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Stemness of T cells and the hematopoietic stem cells: Fate, memory, niche, cytokines

Francesca B Aiello^{1,2}, Laura Graciotti³, Antonio D Procopio³, Jonathan R Keller⁴, and Scott K Durum^{1,*}

¹Laboratory of Molecular Immunoregulation, Frederick, MD 21702, USA.

²Department of Medicine and Aging Sciences, University of Chieti-Pescara, 66013 Chieti, Italy

³Department of Clinical and Medical Sciences, Marche Polytechnic University, 60100 Ancona, Italy.

⁴Laboratory of Cancer Prevention, Frederick, MD 21702, USA

Abstract

Stem cells are able to generate both cells that differentiate and cells that remain undifferentiated but potentially have the same developmental program. The prolonged duration of the protective immune memory for infectious diseases such as polio, small pox, and measles, suggested that memory T cells may have stem cell properties. Understanding the molecular basis for the life-long persistence of memory T cells may be useful to project targeted therapies for immune deficiencies and infectious diseases and to formulate vaccines. In the last decade evidence from different laboratories shows that memory T cells may share self-renewal pathways with bone marrow hematopoietic stem cells. In stem cells the intrinsic self-renewal activity, which depends on gene expression, is known to be modulated by extrinsic signals from the environment that may be tissue specific. These extrinsic signals for stemness of memory T cells include cytokines such as IL-7 and IL-15 and there are other cytokine signals for maintaining the cytokine signature (TH1, TH2 etc) of memory T cells. Intrinsic and extrinsic pathways that might be common to bone marrow hematopoietic stem cells and memory T lymphocytes are discussed and related to self-renewal functions.

*S.K.D.: (to whom correspondence should be addressed): Laboratory of Molecular Immunoregulation, FCRDC, Bldg 560, Frederick, MD 21702, USA. Tel: +1 301-846 1545; Fax: +1 301-846-6720; durum@nih.mail.gov.

Author contacts

F.B.A.: Laboratory of Molecular Immunoregulation, FCRDC, Bldg 560, Frederick, MD 21702, USA. Tel: +1 301-846 6283; Fax: +1 301-846-6720; aiellofb@nih.mail.gov, Department of Medicine and Aging Sciences, University of Chieti-Pescara, via dei Vestini, 66013 Chieti, Italy. Tel +39-0871-357399; Fax: 39-0871-540079; fbaiello@unich.it

L.G.: Department of Clinical and Medical Sciences, Marche Polytechnic University, via Tronto 10, 60100, Ancona, Italy. Tel: +39 071-2206242; Fax: +39-071-2206103; l.graciotti@univpm.it

A.D.P.: Department of Clinical and Medical Sciences, Marche Polytechnic University, via Tronto 10, 60100, Ancona, Italy. Tel: +39 071-2206241; Fax: +39 071 2206103; a.d.procopio@univpm.it

J.R.K.: Laboratory of Cancer Prevention, FCRDC, Bldg 560, Frederick, MD 21702, USA. Tel: +1 301-846-1461, Fax: +1-301-846-6646, kellerjo@nih.mail.gov

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Keywords

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1) Introduction

A concept of “stemness” arises in modern discussions of embryonic stem cells, tissue stem cells and cancer stem cells, but many of its principles were discovered in hematology. Blood cell homeostasis has long been appreciated to require life long production of hematopoietic stem cells (HSC). As we will discuss here there is a similar life long persistence of memory T lymphocytes that is necessary to protect from infections. Long-term protective immune memory has been clearly demonstrated for measles, mumps, yellow fever, and polio. Noteworthy, CD4 and CD8 memory T cells specific for small pox antigens can be detected up to 75 years after vaccination (1). The hypothesis that memory B and T lymphocytes could have stem cell properties was formulated in 2001-2002 (2). It was inspired by the observation that the transcription factor Bcl-6, structurally similar to a repressor of neuronal differentiation in *Drosophila*, inhibits the expression of the transcription factor Blimp-1 which induces the differentiation of B cells in plasma cells and prevents B cell terminal differentiation (3). In keeping with this observation, Bcl-6 expression in lymphoid tissue is restricted to germinal center cells (4), and in memory B cells is 6-8 fold higher than in naive B cells (5). In 2002 transcriptional repressors of the Polycomb family, which regulate segmentation in *Drosophila*, were found to be expressed in hematopoietic stem cells (HSC) and in T cells (6). In HSC their expression declined with differentiation, but their functions were unknown (6).

Bone marrow HSC are distinguished on a functional basis. Cells transplanted into a lethally irradiated mouse that are able to sustain hematopoiesis over some months are defined as “long-term” HSC, whereas those that sustain hematopoiesis for no longer than three months are defined as “short-term” HSC. Long-term HSC give rise to short-term HSC which in turn give rise to multipotent progenitors without self-renewal potential (7). In 2006 gene expression profiles of HSC and T cells were compared. In memory T cells the transcripts differed from those observed in naive and effector cells, and in long-term HSC they differed from those of short-term HSC and multipotent progenitors. Interestingly, similar sets of transcripts were up or downregulated in HSC and memory T cells (8), suggesting similar gene expression profiles. These data reinforced the hypothesis that self-renewal of HSC and memory T cells might be regulated by shared signalling pathways. The aim of this review is to discuss these pathways. We hope that clarifying the mechanisms underlying the “stemness” of memory lymphocytes will improve protocols of vaccination and therapies for infectious disease, and have implications for diseases involving cells of the immune system.

