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T CELLS DEVELOPMENT IN VITRO: A MINIMALIST APPROACH

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ABSTRACT

T lymphocytes are considered an essential and advanced component of the immune system, since these cells are able to discriminate self from non-self, start up an immune reaction and further develop into memory cells. However, therapies based on the use of patient derived newly generated T cells re-inoculated into humans do not exist. This is due to difficulties in replicating the peculiar conditions required for T cell development in vitro. The systems developed so far are based on the use of animal or unrelated human thymic tissue and therefore they would not be adequate to be used in any clinical application. Having conjectured that human skin cells, rearranged in a three-dimensional fashion, would be able to support the development of human T lymphocytes from hematopoietic stem cells, we developed a model consisting of human skin keratinocytes and fibroblasts arrayed on a synthetic matrix so to create a prototype suitable to be translated into the clinic. In this way we were able to induce few hundred cord blood $CD34^+$ haematopoietic stem cells to entirely develop into mature $CD4^+$ or $CD8^+$ T lymphocytes in vitro. However, circulating adult peripheral $CD34^+$ precursors failed to survive in the same conditions. Finally we were able to explain our success as consequence of strong induction of the Notch delta ligand Dll-4 by the keratinocytes cultured in the construct.

In synthesis, we report here for the first time that skin keratinocytes, in the presence of fibroblasts and reconfigured in a three-dimensional arrangement, are able to induce the differentiation of a minimal amount of cord but not adult blood stem cells into fully differentiated T cells by acting through the Dll-4 Notch signaling pathway in vitro.

Keywords:

T cell development, Notch signalling, Dll-4, HSC, stem cells ageing

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

b-FGF basic- Fibroblasts Growth Factor

BM Bone Marrow

CB Cord Blood

CLP Common Lymphoid Precursors

Cx-43 Connexin-43

DC Dendritic Cells

Dll-1 Delta-Like Ligand 1

Dll-4 Delta Like Ligand 4

DN Double Negative Cells

DP Double Positive Cells

ELP Early Lymphoid Progenitors

ETP Early Thymic Precursors

Flt3L Flt3 Ligand

GH Growth Hormone

HSC Haematopoietic Stem Cells

IGF Insulin Growth Factor

IL-15 Interleukin-15

IL-7 Interleukin-7

ISP Intermediate Single Positive Precursors

KGF Keratinocyte Growth Factor

LMPP Lymphoid-Primed Multipotent Progenitors

mTEC Medullary Thymic Epithelial Cells

MHC-I Major Histocompatibility Complex Type I

MHC-II Major Histocompatibility Complex Type II

MPP Multipotent Progenitors

NK Natural Killer Cells

PB Peripherial Blood

RTE Recent Thymic Emigrants

SP Single Positive Cells

TCR T Cell Receptor

TEC Thymic Epithelial Cells

TN Triple Negative Cells

VEGF Vascular Endothelial Growth Factor

2D Two Dimensional

3D Three Dimensional

PUBLICATIONS

Antonio Lapenna, Christopher B-Lynch and R. Aspinall. A simple model system which licenses the production of T cells in vitro from CD34^{+} stem cells derived from cord blood, but not adult blood (submitted to Blood, 2012)

Wayne A. Mitchell, Aina Castells, Pierre Oliver Lang, Emmanuel Matas, Antonio Lapenna and Richard Aspinall. Novel delivery of Interleukin 7; Pulmonary delivery of Interleukin 7 provides efficient and safe delivery to the aging immune system. (accepted for publication in Rejuvenation research 2012)

Sheila Govind, Antonio Lapenna, Pierre Olivier Lang and Richard Aspinall. Immunotherapy of Immunosenesence; Who, How and When? (in press in Open Longevity 2012; 6)

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AIMS AND OBJECTIVES

This study is driven by the hypothesis that human skin cell lines can support the development of mature naïve T cells from umbilical cord blood or from adult peripheral blood stem cells. A previous innovative study demonstrated that this is possible using human skin cells from biopsies rearranged in a scaffold and $CD133^{+}$ bone marrow haematopoietic stem cells (*Clark R.A. et al. 2005*).

It has been also predicted that the environment created within a 3D matrix will be essential for this development. This in part may be due to the ability of the 3D environment to mantain delta ligand expression as previously demonstrated *(Mohtashami M. and Zuniga-Pflucker J.C. 2006*).

Therefore the aims of the study were:

- To find out if stem cells from cord and adult blood can be maintained and develop into mature naïve T cells by a keratinocyte cell line and a fibroblast cell line.
- To test how crucial a 3D structure is to development of T cells and how this is linked to DLL-1 and DLL-4 expression.
- To identify differences between haematopoietic stem cells in regard of the age and source (cord vs adult peripheral blood).
- To demonstrate that the T cells produced by the 3D skin system are the result of haematopoietic stem cells differentiation and not of contaminant T cells amplification.

1 INTRODUCTION

1.1 THYMIC T CELL DEVELOPMENT

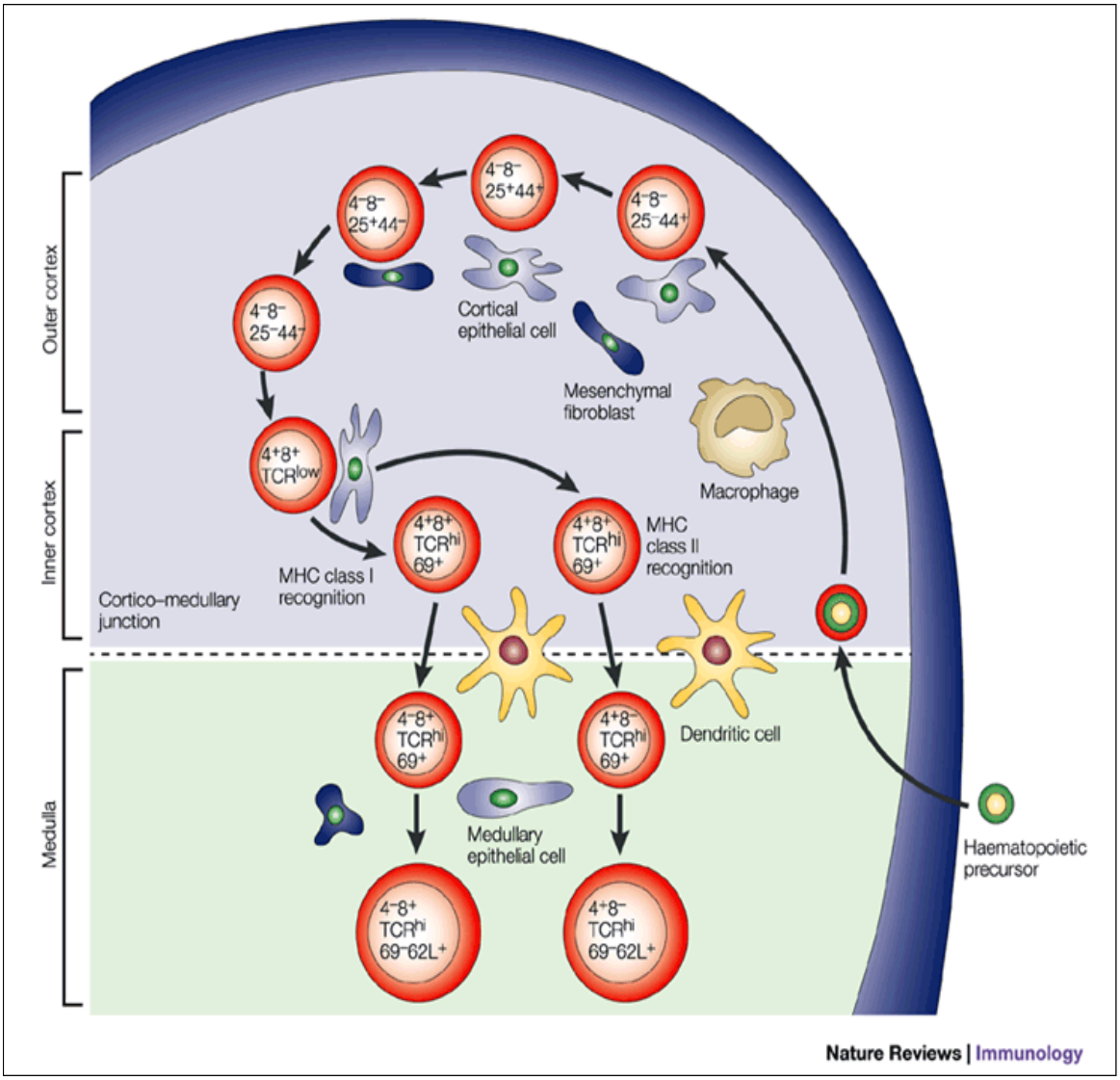

Figure 1: Thymic T cell development. The thymocytes mature through the thymic microenvironment and by interacting with thymic cells, undergo positive and negative selection. The image is taken from Anderson G. and Jenkinson E.J. Nature Reviews Immunology 2001; 1(1): 31-40

1.1.1 Extrathymic T cell precursors

Whilst all other blood cells are constantly produced within the bone marrow from resident haematopoietic stem cells, T cell development requires migration of precursors from the bone marrow into the thymus (*Zlotoff D.A. and Bhandoola A. 2011).*

The exact level of lymphoid commitment among bone marrow (BM) stem cells is still unknown (*Bandoola A. et al. 2007*), although HSC clearly start to differentiate before entering the thymus (*Yang Q. 2010*) and murine BM already contains multiple progenitors with T-lineage potential (*Perry S.S. et al. 2006*). Despite that, long-term primitive hematopoietic stem cells (LT-HSC), which are mainly referred to be $CD133^{+}Flt3^{-}$ (*Bathia M. 2004; Hess D.A. et al. 2006*; *Summers Y.J. et al. 2004; Zeigler F.C. et al. 1994*) demonstrated long-term T-lineage potential when placed into the thymus (*Vicente R. et al. 2010*).

The extrathymic precursors are a pool of $CD34^{+}$ cells (*Saran N. et al. 2010*) which contain $Flt3^{low}$ nonrenewing multipotent progenitors (MPP) (*Adolfsson J. R. et al. 2001*), $Flt3^{hi}$ lymphoid-primed multipotent progenitors (LMPP) (*Adolfsson J.R. et al. 2005*), early lymphoid progenitors (ELP) that already express mRNA for several lymphoid-specific genes such as Rag-1 (*Igarashi H. et al. 2002*), common lymphoid progenitors (CLP) (*M. Kondo et al. 1997*) and T lineage committed progenitors (*Schwarz B.A et al. 2004; Krueger A. and von Boehmer H. 2007*). Although CLP have been suggested to be the major immediate source of circulating thymic progenitors in mice (*Serwold T. et al. 2009*), the exact identity of the thymus-settling progenitors remain still unclear (*Bhandoola A. et al. 2007*) and the most recent studies propose that LMPPs and not downstream progenitors the first precursors to colonize the thymus (*Luc S. et al. 2012*).

In cord blood, a subpopulation of $CD34^{+}CD38^{-}CD7^{+}$ precursors which can generate B, natural killer (NK) and dendritic cells (DC) but lack myeloid and erythroid potential has been identified (*Hao Q.L. et al. 2001*).

1.1.2 Homing of hematogenous precursors to the thymus

Given that the thymus does not contain resident long-term self-renewing precursors, hematogenous stem cells would periodically repopulate the thymus in order to garantee T cell generation. Studies in normal adult mice have shown that this process is intermittent. In fact it was reported that the gate for precursors was opened for about 1 week to allow the niches to fill and afterwards closed for other 2-3 weeks so to allow them to empty (*Foss D. et al. 2001*). Therefore the thymus would constantly present only a limited amount of vacant thymic progenitor niches, and it has been estimated that only about 100-300 of these niches are regularly occupied by progenitors which are then replaced at an average rate of 2–3% per day (*Donskoy E. and Goldschneider I. 1992*; *Spangrude G. J. and Scollay R. 1990)*.

Furthermore another study indicates that the thymus would be able to impress T cell fate on any precursor capable of filling this limited number of niches (*Saran N. et al. 2010*). The mechanisms that determine whether precursors own T cell fate or not rely on their intrinsic capacity to home and settle the thymus. Whereas HSC and MPP are unable to settle the adult thymus, more differentiated LMPP and CLP can do that because they express indispensable homing molecules (*Schwarz B. A. et al. 2007*).

Thymic settling is selective and it depends on the surface expression of determinated molecules on circulating thymic precursors. So far this process of importation has been identified to be regulated by the interactions between p-selectin glycoprotein ligand-1 (PSGL-1)/p-selectin, CCL25/CCR9 and CCL19-CCL21/CCR7 (*Dudakov J.A. and M. R. M. Van den Brink 2011*). Different studies have reported that thymus released chemokine CCL25 and its receptor CCR9 mediate progenitors homing to the thymus (*Uheara S. et al. 2002*; *Wurbel M.A. 2006*). Interestingly, given that CCR9 is restricted to $Flt3^{hi}$

precursors, Flt3 signaling has been suggested to play a role in the generation of $CCR9^{+}$ thymus-settling progenitors (*Schwarz B. A. et al., 2007*). CCR7 has also been found to be required for precursors thymic settling and it has been detected in mice BM precursors which possess the capability to settle the thymus (*Schwarz B.A. et al. 2007*). Studies which have compared both $CCR9^{-/-}$ and $CCR7^{-/-}$ single knockout to $CCR9^{-/-}CCR7^{-/-}$ double knockout mice progenitor cells have shown that each one of this receptor is able to sustain thymic settling in the absence of the other (*Krueger A. et al. 2010*; *Zlotoff D. A. et al. 2010*).

The periodic gated importation of T cell precursors into the thymus is regulated by a molecular gate-keeping mechanism located within the thymic endothelium. This system is able to regulate the temporal and quantitative expression of P-selectin and CCL25 by responding to two distinct negative feedbacks. The first feedback depends on the amount of occupied niches while the second is influenced by the size of the lymphocyte peripheral pool (*Gossens K. et al. 2009)*.

Also BM progenitors lacking CCL-25 and P-selectin molecules show relatively modest defects in T lymphopoiesis (*Rossi F.M. 2005*). Therefore the whole of these data suggest that other unknown redundant factors would contribute to thymic settling and selectivity.

1.1.3 DN thymocytes

1.1.3.1 Human DN thymocytes

In humans $CD4^{-}CD8^{-}$ (double negative) DN thymocytes are characterized by the different expression of CD34, CD38, and CD1a and three distinct steps can be recognized: a $CD34^{+}CD38^{-}CD1a^{-}$ stage that represents the most immature thymic subset and the successive $CD34^{+}CD38^{+}CD1a^{-}$ and $CD34^{+}CD38^{+}CD1a^{+}$ stages (*Spits H. et al. 1998; Plum J. et al. 2000*). The very earliest human thymic progenitors express high levels of CD34 and CD45RA, and lack CD38, CD2, CD5 and CD1a (*Res P. et al. 1996*). Despite already partially T lineage

committed, these progenitors still have the potential to develop into B, NK, dendritic, myeloid and erythroid lineage cells (*Weerkamp F. et al. 2006; Luc S. et al. 2012; Cederig R. 2012*). As these cells mature they progressively lose CD34, and acquire CD38 and CD7 at first followed by CD1a, CD2, CD5, and lastly CD3, CD4 and CD8 (*Galy A. et al. 1993*). In the human thymus CD7 expression levels identify progressive stages of T lineage commitment among the $CD34^{+}CD1a^{-}$ precursors population, as $CD34^{+}CD7^{-}$ cells still retain both multilymphoid and myelo/erythroid potential, $CD7^{INT}$ (intermediate) only multilymphoid (B/NK/T/DC), and $CD7^{++}$ (which include the majority of human thymic $CD34^{+}$ cells) are T/NK/DC committed progenitors (*Hao Q.L. et al. 2008*). Importantly less committed $CD34^{+}CD7^{-}$ thymocytes already express early T lymphoid genes such as Tdt, pTα, and IL-7Rα and are deficient in engraftment capability, therefore suggesting that lymphoid commitment and loss of HSC function begins immediately following the entrance of precursors into the human thymus (*Hao Q.L. et al., 2008*). Within this period $CD34^{+}CD7^{+}CD3^{-}CD4^{-}CD8^{-}$ triple negative (TN) cells start to lose CD34 expression and develop into $CD34^{+}CD7^{+}CD2^{+}CD5^{+}CD1a^{-}$ bipotential T/NK precursors, which subsequently will evolve towards $CD34^{low}CD7^{+}CD2^{+}CD5^{+}CD1a^{+}$ fully committed T cell progenitors (*Sanchez M.J. et al. 1994*).

Concurrently with the decrease of CD34 during the previous stage, thymocytes acquire CD1a (*Galy A. et al. 1993*), and whilst TCR-δ gene rearrangement starts in $CD34^{+}CD1a^{-}$ cells rearrangement of the TCR-γ and TCR-β loci begins in $CD34^{+}CD1a^{+}$ cells (*Blom B. et al. 1999*). $CD34^{+}CD1a^{-}$ precursors still have their TCR-γ and TCR-β loci in germ line position (*Dik W.A. et al. 2001*).

1.1.3.2 Murine DN thymocytes

In mice DN precursors are subdivided in four subsets regarding CD44 and CD25 expression and the following maturation pathway has been described: $CD44^{+}CD25^{-}$ (DN1) > $CD44^{+}CD25^{+}$ (DN2) > $CD44^{-}CD25^{+}$ (DN3) > $CD44^{-}CD25^{-}$ (DN4) (*Godfrey D.I. et al. 1993*). Murine DN1, late DN1/DN2 and

DN3 stages would respectively resemble the human $CD34^{+}CD38^{-}CD1a^{-}$, $CD34^{+}CD38^{+}CD1a^{-}$, and $CD34^{+}CD38^{+}CD1a^{+}$ subsets (*Dik W.A. et al. 2001*).

1.1.3.2.1 DN1 thymocytes

DN1 thymocytes consist of a mixed population of progenitors which hold the ability to generate T, B, NK, and DC. The immediate precursors of DN1 thymocytes, which have been defined early thymic precursors (ETP) and characterized as $Lin^{-}CD25^{-}CD4^{low}CD117(c\text{-}Kit)^{hi}CD44^{hi}$ (*Wu L. et al. 1991a*) are considered to be most immature T cell precursors to settle the thymus (*Moore T.A. et al. 1995*). ETP are not IL-7 responsive and they show marked T lineage but low B and myeloid maturation potential (*Wu L. et al. 1991b*). These precursors are believed to directly derive from bone marrow CLPs although it has been reported that they can also develop via a CLP-independent pathway (*Allman D. et al. 2003)* and only a fraction of these precursors are considered proper ETPs. In fact, although in the adult mouse thymus are present about 10000 $Lin^{-}CD4^{low}$ cells (*Bandhoola A. et al., 2003*), the precursor niches are thought to accept from the periphery only a few hundred settling ETP. Indeed, recent data have separated $CD4^{low}$ ETP from the rest of $CD4^{low}$ precursors. Among $CD4^{low}$ cells, proper ETPs retain both T and B potential, whereas the other $CD4^{low}$ cells are T lineage restricted progenitors which can be further subdivided in DN1a ($CD24^{-}$) and DN1b ($CD24^{low}$) thymocytes by CD24 expression. DN1b originate from DN1a cells as indicated by studies on the OP9-DL1 in vitro system where most of DN1a thymocytes acquire DN1b phenotype following 4 days of culture. The whole of these data suggest that DN1a cells are the earliest mouse intrathymic progenitors that have purely restricted T lineage potential (*Porrit H. et al. 2004*). However, it is still uncertain whether DN1a thymocytes and ETP migrate separately to the thymus or divide into two separate populations after settling the thymus.

1.1.3.2.2 DN2 thymocytes

DN2 thymocytes are precursors which have lost the potential to differentiate into B and NK cells but which can still generate both αβ and γδ T and dendritic cells.

Importantly, DN2 thymocytes begin to express recombinant activating genes (RAG-1 and RAG-2) (*Yannoutsos N. et al. 2001)* and to rearrange the loci for TCR β, γ and δ but not yet α probably because of an intrinsic inactivity of T early α (TEA) promoter during this stage (*Ching-Yu H. and B.P. Sleckman 2007)*. If DN2 thymocytes have already completed TCR γδ rearrangement they receive signals through the pre-TCR γδ receptor that block further TCR β gene rearrangement and drive these cells towards the γδ lineage. Alternatively, if successful β gene rearrangement is completed first, signals transduced through the pre-TCR receptor block further γ and δ gene rearrangement so that DN2 thymocytes become αβ committed (*T. Thagon and E.V. Rothenberg 2008)*. Normally in the thymus less than 5% of DN2 cells develop towards γδ T lineage while most of them are designated to develop to αβ T cells.

