs in the BM decline in an age-dependent manner (Zediak, Maillard et al. 2007).

The age-dependent decline of T-lymphoid potential moreover reflects that HSCs are not a homogenous group of cells with unrestricted differentiation potential. Even phenotypically defined LT-HSCs are a heterogeneous population that differently contribute to myelopoeisis and lymphopoiesis: α-HSC, with robust myeloid but deficient lymphoid potential, and β-HSC with balanced myeloid and lymphoid lineage potential (Dykstra, Kent et al. 2007). During life these HSC-subsets contribute differently to lineage output: While myeloid/lymphoid balanced β-HSCs sustain fetal haematopoiesis, lymphoid-deficient α-HSCs start to contribute to haematopoiesis after birth and become the dominant HSC-subset during early adulthood (Benz, Copley et al. 2012). While the α- and β-HSC-subpopulation have been described in mice, it has been proposed that human LT-HSCs are likewise heterogeneous and that LT-HSC subsets contribute differently to myeloid and lymphoid lineages (Cavazzana-Calvo, Fischer et al. 2011).

(iii) As compared to HSC in the fetal niche, haematopoietic cells in the postnatal BM mostly remain in a quiescent, G_0 state. They have a lower proliferative and self-renewal activity and display different growth-factor requirements for proliferation and lineage decisions (Copley, Beer et al. 2012).

The reasons, why adult HSCs switch lineage potential and loose their self-renewal and proliferation capacity with age are not well understood. Yet, several possible mechanisms have

been proposed. Fetal HSC are more responsive to SCF, which might account for their increased self-renewal activity (Bowie, Kent et al. 2007). In a consistent manner, maintenance and proliferation of adult, but not of fetal HSCs requires the activation of a specific gene programme (i.e. BMI1, GFI1, Tel/Etv6) (Copley, Beer et al. 2012). Some TFs (e.g. GATA-2 and Id1) that maintain the HSC-potential and inhibit their differentiation are expressed at higher levels in CB (and fetal) $CD34^+$ cells than in BM $CD34^+$ cells (Ng, van Kessel et al. 2004).

A possible regulator of the switch from the fetal-to-adult stage is the transcription factor SOX17. SOX17 is found at higher levels in fetal HSC and is a key TF for the in maintenance and self-renewal of fetal, but not of adult HSCs. Overexpression of SOX17 induces adult murine HSC to adopt fetal-like HSC properties (Kim, Saunders et al. 2007; He, Kim et al. 2011).

Posttranscriptional control of the Let-7 micro-mRNA (miRNA) family by the miRNA binding protein Lin28b has been recently shown to contribute to the distinct lymphoid potential at the fetal-to-adult switch of haematopoiesis. Let-7miRNA is only expressed in adult, but not fetal HSC. In contrast, Lin28b is specifically expressed in the fetal liver and the fetal thymus (of both men and mice) and inhibits the processing of Let7 pre-miRNA. Specific Lin28b expression in fetal HSC thereby contributes to the unique lymphoid potential at this ontogenic stage. In consistency, ectopic Lin28b expression in adult HSCs allows to reprogram them to undergo fetal-like lymphopoiesis. This includes, that Lin28b confers IL-7 independency, which is a key feature of the fetal HSC (Yuan, Nguyen et al. 2012).

Finally, I assume that a differential pattern of Notch1 activation in fetal and adult haematopoiesis could explain the low T-lymphoid potential of adult HSCs. T-lineage polarized $CD34^+/CD45RA^+/CD7^+$ progenitors can be found in the fetal BM, in the fetal liver (FL) and in CB. However they are absent in adult BM (Haddad, Guimiot et al. 2006). Since the generation of $CD34^+/CD45RA^+/CD7^+$ progenitors is Notch1 dependent, it might be possible that fetal HSCs encounter first Notch1-activation in the BM (or the FL) due to either distinct expression of ligands in the fetal microenvironment and/or a higher responsiveness of fetal HSC to Notch signals. The observation that haematopoietic progenitors in CB display higher transcript levels of Notch1 and of the Notch target HES1 reinforces this assumption (Panepucci, Oliveira et al. 2010). Moreover, DL-4 is expressed in the human FL and interacts with Notch1 on $CD34^+/CD38^-$ progenitors during fetal haematopoiesis (at least during the first trimester of

gestation) (Dando, Tavian et al. 2005). Whether this might favour emergence of T-lymphoid progenitor in the FL has not been addressed in previous studies.