Self-renewal is regulated by cell autonomous intrinsic factors (like transcription factors) and by extrinsic factors such as cytokines. There is a link between the extrinsic and the intrinsic arms of the process, for instance, when the expression of transcription factors is modulated by cytokines (9). HSC can undergo either “asymmetrical” or “symmetrical” division (10). Asymmetrical division generates one stem cell that remains undifferentiated and one

daughter cell that differentiates, thus it does not change the stem cell number (10). Symmetrical division generates two stem cells and may be required to increase the stem cell pool (10). It was hypothesized that the probability of asymmetrical division of hematopoietic cells could be influenced by cytokines (11) and by signals from cells present in the specific HSC microenvironment, termed the “niche” (12). In 2007 symmetrical and asymmetrical divisions were elegantly demonstrated to occur for HSC and signals from the microenvironment were shown to influence the pattern of division (13).

It is now clear that “self-renewal” of a stem cell is the result of multiple coordinated processes: proliferation, quiescence, survival, and maintaining the capacity to differentiate along a lineage. Memory T cells may share self-renewal pathways with long-term HSC, however there are pathways that can be reasonably expected to be different. For instance, HSC have to maintain an undifferentiated phenotype, while T lymphocytes need to retain the memory of the antigen and of their functional phenotype (Th1, Treg, etc), although plasticity of function appears to require that extrinsic signals maintain a functional phenotype. In the first part of this review the pathways regulating HSC self-renewal that may be also present in memory T cells will be described.

2 Part I

2.1 HSC markers in bone marrow: “stem cell attire”

Long-term HSC represent less than 0.5% of mouse bone marrow cells, thus the identification of a specific phenotype has been crucial for their isolation. There are differences between murine and human surface markers. Murine HSC express the stem cell factor receptor c-Kit, Thy-1(CD90), and stem cell antigen1 (Sca-1/Ly-6A), but not lineage markers (Lin⁻), thus they are frequently termed “KTLS” or “KLS” cells (14). KLS include long-term and short-term HSC (14). Interestingly, Sca-1/Ly6A which belongs to the family of lymphocyte activation (Ly) proteins was originally identified as an antigen upregulated on activated lymphocytes (15). Sca-1, however, does not have a human ortholog (15). Murine, but not human HSC are CD38⁺ (14). Human HSC are Lin⁻, and express c-Kit and Thy-1 (16).

CD34 is a type I transmembrane protein expressed by murine and human HSC. CD34 may play a role in cell-adhesion and lineage outcome (14, 17, 18), and mast cells, megakaryocytes, endothelial cells and fibrocytes are variably positive (14, 17). Expression of CD34 declines with differentiation, however, it may also reflect the activation status of the cell, since it is inducible by G-CSF and flououracil (14). Long-term HSC are CD34 low-negative in mice (19).

In humans HSC with long-term capability of self-renewal are CD34 positive (20). In addition, a rare CD34 negative population with a long-term capability of self-renewal even higher than that of CD34 positive HSC has been very recently described (21).

Human CD34⁺ HSC express CD44 (22), an integral membrane protein that in mice is a specific marker of memory T cells. (discussed in the second part of the review). CD44 exhibits multiple isoforms deriving from the utilization of different exons and from different post-translational modifications. These isoforms bind hyaluronic acid and variety of other

molecules, such as extracellular matrix proteins, growth factors and adhesion molecules such as E selectin and L-selectin, expressed by endothelial cells and leukocytes, respectively (22). HSC CD44 is the most potent E selectin and L selectin ligand. Murine KLS, however, do not express CD44, thus, it has been difficult to study its role in vivo (22).

Human and murine long-term HSC express prominin or CD133, a pentaspan transmembrane molecule which is a useful marker, since both CD34 negative and positive cells are CD133+ (14). The signaling lymphocyte activating molecule SLAM (CD150) is selectively expressed by murine long-term HSC but not by multipotent progenitors (23). It belongs to the family of SLAM related receptors (SRR) that play a role in the maintenance of immunological memory (1, 24). CD150-deficient mice have normal hematopoiesis (23), this could indicate a non-essential role of CD 150 or redundant activities of other SRR. Anti-CD150 permits a great improvement of long-term HSC purification in mice. Human and murine bone marrow cells, however, display different SRR molecules, and human long-term HSC have been reported to be CD150 negative (25). Therefore CD150 in humans may not be a useful marker for improving purification of long-term HSC (26).

Finally, it has been recently found that human HSC coexpressing Thy-1 and the adhesion molecule $\alpha 6$ integrin (CD49f) which is not expressed by multipotent progenitors, were the most effective in long-term engraftment, and a single cell of this type could give rise to all hematopoietic lineages in vivo (27). Murine and human long-term HSC markers are summarized in Fig. 1.

2.2 Pathways regulating proliferation

A number of intrinsic and extrinsic pathways control the proliferative activity of HSC and some of them have been shown to operate in T cells. The most important intrinsic pathways are mediated by products of Polycomb-group (PcG) and Trithorax-group genes (28). These proteins form multimeric complexes that act on chromatin, regulating transcription of homeobox (Hox) genes. PcG products were identified in *Drosophila* as repressors of Hox genes, which encode transcription factors regulating body patterning and organogenesis (28). The 39 mammalian Hox genes are divided in 4 clusters (A-D) located on different chromosomes. Based on the sequence homology of the DNA binding homeodomain, the aligned genes are assigned to 13 groups defined as paralogs (28, 29). Hox gene function is necessary for the formation of the hematopoietic system, (10, 29) and HoxA9-deficient mice display impaired HSC proliferation (30).