1.1.3.2.3 DN3 thymocytes

During the DN3 stage murine thymocytes cease to express both c-kit (CD117) and CD44 and become $CD117^{-}CD4^{-}CD8^{-}CD44^{-}CD25^{+}$ cells. Contrary to the previous stages, now cell proliferation slows down, and these precursors lose multilineage potential and became committed to the T cell lineage (*Rothenberg E.V. 2007*). During the DN3 stage changes in transcription factor genes expression is initiated. For instance the transcriptional repressor Bcl11b starts to be up-regulated during the DN2 stage and plays a putative role in permitting DN3 cells to undergo β selection, given that most of β rearrangement takes place at this stage (*Wakabayashi Y. et al. 2003)*. Then, if the DN3 cells successfully complete the β chain rearrangement they will be able to combine this TCR component to the invariant α chain to form the pre-TCR complex, a molecular structure required by these cells in order to mature towards the DN4 and DP stages. Alternatevely, if these precursors fail successful β chain rearrangement they will undergo p53 dependent apoptosis (*Jiang D. et al. 1996*). Although proliferation and differentiation of DN3 thymocytes need both Notch signaling (*Maillard I. et al. 2006)* and preTCR expression (*Michie A.M. and Zúñiga-Pflücker J.C.* 2002) these events are independent of IL-7 (*Balciunaite G. et al. 2005*).

1.1.3.2.4 DN4 thymocytes

During the transition from the DN3 to the DN4 stage CD25 expression halts and in DN4 thymocytes RAG-1 and 2 become inactivated (*Yannoutsos N. et al. 2001)*. Now these precursors experience rounds of division and rapidly progress to the DP stage. Importantly, only pre-T cells which express a functional pre-TCR and down-regulate p19Arf expression will enter the DP stage. Although it is known that Pre-TCR signaling down-regulates p19Arf through Bmi-1 activity, other mechanisms seem to be involved. For instance, Notch signaling has been found to be essential for the DN-DP transition independently of pre-TCR activation. Indeed p19Arf repression by Bmi-1 seems to be related with Notch signaling activity (*Myiakazi L. et al*. *2008*).

1.1.4 ISP thymocytes

In humans CD34+CD1a+$CD3^-$ thymocytes pass through a CD4+ intermediate single positive (ISP) step. Throughout this stage they first start to induce CD8α (when are defined early double positive thymocytes) followed by CD8β, and finally become DP thymocytes (*Hori T. et al., 1991; Kraft D.L. et al. 1993*). Evidence from studies using human tissue suggests that TCRβ selection starts at the transition of the $CD3^-CD4^+CD8^-$ into $CD3^-CD4^+CD8\alpha^+\beta^-$ stage (*Blom B. et al. 1999*).

CD4+ precursors have been subdivided in a $CD4^+CD1a^+$ and a $CD4^{hi}CD1a^{hi}$ population (*Hori T. et al., 1991)*. Although both dendritic and T cells have been shown to arise from this $CD4^+CD1a^+$ intermediate stage, separate progenitors for either DC or T lineages have been identified on the basis of these surface markers expression in $CD4^{low}CD1a^{low}$ and $CD4^{++}CD1a^{++}$ cells, which would be true ISP T and earlier DC precursors, respectively (*Marquez C. et al. 1995*). CD4 bright expression has been reported on thymic DC (*Sotzik F.et al. 1994*) and these cells have been suggested to derive from thymic $CD34^+$ precursors which had predominantly differentiated through a $CD1a^+$ pathway (*Dalloul A. et al. 1999*). $CD4^+CD1a^+$ cells can be also subdivided in $CD44^+$ DC and $CD44^-$ T precursors (*Marquez C. et al. 1995*) and in mice the switch from $CD44^+$ to

$CD44^-$ is an essential stage in early T cell full lineage commitment (*Barcena A. et al. 1994*).

Some human $CD4^+$ ISP are already TCR β^+, however their frequency is much higher within the $CD4^+CD8\alpha^+$ (early double positive) stage. Indeed, differently from mice, few human precursors undergo β-selection before becoming $CD4^+$ ISP (*Blom B. et al. 1999*) whereas a larger part is β-selected during the DP stage (*Carrasco Y.R. et al. 1999).* Finally a third group of cells starts up β rearrangement only when already $CD4^+CD8\alpha^+$ early DP (*von Boehmer H*. et al. 2003).

Both in rats and mice DN thymocytes before mature to DP cells pass through a $CD4^-CD8^+$TCR $\alpha\beta^{low}$ intermediate single positive stage (*Paterson D.J. and Williams A.F. 1987*; *Shortman K. et al. 1988; McDonald et al. 1988*). Studies in vitro using murine DN thymocytes have also showed the concomitant presence of a $CD3^-CD4^+CD8^-$ ISP population, therefore suggesting the occurrence of two different independent pathways from DN to DP thymocytes in mice, and further observations have showed that a $CD4^+$ ISP population would appear slightly earlier and in lower proportion in comparison to the $CD8^+$ (*Hugo P. et al. 1990*). This choice between a 'CD4 or a 'CD8 intermediate ISP pathway seems to be genetically predetermined (*Matsumoto K*. *et al. 1991*).

1.1.5 DP thymocytes

$CD3^+CD4^+CD8^+$ DP cells are the largest population of cells normally detected within the thymus where they constitute around 75-88% of thymocytes. They are short-lived rapidly proliferating cells, but as soon as they stop dividing RAG-1 and 2 expression increases and the TCR α chain gene rearrangement begins (*Yannoutsos N. et al. 2001)*. It is during this stage that thymocytes undergo negative and positive selection processes (*von Boehmer H*. *et al. 2003*). Once DP cells have rearranged their TCR α chain they also strongly express a complete TCR along with CD3 and ζ chain. However these cells, prior to selection still express a random repertoire which may either recognize any antigenic peptide (self or not self) presented by any MHC (self or foreign) or

may not recognize any combinations of the previous patterns. DP thymocytes have a lifespan of 3-4 days, and just 1 to 3% of them will survive these processes to finally become SP mature T lymphocytes ready to leave the thymus. The DP thymocytes are mainly located within the thymic cortex but after positive and negative selection they migrate to the medulla where they undergo another round of negative selection before exiting the thymus (*Sprent J. and Kishimoto H*. *2002*).

1.1.6 Positive selection and the development of SP phenotypes

Thymic positive selection mainly takes place in the inner cortex. This process is required in order to eliminate those cells which do not express a MHC-self restricted TCR. The crucial role played by MHC bound self peptides in generating self restricted cells has been recognized and the critical parameter determining the post contact thymocytes outcome appears to be the relative amount of TCRs occupied by peptide/MHC complexes, a proportion determined by the level of avidity of the TCR-MHC interaction (*Ashton-Rickardt P.G. et al. 1994*). According to this model those thymocytes whose TCR showed a low affinity for the MHC/antigen complex would die by apoptosis whereas those whose receptor had a high affinity would receive a 'survival signal' (.*Ashton-Rickardt P.G. and Tonegawa S. 1994).* The underlying mechanisms have been in part elucidated. For instance, it has been discovered that thymocytes survival would, at least in part, be triggered through Egr1 activation following TCR engagement in an ERK-dependent manner (*McNeil L. K. et al. 2005).*

It is during the positive selection process that DP cells acquire a single positive mature phenotype, becoming definitely $CD4^+$ or $CD8^+$ functional lymphocytes. Although the precise underlying mechanisms are still unclear, two different models have been proposed (*Ronald N. Germain 2002)*. Presently the majority of evidence tends to support the 'stochastic model' (*Ray K. M. Leung et al*. *2001*). According to this model, DP thymocytes casually lose CD4 or CD8 regardless of their TCR specificity. Afterwards, if a thymocyte co-express CD4 and MHC-II restricted TCR (or vice versa CD8 and MHC-I restricted TCR) it

would survive, otherwise in absence of an adequate stimulation it would die. The alternative model, named ‘instructive model’, assumes that thymic epithelial cells (TEC) interact with thymocytes through MHC-I or II and signal to these precursors to switch off the wrong co-receptor. This interaction subsequently would cause distinct intracellular signals, in part due to the participation of either CD8 or CD4 (*K. Ravichandran and S. Burakoff* 1994).

Over-expression of *Notch* directs T cells into the SP CD8 lineage, so suggesting that this factor can act as an inhibitor of CD4 SP development (*Ellen Robey et al. 1996)*. Moreover, further evidence in mice have shown that when in presence of constitutively activated Notch, MHC-II restricted thymocytes are directed towards CD8 differentiation pathway (*Robey E. et al. 1996)*. In effect it has proposed that TCR MHC-II interaction does not necessitate Notch signaling (figure 2) and there is evidence that continued signaling through the TCR complex triggers to the development of $CD4^{+}CD8^{-}$ rather than $CD4^{-}CD8^{+}$ lymphocytes (*Yasutomo K. et al. 2000)*. Indeed TCR-mediated GATA-3 upregulation it has been found to be required for CD4 T cells generation (*HernÃndez-Hoyos G. et al. 2003*) An interesting interpretation of these data is the so-called 'coreceptor reversal' model, which proposes that during positive selection TCR activation on DP thymocytes induces a shutdown of CD8 expression, without prompting the precursors towards a $CD4^{+}CD8^{-}$ lineage fate.Consequently, MHC-I restricted thymocytes would receive a weak signal because of CD8 transient downregulation, whereas MHC-II restricted cells would be engaged in a uninterrupted strong signal. The precursors that have temporarily harmed CD8 expression would be then induced by IL-7 to differentiate into $CD8^{+}$ T cells by switching off CD4 and up-regulating CD8 *(Brugnera E. et al. 2000*). This model offers a valid explanation for the large number of $CD4^{+}CD8^{dim}$ precursors observed in the MHC-II deficient mouse thymus (*Crump A.L. et al*. *1993*).

1.1.7 Negative selection

Negative selection is required in order to avoid the possibility that potential autoreactive T lymphocytes enter into the periphery. The thymocytes which have passed the positive selection migrate to the thymus cortico-medullary junction where they meet macrophages and dendritic cells which express self peptide-MHC complexes and if they bind self peptide-MHC with high affinity the cells undergo apoptosis and die.

According to the 'differential avidity hypothesis', both low and high TCR affinity-avidity interactions induce cell survival during positive selection, whereas high affinity/avidity TCR MHC-peptide engagements lead to apoptotic signals during negative selection. Consequently the precursors which interact with too strong affinity to self presented antigens at this stage would be eliminated before become mature T cells (*Ashton-Rickardt P.G. and Tonegawa S. 1994).* Indeed it has been demonstrated that TCR dependent prolonged low level activation of the extracellular signal-regulated kinases (ERKs) pathway promotes positive selection, while strong but temporary ERK activation triggers cell death stimuli (*Mariathasan S. et al. 2001*; *McNeil L. K. et al. 2005*). The pro-apoptotic Bcl-2 family member Bim has been reported to be crucial for negative selection by inducing thymocytes apoptosis following a TCR strong signaling (*Bouillet P. et al*. *2002*). Surprisingly, very recently it has been discovered that in the case of ubiquitous self-antigens Bim is not required for the negative selection of self-reactive thymocytes and therefore the existence of additional nonapoptotic mechanisms has been suggested (*Suen A. Y. W. and T. A. Baldwin 2012)*.

Alternatively to the previous model, the 'differential signaling hypothesis' proposes that qualitatively but not quantitatively different signals are delivered during positive and negative selection (*Aberola-Ila J. 1996)*. However, the 'differential avidity hypothesis' seems to be more realistic because some signaling pathways involved in the two selection processes are common *(Mariathasan S. et al. 2001)*. Moreover the avidity model has been supported by experiments in which the avidity of signaling in thymus organ cultures was

controlled by manipulating the amount of peptide presented in TAP-deficient TCR-transgenic mice (*Ashton-Rickardt P.G. et al*. *1994*).

Medullary TEC (mTEC) play a fundamental role in negative selection and a variety of tissue related antigens (TRA), are presented to developing thymocytes by these cells in the medulla (*Derbinski J. et al. 2001*). A strong correlation between intrathymic expression of certain autoantigens and susceptibility to autoimmune diseases, such as type I diabetes (IDDM) it has been reported (*Pugliese A. et al. 1997*). Medullary TEC require the expression of the transcription factor AIRE in order to transcript adequate levels of organ-specific antigens (*Anderson M.S. et al. 2002)*. Additionaly it has been recently reported that another mechanism through Aire controls thymic negative selection is by regulating the transfer of TRA from mTEC to thymic dendritic cells (*Hubert F.X. et al. 2011*). Finally, mutation or deletion of the AIRE gene is linked to autoimmunity respectively in humans and mice (*Aaltonen J. et al. 1997; Ramsey C. et al. 2002*). However only about one third of TRA expression is controlled by Aire and it has been suggested that other factors exist in order to induce the expression of Aire-independent TRA by mTEC (*Derbinski J. et al. 2005*). Interestingly, the experimental loss of a single TRA gene expression in mice thymus, even if Aire is present and functional, is able to remove central tolerance for that antigen and cause development of specific autoimmunity (*DeVoss J. et al. 2006*).

Given that some precursors are able to escape from thymic negative selection (*Bouneaud C. et al. 2000*), there are other mechanisms outside the thymus which are able to induce self tolerance, such as T cells functional anergy induced by regulatory cells (*Ronald H. Schwartz 2003)*.

Figure 3 reviews thymic T cell development in mice and humans.

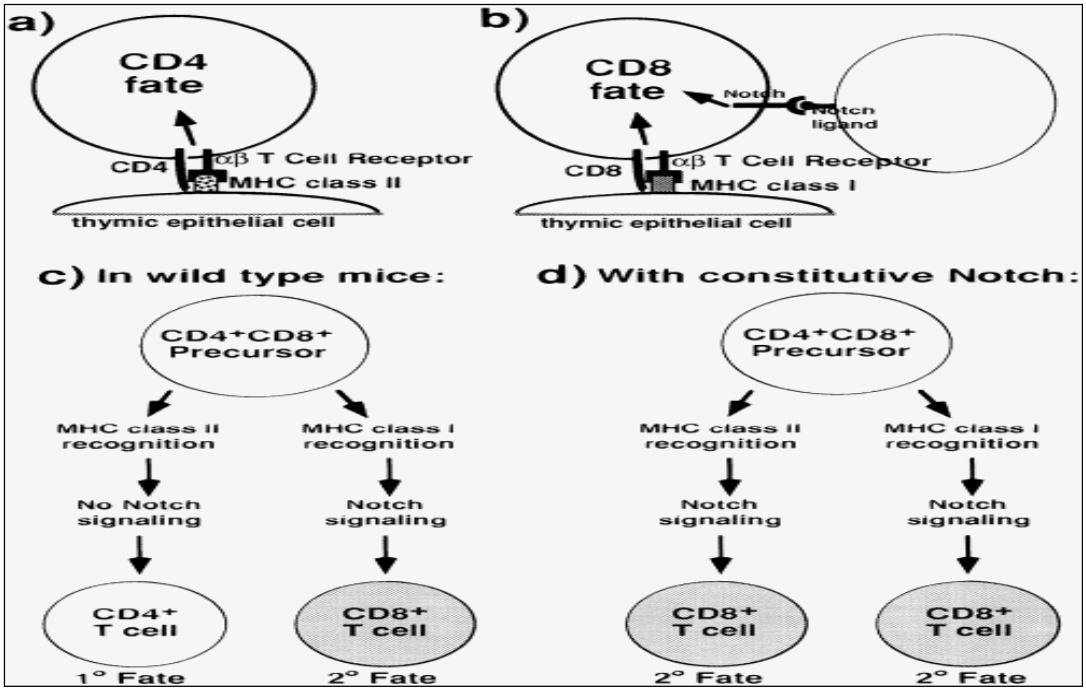

Figure 2: Signal Integration in the CD4 versus CD8 Lineage Decision. The image is taken from Robey E. et al. Cell 1996; 87(3): 483-492

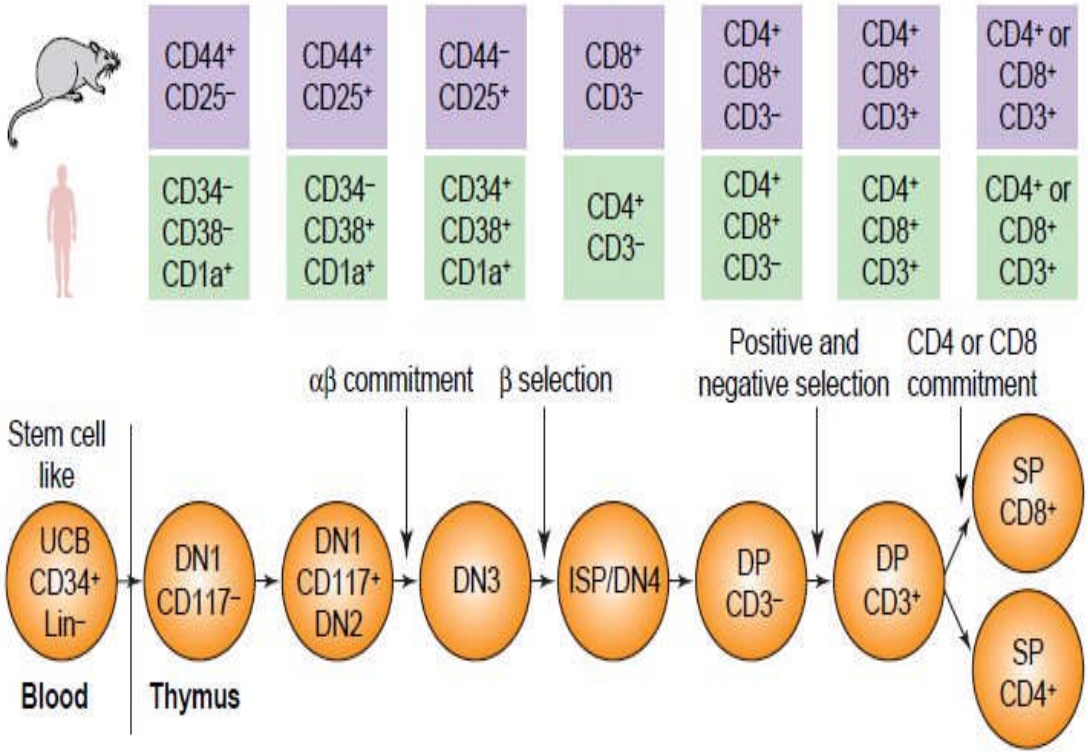

Figure 3: T cell development in mice and humans. The image is taken from Weerkamp et al. Trends in Immunology 2006; 27:125-131

1.1.8 Naive T lymphocytes

When the thymocytes have successfully passed through all the developmental and selection processes they are finally mature to exit the thymus and enter into the circulatory system as naïve T lymphocytes. Although these newly generated immune cells are considered mature they have not yet encountered any antigen and this render them different from activated and memory T lymphocytes. Given that these cells express a vast diverse repertoire they are able to respond to novel pathogens that the immune system has never encountered before. Their presence it is essential for the organism in order to harbour an adequate response to new pathogens. However, as the thymus atrophies with age, thymic output decreases and in turn, there is an increase in infection susceptibility and reduced vaccine responsiveness (*Grubeck-Loebenstein B 1998)*. Naive T cells are usually characterized by the surface expression of L-selectin (CD62L) and lack of markers typical of activated and memory T cells, such as CD25, CD44 and CD69 and shortened CD45 isoforms. These cells need L-selectin (CD62L) to enter into the lymph nodes (*Bradley L.M. et al. 1994)* which is the place where they encounter their cognate antigens and start to differentiate into effector T cells. When naive T cells encounter a cognate antigen and they are activated they become $CD25^{+}CD44^{+}CD62L^{low}CD69^{+}$ cells. Later ahead some of these cells further differentiate into peripheral memory T lymphocytes. Naive T cells require to be stimulated by antigen presenting cells (APC) through cell-cell contacts (immune-synapse) to be activated. The immune-synapse is a dynamic and strong structure constituted by a multiplicity of adhesion and co-stimulatory molecules such as, on the T cells surface, LFA-1 and CD28 (*P. Anton van der Merwe and Simon J. Davis 2002)*. The constitution of an immunological synapse guarantees that the cytokines released during lymphocytes activation do not act against bystander cells (*Grakoui A*. *et al. 1999*). The APC cells that activate naive T lymphocytes are constituted by dendritic cells (DCs) which migrate to lymph nodes after encountering an antigen, and there these cells activate both naive and memory T cells, whereas both macrophages and B cells are able to stimulate only memory T cells. Therefore DCs drive the

differentiation of naive T cells into effector, memory or tolerant cells (*Lanzavecchia A. and Sallusto F*. 2001). In fact DCs are also able to tolerize peripheral naïve T cells to self-antigens (*Banchereau J. and Steinman R.M*. *1998*). Moreover these cells polarize CD4+ naive T lymphocytes into either Th1 or Th2 T helper effectors (*Moser M. and Murphy K.M. 2000).* Moreover systemic Interleukin 7 (IL-7) mediates the expression of MHC-II on IL-7Rα^+ plasmacytoid DCs and in turn naive $CD4^+$ T cell population expansion *(Guimond M. et al*. *2009*). If the naïve T cells after having scanned dendritic cells during their permanence within the lymph node, have not encountered any cognate antigen, they then return to the blood stream via efferent lymphatics and further continue blood and lymph node recirculation until they die (*von Andrian U.H. and Mackay C.R. 2000).*