7 STRATEGIES TO IMPROVE THE GENERATION OF T CELL PROGENITORS FROM BM CD34$^+$ CELLS

Given that prenatal HSCs harbour a higher intrinsic T-lymphoid potential, it is tempting to think of strategies that might confer a more juvenile phenotype to adult HSCs to increase their capacity to differentiate into T-lymphoid progenitors.

Overexpression of SOX17 or Lin28b in adult (murine) HSCs has allowed their successful reprogrammation (He, Kim et al. 2011; Yuan, Nguyen et al. 2012). Lin28b appears to be capable to confer a fetal-like T-lymphoid differentiation pattern with emerging T cell harbouring innate like-properties. However, the proliferation capacity of these reprogrammed cells is not increased.

When considering reprogrammation of adult towards fetal HSCs, one has to keep in mind that fetal HSCs themselves do not necessarily represent a homogenous population but might change their cell intrinsic differentiation potential during gestation. For instance, CD34$^+$/CD45RA$^+$/CD7$^+$ T-lineage progenitors described by Haddad et al. were found at highest frequencies between 15 an 23 weeks of gestation (Haddad, Guimiot et al. 2006). Reprogrammation towards the precise ontogenic stage when HSCs are most likely to become such T-lymphoid progenitors therefore appears difficult to achieve. From a safety point of view, reprogrammation would imply the overexpression of potential oncogenes and thereby the risk to generate malignant cell clones. For instance, long-term overexpression of SOX17 in adult HSC can lead to leukaemogenesis (He, Kim et al. 2011).

Besides direct reprogrammation, another approach might consist in establishing culture conditions that allow the *in vitro* expansion of the most immature LT-HSCs. As outlined before, self-renewal activity and proliferative capacity of LT-HSC are key features of fetal HSCs. Culture techniques enabling expansion of LT-HSCs might concomitantly confer more juvenile properties to these cells. A part from this hypothetical effect, expansion of the LT-HSC pool would allow amplification of most immature and multipotent subsets and thereby increase the number of progenitors for the initiation of the DL-4 culture.

A number of recent studies have explored the potential of small molecules, novel cytokines and transcription factors to *in vitro* expand HSCs and in particular the most immature LT-HSCs. Inhibition of the Aryl hydrocarbon receptor (Ahr) by the small molecule Stemregenin (SR1) improved the LT-HSC and the thymopoietic potential of CB and postnatal HSCs (Boitano, Wang et al. 2010). Angiopoietin, IGFBP1 or Pleiotrophin were demonstrated to regulate regeneration and expansion of HSCs (Zhang, Kaba et al. 2008; Himburg, Muramoto et al. 2010). Wnt signalling regulates HSCs' self-renewal and plays an important role at early stages of thymopoiesis (Weerkamp, van Dongen et al. 2006). Ectopic expression of HOXB4-in or culture with a TAT-HOXB4 fusion protein improves the *in vivo* engraftment potential of adult HSCs (Amsellem, Pflumio et al. 2003; Krosl, Austin et al. 2003). This might involve modification of Notch1 signalling (Schiedlmeier, Santos et al. 2007). Complementation with such factors might therefore be helpful to extend the applicability of the DL-4 system to adult HSC.

8 POTENTIAL CLINICAL APPLICATION

In contrast to OP9-DL-1 system, the proposed DL-4 system does not contain genetically modified murine cells and thus circumvents a considerable safety risk. The use of magnetically sorted CD34$^+$ cells (as routinely performed in HLA-mismatched human transplantation settings), with no enrichment of the more immature subsets (i.e. CD34$^+$/CD38$^-$ cells) resolves a further obstacle to human application. A very short DL-4 culture period of just one week allowed the generation of sufficient progenitor numbers to envisage their adoptive transfer *in vivo*. T cell progenitors generated during DL-4 culture accelerated thymic reconstitution and emergence of peripheral T cell in NSG mice. Thymopoiesis and peripheral T cell reconstitution were further improved when DL-4 cells were co-injected with untreated CD34$^+$ cells.