Two PcG complexes, 1 and 2, have been identified and, impressively, members of both complexes participate in programs of self-renewal. At least 12 mammalian homologues of *Drosophila* proteins are members of PcG complex 1, including Bmi-1 and Mel-18 proteins (28). Bmi-1, highly expressed by mouse and human HSC, is necessary for maintenance of adult HSC and neural stem cells (31, 32). Bmi-1-deficient mice exhibit a hypocellular bone marrow with a markedly reduced number of long-term HSC (31). Bmi-1 represses the expression of the Ink4A locus products: the cell cycle inhibitor p16^{INK4a}, and p19^{ARF} which controls apoptosis through the tumor suppressor gene p53 (33, 34). Deletion of these genes, however, only partly reverses the self-renewal defect of Bmi-1-deficient stem cells (10, 33,

34). Since Bmi-1 promotes many functions involved in self-renewal, it is reasonable that these two pathways are not sufficient to mediate all Bmi-1 effects.

Mel-18 is highly homologous to Bmi-1 (28), and since PcG proteins negatively regulate Hox genes, the expression of Hox genes was examined in Mel-18-deficient mice. Only the expression of HoxB4 was found to be markedly increased (35). This was associated with an increased number of HSC in the G₀ phase of the cell cycle with long-term reconstitution activity (35). Thus, Mel-18 is a negative regulator. More importantly, it has been found that embryonic stem cells transduced with HoxB4 or with mammalian paralog group 4 members (Hox A-D) acquire the capability to differentiate in HSC with long-term reconstitution activity (29, 36), and this observation may be of future clinical relevance.

The Mixed Lineage Leukaemia gene family of transcription factors comprises 5 members, MLL (also called MLL1) is the mammalian homologue of the Trithorax Drosophila gene and it is commonly rearranged in childhood leukaemia (10). It is required for the expression of multiple Hox genes (37). MLL-deficient mice die before embryonic day 17, and cells from the aorta-gonad-mesonephros region from these embryos, unlike the cells from control embryos, are unable to repopulate the bone marrow of irradiated mice (38). The function of MLL has been studied in conditional deletion models in murine fetuses and in adult mice. Fetal liver cells show a reduction in the number of long-term HSC, particularly in the quiescent fraction (39) and adult bone marrow cells show a reduced number of KSL, with inappropriate cell cycle entry and depletion of quiescent cells (40).

The Notch pathway also regulates expression of Hox genes during embryogenesis (10, 41). This extrinsic pathway is highly conserved throughout evolution, and controls self-renewal at different levels. Notch receptors and ligands are transmembrane proteins, thus signaling derives from cell-cell interactions. Notch signaling is essential for the generation of embryonic HSC and for T cell development (42, 43). It has been proposed that in adult bone marrow HSC Notch activation promotes proliferation and inhibits differentiation (10). For this reason this pathway will be discussed in the paragraph concerning the maintenance of stem cell identity.

The Wnt pathway also regulates Hox gene expression (41). In humans there are 19 secreted Wnt proteins that can bind 10 surface receptors of the Frizzled receptor family, which are homologous to G protein coupled receptors (44). Ligand-receptor interaction activates the cytoplasmic phosphoprotein protein Dishevelled (Dsh), a modular protein utilizing distinct domains to activate three pathways: the canonical, β -catenin dependent pathway, and the “non canonical” pathways. The non canonical pathways are so termed because they are β -catenin-independent (44, 45) and include the Planar Cell Polarity (PCP) and the Wnt/Ca²⁺ pathways. In the canonical pathway the binding of Wnt proteins to their receptors protects β -catenin from proteosomal degradation in the cytoplasm. β -catenin then translocates to the nucleus, forming a complex with transcription factors also expressed by T lymphocytes: T cell factors 1-4 (TCF-1-4) or Lymphoid-enhancer binding1 (Lef-1) (44, 45). These complexes activate Wnt downstream target genes. Wnt ligands are expressed by HSC and by bone marrow stromal cells suggesting both paracrine and autocrine signalling (41). Transduction of HSC with constitutively active β -catenin or stimulation with recombinant

Wnt3A promotes HSC proliferation in vitro and increases expression of HoxB4 and Notch (46). Transgenic mice with activated β -catenin develop thymic lymphoma, and β -catenin activity promotes initiation and maintenance of cancer stem cells in myeloid chronic leukaemia (reviewed in 47). In agreement with these results, Wnt3A-deficient mice, which die prenatally, have reduced numbers of HSC in fetal liver with impaired long-term HSC function (48). Surprisingly, β -catenin- or β - γ -catenin-deficient HSC exhibit normal hemopoiesis (49, 50), and enforced constitutive activation of β -catenin induces failure of hematopoiesis (41) and apoptosis of HSC (51). These results, apparently contrasting, may be explained by the observation that the Wnt3A-mediated canonical pathway is physiologically inhibited by the Wnt5A-mediated non-canonical pathway, which, for instance, causes decreased β -catenin expression (52). Another explanation may be that Wnt/ β -catenin promotion of HSC self-renewal may be predominantly intrinsic to stromal cells rather than HSC themselves (53). Wnt-mediated stromal signals are complex, they could promote an increase of HSC cell number (53), but also quiescence, and will be discussed in the section concerning quiescence.