1.1.9 Homeostasis of naïve T lymphocytes

Naive T cells are considered to be quiescent non-dividing cells and require IL-7 and IL-15 (and in minor proportion other cytokines such as IL-2, IL-4, IL-12 and IL-21) for their survival (*Hassan J. et al. 2001*; *C.D. Surh and Sprent J. 2005; Marrack P. et al*. *2000*). These cytokines are also responsible for naïve T cells proliferation in response to peripheral T cell lymphopenia, a phenomenon nominated lymphopenia-induced proliferation (LIP) (*Takada K. and Jameson S.C. 2009).* IL-7 requirement for both T cell naïve homeostasis and LIP is supported by studies in $IL7^{-/-}$, $IL\text{-}4^{-/-}$ and IL-15 $^{-/-}$ mice (*Tan J.T. et al. 2001)*. For instance it has been observed a gradual disappearance of naïve T cells after 1 month since their adoptive transfer into $IL\text{-}7^{-/-}$ hosts (*Tan J.T. et al. 2001)*. The whole of previous experiments suggests that naïve T cells necessitate IL-7 to survive, and, moreover, that low basal levels of circulating IL-7 are generally able to regulate the entire size of the naïve peripheral T cell pool (*Rathmell J.C. et al*. *2001*). However, an optimal stimulation of naïve T cells requires the cooperation of sustained low affinity TCR triggering and strong IL-7 signaling (*Surh and Sprent J. 2008)*. IL-15 activity appears to play a major role in both

naïve and memory $CD8^+$ T cells homeostasis (*Berard M. et al. 2003)* as suggested by studies in mice defective for this cytokine which showed a selective deficiency of the previous $CD8^+$ subsets together with TCR γδ TILs, NK and NKT cells (*Lodolce J.P. et al. 1998*). In spite of that, IL-7 alone is able to induce proliferation of naïve and memory $CD8^+$ T cells (*Schluns K.S. et al. 2000*) whereas IL-15 without IL-7 has only a little effect on naïve CD4+ cells *(Surh and Sprent J. 2008)*. Moreover, IL-7 also drives the differentiation of effector $CD4^+$ into memory T lymphocytes (*Bird L. 2004).*

Recently it has been discovered a role of IL-21 in naïve T cells homeostasis. In fact this cytokine not only contributes to proliferation of naïve T cells without affecting memory cells but it is also important in order to retain the expression of naive T cells specific surface markers (such as CD45RA, CD27, CD62L and CCR7 on $CD4^+$ T lymphocytes). Despite a similar structure, the role of IL-21 strongly diverges from IL-2 since this cytokine drives proliferation and differentiation of naïve $CD4^+$ T lymphocytes into activated memory T cells (*Ferrari-Lacraz S. et al. 2008*). On the contrary, IL-2 stimulates the development of regulatory T cells (*Malek T.R. and Bayer A.L. 2004*) and in combination with IL-7 it is able to sensitize proliferating naïve (but not memory) T cells to Fas-induced apoptosis (*Jaleco S. et al. 2003*). Finally IL-21, in concert with IL-15, can induce the expansion of $CD8^+$ memory T cells (*Nguyen H. and Weng N.P. 2010).*

The lifespan of naive $CD8^+$ T cells in TCR transgenic mice has been determined. These cells did not divide in secondary lymphoid organs and their minimum life span was about eight weeks (*von Boehmer H. and Hafen K. 1993).* Other studies in mice have shown that the T cell naive pool would be self-sustaining also in the absence of proliferation due to lack of antigen recognition and stimulation (*Di Rosa F. et al. 1999*). Moreover, since the thymus supplies a constant output, the number of naïve T cells it is in some way maintained stable by a sort of competition for the available space in the periphery (*Freitas A.A. and Rocha B. 2000*). In fact, when thymic output

decreases, as it happens in the elderly, a compensatory expansion of the memory T cell compartment takes place. Moreover it has been reported that with aging peripheral naive $CD4^+$ T cells become longer lived (*Tsukamoto H. et al. 2009*). Recently it has been determined that the naive T cells lifespan is about 6-10 years in men and it has been found that despite mice, whose naive T cell pool is almost exclusively sustained by thymus output, in humans this is maintained throughout the lifetime by cell division (*den Braber I. et al. 2012*). Therefore in humans the TREC content and the TCR repertoire diversity of peripherial T cells which present a naïve phenotype declines with age (Figure 4) (*Zhang S.L. and Bhandoola A. 2012*). Although it has been reported that in mice the homeostatic proliferation of naïve T cells give arise to T cells with different propierties (*Haluszczak C. et al. 2009*), it is presently still unknown wheter the human naïve T cells which proliferate in the periphery are functionally equivalent to RTE.

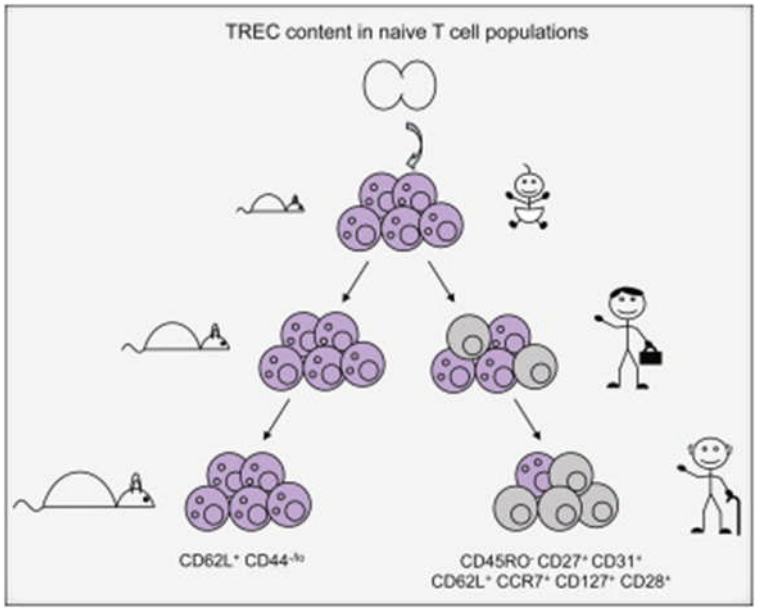

Figure 4: TREC content in human naïve T cells declines with age. The image is taken from Zhang S.L. and Bhandoola A. Immunity 2012; 36(2): 163-165

1.1.10 Determination of thymic output

The concentration of T cell receptor excision circles (TREC) is one method to quantify thymic output in humans (*Douek D.C*. *et al. 1998)*. TREC are small portions of circular DNA that have been cut out from the genome during TCR α and β gene rearrangement. Since episomes do not replicate with the cell, as the T lymphocytes divide the TREC are passed to just one of the pair of descendent cells. Given that TREC amount is obviously higher among recent thymic emigrants (RTEs) TREC/T cell ratio efficiently reflects thymic lymphocytes de novo generation and output (*Douek D.C*. *et al. 1998*). The most suitable TREC for analysis is δRec-Ψ(J)α or signal joint (sj)-TREC which results from δRec-Ψ(J)α rearrangement (TCRD deletion), an event that happens before V(α)-J(α) rearrangement and that indicates definitive commitment of thymocytes to the αβT cell lineage. This deletion leads to the generation of a sj-TREC and can be observed in about 70% of all newly generated αβ T cells (*Douek D.C*. *et al. 1998; De Villartay J.P. et al*. *1988*; *Verschuren M.C*. *et al. 1997*). TREC are stable during a long period (*Sempowski G. et al*. *2001*) and a maximum of two sjTREC can be present within one αβ T cell if the corresponding rearrangement takes place in both alleles and if the cell does not proliferate after the rearrangement (*Verschuren M.C*. *et al. 1997*).

Assess of TREC/T ratio in blood samples shows some limitations. The most restrictive one is the absolute requirement of the flow cytometry analysis in order to quantify CD3 number in combination with the quantitative real-time polymerase chain reaction (qPCR) to detect TREC levels (*Mitchell W.A. et al. 2010*). A new method it has been recently developed with the aim of surmounting these limits and evaluating the TREC/T ratio by using only qPCR. This mono-assay adds to TREC quantification the possibility to quantify the sample T cell number by using albumin as a housekeeping gene which reflects the total cell quantity, and VD-J as an index of unrearranged TCR-β count and in turn the total germline cell number. Consequently the unrearranged TCR-β

cell count subtracted from the total cell number correspondes to the final rearranged TCR- β count and therefore the T-cell sample number. This innovative method is also able to accurately quantify theTREC/T ratio in dried blood spot samples (*Lang P.O. et al. 2011*).

An alternative parameter to determine human thymic output is CD31 (PECAM-1) surface molecule expression. $CD31^+$ T cells are believed to correspond to a subset recently emigrated from the thymus (RTEs), whereas the $CD31^-$ subset is thought to derive from $CD31^+$ T cells after homeostatic cell division (*Kimmig S. et al. 2008*). CD31 can therefore validly be used to distinguish $CD31^+$ RTEs or thymic naive from $CD31^-$ central naive $CD45RA^+$ T cells. Indeed it has been demonstrated a significantly higher TREC content in the first compared with the latter cell population (*Kimmig S. et al. 2008*). Moreover, similar to TREC level, absolute numbers of thymic naive $CD31^+$ $CD45RA^+$ T cells decrease in peripheral blood during ageing *(Junge S. et al. 2007*). However a slight decline in TREC levels among the thimyc naive $CD31^+$ $CD45RA^+$ T population happens with time therefore suggesting a certain degree of in vivo turnover (*Kilpatrick R.D*. *et al. 2008*). This trend appears to be caused by extrathymic proliferation of the $CD31^+$ thymic naive $CD45RA^+$ T cells which still maintain CD31 expression. In effect it has been reported that homeostatic levels of IL-7 in absence of TCR engagement induce RTE proliferation (*Azevedo R.I. et al*. *2009*). Thus transition from $CD31^+$ thymic naive $CD45RA^+CD4^+$ T cells towards $CD31^-$ central naive CD45RA T cells would require TCR engagement and would be characterized by intensive cell division, whereas cytokine driven homeostatic proliferation might result in low level autoreplication of $CD31^+$ thymic naive $CD4^+$ T cells (*Kohler S. and Thiel A. 2009).* However, almost all $CD31^+$ T cells are RTE and therefore the assessment of absolute numbers of $CD31^+$ among T cells can be a suitable method to evaluate human thymic output, altough it would be better if performed along with TREC levels measurement (*Bains I. et al. 2009*). Recently protein tyrosine kinase 7 (PTK7) has been introduced as a novel marker for $CD4^+$ RTEs enumeration. $PTK7^+$ $CD4^+$ cells are true RTEs which contain high

levels of sj-TRECs, rapidly decrease after complete thymectomy and show reduced IL-7 responsiveness, capacity to become activated, proliferate, and produce IL-2 and IFN-γ compared to the older PTK7$^-$ CD31$^+$CD4$^+$ naive T cells (*Haines C. et al*. *2009*). Over the years, other systems to identify RTEs in mice have been established such as labeling thymocytes with BrdU (*Tough D.F. and Sprent J.1994*), the intrathymic injection of FITC (*Scollay R. et al. 1984*) and development of RAG2–GFP transgenic mice *(Yu W. et al*. 1999) Since GFP signal is still detectable after RAG expression ends, GFP$^+$ peripheral T cells are RTEs and GFP signal intensity correlates with time since thymic exit. *(Yu W. et al*.1999). Since mouse RTEs are enriched of QA2^{lo}CD24^{hi} cells they can be detected by flow citometry, but this method excludes about 85% of RTE (*Fink P.J. and Hendricks D.W. 2011*). Finally, in rats RTE are phenotipically Thy$^+$CD45RC$^-$RT6$^-$ and can be easily quantified by flow citometry (*Hosseinzadeh H. and Goldschneider I. 1993*).

1.2 FACTORS INVOLVED IN T CELL DEVELOPMENT

1.2.1 NOTCH

1.2.1.1 Notch protein

Notch proteins are a family of transmembrane receptors that control embryonic development and cell fate, determination, number and migration (*Artavanis-Tsakonas S. et al. 1995*; *Weinmaster G. et al. 1991*). The Notch signalling pathway has been conserved through evolution and four Notch receptors (Notch 1-4) have been isolated in mammals (*Weinmaster G. 1997*). These proteins have a large extracellular domain constituted by 29-36 copies of epidermal growth factor (EGF) motifs and three Lin-12/Notch repeats (LNR) (*Wharton K.A. et al. 1985*; *Sakamoto K. et al. 2005*). The EGF motifs are required for ligand induced Notch proteins homodimerization and protection

from the action of proteases which may determine unspecific constitutive activation (*Sakamoto K. et al. 2005).* The intracellular part contains a polyglutamine tract called OPA domain, six CDC10/ankyrin repeats (*Breeden L. and Nasmyth K. 1987*), one or two nuclear localization sequences and a C-terminal PEST sequence which controls protein degradation (*Blaumueller C.M. et al. 1997*). Notch is constitutively processed by a calcium dependent furin-like serin protease and this is an indispensable step for its maturation (*Logeat F. et al. 1998*). In fact, following this proteolytic processing, the extracellular and intracellular Notch protein portions reassociate through disulfide bonds and form a heterodimer which is the functional receptor presented on the cell membrane (*Blaumueller C.M. et al. 1997*). After that the initial proteolytic cleravage of Notch generates a functional receptor, a contact between Notch and the ligand is required for its activation. This binding determines a sequence of cleavages that result in the generation of a Notch intracellular domain (NIC) which migrates to the nucleus where through the interaction with CBF-1 and other transcriptional factors induce target genes transcription (*Struhl G. and Adachi A 1998*; *Schroeter E.H. et al. 1998*). Figure 5 shows Notch receptors and ligands and figure 6 the Notch activation pathway.

Within the Notch family, only Notch1 appears to play a role in T cell development as demonstrated by studies in mice (*Wilson A. et al. 2001*). Indeed it is well recognized that Notch-1 signalling halts B cell commitment and both induce and maintain T lineage potential of thymic precursors (*Radtke F. et al. 2004*).

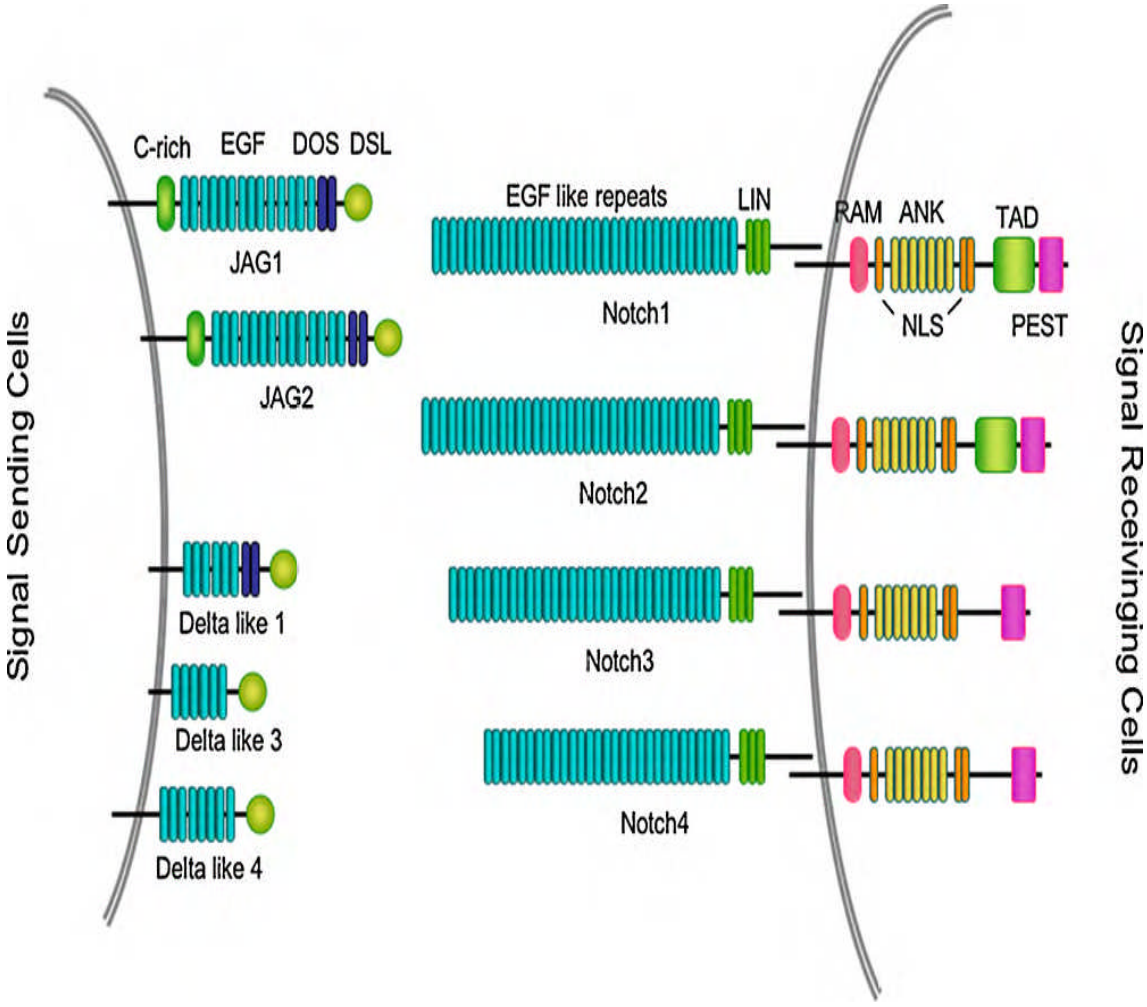

Figure 5: Notch receptors and ligands. The image is taken from Yashiro-Ohtami Y. et al. Seminars in Immunology 2010; 22: 261-269

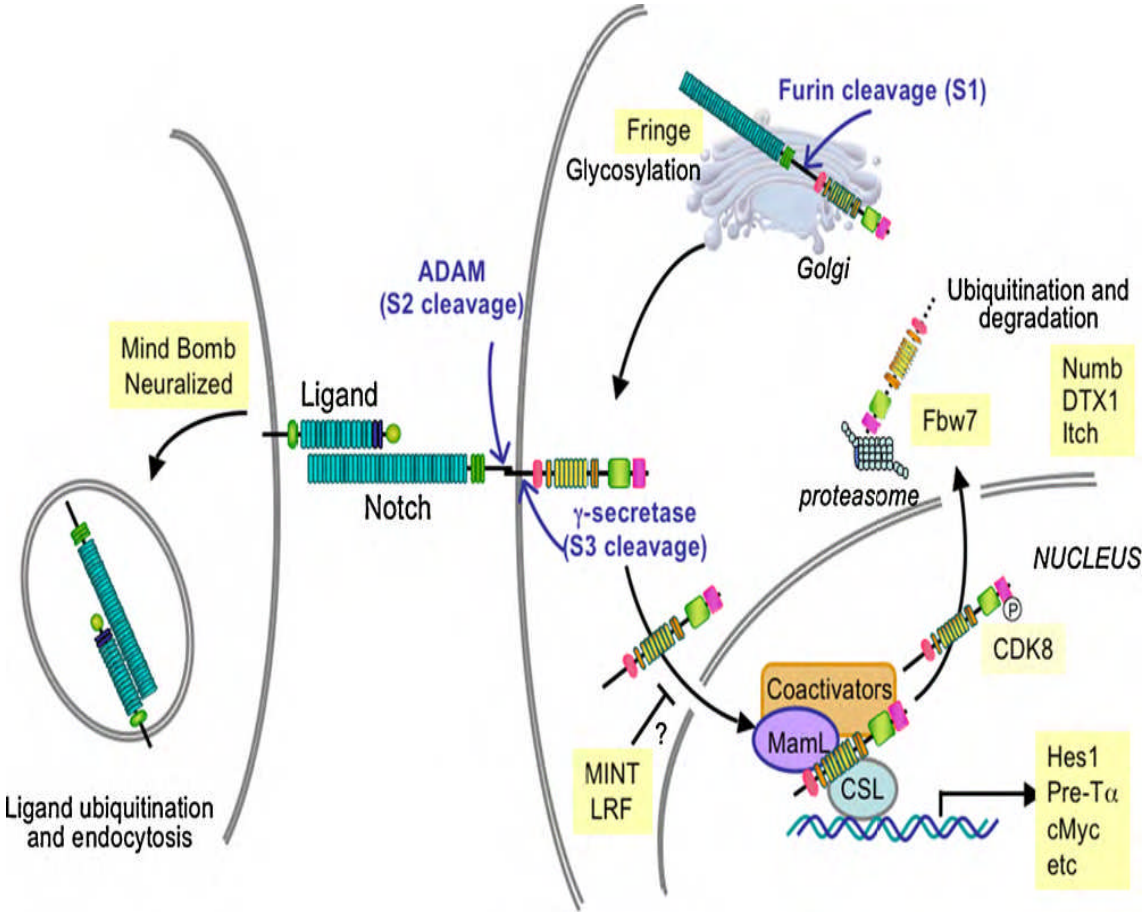

Figure 6: Notch activation pathway. The image is taken from Yashiro-Ohtami Y. et al. Seminars in Immunology 2010; 22: 261-269

1.2.1.2 Notch target genes

A variety of Notch induced genes are involved in T cell development. Among them, Hes-1, a member of the helix-loop-helix (bHLH) proteins family, is required for T lineage specification at early stages of T cell development whereas it is dispensable for further thymocyte maturation through and after the β selection checkpoint (Wendorff A.A. et al. 2010). Indeed, Hes-1 overexpression in bone marrow inhibits B-cell development (*Weerkamp F. et al. 2006*).