The additive effect of DL-4 primed and untreated CD34$^+$ haematopoietic progenitor cells introduces the most evident clinical application of DL-4 cells. In this situation, a single CB graft could be divided into two parts. DL-4 culture could be initiated with one part of the graft. At the time of transplantation, the culture product could be co-infused with the untreated CB unit or shortly after. In this straightforward instance, DL-4 progenitors could bypass the need for *de novo* T cell progenitor generation in the patient's BM and provide a first wave of thymopoiesis. This should accelerate the emergence of donor derived T cell from the recipients' thymus, confer improved T cell immunity and reduce infection-related post-

transplantation morbidity and mortality. Having undergone immunological education in the patients' thymus, the emerging mature T-cell pool should be host-tolerant and lack GvH activity. At the same time, the untreated $CD34^+$ cells would assure engraftment of non-T-lymphoid lineages in the BM and eventually generate T-lymphoid progenitors to sustain long-term thymopoiesis. The improved donor T cell function after the treatment with T progenitors might furthermore reduce host-versus-graft activity and thereby graft rejection (Zakrzewski, Kochman et al. 2006; Zakrzewski, Suh et al. 2008).

The low $CD34^+$ cell content in individual CB units is a major limitation for CB transplantation (CBT) since injection of low cell doses (< $2x10^5$ $CD34^+$/kg bodyweight) leads to increased graft failure (Wagner, Barker et al. 2002). Low cell doses would therefore be a restriction for the use of split-use of individual CB units in therapeutic DL-4 cultures and would only make it a treatment option for small children with a low body weight. In the past years, the combined transplantation of two unrelated CB units (double unit CBT (dUCBT)) has turned out as an effective strategy to circumvent cell dose limitations and has allowed to extent CBT to an increasing number of adult patients. (Brunstein, Gutman et al. 2010).

The co-transplantation experiments performed in this study showed that DL-4 progenitors enhanced T cell reconstitution even when injected with a second HLA-incompatible graft. These experiments quite closely mimic the HLA-mismatched dUCBT approach and suggest the application of DL-4 in such a setting. This would make DL-4 progenitors a valid treatment option also for adult patients. In this case, DL-4 culture could be performed with one CB unit and the culture product could be co-injected with a second, untreated CB graft. It is further conceivable that in such an instance, DL-4 progenitors generated from a CB graft could serve as a complementary therapy in HSCT with grafts from other HSC sources, such as $CD34^+$ enriched BM cells or mobilized peripheral blood cells.

When considering, how DL-4 progenitors might accelerate T cell reconstitution, the most obvious mechanism is that DL-4 progenitors directly populate the thymus and give rise to a first wave of *de novo* thymopoiesis. This would lead to the faster egress of mature T cells and regeneration of the peripheral T cell compartment. A less evident, but convincing mechanism is be that DL-4 progenitors might also provide beneficial effects to the regeneration of thymic stroma and might thereby help to regenerate thymic function.

The hypothesis that the dependency of differentiating T cells on an intact thymic microenvironment is not unidirectional but symbiotic is sustained by a number of studies. At several differentiation stages, thymocytes provide essential signals for the development and

maintenance of thymic cortical and medullary thymic stromal cells: DN1 and DN2 thymocytes are required for the development of the thymic cortex (Hollander, Wang et al. 1995). The presence of TCR $\alpha\beta^+$ thymocytes is necessary for the development and maintenance of the thymic medulla. During negative selection, interaction of auto antigen reactive CD4 SP with mTECs contributes to the maturation of $Aire^+$ mTECs, which are essential for conferring central tolerance (Irla, Hugues et al. 2008). Finally, intrathymic ILC have been recently shown to contribute via IL22 and IL23 to TEC repair after stress induced damage of the thymic microenvironment (Dudakov, Hanash et al. 2012).

Therefore, it is tempting to hypothesize that *in vitro* generated lymphoid progenitors likewise enhance the generation of thymocyte subsets that provide beneficial effects to the thymic stroma. This point of view is sustained by observations, the van den Brink group made when first using *in vitro* generated T-lymphoid progenitors in murine allogeneic HSCT. In these experiment they found that improved thymopoiesis was maintained even after the T-progenitor derived cells had left the thymus. DL-4 progenitors are capable of inducing thymopoiesis even in the highly disturbed thymic microenvironment of irradiated, immunodeficient mice. Co-transplantation of DL-4 cells with untreated $CD34^+$ cells leads to more robust thymic recovery and further improvement of peripheral T cell reconstitution. These observations allow the legitimate hypothesis, that DL-4 cells themselves could provide beneficial signals to the altered thymic stroma. This would enhance the functional regeneration of the thymus, which could aftermath be repopulated more efficiently by T-lymphoid progenitors emerging from the untreated $CD34^+$ HSC unit.

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