Growth factors and cytokines produced from a variety of cells of the hematopoietic stem cell niche positively influence HSC proliferation: Stem cell factor (SCF), Thrombopoietin (TPO), FGF proteins, angiopoietin-like proteins, IGF-2, Flt-3 ligand and others (54). SCF is a major regulator of HSC self-renewal, promoting both proliferation and survival. SCF, alone or more effectively in combination with other cytokines, induces proliferation of fetal and adult HSC, and its receptor, c-Kit, is a tyrosine kinase (55, 56). SCF binding induces receptor homodimerization and cross-phosphorylation, creating docking sites for recruitment of other kinases, in particular, the p85 subunit of phosphatidylinositol-3-kinase (PI3-Kinase) which partly mediates the proliferative signal (56). SCF also activates the Janus kinase 2 (Jak-2), and this is followed by phosphorylation of the transcription factors Stat3 and Stat5A, known to positively regulate proliferation of murine and human HSC (55-57). In mature T cells the pathways induced by SCF are activated by other cytokines, and similarly promote proliferation and survival.

IL-10 exerts pleiotropic effects on T and B lymphocytes. Surprisingly, Lin⁻Sca-1+cKit⁺ cells express IL-10 receptor and were reduced in IL-10-deficient mice. IL-10 induced an increase of HSC number in short-term culture and HSC cultured on IL-10 secreting stroma display enhanced bone marrow repopulating activity (58). Thus, IL-10 has activities on both HSC and lymphocytes.

2.3 HSC quiescence

During early G1 phase, bone marrow HSC can exit from the cell cycle and enter a dormant, quiescent state termed the “G0” phase. In early studies it was shown that approximately 75% of murine KLS were quiescent and that 99% of them divided on average every 57 days (59). Subsequently, the CD34-CD150⁺ fraction of murine KLS has been found to divide once every 145 days, corresponding to 5 division per mouse lifetime (60). Importantly, in mice treated with 5-FU or G-CSF these cells can enter the cell cycle and then switch to dormancy again (60). However, in serial transplantation, the ability to reconstitute the hematopoietic system eventually declines, and this loss of function is defined as “exhaustion” (reviewed in

61). The cyclin-dependent kinase inhibitor p21 regulates the transition through the late G1 phase by inhibiting cyclin E-CDK2 complexes. In p21^{-/-} mice there is a great reduction in the potential of reconstituting the hematopoiesis after serial transplantation and resistance to repeated administration of 5-FU is greatly reduced, and this is associated with a decrease of HSC in the G0 phase (61). In a different murine genetic background, however, p21 function is required only under condition of cellular stress (62). Thus, quiescence is important to prevent exhaustion due to stress induced proliferation. Quiescence can protect from acquiring mutations, from apoptosis and from “senescence”, a state of permanent cell-cycle arrest (63). Last but not least, quiescence can also prevent differentiation, since proliferation is associated with commitment and differentiation. Mice deficient for growth factor independent-1 (Gfi-1) transcriptional repressor, or for the transcription factor E47, show reduced p21 expression and a phenotype similar to that of p-21-deficient mice 61, 64). E47-deficient KLS show a 50% decrease in p21 expression associated with hyperproliferation (64). In mammals E proteins (E47, E12, HEB and E2-2), belonging to the family of helix-loop-helix transcription factors, can act as transcriptional activators or repressors depending on the cellular context (64). E47 and E12 (splice variants of the gene E2A) and HEB are expressed in T cells (65). Inhibitors of DNA binding (Id) proteins also display a helix-loop-helix transcription domain and inhibit the transcriptional activity of E proteins forming with them inactive heterodimers (66). Id proteins have been recently shown to play an important role in memory T cell differentiation (discussed in part II). The role of Id proteins in HSC, however, is less clear. Loss of Id1 was associated, as expected, with an increased expression of p21 (66) but the number of cycling HSC was increased (66) and there were fewer long-term HSC (67). It was subsequently observed that Id1-deficient bone marrow cells developed normally in Id positive irradiated wild type mice, whereas HSC development was impaired in Id-deficient mice, indicating that the HSC defects were due to the microenvironment (68). Interestingly, in these mice stromal cells showed an altered cytokine production that could be at least partially responsible for the HSC defects (68).

The interactions of HSC with cells of the stem cell niche can induce quiescence. An important role of the Wnt-mediated pathway has been demonstrated using a paninhibitor of the canonical signalling. The protein Dickkopf1, produced by osteoblasts, blocks the binding of Wnt proteins with their receptors. Mice overexpressing Dickkopf1 in osteoblasts show reduced expression of p21 associated with an increased cycling of KLS cells and a decline in their hematopoietic reconstituting activity (69). Consistent with this result is the evidence that Wnt5A inhibits the activity of Wnt3A by increasing the percentage of HSC in G0 phase (52).

Low levels of reactive oxygen species (ROS) can maintain quiescence and block differentiation whereas high levels of ROS can induce HSC exhaustion through excess differentiation. The phosphoinositide 3-kinase (PI3K) pathway is critical for the activation of AKT, a family of serine-threonine kinases promoting survival in multiple cell types including lymphocytes. AKT 1 and 2 double deficient long-term HSC showed increased quiescence (cells in G0) accompanied by low levels of ROS, whereas artificially elevating ROS induced HSC differentiation in these cells (70).