Similarly to Hes-1, T-cell factor 1 (TCF-1) is upregulated by Notch signalling and it has been identified as an important regulator of early T cell lineage

specification (*Verbeek S. et al. 1995*). This transcription factor is highly expressed in the earliest thymic progenitors, and it and it has been reported that its overexpression in BM progenitors induce T lineage specification in absence of Notch (*Weber B.N. et al. 2011*). Further analysis of TCF-1 transduced precursors showed expression of T cell specific transcription factors such as Gata-3, Bcl11b, and components of the T-cell receptor, therefore suggesting that Notch signalling, at least in part, would induce T cell fate commitment by inducing TCF-1, and in turn, upregulating other T cell genes (*Weber B.N. et al. 2011*). Although lack of TCF-1 strongly reduces thymic cellularity in mice (*Schilham M.W. et. al. 1998*), its role in early T cell development seems to be redundant and the related transcription factor LEF-1 would normally compensate for its absence (*Okamura R.M. et al. 1998*).

GATA-3 is a zinc finger transcription factor whose expression among hematopoietic cells is limited to mature T cells and thymocytes and GATA-3 binding sites are present in a variety of T cell specific genes (*Ting C.N et al. 1996)*. GATA-3 action is considered important during early stages of T cell development and in the differentiation of LMPP to ETP (*Hosoya T. et al*. 2009). Both adult and fetal ETPs express high levels of GATA-3 and adult mice in which GATA-*3* was deleted generated <3% of ETPs and DN2 cells when compared with control mice *(Hosoya T. et al*. *2009*). Despite it is not yet clear which early progenitor require GATA-3 activity for ETP generation, it has recently reported that the VCAM-1^{-} pool of adult LMPPs already express low levels of GATA-3 mRNA (*Lai A.Y. and Kondo M. 2007*). However, reintroduction of GATA-3 in HSC derived from GATA3-deficient mice before Notch signaling activation failed to induce T cell development (*Hozumi K. et al. 2008*). Importantly it has been observed that Notch signaling is able to block B cell development independently of GATA-3, a data which indicates that GATA-3 would not be required for inhibition of B cell development but only for T cell lineage orientation (*Hozumi K. et al. 2008*). Interestingly when GATA-3 is deleted in mice, only bone marrow NK but not thymic NK cells are generated *(Vosshenrich C.A. et al. 2006).* Therefore the whole of these data suggest that

GATA-3 action would be crucial in haematopoietic progenitors which have already entered the thymus and lost B cell potentiality following Notch signalling activation. Finally GATA-3 is fundamental for T cell development at the β selection stage and for the generation of CD4 SP T cells (*HernÃndez-Hoyos G. et al. 2003; Pai S.Y. et al. 2003*).

The pre-TCR α chain gene (pTa) is also a target of Notch signaling in T cell precursors (*Deftos M.L. et al. 2000*). The pTa gene encodes a transmembrane protein that couples with the newly rearranged TCRβ chain so to produce the pre-TCR complex in thimic precurosors (*von Boehmer H. and Fehling H.J 1997*). The pTa gene is expressed in thymocytes, and its up-regulation corresponds with irreversible T cell lineage commitment in both murine and human T cell precursors (*Res P. and Spits H. 1999*). Moreover, pTa is required for αβ commitment but not γδ T cell development (*Fehling H.J. et al. 1995*; *Aifantis I. et al. 1998*). The pTa gene expression depends on Notch activity as mutation of this gene CSL-binding sites abolished its induction in mice DN2 thymocytes, although it did not impaired its subsequent expression (*Reizis B. and Leder P*. 2002).

1.2.1.3 Notch ligands

The Notch ligands are transmembrane glycoproteins with an extracellular domain containing a variable number of EGF-like repeats and a conserved region unique to this family named DSL (Delta/Serrate/Lag-2) domain which is required for Notch binding and activation (*Fleming R.J. et al. 1997*; *Fitzgerald K. and Greenwald I. 1995*). This family of proteins is divided into two subclasses: the Serrate/Jagged family including Jagged-1 and Jagged-2 and the Delta family consisting of Delta-like ligands (Dll) Dll-1, Dll-2 and Dll-4 (*Harman B.C. et al. 2003).* These factors are shown in figure 5.

1.2.1.3.1.1 Dll-4

Among these factors, Dll-4 it has recently been identified as the fundamental and nonredundant Notch1 ligand responsible for inducing T cell development of thymic precursors (*Koch U. et al. 2004; Hozumi K. et al. 2008*). Indeed, DII-1 is

not necessary for normal T cell development in mice (*Hozumi K. et al. 2004*). Moreover a recent study evidenced the existence of a hierarchy in Notch-Delta ligands strength interactions, with Notch1-Dll-4 at the top of the rank and with the maximum capability in inducing and sustaining T cell development of haematopoietic precursors (*Besseyrias* V. *et al. 2007*). However, it is well accepted that Dll-1 alone is able to induce T cell development (*Ohishi K.et al. 2002)* as demonstrated by several studies with in vitro models (*Schmitt T.M. and Zúñiga-Pflücker J.C. 2006)*. Despite its crucial role in T cell development, little is known about thymic Dll-4 gene regulation, as knoweldge in this area mainly regards endothelial cells given that many studies have associated Dll-4 to vascular development and neo-angiogenesis while the role of this factor in thymopoiesis has been discovered only recently.

1.2.1.3.1.2 Dll-4 gene regulation by hypoxia, grow factors and the link with Foxn-1

Dll-4 knockout mice shows embryonic lethality from severe vascular defects (*Gale N.W. 2004)* and these phenotypes have been also described in VEGF knockout mice (*Ferrara N. 1996).* Moreover a certain number of studies have shown that Dll-4 is up-regulated at sites of physiologic and pathologic angiogenesis (*Mailhos C. et al. 2001*; *Claxton S. and Fruttiger M*. *2004*). In fact it has been shown in vitro that Dll-4 is a hypoxia-regulated gene (*Mailhos C. et al. 2001)*, and that an hypoxic induction of this gene occurs through the direct action of either HIF-1 or HIF-2 on at least one RTE element somewhere between - 1587 and - 931 bp upstream the transcription starting site within the Dll-4 gene promoter (*Diez H*. *et al. 2007*). Although VEGF-responsive elements within the Dll-4 gene promoter have not yet been characterized, it has been shown that both the transcription factors Foxc1 and Foxc2 can directly activate Dll-4 in endothelial cells in response to VEGF by acting via the phosphatidylinositol 3-kinase pathway (*Hayashi H. and Kume T. 2008).* In effect a first study in human endothelial cells demonstrated that VEGF signaling is responsible for both Dll-4 and Notch-1 induction through VEGF receptors 1 and 2 and that this action is actually transmitted via the phosphatidylinositol 3-

kinase/Akt pathway. However this pathway seems to be necessary but not sufficient for VEGF-induced Notch1/Dll4 expression and some other signaling pathway(s) would participate in this process. Even though the characteristics of such an additional pathway(s) are still unknown, it is unlikely that either MAPK or Src family tyrosine kinase would partecipate. A mutual induction between Notch-1 and Dll-4 has been excluded, given that only Dll-4 but not Notch-1 is induced by b-FGF in HFAEC (*Liu Z.J. et al.* 2003). A synergistic induction of Dll-4 by VEGF and b-FGF it has also been observed in HUVEC, and their combination in these cells leaded to a further increase in Dll-4 expression (*Patel N.S. 2005*).

The transcription factor Foxn1 is required for morphogenesis and maintenance of the functional three-dimensional thymic microstructure as indirectly demonstrated by the phenotypic effects of this gene mutation both in mice and humans (*Cunningham-Rundles C. and Ponda P.P. 2005).* It could be possible that Foxn-1 can induce Dll-4 because nude mice which harbor a point mutation in Foxn1 gene (*Nehls M. et al. 1994)* lack Dll-4 expression (*Tsukamoto N*. *et al. 2005*). A more recent study (*Tsukamoto N. et al. 2005*) has confirmed that Foxn1 is required to induce the expression of Dll4 in the early thymus anlage TEC but has also indicated that its expression is no more necessary in postnatal thymus given that at this stage some Dll-4^{+} TEC were Foxn1^{-} (*Itoi M*. *et al. 2007*). Therefore Foxn-1 may be required for the induction but not for the maintenance of Dll-4 expression. These data are of extreme interest because they would suggest that Foxn-1 induced three-dimensional thymus structure is in some way responsible for the expression of Dll-4 by TECs. Moreover Foxn1 (*Nehls M. et al. 1994)* induces b-FGF by directly binding a regulatory element on this gene promoter (*Weiner L. et al. 1994)* and thereore in turn may also induce Dll-4. Finally Foxn1 is crucial in postnatal TEC maintenance and the changes which occur in Foxn1 thymic expression with time may be the cause or a downstream effect of the mechanisms which underlie the involution of the thymus in the elderly (*Chen L. et al. 2009).*

1.2.1.3.1.3 Dll-4 gene regulation by integrins

Since cells in the thymus are organized in a three-dimensional fashion it is reasonable to assume that molecules involved in cell-cell and cell matrix interactions have a role in determine TEC phenotype. The integrin signaling is a bidirectional network which responds both to changes in ECM composition and growth factor stimulation (*Hynes R.O. et al. 2002*). Moreover growth factor induced responses are often modulated by cellular adhesion through integrins mediated outside-in signals (*Ross R.S. et al. 2004*). This phenomenon could depend on physical interaction between several growth factor receptors and activated integrins. For instance the interaction between VEGFR-2 and the vß3 integrins (*Borges E. et al. 2000*) are reciprocally stimulatory in endothelial cells (*Brakenhielm E. 2007*). The vß3 integrins are also necessary for the second wave of MAP kinase activation which occurs after b-FGF stimulation in HUVEC (*Eliceiri B.P. et al. 1998*) and occupancy of Vß3 integrins by ligands such as vitronectin and fibronectin is able to activate the Insulin like grow factor I receptor (IGF-IR) in a variety of cell types (*Legate K.R. et al. 2009*). Despite that it is presently unknown if these factors regulate Dll-4 expression in TEC.

1.2.1.3.1.4 Dll-4 gene regulation by cytokines

Although Dll-4 expression has been detected in TECs and this factor is essential for T cell development, putative regulatory roles of hematopoietic cytokines on its expression are still poorly understood. However, recent data derived from studies with the highly Dll-4 expressing murine stromal cell line SC9-19 have provided some indications (*Suzuki M. et al. 2006*). The authors of these works tested the action of SCF, GM-CSF, IL-6, M-CSF or VEGF on Dll-4 induction and all of these cytokines except M-CSF increased Dll-4 expression in this system (figure 7). The stronger induction of Dll-4 protein was observed by IL-6 treatment and VEGF was used as a positive control given its recognized activity on endothelial cells. IL-6 did not induce VEGF indicating that IL-6 induced Dll-4 expression is not a VEGF-mediated effect. Finally, they were able to show that these cytokines induce Dll-4 acting through the JAK/STAT pathway

by inducing STAT3 phosphorylation. Surprisingly VEGF as well acted through the PI3K/AKT but not the JAK/STAT pathway.

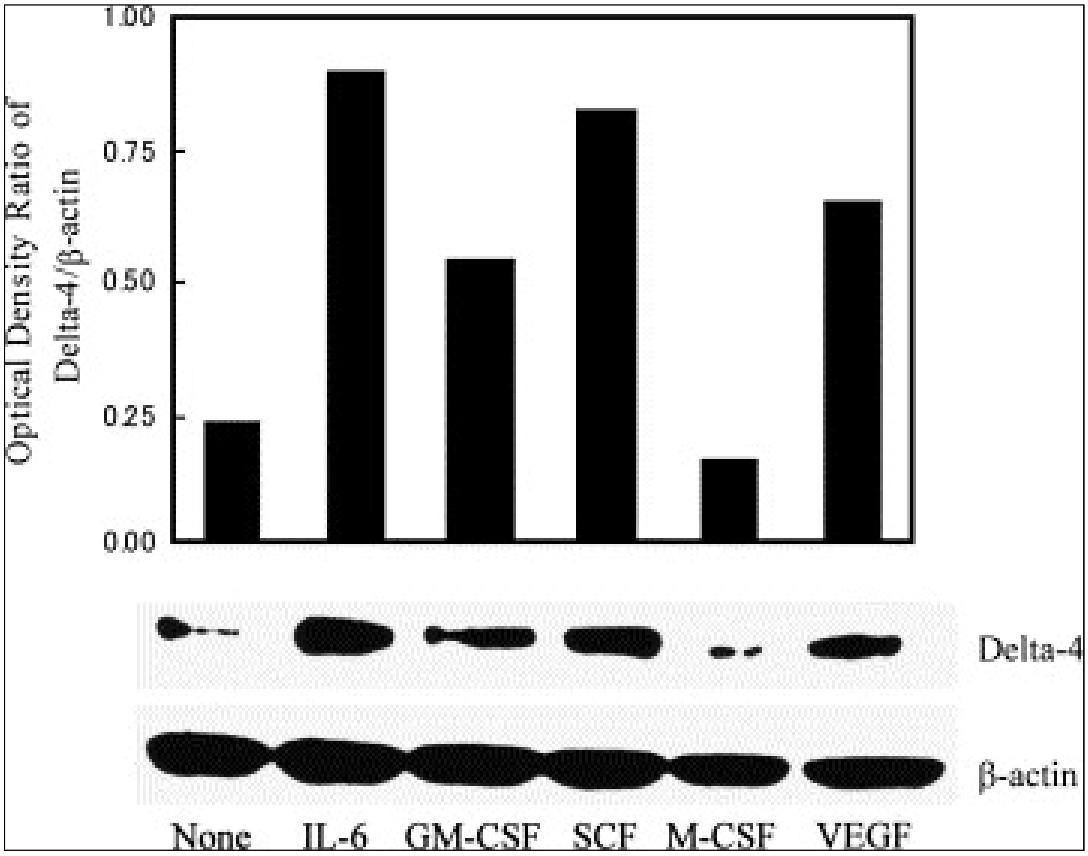

Figure 7: Dll-4 induction by different cytokines in SC9-19 cells. Taken from Suzuki M. et al. Exp. Hematol. 2006; 34: 1143-1150

1.2.1.3.1.5 Dll-4 gene regulation by hormones

Another interesting study regards the human endometrium (*Mazella J. et al. 2008*). In a similarl way to the thymus endometrial cells form a sort of three-dimensional syncytium and are regulated by hormones. This study showed that Dll4 is expressed in the human endometrium in a spatiotemporal dependent manner with the higher amount of Dll-4 present during the mid/late proliferative and early secretory phase when the growth of the glandular epithelium is maximum. Additionally the authors studied some hormones effect in primary cultures of isolated glandular epithelial and stromal cells. In glandular cells medroxyprogesterone acetate significantly reduced while relaxin induced Dll4

mRNA. In stromal cells, both estradiol and medroxyprogesterone acetate reduced the level of Dll4 mRNA. Despite progestine and estradiol do not directly act on endometrial cells they produce a variety of growth factors and peptide hormones that may be involved in this hormone mediated mechanism (*Tseng L. et al. 2002*). On the contrary relaxin receptor is expressed by endometrial cells (*Mazella J. et al. 2004*) but this effect may be mediated by VEGF as in these cells this grow factor has been found to be induced by relaxin (*Palejwala S. et al. 2002*). Relaxin may exert parallel effects on both VEGF and Dll4 genes through the activation of a cAMP response element-binding protein as another study could indirectely suggest (*Tang M. et al. 2005*).

1.2.1.4 Notch activity

Although it has not yet completely clarified whether Notch-dependent T cell commitment starts before or after the entrance of the haematopoietic precursors into the thymus, recent studies tend to strongly support the second hypothesis (*Harman B.C. et al. 2003).* Given that T cell development has been much more extensively studied in mice than in humans, most of knowledge about Notch activity on T cell development regards mice. For instance it has been identified that in mice Notch signaling supports β chain rearrangement in DN3a ($CD44^{-}CD25^{+}CD27^{-}$) thymocytes. An important increment in Notch activity begins during the DN2 stage and it continues in DN3a but, as the thymocytes successfully undergo β selection and develop into DN3b ($CD44^{-}CD25^{+}CD27^{+}$) cells, Notch signaling declines although still active during all the following stages (*Taghon T. et al*. *2009*). In fact , while Notch signaling plays an important role regardless of pre-TCR activity at the β check-point (*Michie A.M. et al. 2007*; *Maillard I. et al. 2006)*, later its role seems to be much less important, although studies with in vitro systems demonstrates that continues to contribute to T cell development (*Annette I et al. 2006*). On the contrary, Notch signaling is not required for β selection in humans (*Taghon T. et al*. *2009*).

In humans Notch signalling has beed found to cooperate with IL-7 for the expansion of early thymic $CD34^+$ progenitors (*Garcia-Peydro M. et al. 2006)* and differentiation of these very early precursors is slower when in presence of Notch ligand then when in its absence (*Taghon T. et al*. *2009*). Therefore, Notch signaling may be important to expand the thymic early precursor pool first, and to maintain equilibrium between cellular proliferation and T cell differentiation after. In the adult mice thymus the higher expression of Notch ligand has been found next to the thymic cortico-medullary junction that is where haematopoietic progenitors enter the thymus (*Lind E.F. et al. 2001).* In line with this evidence, data regarding the expression of Notch-1 and its target genes Hes-1, Gata-3 and Myc in thymocytes show a very strong Notch activity after that these cells enter the thymus (*Van de Walle I. et al. 2009*). Despite all these data, in humans the timing of Notch signaling requirement for T cell commitment has not yet been precisely established whereas it has been demonstrated that Notch signaling would be crucial to preserve T cell commitment until the DN3 stage in mice (*Schmitt T.M. et al. 2004*). A recent study has shown that murine DN1 cells can still give arise to few B and NK cells and DN2 to even more NK cells, when cultured in the absence of Dll-1. On the contrary, when the precursors were cocultured on the OP9-Dll1 stroma, both B and NK cell development was abolished (*Schmitt T.M. et al. 2004*). Importantly, these studies have clearly established that though low levels of short Notch signaling are sufficient to stop B cell lineage potential, stronger sustained signal would be required in order to guarantee subsequent T cell commitment. Furthermore, since bone marrow stromal cells can provide low levels of Notch signaling it has been also proposed that T cell precursors may start to lose B cell potential before entering the thymus (*Schmitt T.M. et al. 2004*). To conclude, at least in mice, there would be two different phases of Notch signaling during T cell development.