The transcription factors of the forkhead box (Fox) family are characterized by a forkhead or winged DNA binding domain and are divided in subfamilies based on their sequence homology (71, 72). The transcription factors of the O subfamily (Fox O 1, 3, 4 and 6) are regulated downstream the PI3K pathway. In quiescent cells they are localized into the nucleus, where they are hypophosphorylated and constitutively active. T cells express FoxO1, 3 and 4 (72). In FoxO3a-deficient mice HSC show elevation of ROS. The ROS effect, inducing differentiation, may in turn be mediated by activation of the mitogen-activated protein kinase (MAPK) p38 since inhibiting p38 MAPK improves the capability of HSC to form colonies in long-term cultures (73). The tumor suppressor gene Pten is a phosphatase which dephosphorylates PI3K, inactivating it (74). By inhibiting PI3K activity it acts indirectly, activating FoxO family members and therefore maintaining quiescence. Importantly, Pten is also a negative regulator of mammalian target of rapamycin complex 1 (mTORC1) which must be repressed to maintain quiescence in HSC (75). The mammalian target of rapamycin mTOR is a crucial regulator of multiple lymphocyte functions. It is a serine-threonine kinase existing in two multi-protein complexes mTORC1 and mTORC2, and its activation can occur via PI3K signaling (82). Its role in the generation of memory T cells will be discussed in the second part of the review.

2.4 HSC survival

Control of survival is essential for the homeostatic maintenance of HSC number, which is particularly important considering the limited life span of differentiated blood cells. A variety of intrinsic and extrinsic pathways protect HSC from apoptosis. The most studied intrinsic pathway is the mitochondrial apoptotic pathway, regulated by the anti-apoptotic Bcl-2 family members. The anti-apoptotic Bcl-2 family includes Bcl-2, Bcl-x, Bcl-w, Mcl-1 and other members that share regions of structural homology (Bcl-2 Homology Regions, or BH regions 1-4) (76). Early studies have shown that constitutive expression or overexpression of Bcl-2 enhances progenitor cell survival (reviewed in 77), and that mice lacking Bcl-x_L/x_S undergo massive HSC death (78). Subsequently, it has been shown that inducible ablation of Mcl-1 results in non-viable HSC with failure of hematopoiesis (77). Extrinsic signals for survival of HSC and progenitor cells have been characterized in vitro to include cytokines. For instance SCF and IL3, which can act synergistically, were shown to sustain survival of HSC (56, 79, 80). IL-6 and Flt3 ligand also were able to promote HSC survival (Note that Flt3 is expressed by human but not by murine long-term HSC) (81). It is now clear that all these cytokines increase the expression of Bcl-2 family members. Mcl-1 RNA expression was inducible by SCF, IL-6 and Flt3 ligand, while Bcl-2 and Bcl-x_L RNA expression was upregulated by IL-3 (82). Cytokine synergistic activity can thus be explained by their specific influences, and a number of observations support this hypothesis. In IL-3-dependent hematopoietic cell lines IL-3 deprivation upregulates the protein level of the pro-apoptotic protein Bim whereas Bcl-x_L protein level is downregulated (83). SCF deprivation of KLS induces Bim and downregulates Bcl-2 mRNA, but does not affect Bcl-x. (84). IL-3 but not SCF increases Bcl-2 and Bcl-x protein expression in primary Lin⁻ progenitor cells (82).

Another pathway important for survival is sustained by the intracellular serine protease inhibitors, called “ov-serpins” based on their homology with ovoalbumin. In early studies

these enzymes were shown to be expressed in hematopoietic precursors and in mature peripheral blood cells (85). They inhibit the activity of serine proteases, or granzymes contained in cytotoxic granules, such as Granzyme B in cytotoxic T cells and protect from the apoptosis initiated by proteolysis (85, 86). Serpin (Spi) 2A was shown to be expressed in a cell line commonly used as a model of hematopoietic stem cells and in hematopoietic progenitors (85). Differentiation induced downregulation of Spi2A mRNA expression whereas enforced expression was associated with increased quiescence (85). In later studies Spi2A has been shown to favour the long-term maintenance of memory T cells (87).

The PgC protein Bmi-1 contributes to the maintenance of mitochondrial integrity. In Bmi-1-deficient mice there is accumulation of mitochondrial ROS, engagement of the DNA damage response (DDR) (88) and loss of viability (89). As noted previously, activation of p38MAPK in FoxO3-deficient mice is associated with loss of quiescence (73). In addition, activation of p38MAPK has been recently shown to induce KLS apoptosis and senescence in mice (90), but the mechanism leading to apoptosis has not been investigated. Interestingly, in memory T cells the apoptosis due to deprivation of the cytokine IL-7, which is essential for their survival, is mediated by the activation of p38MAPK, responsible for phosphorylating a protein inducing intracellular alkalinisation (91).