Notch signaling seems to play an opposite role in humans and mice in regard of TCR αβ versus γδ T cell lineage decision. Studies in mice have shown that TCR αβ development require higher levels of Notch signaling than TCR γδ whereas decreased Notch activity produced an increase in TCR γδ at the expense of

TCR αβ development (*Washburn T. et al. 1997*). In addition, mice defective in Notch signaling presented increased TCR γδ and lower TCR αβ cells number (*Tanigaki K. et al. 2004*). These data have been also confirmed by further observations in models in vitro (*Ciofani M. et al. 2006*; *Garbe A.I. et al. 2006*). Furthermore TCR γδ development seems to be related to Jagged rather than Delta like (Dl) dependent Notch signaling (*Jiang R. et al. 1998*; *Jaleco A.C. et al. 2001*) and Jagged ligands produce a lower level of Notch activation compared to Dl ligands (*Brückner K. et al. 2000*). Differently from mice, low Notch signaling is required at $CD34^{+}CD1^{+}$ and ISP $CD4^{+}$ stages (*Garcia-Peydro M. et al. 2003*). Moreoever, it has been observed that in the presence of a high and sustained Notch activity before the pre-TCR checkpoint the αβ T cell differentiation pathway is impaired whereas the development of TCR γδ T cells is promoted (*Garcia-Peydro M. et al. 2003*). In fact, a diminution in Notch signaling seems to be needed in order to consent the TCR α chain gene rearrangement, a prerequisite for complete TCR αβ formation. In addition low Notch signal levels in vitro have been associated to a higher number of TCR αβ thymocytes and TCR γδ T cells production increased when Notch activity was high (*Van de Walle I. et al. 2009*). Interestingly, IL-7 addition was not able to enhance TCR γδ T cells production in the presence of low Notch activity (*Van de Walle I. et al. 2009*). Given that IL-7 is crucial for TCR γδ cells growth it is believable that Notch signaling regulation plays a major role in TCR γδ T cells development (*Laky K. et al. 1998*). Finally, an absolute lack of Notch signaling has been recently repoted to impair TCR γδ T cell development from ISP $CD4^{+}$ thymocytes, a data which implies that Notch signalling would be also essential during the final stages of these cells differentiation (*Taghon T. et al. 2009*). Therefore diverse levels of Notch signalling would differently affect the expression of its target genes as, for instance, Hes-1 and Myc are down-regulated in $CD4^{+}$ ISP and DP thymocytes whereas Hes-1, Dtx-1, Myc and other Notch target genes such as NRARP and RUNX-3 are all up-regulated in TCR γδ T cells (*Van de Walle I. et al. 2009*). Finally, as mentioned previously, Notch signaling is needed to stimulate the development of SP $CD8^{+}$ T

lymphocytes whereas its activity seems to lack, possibly because of a MHC-II negative interference, in DP progenitors designated to become SP $CD4^+$ T cells (*Robey E. et al. 1996*).

1.2.2 IL-7

1.2.2.1 IL-7 protein and receptor

The human IL-7 is a four α-helical glycoprotein of 25 kDa with a hydrophobic core which binds to a dimeric receptor formed by a specific α (CD127) and a common γ (CD132) subunit (*Jiang Q. et al. 2005; Fry T.J. and Mackall C.L. 2002*). The γ subunit is common to other cytokine receptors (IL-2, IL-4, IL-9, IL-13 and IL-15) even though the role played by IL-7 signaling through its specific receptor in lymphocyte development and homeostasis remains exclusive of this cytokine (*Jiang Q. et al. 2005*).

1.2.2.2 IL-7 function

IL-7 is a T cell growth factor which plays an essential non-redundant role in T cell development as impaired TCR αβ T cell production and absence of TCR γδ T cells in a variety of studies in mice lacking IL-7, IL-7Rα or γ chains indirectly demonstrate (*Von Freeden-Jeffry U. et al*. 1995; *Peschon J.J. et al. 1994*). Although IL-7 has been initially identified as a B cell growth factor, it is presently well know that in the presence of Notch signaling this cytokine induces both proliferation and T cell lineage commitment (*Takeda S. et al. 1989*). Moreover it seems that humans, differently from mice, do not need IL-7 for normal B-cell development (*Puel A. et al. 1998).* There is a strong up-regulation of IL-7 receptor (IL-7R) in common lymphoid precursors (CLP), blood progenitors which still have both T and B potential (*Akashi K. et al. 1998)* and IL-7 is also essential for the differentiation of DP into $CD8^+$ SP mature lymphocytes (*Hongfang W. et al. 2005)* as well as for TCR T lineage development since γ chain expression depends on IL-7 signaling (*Maki K. and Ikuta K. 2008*). Similarly with Notch, low IL-7 activity is required for TCR- β T cell development while high IL-7 activity inhibits TCR- β in favor of TCR γδ T cell maturation

(*Offner F. and Plum J. 1998).* Contrary to Notch, that is crucial to suppress B cell lineage differentiation and to promote T cell lineage commitment, IL-7 alone is not able to fully support T cell development. Therefore a correct balance between Notch and IL-7 activities would be required in order to guarantee a correct T cell development (*Garcia-Peydro M. 2006).* IL -7 signaling is able to sustain the survival of developing and mature T cells by up-regulating the expression of pro-survival transcription factors such as Bcl-2 and Mcl-1 and, on the other hand by inhibiting pro-apoptotic factors such as Bax, Bad and Bim (*Qiong J. et al. 2005).*

1.2.2.3 IL-7 sites of production and action

IL-7 is produced by a variety of cells in humans such as intestinal epithelial cells (*Watanabe M. et al. 1995*), keratynocytes (*Heufler C. et al. 1993*), and other non epithelial cells such as follicular dendritic cells, endothelial cells, smooth muscle cells and fibroblasts (*Kroncke R. et al. 1996*). The sites of IL-7 thymic production have been recently identified in adult mice and it has been found that there is heterogeneity in IL-7 expression within thymic epithelial cells (TECs) as well as time dependent modifications in its levels within these cells (*Alves N.L. et al. 2009*). These studies have evidenced a spatial correlation between TECs which produce high levels of IL-7 and thymocyte development. Two IL-7^{hi} TEC subsets have been described, one bearing cortical ($Ly51^{+}CDR\text{-}1^{+}MTS10^{-}$) and one medullary ($Ly51^{-}CDR\text{-}1^{-}MTS10^{+}$) traits, respectively been scattered within the cortex and the medulla, and a very big number of both have been found in the cortico-medullary junction (*Alves N.L. et al. 2009*). The latter evidence is of great interest, as HSCs enter the thymus in this location and immediately succeeding early T progenitors are believed to necessitate a high amount of IL-7 for their amplification. Studies with OP9 and OP9-DL1 stroma have also demonstrated that maturation from DN to DP thymocytes necessitates a low amount of IL-7, whereas the differentiation from the DP to SP T cells needs a higher level of IL-7 (*Hongfang W. et al. 2005*). This is concordant with the evidence that the precursors which fail the positive selection down-regulate the IL-7R, despite those which successfully undergo positive selection which

maintain its expression (*Kroncke R. et al. 1996*). IL-7 receptor has been found expressed on hematopoietic cells, mainly of the lymphoid lineage such as NK/dendritic precursors, common T/NK/B lymphoid precursors, developing T and B cells, mature T lymphocytes and macrophages. The level of IL-7R expression is tightly regulated, depending on the stage of lymphoid activation and differentiation. For instance it is up-regulated on memory T cells, so to facilitate their persistence, while it is down-regulated on IL-7 independent DP thymocytes probably to do not deprive neighboring IL-7 dependent DN cells from this cytokine signaling.

1.2.2.4 IL-7 in embryonic thymus

In the embrional thymus, IL-7-expressing TECs are present throughout the early thymic rudiment, where they are organized into discrete zones overlapping with Foxn1 (*Zamisch M. et al. 2005*). Although there are notable similarities in the temporal and spatial expression of IL-7 and Foxn1 during fetal thymic development its induction in fetal TECs occurs independently of Foxn1 (*Zamisch M. et al. 2005*). However analyses of Foxn1 negative compared with wild-type fetal thymus would suggest that Foxn1 may positively modulate the level of IL-7 expression (*Zamisch M. et al. 2005*). During mouse embryogenesis IL-7 expression is initiated in the thymic fated domain of the early primordium by embryonic day 11.5 and is expressed in the recently described $K8^{+}K5^{+}MTS24^{+}$ common TECs progenitors so to be considered as the earliest known marker of TEC fate commitment during thymic ontogeny (*Gill J. et al. 2002*). In the thymic rudiment IL-7 promotes thymocytes development but does not act as an autocrine factor for TEC progenitors (*Zamisch M. et al. 2005*).

1.2.3 Other factors important for T cell development

A variety of pleiotropic hormones, neuropeptides, and growth factors produced by different organ systems have been found to be associated with effects on both circulating immune cells and the thymus.

1.2.3.1 Grow Hormone (GH) and Insulin Like Grow Factor I (IGF-I)

It is now widely established that GH exerts a variety of pleiotropic effects upon the thymus especially by regulating the thymic microenvironment. This modulation occurs by different mechanisms such as induction of cytokines and hormones by the thymus. A superior secretion of IL-1α, IL-1β and IL-6 in thymic bovine cell cultures (*Savino W. et al. 2003*), a more elevated production of the chemokine CXCL12 in mouse TEC cultures (*Smaniotto S. et al. 2005*), and increased levels of serum thymulin in both humans and mice (*Savino W. et al. 2003*) have all been detected following experimental GH treatment. Unfortunately it has not yet been determined if IL-7 is also under the influence of GH. In addition to cytokines and hormones, increased deposition of both laminin and fibronectin by TECs is also augmented by GH, as observed both in vitro and in vivo (*Smaniotto S. et al. 2005*; *Mello-Coelho V. et al. 1997*). Last but not least, it is significant that GH induces proliferative effect on TECs through the stimulation of various cyclins and cyclin-dependent kinases in vitro (*Savino W. et al. 2003*). GH acts as a thymopoietic factor in vivo as proved by studies and observations both in mice and humans although its action may be at least partially dependent of other stimuli (*Savino W. and Dardenne M. 2010*). In addition it has been found that GH exerts an action upon the entrance of HSCs into the thymus, thymocytes intrathymic migration and mature thymocytes export (*Savino W. and Dardenne M. 2010*). GH seems also to act in vivo as an autocrine/paracrine factor because is produced and released by human thymocytes and TECs even in the absence of any stimulation (*Savino W. and Dardenne M. 2000*).

Many GH thymic effects such increase in TEC numbers, proliferation, and chemokine expression seem to be IGF-I mediated (*Timsit J. et al. 1992*; *Chu Y.W. et al. 2008*). This data is supported by the observation that GH itself enhances the expression of IGF-1 and IGF-1 receptor in cultured TECs (*Mello-Coelho V. et al. 2002*). IGF-1 administration is able to enlarge the murine LSK (lin^- c-kit^+ Sca-1^+) T cell peripheral precursor population in vivo (*Bhandoola A. et al. 2007*) but it is still unknown if IGF-I enhances their proliferation/release

from the bone marrow or directly increases their proliferation once released into circulation (*Chu Y.W. et al. 2008*). Finally, the functional integrity of the somatotropic GH/IGF-1 axis seems to be important for the maintenance of a valid thymopoiesis in adults (*Morrhaye G. et al. 2009*). Because of all these propierties GH and IGF-I administration have been evaluated in the treatment of thymic atrophy related immunosenescence (*Savino W. and Dardenne M. 2010*).

1.2.3.2 Ghrelin and Leptin

Ghrelin is an orexigenic peptide hormone predominantly secreted by enteroendocrine stomach cells which acts as a GH secretagogue (GHS) by binding to its 7 transmembrane G protein coupled receptor, the GH secretagogue receptor (GHS-R) (*Kojima M. et al. 1999*). Recently ghrelin and GHS-R have been found to be expressed in circulating T cells and also inside the thymus (*Taub D.D. 2008*). Ghrelin has been shown to induce thymic GH expression, a mechanism which may be responsible for the ability of the latter factor of enhancing thymopoiesis (*Kojima M. et al. 1999*). Moreover, ghrelin has been reported to increase early thymic progenitor number, thymic output and peripheral T cell receptor (TCR) diversity and a reduction of this factor and receptor expression is correlated to decreased thymic output in aged mice (*Dixit V.D. et al. 2007*).

Leptin is an anorexigenic hormone predominantly produced by adipocytes and induces significant effects on immune responses such as the induction of proinflammatory cytokines expression (*Aw D. et al. 2007*). Despite their metabolic opposite effects, leptin and ghrelin exert similar actions in the thymus, as it has been shown that both increase thymocytes and TEC numbers, reduce precursor apoptosis, and raise thymic output and peripheral TCR repertoire diversity in aged mice (*Dixit V.D. et al. 2007*). However only leptin, unlike ghrelin, has been shown to increase peripheral IGF-1 levels, to upregulate keratinocyte growth factor (KGF) and to induce prosurvival effects in elderly thymocytes, and only ghrelin, unlike leptin, has been found to be able to inhibit TNF-α and to induce Stem Cell Factor (SCF) in mouse aging thymus (*Dixit V.D.*

et al. 2007). Therefore a fine balance between these two metabolic hormones seems to be critical in the regulation of thymopoiesis.

1.2.3.3 Sex hormones

The link between the reproductive system and immunity was initially reported in 1904, in a study on castrated cattle, which presented an enlarged thymus (*Henderson J. 1904*). Many other studies in several animal models over the years have then demonstrated enhanced thymic growth and immune reconstitution togheter with increased thymic output following sex steroid ablation, while testosterone re-administration suppressed these effects (*Hince M. et al. 2008*). Despite that, only recently the specific actions of sex hormones on T cells development have been elucidated in detail.

For instance the hypothalamic decapeptide luteinizing hormone-releasing hormone (LHRH) has been shown to control thymopoiesis both directly and indirectly, the latter through the regulation of sex steroids production in the gonads (*Marchetti B. et al. 2000*). The LHRH direct effect, depend on the binding of this factor to its receptors on thymocytes and thymic stromal cells. This interaction activates downstream protein kinase C translocation, consecutively resulting in IL-2 receptor (CD25) up-regulation and increaseof thymocytes proliferation (*Batticane N. et al. 1991*).

It is presently well established that sex steroids induce age realted thymic atrophy and surgical or chemical (using LHRH analogues) gonadectomy is able to reverse this process both in mice and humans (*Sutherland J.S. et al. 2005*). The action of testosterone can induce thymic atrophy very rapidly (within hours) and studies in mice have suggested that direct interaction of androgens with their receptors (AR) on TECs cause thymic degeneration (*Olsen N.J. et al. 2001*). Furthermore, administration of testosterone results in DP thymocytes apoptosis both in vitro and in vivo by inducing, at least in part, TNF release (*Guevara Patino J.A. et al. 2000*). A recent study has then showed that androgen withdrawal stimulates TECs proliferation and CCL25 expression in mouse, an effect that increases early thymic precursors (ETPs) uptake and

accelerates the maturation of DN thymocytes (*Williams K.M. et al. 2008*). Finally, very recently it has been discovered that androgens can indirectely induce thymic involution stimulating the synthesis and release of glucocorticoids (GCs) by the thymic stroma (*Chen Y. et al. 2010*).

In females, periodic increases in estrogen levels, as it happens during pregnancy, puberty or exogenous administration, have been reported to enhance thymic atrophy (*Olsen N.J. and Kovacs W.J. 1996*) and are characterised by a reduction in the number of DP thymocytes (*Brunelli R. et al. 1992*). Moreover, it has been observed that elevated estrogen levels in mice directly inhibit the production of $Flt3^+$ LSK (Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$) precursors in the bone marrow plus ETPs and $CD4^-CD8^-$ double negative (DN) thymocytes in the thymus (*Zoller A.L. and Kersh G.J. 2006*). Finally, studies in mice have shown that the presence of the estrogen receptor alpha (ER-α) is required in order to promote initial thymus development early in life (*Erlandsson M.C. et al. 2001*) but in contrast, activation of ER-α following exposure to estrogen in post puberty induces significant thymic atrophy (*Li J. and McMurray R.W. 2006).* Therefore, among with the organism development would take place a shift in the estrogen signaling pathways.

1.2.3.4 Keratinocyte growth factor (KGF)

KGF-1 (FGF-7) belongs to the family of the structurally related FGFs and is a potent epithelial cell mitogen (*Finch P.W. and Rubin J.S. 2004*) found expressed under physiological conditions in the thymus both by thymocytes and mesenchymal cells during specific developmental stages (*Jenkinson W.E. et al. 2003*). KGF exerts its biological activity by binding the IIIb variant of the FgfR2 receptor (FgfR2IIIb), which is expressed in the thymus exclusively on TECs surface (*Guo L. et al. 1996*). Currently, it has widely well established that KGF (or other FGF family members that signal through the FgfR2IIIb) plays a role in thymic organogenesis. This knowledge comes from studies in FgfR2IIIb deficient mice and from the in situ demonstration of KGF mRNA in the fetal thymus (*Revest J.M. et al. 2001*; *Finch P.W. et al. 1995*). Moreover, a few studies indicate that the postnatal thymic epithelial compartment requires

persistent KGF stimulation, and the thymic expression of this growth factor is maintained throughout life (*Alpdogan O. et al. 2006*). Another group of experiments have then demonstrated that KGF administration before then inducing thymic damage in animal models is able to reduce injury and/or stimulate repair of TEC (*Min D. et al. 2002*; *Rossi S.W. et al. 2007*). In murine models of aging, similar effects have been associated to a KGF mediated intrathymic elevation of IL-7 (*Min D. et al. 2007*). Furthermore, KGF induces in vivo expansion of both mature ($MTS24^{-}$) and immature TEC ($MTS24^{+}$) and is also able to support the transition of immature into mature TEC (*Rossi S.W. et al. 2007*). In agreement with that, following exposure to exogenous KGF, a transiently enlarged but regularly structured thymic microenvironment is generated and this in turn, results in a higher thymic output (*Rossi S.W. et al. 2007*).

Some of the mechanisms that underlie these effects have been elucidated. KGF binding to the FgfR2IIIb on fetal and adult TECs increases the transcription of BMP2, BMP4 and Wnt10b genes (*Rossi S.W. et al. 2007*). Additionally, it has been found a link between KGF signaling and activation of the PI3K/Akt/NF-κB and p53 pathways. The induction of the PI3K/Akt/NF-κB pathway constitutes a typical feature of KGF signaling in some epithelial cells, but intriguingly there are no other reports of p53 pathway activation through KGF signalling (*Bao S. et al. 2005*) whereas a link between the PI3K/Akt/NF-κB and the p53 pathways is possible because in several cell systems Akt can modulate p53 activity via a positive feedback loop (*Harris S.L. and Levine A.J. 2005*). Surprisingly, the activation of the Ras/MAP kinase pathway has not been found to be required for the KGF-mediated up-regulation of Wnt10b (*Rossi S.W. et al. 2007*). This result was unexpected because FGF signaling is normally expected to trigger the activation of the Ras/MAP kinase pathway (*Kouhara H. et al. 1997*). Finally, it has been discovered that thymocytes (with the exception of the more immature TN $CD3^{-}CD4^{-}CD8^{-}$ subsets) are able to produce KGF and the highest levels have been detected in the mature SP subsets, whereas the DP population has been associated to intermediate values (*Erickson M. et al. 2002*). Given that the

existence of a crosstalk between TECs and developing thymocytes is realistic and it could provide a feedback mechanism with the aim of precisely regulate KGF levels within the intrathymic milieu and in turn guarantee a correct thymopoiesis.

1.2.3.5 Connexin-43

The thymic epithelial network can be considered as a physiological syncytium. The existence of this sort of cellular organization has been effectively demonstrated in vitro in human and murine cultures of TEC by immunohistochemical labelling, fluorochrome injection and double whole-cell patch clamp experiments (*Alves L.A. et al. 1995*). Immuno- and Northern blot studies revealed that TECs direct cell-cell communications are constituted by gap junctions and both the gap junction protein connexin 43 (Cx-43) and its mRNA are present in TECs (*Alves L.A. et al. 1995*). Some in vitro experiments have then demonstrated that gap junctions have role in modulating TEC functions. For instance by using dye-coupling assays it has been shown that treatment of rat TECs with different factors such as IL-1, GH, ACTH, steroid hormones and neuropeptides known to inhibit thymulin production causes decreased dye-coupling, (*Head G.M. et al. 1997; Head G.M. et al. 1998*). In addition, treatment of these cells with the gap junction inhibitor octanol is able to reduce thymulin production (*Alves L.A. et al. 1995*). Finally, as observed in other epithelial systems, the factors found to reduce dye-coupling, increased cell proliferation (*Head G.M. et al. 1998*).

Another set of experiments have then demonstrated that, likewise in other cellular systems, cAMP and PKC intracellular pathways are involved in the homeostatic control of gap junction mediated communication in TEC, respectively exerting a positive and negative role upon cell coupling (*Nihei O.K. et al. 2010*). Furthermore, epinephrine, by inducing cAMP, leads to an increase of dye coupling in both humans and mice TEC cultures (*Nihei O.K. et al. 2010*). Some reports have suggested a role for catecholamines on TEC proliferation, thymic cytokines secretion and T cell development (*von P.B. et al. 1999*; *Kurz B. et al. 1997*). In the uterus Cx-43 expression is markedly enhanced in

response to estrogen and its loss results in aberrant differentiation of uterine stromal cells and impaired production of several angiogenic factors, such as VEGF (*Laws M.J. et al. 2008*). A previous observation comes in support of this concept, as cAMP and phorbol ester-dependent signaling pathways were found to enhance VEGF expression by human endometrial stromal cells (*Popovici R.M. et al. 1999*). This data may be quite important if we consider that, as discussed before, VEGF is known to induce Dll-4 in endothelial and bone marrow stromal cells.

Studies of mice carrying a targeted disruption of the Cx-43 gene (*Reaume A.G. et al. 1995*) indicate a physiological role for this gap junction component in both bone marrow and thymus microenvironments (*Montecino-Rodriguez E. et al. 2000*). The idea of a hypothetical Cx-43 role in hematopoiesis has arise from the initial observation that this type of gap junction is up-regulated during the development and the regeneration of the hematopoietic system (*Rosendaal M. 1995*; *Krenacs T. et al. 1998*). Therefore Cx-43$^{-/-}$, Cx-43$^{+/-}$ and Cx-43$^{+/+}$ mice were subsequently analyzed in regard of their hematopoietic functionality (*Montecino-Rodriguez E. et al. 2000*). Since Cx43$^{-/-}$ mice die immediately after birth because of a ventricular malformation (*Reaume A.G. et al. 1995*), there are only data from embryos and newborns but not adult Cx43$^{-/-}$ mice. An interesting observation was that thymic cellularity was on average higher in Cx43$^{-/-}$ and Cx43$^{+/-}$ embryos and neonates than in Cx43$^{+/+}$ littermates (*Montecino-Rodriguez E. et al. 2000*). The frequency of CD4^{+}CD8^{+} DP thymocytes was higher whereas the frequency of CD4^{+} single-positive cells significantly lower in the Cx43$^{-/-}$ and Cx43$^{+/-}$ mice compared to their Cx43$^{+/+}$ littermates. There was also a decrease in the absolute number of TCR αβ T cells in both Cx43$^{-/-}$ and Cx43$^{+/-}$ neonates. DP thymocytes development into SP T cells would be therefore dependent on the expression of Cx-43. This expression seems to be also important for bone marrow B lymphopoiesis as a reduction in the frequency of mature sIgM^{+} cells in Cx43 $-/-$ and Cx43+/ $-$ embryos and neonates has been observed. However, since thymic cellularity and frequency of thymic subpopulations in Cx43$^{+/-}$ adult mice were normal, the

expression of Cx-43 at normal levels would be critical for the establishment or the regeneration of blood cell formation but not during steady-state hematopoiesis, as also suggested by other precedent studies (*Krenacs T. and Rosendaal M. 1998*; *Rosendaal M. et al. 1994*) and by the observation that recovery of bone marrow cellularity in $Cx43^{+/-}$ mice is not as efficient as in $Cx43^{+/+}$ animals (*Montecino-Rodriguez E. et al. 2000*). The observed effects on T lymphopoiesis depend on diminished Cx-43 expression on TEC as indicated by analyses with chimeras generated by reciprocal bone marrow transplantation between $Cx43^{+/-}$ and $Cx43^{+/+}$ mice (*Montecino-Rodriguez E. et al. 2000*). The analysis of the experiments described above also implies that these effects are dose dependent and that the lost of a single Cx-43 allele is sufficient to impact blood cell production (*Montecino-Rodriguez E. et al. 2000*). Finally, given that dye transfer between hematopoietic precursors and stromal cells has been reported both in thymus (*Alves L.A. et al. 1995*) and bone marrow (*Krenacs T. and Rosendaal M. 1998*) heterologous cell couplings may be involved in the regulation of haematopoiesis (*Rosendaal M. et al. 1997*).

1.3 STEM CELL AGEING AND T CELL DEVELOPMENT

1.3.1 Ageing of haematopoietic stem cells

It is assumed that the maximum self-renewal capacity of haematopoietic stem cells (HSCs) is finite and genetically determined as well as it is presupposed that cell proliferation, unless asymmetrical, leads to loss of stem cell quality (*Kamminga L.M. et al. 2005*).

Some measurable effects of aging on human HSCs proliferation have been described however the functional consequences on the aged hematopoietic system have not yet been defined (*Fuller J. et al. 2002*). A few studies in mice have shown that aging affects the number and cell cycle status of HSCs. These precursors are rarely in the S/G2/M phases of the cell cycle in young and middle-aged mice, whereas in old mice are frequently in cycle (*Morrison S.J. et*

al. 1996). This pattern could reflect a progressive shift from a quiescent state necessary to maintain long-term self-renewal and to prevent stem cell exhaustion, towards another where only few stem cells are in a quiescent state.

An additional study would suggest that stem cell quiescence is able to attenuate DNA damage response and to permit DNA damage accumulation during aging (*Rossi D.J. et al. 2007*). Consequently, stem cell reconstitution and proliferative potential would be impaired in the elderly because of diminished self-renewal capacity and increased cellular apoptotic rate, phenomena which in turn conduct to cellular functional exhaustion (*Rossi D.J. et al. 2007b*).

Furthermore it has been demonstrated that with ageing HSC show increased propensity to differentiate towards myeloid rather than lymphoid lineage (figure 8). This event may contribute to the decline in lymphopoiesis typical of aging and comparison of the gene expression profile of young and old HSC show up-regulation of myeloid and down-regulation of lymphoid lineage associated genes in aged cells (*Rossi D.J. et al. 2005*). An intrinsic drop in the diverse potential of aged HSC could be secondary to the cumulating of genomic damages with time. However, since lymphoid specific genes expression in HSCs declines with aging (mainly IL-7R gene) a lineage skewing may, at least in part, be consequence of transcriptional changes (*Rossi D.J. et al. 2005*). Even though, the epigenetic modifications that underlie altered gene homeostasis in aged HSCs have not yet been clearly recognized, it has been recently demonstrated that some transcription factor modifications take place in the elderly (*Issa J.P. 2003*).

The typical marker of cell senescence is telomere shortening, though it has been recently proposed that telomere length as such is less important for the process of aging than specific molecular alterations in telomere structure. In fact despite mouse HSCs over-expressing mTERT show maintenance of telomere length after serial transplantation, these cells have not extended replication capacity compared to whose from wild-type mice (*Allsopp R.C. et al. 2003*). However these studies are difficult to translate to humans as mice have longer telomeres and telomere dysfunction is not believed to be cause of stem

cell exhaustion in many mouse strains.Moreover, haematopoiesis in telomere knockout mice is surprisingly normal (*Rudolph K.L. et al. 1999*). However, telomeres length in human adult blood leucocytes is shorter than in germline cells from the same donor and stem cell subsets isolated from human adult bone marrow show shorter telomeres than similar cells collected from fetal liver or cord blood (*Cook H.J. and Smith B.A. 1986*). The latter data implies that a progressive decline in telomere length in HSCs takes place with age (*Vaziri H. et al. 1993*). Furthermore, it is considered that transplanted HSCs derived from aged donors may reach their proliferative limit during the lifetime of the recipient, as several cases with graft failure after HSC transplant have been reported to have unusual telomere shortening (*Wynn R.F. et al. 1998*).

The role of factors involved in cell life regulation has also been investigated (*Lundberg A.S. et al. 2000*). For instance, it has been observed that, while on one hand basal p21 expression levels are required to maintain stem cell quiescence, on the other hand, increasing p21 expression induces loss of stemness and self-renewal capacity and, as a consequence, removal of HSCs out of the stem cell pool. It has been also proposed that p21 up-regulation would take place in response to telomere dysfunction.

The relevance of p21 action during normal aging has yet to be completely elucidated (*Zhenyu J.U. et al. 2006*). Recently it has been discovered that in mice HSCs the levels of p16 mRNA increase with age (*Janzen V. et al. 2006*). This occurrence not only had a negative impact on the HSCs pool size and on these cells self renewal capability, but was also related with reduced expression of Hes-1, a downstream effector of Notch activation involved both in HSCs self renewal (*Kunisato A. et al. 2003*) and T lineage commitment (*Tomita K. et al. 1999*; *Stier S. et al. 2002*). A recent study in mouse has then reported that the deletion of p16 gene in T lineage cells is able to restore several aging related defects such as thymic involution and decreased naïve T cells production, without inducing T cell neoplasms. However the ablation of this gene is linked to a strong incidence of systemic high grade B-cell lymphomas (*Liu Y. et al. 2011*). Therefore, it is reasonable to assume that the expression of p16 can either

promote aging or prevent cancer development in the progeny of a common stem cell. So far, all the mechanisms which drive the action of this and other tumor suppressor genes in aged stem cells have not yet been entirely understood (*Leviand B.P. and Morrison S.J. 2009*).

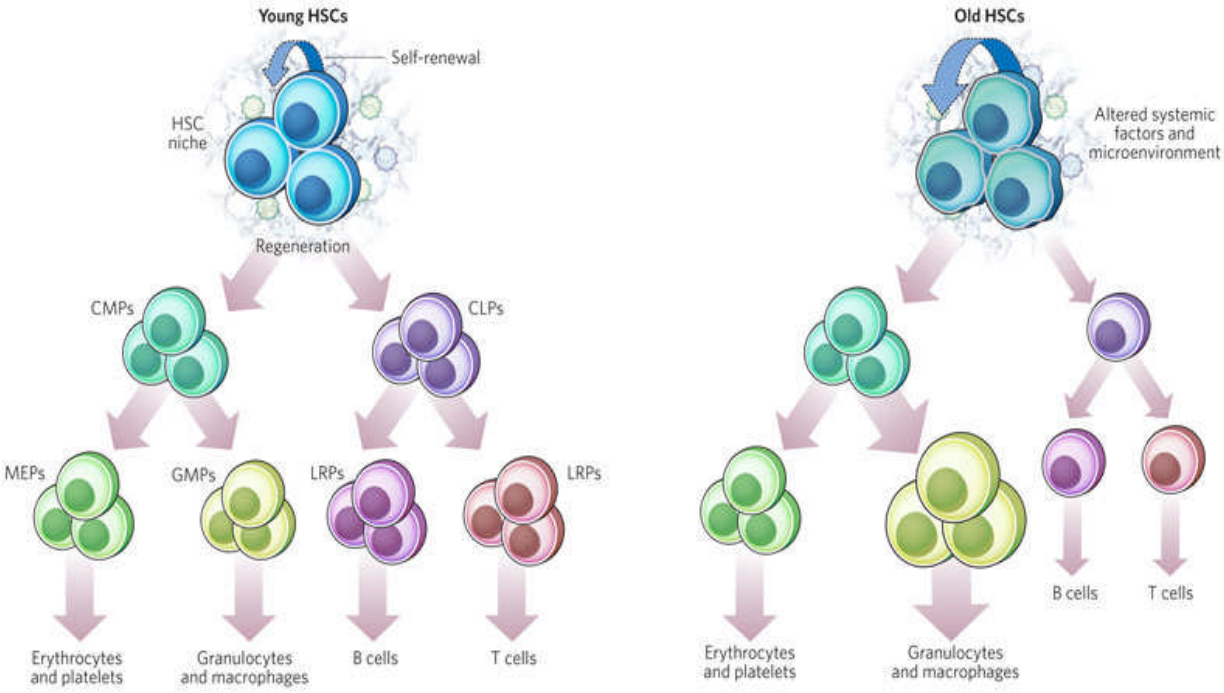

Figure 8: myeloid lineage skewing with aging. The image is taken from DePinho R.A. and Sahin E. Nature 2010; 464: 520-528

1.3.2 Difference between cord and adult blood haematopoietic stem cells populations

As previously mentioned, a progressive shift from lymphoid to myeloid potential of stem cells seems to occur with ageing and this could impact on the possibility of generating T cells in vitro, depending on the age of the donor stem cells used. Variations in the frequency of precursors with divergent lineage potentials have been effectively described among blood samples from sources of different age.

A study from bone marrow of either young or old mice has shown an age dependent redistribution of the lineage committed precursors and the frequency

of $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD34^{+}Flt3^{+}$ early lymphoid precursors/lymphoid-primed multipotent progenitors (ELP/LMPP) was found decreased with age (*Rossi D.J. et al. 2005*). This population lies upstream of common lymphoid precursors (CLP), largely contributes to the reconstitution of lymphoid cell pool and possess only limited myeloid potential (*Adolfsson J. et al. 2005*). CLP are known to up-regulate the expression of both FLT3R (CD135 or FLK2) and IL-7Rα (CD127), two cytokine receptors critically involved in lymphoid commitment (*Peschon J.J. et al. 1994*; *McKenna H.J. et al. 2000*). Flt3R (CD135) up-regulation within the $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD34^{+}$ stem cell compartment is also accompanied by loss of self-renewal capacity (*Adolfsson J. et al. 2001*). Moreover, a synergistic interaction between Flt3 ligand and IL-7 on uncommitted progenitors has been reported (*Veiby O.P. et al. 1996*). Finally, a recent report has demonstrated that CLP are significantly decreased in old mice and that these aged cells exhibit a diminished responsiveness to IL-7 (*Miller J.P. and Allman D. 2003*).

In humans, early lymphoid progenitors (ELP) have been phenotipically characterized in both cord blood and bone marrow as $CD34^{+}CD38^{-}Thy\text{-}1(CD90)^{-/lo}CD45RA^{+}$ cells and have been located within the $CD34^{+}CD38^{-}$ compartment (*Doulatov S. et al. 2010*). These cells generally express CD10 but not CD24 and in cord blood can be further subdivided into a $CD7^{-}$ and a $CD7^{+}$ subset whereas in bone marrow ELP are uniformly $CD7^{-}$. Although bone marrow $CD34^{+}CD38^{-}CD7^{-}$ cells demonstrate some T cell potential on OP9-DL1 stroma, cord blood $CD34^{+}CD7^{+}$ HSCs show a much higher cloning efficiency and proliferative potential (*Doulatov S. et al. 2010*). This and other studies demonstrate that different thymic precursors appear throughout the life. One precursors population, characterized as $Lin^{-}CD34^{+}CD10^{+}CD24^{-}$ has a very low myeloid potential but can generate B, T, and NK cells and co-express RAG-1, TdT, PAX-5, IL-7Rα and CD3ε (*Six E.M. et al. 2007*). This population is present in cord blood and bone marrow but can also be found in adult peripheral blood (*Six E.M. et al. 2007*).

Another population, characterized as $CD34^{hi}CD45RA^{hi}CD7^{+}$, it is peculiar of the cord blood, it is T/NK lineage polarized and it has been proposed to correspond to T lineage committed precursors (*Hao Q.L. et al. 2001*). These cells originate directly from $CD45RA^{INT}CD7^{-}$ precursors and RNA expression analyses showed that they express PU.1 and GATA-3 but not Pax-5, TdT, IL-7Rα and CD3 (*Haddad R. et al. 2004*). Studies using fetal thymic organ cultures and OP9-DL1 stroma have shown that these precursors can generate cells of all the three lymphoid lineages and that their potential is biased towards the T/NK cells lineage whereas $CD34^{+}CD45RA^{hi}Lin^{-}CD10^{+}$ precursors predominantly exhibit B-cell potential and are considered pro-B cells (*Haddad R. et al. 2004*).

These results imply that differences among precursors frequency between cord and adult blood stem cells can produce divergent results when used to try to generate T cells in vitro systems.

1.4 IN VITRO T CELLS DEVELOPMENT: PRIOR SYSTEMS

1.4.1 FTOC (Fetal Thymic Organ Culture) System

The first T cells to be developed ex vivo were produced by using Fetal Thymic Organ Culture (FTOC), a system which was developed in the mouse and allowed HSC to generate T cells, within a three-dimensional environment (*Mohtashami M. and Zuniga-Pflucker J.C. 2006*).

Importantly, this system demonstrated how the maintenance of a three-dimensional structure it is indispensable in order to allow T cell maturation, and it has been very useful to investigate thymic T cell development in a large variety of studies. In fact previous studies with thymocytes in suspension or cocultured with monolayers of thymic stromal cells have suggested that the responses of the T cell precursors under such conditions may be different to those within an intact microenvironment. Although this system is still the gold

standard to study thymocyte development and selection in a manner comparable to that seen in vivo it is not a valid system to be translated into any clinical application. In fact this system requires a large amount of fetal lobules from a xenogenic source, and therefore this approach would not be able to support the development of self-restricted and tolerant T cells.

1.4.2 OP9-DL1 Monolayer System

Another system to develop and investigate T cell differentiation in vitro was established with human cord blood derived HSCs ($Lin^- CD34^+CD38^-$) cultured in the presence of Delta-like 1 expressing OP9 mouse bone marrow stromal cells (*Schmitt T.M. and Zuniga-Pflucker J.C. 2002*). In this system HSCs displayed many of the early differentiation steps associated with normal human T cell differentiation, including the sustained generation of $CD7^+CD1a^+$ thymocytes as well as the efficient generation of $CD4^+CD8^+$ double positive (DP) αβ-TCR lineage T cells. In addition stimulation of in vitro generated DP cells with anti-CD3 plus anti-CD28 induced cell activation, as demonstrated by the expression of CD69 and CD27 and by the up-regulation of the CD28 and CTLA-4 costimulatory receptors. The main limit of this system was that very few $CD4^+$ or $CD8^+$ single positive (SP) cells arose from $CD4^+CD8^+$ DP cells (*Schmitt T.M. and Zuniga-Pflucker J.C. 2002*). The inability of this system to support positive selection it is probably due to the fact that OP9 cells lack additional factors that are present on thymic stromal cells from many species. In fact it has been demonstrated that murine MHC molecules are able to support the positive selection of human T cells (*Res P. et al. 1997*). In some cases the limit of the OP9-DL1 monolayer to produce terminally differentiated SP T cells has been overcame by using precursors transduced with TCR α and β viral constructs and in one of these studies the authors were able to observe generation of a few CD8 SP mature T cells (*Zhao Y. et al. 2007*). However, it is unclear whether development of such double-transduced precursor cells follows physiological pathways of TCRαβ differentiation or rather, as described in TCRαβ double-transgenic mice, differentiate along a TCRγδ-like pathway without the need for MHC-driven positive selection (*Von Boehmer H. et al. 1991*). Moreover there

was no evidence that the cells generated were conventional positively selected T cells. Successively another group was able to develop antigen specific $CD4^+$ SP T cells from murine adult BM cells transduced with an OT2 CD4 TCR and cocultured on OP9-Dll1 stroma (*Dai B. and Wang P. 2009*). Another group was able to produce some SP T cells from EML (a multipotent hematopoietic cell line) using the OP9-DL1 cocolture system (*Kutlesa S et al. 2009*). Finally another group produced both $CD4^+$ and $CD8^+$ functional T cells from human $CD34^+$ cord blood cells on OP9-DL1 system. However these cells mainly consisted of $TCR\gamma\delta^+$ and $IL\text{-}2R\beta^+$ $CD8_{\alpha\alpha}^+$ $TCR_{\alpha\beta}^+$ cells whereas only few were conventional $CD4^+$ and $CD8^+$ $TCR_{\alpha\beta}^+$ SP T cells (*Van Coppernolle S. et al. 2009*). Interestingly OP9-DL1 expresses only low levels of murine class I and no class II MHC and no DC cells were detected. In addition, induction of murine or human MHC class I expression on OP9-DL1 cells had no influence on the differentiation of mature $CD8^+$ cells. Thus, the authors supposed that T cell precursors that expressed HLA class I and some HLA class II may have interacted with DP cells and in this way would have caused positive selection. Even though there is evidence that this process may induce positive selection of conventional T cells, it may be less efficient than cortical TEC-mediated positive selection. Furthermore, the nature of the T cells generated in these cultures has not yet been studied in detail.