2.5 Maintenance of stem cell identity: the mysterious undifferentiation of bone marrow HSC and the role of the niche.

The Notch pathway is essential to maintain neural cell progenitors and to inhibit their differentiation. It also plays an essential role in T cell development (reviewed in 92). As previously mentioned Notch is required for the generation of HSC in the embryo whereas at later stages it determines lineage outcomes, including specification of lymphopoiesis (12, 93). There are studies supporting the hypothesis that Notch may promote proliferation of bone marrow HSC maintaining an undifferentiated phenotype. Importantly, however, in different types of Notch-signalling-deficient-mice, the number of bone marrow HSC is not decreased, indicating that this pathway is dispensable for HSC self-renewal (43). In mammals there are four Notch receptors (Notch1-4) and five structurally similar ligands: Delta-like 1, 2 and 3 and Jagged 1 and 2, that are single pass transmembrane proteins, thus, Notch signalling derives from cell-cell interaction (93). Osteoblasts in the stem cell niche and hematopoietic cells express both receptors and ligands, thus osteoblasts can signal osteoblasts and HSC, and HSC can signal HSC and osteoblasts (12).

Ligand binding induces cleavage in the extracellular domain and then in the transmembrane domain. The second cleavage is performed by the enzyme γ -secretase, which has two catalytic subunits, Presenilins 1 and 2. Following the second cleavage Notch intracellular domain (NICD) translocates into the nucleus, where in association with the transcriptional coactivator Mastermind like-1 (MAML-1) they convert the transcriptional repressor CBF-1 (also called RBPJ or CSL) in a transcriptional activator (93, 43). The target genes include the basic helix-loop helix transcription factors HES and their homologues HERP that, in general, function as transcriptional repressors and inhibit cell differentiation, although they can participate in other functions (42, 43). Other target genes, such as GATA3, CD25 and pre-Ta are involved in driving T cell development (42). The endocytosis of the ligands in

endosomes by the signal-sending cell (before they are presented on the cell membrane) is necessary to activate signalling in the receiving cell (12, 42). This process is regulated by ubiquitination, and Mib-1 ubiquitin ligase is one of the key enzymes that regulate this process: Mib-1-deficient mice exhibit pan-Notch defects in development and organogenesis (42).

Early in vitro studies showed that constitutive activation of Notch resulted in the inhibition of granulocytic differentiation in a myeloid cell line (94). Activating Notch mutations are frequently found in human acute T cell lymphoblastic leukemia (42), and transplantation of bone marrow stem cells transduced with activated Notch induce development of T cell leukaemia in mice (95). Thus, to perform in vivo studies, KLS from RAG-1-deficient mice, which lack mature T and B cells, were transduced with activated Notch and transplanted into lethally irradiated C57BL/6 mice. In these mice the number of KLS was higher than that of mice transplanted with KLS carrying a control vector, while the number of differentiated granulocytic and erythroid cells was lower (96). KLS from the two groups of mice showed a similar cell cycle kinetics indicating an anti-differentiative rather than a proliferative effect (96). In addition, inhibition of CBF-1-dependent Notch pathways, obtained introducing in KLS a retroviral expression vector encoding for a CBF-1 dominant negative, resulted in a marked decrease of KLS and an increase of Lin⁺ cells (97).

A role of the stem cell niche in Notch signalling was proposed. In mice expressing a constitutively active parathyroid hormone receptor in the osteoblastic lineage, osteoblasts upregulated Jagged-1. An increase in the number of both osteoblasts and HSC was observed, and HSC showed an elevated NICD level (98). However parathyroid hormone stimulates osteoblasts to produce multiple growth factors, not just Jagged-1, that are able to influence HSC proliferation. The results subsequently obtained in Mib-1-deficient mice, however, have supported the concept. These mice, unable to transmit any Notch signal, develop a myeloproliferative disease (MPD) that eventually kills them. Surprisingly, MPD is not due to the absence of Notch signalling in KLS, because wild type KLS transplanted in these mice also undergo MPD despite being able to transmit and receive Notch signals. Thus, Notch-deficient niche cells support an abnormal HSC proliferation in the absence of a mutation in HSC, and a possible explanation is that niche cells are missing a Notch ligand (99).

Other studies have recently shown that Notch 2 is important for the generation of KLS at the expense of myeloid differentiation (100), and it has been demonstrated that KLS expanded by endothelial cells expressing multiple Notch ligands are able to reconstitute lethally irradiated mice (101). Thus, Notch signalling may promote bone marrow HSC proliferation and the maintenance of an undifferentiated phenotype. Nevertheless, the evidence that at least several components are dispensable for HSC self-renewal indicates that the physiological mechanisms keeping bone marrow HSC undifferentiated may be redundant or yet to be discovered.

We would like to conclude this part of the review emphasizing that all of the mentioned pathways may act in cooperation. The Wnt/ β -catenin and Pten/PI3K/AKT pathways have been recently shown to cooperate to drive long-term HSC expansion inducing proliferation, inhibiting apoptosis and blocking differentiation (51). In this case cooperation

simultaneously influences more than one function to promote self-renewal. In Table 1 we have summarized the molecules involved in the different functions contributing to HSC self-renewal with the related references.

3 Part II

3.1 T memory cells and T memory stem cells

The identification of markers distinguishing naïve from memory T cells is still an area of active research. Most markers are common to both mice and humans, but there are some differences between the species, and among CD4 and CD8 T cell subsets, likely attributable to their diversity in binding and functions.

The encounter of murine T cells with antigens results in a permanent expression of the adhesion molecule CD44 which persists when the T cells are transferred into antigen-free recipient mice (102). In mice a high CD44 expression is considered an accurate marker of memory, especially when used in conjunction with other markers. In humans naïve T cells express the high molecular weight isoform of the tyrosine phosphatase CD45 (CD45RA) while memory cells express the low molecular weight isoform (CD45RO), CD45RA however, can be re-expressed both by CD4 and by CD8 positive cells (102).