1.4.3 Xenogenic 3D Systems

Until recently, there had been comparatively little done to culture human HSCs with human supporting cells. The development and investigation into the possibility of using three-dimensional structures to create organ cultures capable of supporting T cells has happen on in recent years. A large amount of research has been put into engineering of smooth muscle and bone tissue and this technology is now being applied to the thymus. These advances in technology gave origin to the development of a three-dimensional framework, made up of a tantalum-coated carbon matrix able to support reconstitution of functioning thymic tissue. The three-dimensional matrices has been seeded

with murine thymic stroma and co-cultured with human BM derive HSCs. Within this xenogenic environment, mature and functional T cells were generated within 14 days. The proportional T cell yield from this system was highly reproducible, generating over 70% $CD3^+$ T cells from either $CD133^+$ or $CD34^+$ progenitor cells. Cultured T cells expressed a high level of T cell receptor excision circles (TREC), demonstrating de novo T lymphopoiesis and functionally mature T cells development (*Poznansky M.C. et al. 2000*).

1.4.4 The Human Three-dimensional Skin System

In a very recent study it has been successfully tested the hypothesis that cellular elements of skin, reconfigured in a different three-dimensional arrangement can support the differentiation of T cells from HSC (*Clark R.A. et al. 2005*).

The three-dimensional scaffold was developed by using Cellfoam (Cytomatrix), an artificial three-dimensional tantalum coated carbon matrix originally designed as an artificial bone matrix (figure 9).

Matrices seeded with keratinocytes and fibroblasts from human skin donors were cultured together and after 5-6 days human bone marrow derived $CD133^+$ hematopoietic precursors were introduced into the colonized matrices. These complete constructs were maintained in culture for 3-4 weeks in the presence of prolymphopoietic cytokines IL-7 and IL-15 as well as Flt-3 ligand.

Interestingly this system was able to provide new T cells containing TRECs, with a diverse T cell repertoire and functionally mature and tolerant to MHC, therefore indicating that the cells produced had successfully undergone positive and negative selection. Even though skin and gut do support the development of TCR $\gamma\delta^+$ T lymphocytes (whereas $\alpha\beta^+$ T cells are primarily produced by the thymus) consistently more than 95% of the $CD3^+$ T cells produced through the skin construct were TCR $\alpha\beta^+$ T cells (*Clark R.A. et al. 2005*). Moreover the fibroblasts they used expressed AIRE, a factor believed to be crucial for deletion of potentially auto-reactive T cells (*Anderson M.S. et al. 2002*; *Ramsey C. et al. 2002*).

Although this method appears to be an effective method of generating T cells ex vivo, the use of stem cells harvested from bone marrow would make this technique difficult to use in a clinical setting.

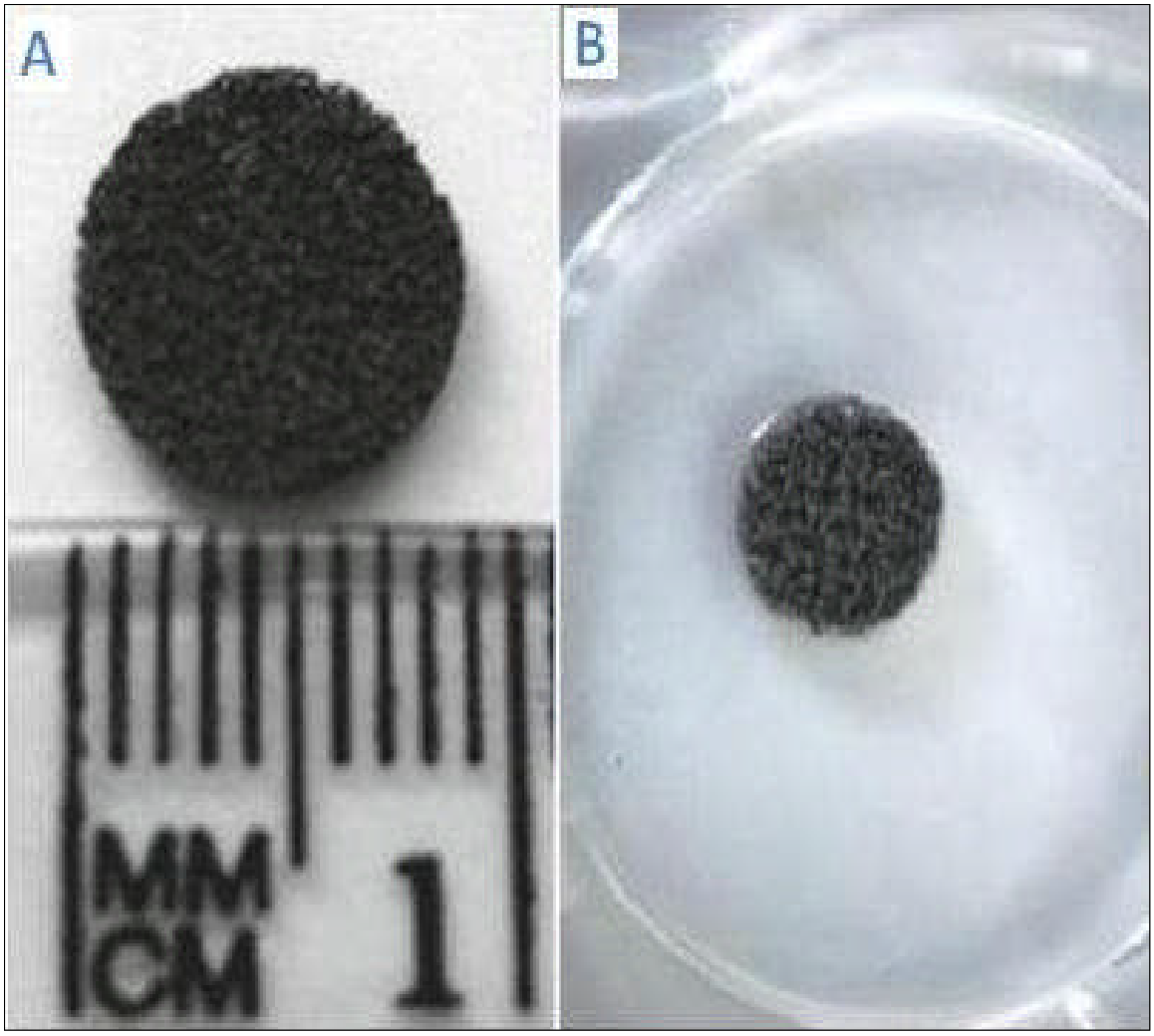

Figure 9: The Cellfoam three-dimensional matrix. Matrices are made of carbon and are coated in tantalum. (A) Matrix diameter is 0.9cm. (B) Cellfoam matrix in a well of a 24-well plate.

2 MATERIALS & METHODS

2.1 Cell cultures

2.1.1 Hacat Keratinocytes culture

The spontaneously immortalized human keratinocytic HaCaT cell line was purchased from CLS, Germany (*Boukamp P. et al. 1988*). These cells were cultured in DMEM medium (Sigma-Aldrich, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, UK), 2 mM L-glutamine (Sigma-Aldrich, UK), 10% antibiotics (penicillin 100U/ml and streptomycin 100mg/ml) (Sigma-Aldrich, UK) in an air-humidificated incubator at 37°C with 5% CO2. Two times weekly the cells were regularly passaged when still less than 80% confluence. The medium was removed, the flask rinsed with PBS, and the cells incubated in 5 ml of 1X trypsin/0.025% EDTA solution (Sigma-Aldrich, UK) at 37°C until they all detached. A 1:10 cell aliquote was then dispensed into a new flask with fresh medium. Some cell aliquots were stored in liquid nitrogen. Once detached the cells to store were spun for 10 minutes at 300 x g at 4°C, supernatant removed and the pellet re-suspended in 0.5 ml of freezing buffer consisting of 90% FBS and 10% DMSO in a cryovial which was placed in a freezing box in a freezer at -80°C for 24 hours to slow down the rate of freezing (~1°C/min). The day after the cryovial was moved to -180°C in a nitrogen bin for long-time storage.

2.1.2 Fibroblast Culture

Human dermal fibroblasts (Invitrogen, UK) were cultured in Medium 106 (Invitrogen, UK) supplemented with 2% heat-inactivated FBS (Invitrogen, UK), 1 µg⁄ml hydrocortisone (Invitrogen, UK), 10 ng⁄ml human epidermal growth factor

(Invitrogen, UK), 3 ng⁄ml human basic fibroblast growth factor (Invitrogen, UK), 10 µg⁄ml heparin (Invitrogen, UK) and 1x gentamycin/amphotericin (Invitrogen, UK). The medium was changed every 2 days and when about 90% of confluence the cells cells were detached with 1X trypsin-EDTA (Sigma-Aldrich, UK), as previously described for Hacat cells with the only exception of one minute incubation at 37°C and passed at 1:10 ratio. Some aliquots were also prepared to be stored at -180°C. The cells used in the experiments were always less than 15 passages.

2.1.3 Two-dimensional cocultures

Fibroblasts and HaCaT keratinocytes were cultured together 1:2 (40,000 fibroblasts and 80,000 HaCaT cells per well of a 24-well plate) in T2 media (equal volumes of DMEM and M106 media supplemented as before).

2.1.4 Three-dimensional cultures

The 3D cultures were performed on round, cylindrical tantalum coated carbon Cellfoam matrices (Cytomatrix, USA) which were kindly donated by the Maastricht group (figure 9). Matrices were placed in PBS in a glass tube and autoclaved. Afterwards, the glass tube with PBS and the matrices was placed overnight in an incubator at 37°C 5% CO_2 to remove any toxin. To enable cell adherence, the matrices were coated with collagen. First, the matrices were air-dried by placing them in seperate wells of a 24 well plate under a laminar flow hood to maintain sterility. Subsequently, each matrix was incubated for 30 minutes with 100 µl of 100 µg/ml rat tail collagen (Sigma-Aldrich, UK) at 37°C and after that air-dried under the laminar flow hood for 10 minutes at room temperature. Once dried, collagen coating was repeated and the matrices seeded with 1 x 10^5 HacaT cells and 5 x 10^4 fibroblasts suspended in a volume of 100 µl of T2 medium, placed in the 37°C 5% CO_2 incubator for 5 hours to

allow the cells to adhere to the collagen coating the matrix, and finally other 2 ml of T2 media was added and this media changed every two days. When cultured alone 1.5 x 10^5 either Hacat or fibroblasts were seeded respectively in 100 µl DMEM or M106 media. Different amounts of cells were tested and for the cocoltures a Hacat/fibroblasts ratio of 2:1 was always used.

2.1.5 Cell counting

Cells were counted under a light microscope with cell counting chamber slides (Invitrogen, UK) and with an automated cell counter (Invitrogen, UK). In the first case the counting was achieveded manually using the following formula:

N. of cells / ml = n. cells per Burker square x 10^4 x trypan blue dilution factor

2.2 Protein extraction

For protein extraction the cells were washed twice with cold PBS, detached with Accutase, spun at 300 x g for 10 minutes at 4ºC and the supernatant completely removed.The cell pellet was then lysed by incubating in 80 µl NP-40 (Nonidet P-40) cell lysis buffer (consisting of 50 mM TRIS buffer PH 7.4, 250 mM NaCl, 5 mM EDTA and 1% NP-40) on ice for 30 minutes. The cell lysis was helped by vortexing the cell pellet in NP-40 any 10 minutes. After lysis the extract was transferred to a new microcentrifuge tube and centrifuged at 2000 x g for 10 minutes at 4ºC. The clean lysate was then moved to a clean microcentrifuge tube, supplemented with 1.25% of protease inhibitor, and stored at -20/-80º C.

5 µl of each sample were collected to determine the amount of proteins extracted using a modified BCA (bicinchoninic acid) Lowry protein assay. This is a colorimetric, reagent-based assay and the colour change intensity is proportional to the protein concentration of the samples supplemented with BCA as this compound induces a reaction which results in the formation of protein-copper complexes which exhibit high absorbance at 562nm. To determine the samples protein concentration a standard curve was prepared by diluting bovine

serum albumin (BSA) with milliQ water and loading 150 µl of the solution in duplicate at the following BSA final concentrations: 100, 80, 40, 20, 10, 5, 2.5, 0 µg/ml. The samples were diluted 1:75 (2ul of sample and 148ul of milliQ water) and a final volume of 150 µl of diluted sample loaded in duplicate. After that 150ul of micro BCA reagent (Thermo Scientific, UK) was added to samples and standard curves and the plate covered with sealing tape, agitated on a plate shaker for 30 seconds, incubated for 1 hour at 37ºC and finally read at 562nm by a Varioscan Flash spectrophotometer (Thermo Scientific, UK).

2.3 DNA extraction

DNA extraction was performed for subsequent TREC analysis of blood and newly generated T cells using Trizol reagent, a mixture of guanidine thiocyanate and phenol in a monophase solution which is able to lise cells and to dissolve RNA, DNA and proteins from a same sample (*Chomczynski P. 1993*).

The cells were pelleted by 10 minutes centrifugation at 300 x g at 4°C and then lysed by incubating the cell pellet for 5 minutes with 750 µl of Trizol LS reagent (Invitrogen, UK), at room temperature. Subsequently 0.2 ml of chloroform (Sigma-Aldrich, UK) were added to each sample, the tubes mixed well by inverting and shaking vigorously and the samples incubated at room temperature for another 5 minutes. After that the samples were centrifuged at 2000 x g for 15 minutes at 4°C so to separate the sample in three phases consisting of an upper colourless aqueous layer containing RNA, a white dense interphase containing DNA and a pink organic phase containing proteins. The upper layer aqueous phase overlying the interphase was carefully completely aspirated as the removal of any residual aqueous phase is critical for the quality of the isolated DNA. Then 0.3 ml of 100% ethanol (Acros organics, UK) were added to the remaining interphase and organic phase, the samples mixed well by inversion, incubated 2-3 minutes at room temperature and centrifuged at 2000 x g for 5 minutes at 4°C to precipitate the DNA. After that the phenol-

ethanol supernatant containing proteins was removed and the DNA pellet washed by resuspending and incubating 30 minutes with periodic mixing to help solubilisation in a solution consisiting of 1 ml of 0.1 M Trisodium Citrate (Sigma-Aldrich, UK)/10% ethanol (Acros organics, UK) and centrifuged at 2000 x g for 5 minutes at 4ºC. This washing step was repeated and then a final third wash step was performed resuspending the DNA pellet in 2 ml 75% ethanol, incubating for 15 minutes at room temperature with periodic mixing and spinning the DNA at 2000 x g for 5 minutes at 4ºC. The ethanol was then removed, the DNA pellet air-dryed by keeping the tubes open for about 5-10 minutes at room temperature and the DNA finally redissolved in a suitable volume of buffer TE (Qiagen, UK) consisting of 10 mM Tris·HCl (pH 8.0) + EDTA 1 mM and placed on ice.

The amount and quality of DNA was then valuated with a spectrophotometer (Picodrop, UK) and subsequently stored at -20ºC. The DNA extraction was considered acceptable if the A260/A280 ratio was comprehended within a 1.7-2.1 range (*Glasel J.A. 1995*).

2.4 RNA extraction

RNA extraction was performed with a guanidine isothiocyanate-phenol: chloroform (GTC) method combined to a Silica-gel column (SGC) technology (Qiagen RNeasy Mini column). The GTC salt is able to denature the cellular proteins and to inactivate RNases so to preserve the isolated RNA from degradation (*Chomczynski P. and Sacchi N. 1987*) while the SGC technology combines the very selective binding properties of silica-based membranes with the high speed of microspin technology.

The experiment bench, the pipettes and all equipment used were sprayed with RNaseZap decontamination solution (Applied Biosystems, UK) in advance, and

filter tips were always used. The culture media was removed, the cells washed twice in PBS, detached with Accutase (Sigma-Aldrich, UK), moved to a 1.5 ml RNAse free sterilized Eppendorf tubes (Eppendorf, UK) and finally pelleted by spinning at 300 x g for 10 minutes at 4°C. The three-dimensional cultures required two additional steps with fresh Accutase in order to reach and detach the cells growing in the centre of the matrix. The detached cells were then lysed by redissolving the cell pellet with 750 µl of Trizol LS reagent (Invitrogen, UK) following 5 minutes of incubation at room temperature. Subsequently 0.2 ml of chloroform (Sigma-Aldrich, UK) were added to each sample, the tubes mixed well by inverting and shaking vigorously and the samples incubated at room temperature for another 5 minutes. After that the sample was centrifuged at 12000 x g for 15 minutes at 4°C so to separate the sample in three phases consisting of an upper colourless aqueous layer, a white dense interphase and a pink organic phase. The top aqueous phase containg RNA was then carefully removed and transferred to new sterile Eppendorf tubes. Immediately after that an equal volume of 70% ethanol was added to the collected aqueous phases and the whole solution mixed by pipetting and transferred to silica-membrane based columns (Qiagen RNeasy Mini Kit, UK). The columns were then centrifuged 30 seconds at 12000 x g at 4°C, the collection tubes surrounding the RNeasy columns replaced with a new one and 500 µl of ethanol-based washing buffer (RPE) were added. The columns were then centrifuged twice at 12000 x g for 2 minutes at 4°C. Finally, the columns were placed onto new 1.5ml Eppendorf tubes, 50ul of nuclease-free water* added to the centre of the columns, the samples incubated at room temperature for 1 minute and then centrifuged at 12000 x g for 1 minute at 4°C to elute the RNA.

The tubes with eluted RNA were immediately placed in ice and in order to evaluate the quality and amount of the extracted RNA. The RNA concentration and purity were measured on a Picodrop spectrophometer (Picodrop, UK), and the latter determined through the A260/A280 nm ratio of absorption (*Glasel J.A. 1995*). Purity was considered adequate when the ratio was within a 1.8-2.2 range (*Manchester K.L. 1996*). The intactness of RNA was then assessed by

analyzing 28S and 18S subunits of ribosomal RNA on ethidium bromidium stained 1% agarose gel electrophoresis (*Vendrely R. et al. 1968*). Given that high quality RNA is mandatory for the proper setting of downstream molecular analysis, only if in presence of pure and intact RNA the samples were stored at - 80°C and successively used.

* *The RNase free water was provided with the Qiagen RNeasy Mini Kit. Alternatively RNase free water can be prepared by adding 1 ml Dyethil Pirocarbonate (Sigma-Aldrich, UK) to 1 liter of mq H2O (total vol 0.1%), incubating at 37°C for at least 12 hours and autoclave.*

2.5 cDNA synthesis from RNA

Reverse Transcription PCR is a technique that permits to generate and amplify complementary DNA sequences from RNA.

In order to obtain cDNA from RNA a high capacity cDNA Reverse Transcription kit was used (Applied Biosystems, UK). 10 µl of Master Mix was prepared on ice as shown below (Table 2-1) and an equal volume (10 µl) of RNA was added. All the surfaces and consumables were previously decontaminated with RNaseZap decontamination solution (Applied Biosystems, UK)

After adding the RNA to the Master Mix, the so prepared reaction samples were briefly centrifuged to ensure that contents were spun down and air bubbles were eliminated. For the reverse transcription reaction a PCR thermal cycler (PTC-200 Peltier Thermal Cycler MJ Research, UK) was programmed as shown below (Table 2-2). After the reaction the tubes with the cDNA were stored at - 20° C.