In humans memory T cells have been divided in two subsets: central (T_{CM}) and effector (T_{EM}) cells, based on the expression of two receptors: the lymphoid homing receptor L selectin, or CD62L, engaging vascular addressins, and CCR7, the receptor for the chemokine CCL21 (103). $CD45RO^+ CCR7^+ CD62L^+$ are defined as T_{CM} . This phenotypic distinction apply to $CD8^+$ cells, since only about 30% of $CD4^+$ memory cells is $CD62L^+$ following polyclonal stimulation (104), and bone marrow $CD4^+$ memory cells are $CD62L^+$ (105). T_{CM} home in secondary lymphoid organs and have little effector function (102, 103), but convert to effector cells after restimulation (106). T_{EM} are found in inflamed peripheral tissue, they are $CCR7^- CD62^-$ and part of them expresses CD45RA (103). Their lifespan is shorter than that of T_{CM} (107), and their number gradually decreases over time (108).

T_{EM} produce $IFN-\gamma$, TNF and IL-2, and contain a high amount of perforin, thus, they can exert an immediate cytolytic function (102). T_{CM} produce $IFN-\gamma$, TNF and IL-2, but the amount of IL-2 is higher than that produced by T_{EM} and is considered responsible for their high proliferative capability (102). Among human virus specific $CD8^+$ cells T_{CM} are perforin negative, while about one third of the T_{EM} are positive (108). Since naïve T cells are $CCR7^+ CD62L^+$, the absence of CD45RO expression is used to distinguish them from T_{CM} (102).

Different groups have shown in mice that the surface “killer cell lectin-like receptor G1” (KLRG-1), a receptor with inhibitory function expressed by natural killer (NK) cells, marks shortlived $CD8^+$ effector T cells (109-111). KLRG-1 expression is inversely correlated with that of the receptor for the potent pro-survival cytokine interleukin-7 (IL-7R). $KLRG-1^{high} IL-7R^{low}$ cells are short-lived effector cells, whereas $KLRG-1^{low} IL-7R^{high}$ cells, displaying long-term survival after the completion of the primary immune response, have been designated as memory precursor cells (109). These markers, thus, allow the isolation of

CD8⁺ memory cells on a functional basis. In virus specific murine CD4⁺ T cells, however, a low expression of IL-7R by effector cells is transient, and not indicative of cells with short life span (112).

Murine and human memory T cells express the signalling lymphocyte activating molecule SLAMF6 or CD150 (24). In mice deficient in the adaptor SAP, which mediates part of SLAMF6 family member signalling, CD4⁺ T cells exhibit impaired adhesion to antigen presenting B cells and are not properly recruited in the germinal center (113).

The bone marrow is the niche of CD8⁺ (114) and CD4⁺ memory T cells (105). Memory T cells co-localize with stromal cells expressing IL-7 (105, 115), suggesting that after their generation in secondary lymphoid organs they migrate to the bone marrow as the most appropriate location for homeostatic maintenance. CD4⁺ memory cells in bone marrow are CD44⁺ and their homing to bone marrow depends on the surface expression of α 2 integrin and Ly6C (105). Ly6C is also expressed by CD8⁺ memory cells (116).

Within the T_{CM} population, is there a “memory stem cell” subset? Murine memory cells with stem cell properties showing, unexpectedly, a low CD44 expression have been identified within the murine CD8⁺ subset by two different laboratories. These cells, designated “T memory stem cells” (T_{SCM}) are Sca-1/Ly6A⁺ (like HSC) and express high levels of Bcl-2 and CD122, the β subunit common to IL-15 and IL-2 receptors (IL-2/IL-15R β) (117, 118). Recently, the human T_{SCM} counterpart has been characterized (119, 120). Human T_{SCM} coexpress CD45RA and CD45RO (120) and are CCR7, CD62L and IL-7R positive (119, 120). In addition, they express CD95 (or Fas), known to be upregulated by naïve T cells after T cell receptor (TCR) stimulation, and the IL-2/IL-15R β (Fig. 2) (119, 120). Cytokines are crucial to generate human T_{SCM}. IL-7, -15, -2, -4, -9, and -21 belong to the γ common (γ -c) family of cytokines, so called because their receptors share the γ -chain, essential for signalling. IL-15 is required for T_{SCM} expansion while IL-7 is critical to maintain the CD45RA⁺ CD62L⁺ phenotype (120). Thus, interestingly, IL-7 and IL-15 are non redundant, although they are both γ -c cytokines essential for memory T cell survival (102, 121). In association with IL-7, IL-2 can also support the expansion of T_{SCM} but less effectively than IL-15. Murine and human T_{SCM} exhibit self renewal ability and can generate T_{CM} (117-120).

There is also some evidence for stem cell properties within the naïve CD4⁺ T population. A subset with very low CD44 expression has been recently found to be more efficient than CD4⁺ cells with low and intermediate CD44 expression in reconstituting the complete CD4⁺ T cell pool when injected in severe combined immunodeficiency (SCID) mice (122). These cells show enhanced expression of Bcl-2 and IL-7R but behave as naïve cells in terms of proliferation and IL-2 production (122).