Table 2.1: cDNA Reverse Transcription Reaction Master Mix Preparation

Component	**Volume (µl)**	**Final Concentration**
10X RT Buffer	2.0	1 X
25X dNTP Mix (25 mM)	0.8	1 X (1 mM)
10X Random Primers	2.0	1 X
MultiScribe Reverse Transcriptase (50U/µl)	1.0	50 U
Nuclease-free water	4.2	
RNA	10	up to 2 µg
Total volume	20	

Table 2.2: PCR cycler conditions for Reverse Transcription PCR reaction

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25^{o}C	37^{o}C	85^{o}C	4^{o}C
Time	10 min	120 min	5 min	forever

2.6 PCR reaction

For normal PCR reactions Green GoTaq Flexi DNA Polymerase kit (Promega, UK) was used and a master mix prepared at the concentrations shown below (Table 2-3). The gene primers were designed considering spanning exon-exon junctions so as to avoid genomic DNA amplification and primer dimer formation (*Dhanasekaran et al. 2010*) by using the Primer3 software and then purchased from Sigma-Aldrich, UK.

Table 2.3: Normal PCR Reaction Master Mix Preparation

Component	Volume (µl)	Final concentration
5X Green GoTaq Flexi Buffer	10 µl	1X
$MgCl_2$ solution (25 mM)	2-8 µl	1-4 mM
PCR Nucleotide Mix (10 mM)	1 µl	0.2 mM
Upstream Primer (10 mM)	X µl	0.1-1 µM
Downstream Primer (10 mM)	Y µl	0.1-1 µM
Go Taq DNA Polymerase (5U/ µl)	0.25 µl	1.25 U

Template cDNA	Z µl	< 500 ng/50 µl
Nuclease Free Water	q. b. to 50 µl	
Total volume	50 µl	

Once prepared the 1.5 ml Eppendorf tubes containing the master mix and the DNA were centrifuged in a microcentrifuge for a few seconds and then placed into a thermal cycler where the reaction started. Given that the inserts amplified were of similar length and the primers annealing temperature were 60°C the same following conditions were used for each reaction:

Condition	**Number of cycles**	**Step**
95°C 2 minutes	1 cycle	Initial denaturation
95°C 1 minute	35 cycles	Annealing
60°C 1 minute		
72°C 30 seconds		

72°C 5 minutes	1 cycle	Final extension
4°C indefinite	1 cycle	

Table n. 2.4 - primers for PCR/RT-PCR

PRIMER	**SEQUENCE**	**Annealing Temp (ºC)**	**cDNA Amplicon size (bp)**	**Gene Accession Number**
DLL-1 forward	CTGATGACCTCGCAACAGAA	60	313	NM_005618.3
DLL-1 reverse	ATGCTGCTCATCACATCCAG	60		
DLL-4 forward	ACTGCCCTTCAATATTCACCT	60	176	NM_019074.3
DLL-4 reverse	GCTGGTTTGCTCATCCAATAA	60		
IL-7 forward	TGAAACTGCAGTCGCGGCGT	60	172	NM_000880.3
IL-7 reverse	AACATGGTCTGCGGGAGGCG	60		
Cx-43 forward	ACTTGCCTTTTCATTTTACTTC	60	219	NM_000165.3
Cx-43 reverse	CCTGGGCACCACTCTTTT	60		
AIRE forward	GATGACCTGGAGTCCCTTCT	55	252	NM_000383.2
AIRE reverse	CTCATCAGAGCTGCATGTCC	55		
CK-1 forward	ATTTCTGAGCTGAATCGTGTGATC	60	130	NM_006121.3
CK-1 reverse	CTTGGCATCCTTGAGGGCATT	60		

CK-10 forward	TGATGTGAATGTGGAAATGAATGC	60	145	NM_000421.3
CK-10 reverse	GTAGTCAGTTCCTTGCTCTTTTCA	60		
GATA-3 forward	CGAGATGGCACGGGACACTA	60	141	NM_001002295.1
GATA-3 reverse	TGGTCTGACAGTTCGCACAGG	60		
Hes-1 forward	TCTTTTTCGTGAAGAACTCCAA	60	240	NM_005524.3
Hes-1 reverse	TCAGCTGGCTCAGACTTTCA	60		
RPS-29 forward	GCTGTACTGGAGCCACCCGC	55-60	142	NM_001030001.1
RPS-29 reverse	TCCTTCGCGTACTGACGGAAACAC	55-60		

2.7 DNA electrophoresis

To confirm the size and presence of the PCR products after their amplification, an agarose gel electrophoresis was performed. A 1.5 % agarose gel was prepared by melting 1.5 gr of agarose (Sigma-Aldrich, UK) in 100 ml TAE 1X buffer. Once the gel was cooled and solidified 10 μl of marker (100 bp plus ladder, Invitrogen, UK) and 15 μl of each sample were added onto the gel wells and this was run at 110V for approximately 45 minutes. After running the gel was stained into a 500 ng/ml Ethidium bromide (Invitrogen, UK) solution for 15 minute and finally observed under the UV.

2.8 Quantitative Real-Time PCR

The quantitative real-time PCR is a technique required in order to quantify a gene transcript level from a biological sample after that the RNA has been extracted and converted to complementary DNA. As the amplification of double stranded DNA proceeds during this PCR reaction the fluorescent signal of the

intercalating dyes added to the reaction increases proportionally to the accumulation of the PCR product and can be detected by the instrument used for the amplification (*Peters I.R. et al. 2004*).

The absolute number of initial cDNA molecules (which reflects the number of sample mRNA molecules) loaded into a reaction can be precisely quantified by the concomitant setting of a calibration curve prepared by serial dilutions of known concentrations of a standard consisting of the same PCR product to analyze (Figures 10-11). The standards were prepared by normal PCR using Green GoTaq Flexi DNA Polymerase kit (Promega, UK), and the expected product correct size checked by agarose gel analysis. After that the PCR products were cleaned up with the QIAquick PCR Purification Kit (Qiagen, UK) in order to purify the PCR products from primers, nucleotides, polymerases and salts 5 volumes of binding buffer PB were added to each sample, placed in the provided spin columns and centrifuged at 12000 x g for 1 minute at 4ºC. Then the columns were washed with 0.75ml of washing buffer PE by 1 minute of centrifugation at 12000 x g at 4ºC and finally placed onto 1.5ml Eppendorf tubes. 20 µl of elution buffer EB was added to the centre of the columnes, these incubated for 1min at room temperature and spun at 12000 x g for 1 minute at room temperature to elute the cleaned DNA. DNA amount (µg/µl) and purity (A260/A280 ratio) were then determined with a picodrop spectrophotometer (Picodrop, UK) and copy numbers calculated by the following formula (Gordones et al., 2007):

$$\text{Number of copies}/\mu\text{l} = \frac{6.022 \times 10^{23}(\text{molecules/mole}) \times \text{DNA concentrations}\,(\text{g}/\mu\text{l})}{\text{Number of bases pairs} \times 660\ \text{daltons}}$$

Where:

6.022x1023 (molecules/mole) = Avogadro's number

660 Dalton = Average weight of a single base pair

Once the number of cDNA copies/µl was detected it was possible to prepare the standard dilutions to generate the calibration curves.

Given that relative changes depending on total mRNA content can be present among the samples to analyse any sample must be normalized to a housekeeping gene which was RSP-29 in this study (*de Jonge H.J.M. et al. 2007*). A valid housekeeping gene is usually chosen from those fundamental for cellular basic functions because their expression is generally maintained constant regardless of any environmental stimuli. However, in some cases the concentrations of even widely used and tested housekeeping genes may still vary with the experimental conditions and therefore a detailed selection procedure and a new validation is highly recommended before using qRT-PCR for a new study (*Thellin O. et al. 2009*).

The reactions were performed on a 96 microwell plate (Bio-Rad, UK) and 1 µl of samples cDNA diluted 1:50 with nuclease free water added to the reaction master mix wells while for standard curves 1/10 dilutions of PCR product were produced by serial dilution in nuclease-free water and 1 µl of each aliquotted into the master mix wells. A graph of threshold cycle (Ct) versus $\log_{10}$ copy number of the sample from the dilution series was generated (Figure 15). A Negative Template Control (NTC) per each gene was also prepared by adding 1ul of nuclease free water to the reactions master mix.

The reactions were prepared by using the same primers used for normal PCR (Table) and Sybr Green reagent (QIAGEN, UK) as shown below (Table 2.5) and analyzed on a CFX96 Bio-Rad Real-Time System (Bio-Rad, UK).

TABLE 2.5 qRT-PCR Reaction Master Mix Preparation

Component	**Volume (µl)**	**Final concentration**
Power SYBR Green PCR 2X	5 µl	1X
Upstream Primer (10 µM)	0.25 µl	0.25 µM
Downstream Primer (10 µM)	0.25 µl	0.25 µM

Template cDNA (1:50)	1 µl	(< 500 ng/µl)
Nuclease Free Water	3.5 µl	
Total volume	10 µl	

Reactions were subsequently performed following this schedule:

Initial activation	*95ºC*	*15 minutes*	*1 cycle*
Denaturation	*94ºC*	*10 seconds*	*35 cycles*
Annealing	*60ºC*	*30 seconds*	
Extension	*72ºC*	*30 seconds*	
Final extension	*72ºC*	*5 minutes*	*1 cycle*

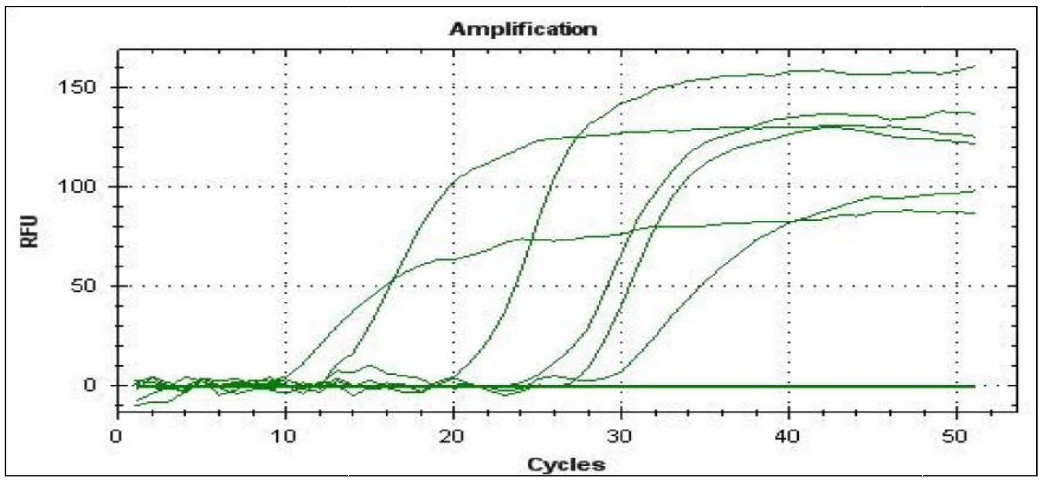

Figure 10: Gene amplification by RT-PCR. Fluorescence detection of different known concentration of target gene Dll-4 occurs at a different number of cycles depending on the inItial template concentration.

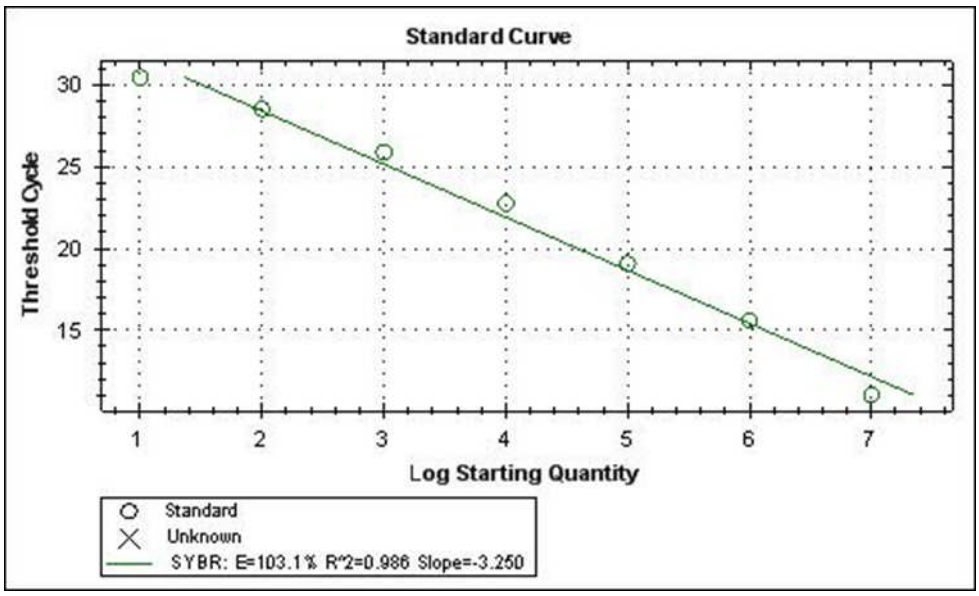

Figure 11: RT-PCR calibration curve. The image shows a calibration curve for the target gene Dll-4 generated by serial dilutions of cDNA template.

A melting curve analysis was produced after the quantitative real-time PCR reaction was completed at the following conditions (Table 6). The melting temperature is the temperature at which the DNA helix of a certain PCR product is completely denaturated and corresponds to the highest amount of

intercalated dye fluorescent signal. This was determined with the CFX96 Touch Systems Software (Bio-Rad, UK) using a rate of change of fluorescence versus temperature graph (*−d(RFU)/dT*) (Figure 12). Despite the area under the curve (AUC) reflecting the total amount of product amplified, the melting curve analysis was not used for gene expression quantification but rather to check whether the fluorescence signal detected by the instrument in real time during the PCR reaction effectively derived from gene amplification or from generation of non-specific products such as primer dimers. Given that the latter are much smaller (~25-35 bp) the resulting melting temperature is usually < 77ºC whereas since the gene products are made by at least 60 bp their Tm is generally > 78ºC. This difference permits to distinguish real gene amplification from detection of unspecific products (*Ririe K.M. et al. 1997*). The melting curves (Figure 16) were generated using a CFX96 Bio-Rad real time PCR system (Bio-Rad, UK).

Table 6: Real-Time PCR cycler conditions for the melting point analysis

Phase	**Temperature**	**Duration**	**Cycles**
Initial Activation	95^{o}C	30 seconds	1
Gradual temperature increase in 0.5^{o}C increments	65^{o}C-95^{o}C	0.5 seconds per increment	1

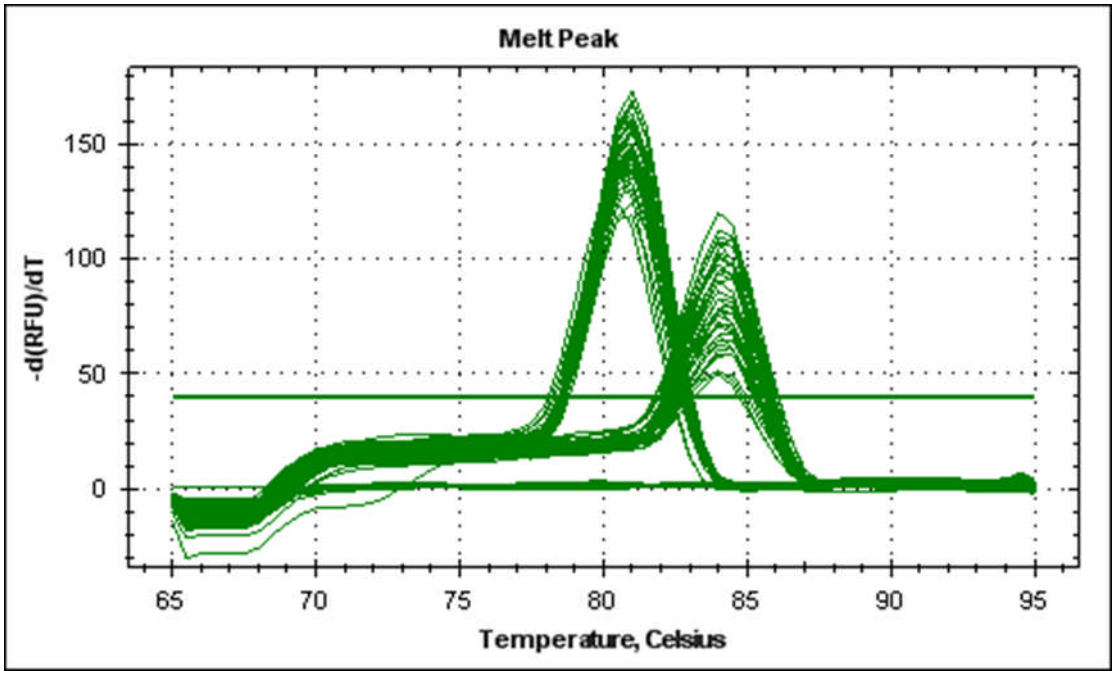

Figure 12: RT-PCR Melting Curve. The image shows melting curve analysis of Dll-4 and VEGF. DLL-4 presents the highest peaks (83ºC) and VEGF the lower (81ºC) temperature of melting. If primer-dimer effect was present the curve peaks would have appeared at lower temperatures.

Analysis of gene expression data was performed after that the specificity and efficiency of the reaction was evaluated. The specificity of the qPCR reaction was validated by melting curve analysis while the efficiency calculated by the CFX96 Touch Systems Software (Bio-Rad, UK) through the standard curve slope (*Peters I.R. et al. 2004*) according to the following equation:

$$Efficiency = [10\,(-1/slope)] - 1$$

The perfect efficiency of a PCR reaction is 100% when 2 molecules of DNA are produced from 1 per cycle (*Pfaffl M.W. 2001*). Data analysis was then achieved through the CFX96 Touch Systems Software (Bio-Rad, UK) by comparing the samples Ct values to the relative standard curve and the final results expressed as DNA absolute copy number on a 10 base logarithm scale.

In this study qRT-PCR analyses were carried out to investigate the effect that the three-dimensional environment has on the expression of genes important

for T cell development in the skin cells which used to generate T cells from HSC in vitro

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2.9 Western Blot Analysis

2.9.1 Samples preparation

The samples were mixed 1:1 with a combination of 1X reducing agent (Invitrogen, UK) and 1X Laemmli blue (Invitrogen, UK) in a final volume of 40 µl (Table 7) and incubated for 10 minutes in an air dry bath at 70° C.

Table 7: protein samples preparation for Western Blot

COMPONENT	**VOLUME (µl)**	**FINAL CONCENTRATION**
Protein	X (up to 26 µl)	30 µg
milliQ water	Y	
Reducing agent 10X	4 µl	1X
Laemmli blue 4X	10 µl	1X
TOTAL VOLUME	40 µl	

2.9.2 Buffers and Gel preparation

Foe Western Blot the following buffers were prepared:

- Tris-HCl 1.5M pH 8.8 (1L):

181.64 gr of Tris Base (Fisher Bioreagents, UK) were slowly added to 800 ml milli-Q water and the solution mixed dropping a a stir bar into the glass bottle and placing it on a rotating magnet. When all the TRIS Base was solubilized

and the solution appeared clear a washed pH meter probe was placed into the solution and the pH adjusted to 8.8 by adding 12N HCl dropwise into the stirring solution until the pH reached the 9.0-9.1 range and then 6N HCl dropwise until the pH was 8.8. Afterwards the pH meter probe was left another 5 minutes to ensure that the pH reading was stable and finally milli-Q water was added up to 1 L. This buffer was immediately used or stored at 4ºC.

- Tris-HCl 1M pH 6.8 (1L):

121.1 gr of Tris Base (Fisher Bioreagents, UK) were added to 800 ml of Milli-Q water and the solution mixed dropping a a stir bar into the glass bottle and placing it on a rotating magnet. When all the TRIS Base was solubilized and the solution appeared clear a washed pH meter probe was placed into the solution and the pH adjusted to to 6.8 by adding 12N HCl dropwise into the stirring solution until the pH reached the 9.0-9.1 range and then 6N HCl dropwise until the pH was 6.8. Afterwards the pH meter probe was left another 5 minutes to ensure that the pH reading was stable and finally milli-Q water was added up to 1 L. This buffer was immediately used or stored at 4ºC.

- 10% SDS (50ml):

5 gr of SDS (Fisher Bioreagents, UK) were placed in a 50 ml falcon tube then milli-Q water was added up to 50ml, the tube mixed vigorously and the solution stored at room temperature.

- 0.1% SDS (50ml):

50 mg of SDS (Fisher Bioreagents, UK) were placed in a 50 ml falcon tube then milli-Q water was added up to 50ml, the tube mixed vigorously and the solution stored at room temperature.

- TBS 5X (1 L):

12.1 gr of Tris Base (Fisher Bioreagents, UK) and 43.8 gr of NaCl (BDH Prolabo, UK) were added to 800 ml ml of Milli-Q water and the solution mixed dropping a a stir bar into the glass bottle and placing it on a rotating magnet.