3.2 Development of immunological memory

Memory lymphocytes provide important protection against infections. The frequency of memory T cells against specific pathogens is higher than in the naïve population, the response is more rapid and vigorous, and lasts longer (102). The primary immune response of naïve T cells against pathogens develops in 4 phases: priming, clonal expansion,

differentiation of effector and regulatory T cells and contraction (102). Signals present at the time of priming crucially influence both expansion and differentiation. During priming T cells recognize antigen-derived peptides bound to MHC molecules on antigen-presenting cells (APC) by a specific T cell receptor (TCR) (signal 1). Expansion and differentiation result from the integration of signal 1, plus signals provided by costimulatory membrane molecules (signal 2), and by cytokines produced by APC and responding T cells (signal 3). Different cytokines induce specific transcription factors and lead to chromatin remodelling and stable changes in gene expression which are necessary for appropriate differentiation. Effector cells generated by naïve CD4⁺ cells comprise five major T helper subsets: TH1, TH2, TH17, TH follicular and T regulatory cells. Effector cells generated by naïve CD8⁺ cells include cytotoxic and regulatory cells. Helper and cytotoxic cells cooperate to eliminate pathogens, while regulatory cells down regulate the activation of T cells and antigen presenting cells. Following the elimination of an antigen, effector cells undergo apoptotic death during the contraction phase, which restores the physiological cell number, so that 5-10% of the cells will survive as memory subsets (reviewed in 102).

It is debated as to which phase of the primary immune response generates the memory program, and there are several models for memory cell differentiation. The “linear” model proposes that memory cells derive from effector cells, thus, commitment to the memory phenotype would occur during the phase of contraction. (123-126). In contrast, according to the “asymmetric, divergent, or branched” model, memory cells would derive directly from naïve cells, in parallel to the effector cells but along a separate lineage, thus, commitment would occur during priming (127-130). The “progressive” model predicts that the signal strength, (TCR-MHC-peptide affinity, degree of costimulation and cytokine production) during the phase of priming would influence lineage commitment so that naïve cells receiving a strong signal would become effectors while those receiving a weak signal would become memory (131-133). Another model proposes that CD8⁺ memory cells would develop from the subset of cells defined as memory progenitor effector cells, KLRG-1^{low}IL-7R^{high}. These cells, capable of long-term survival, would not undergo contraction (109, 110).

Many results supporting each model have been produced, and, for example, two papers reporting data in favour of the linear (126) and the asymmetric (130) models were published in the same journal issue. The evidence that human T_{SCM} challenged with antigen differentiate in T_{EM} and T_{CM} (120) could support a linear model, alternatively, naïve T cells may simultaneously generate T_{SCM}, T_{EM} and T_{CM} by asymmetric division. It has been shown that both CD8⁺ and CD4⁺ naïve TCR-transgenic T cells, are able to undergo asymmetric division, with a daughter cell displaying an effector profile and the other a memory profile (129). In addition, previous data indicated that a single naïve TCR-transgenic T cell transplanted into normal recipient mice, after TCR stimulation generated heterogeneous subsets of effector and memory cells (128). If we hypothesize that not only naïve cells can undergo asymmetric division all of these models might not be mutually exclusive. In support of this hypothesis, recent data show that CD8⁺ memory cells are capable of asymmetric division, producing two daughters resembling effector and central memory cells, respectively (134).

In the following sections signalling molecules utilized by HSC and involved in the development of memory T cells will be considered.

3.3 The powerful network of transcription factors: Tbet, Eomes, TCF, FoxO1, Ids, Bcl-6, Blimp1, Stat3

Pathways relevant for the development of memory T cells have been identified during different phases of the primary immune response. Interestingly, transcription factors regulating *quiescence* in HSC are involved in the *generation* of memory T cells, perhaps because they operate in a different molecular context.

Tbet and Eomesodermin (Eomes) are members of the T-box family of transcription factors (135). It is well known that Tbet is essential for lineage commitment of CD4⁺ T helper 1 (TH1) cells (102). In CD8⁺ cells, Tbet and Eomes regulate cytolytic effector mechanisms, including the transcription of perforin and granzymes (135), and are responsible for the expression of IL-2/IL-15R β (102). Responsiveness to IL-15 is required for CD8⁺ memory cell proliferation and survival, thus, Tbet and Eomes double deficient mice lack CD8⁺ memory cells (102), and Tbet expression is inversely correlated with the generation of long-lived memory CD8⁺ T cells (109).

Wnt proteins play a role in the generation of CD8⁺ memory T cells in part regulating the balance between Tbet and Eomes transcriptional activity. However, which Wnt pathway is involved is not clear. Naive T cells highly express TCF-1 and Lef-1, the transcription factors activated by Wnt proteins (136). TCF-1 expression is downregulated by antigen stimulation (137, 118), and subsequently upregulated during the transition of effectors in memory cells (136). TCF-1-deficient mice display a decrease of KLRG-1^{low} IL-7R^{high} memory precursor cells (138). Wnt3a induces Eomes expression via TCF (139), since TCF-1-deficient T cells show decreased Eomes expression (138). Eomes mediates some TCF effects since reintroduction of Eomes in TCF-1-deficient cells by a retroviral vector restores the expression of IL-2/IL-15R β and improves cell viability, but does not completely reconstitute the pool of TCM, revealing the presence of other Wnt-TCF-dependent pathways (138). In memory T cells, as in HSC the role of β -catenin is controversial. After 4 days, in T cells primed in the presence of Wnt3 there is no accumulation of β -catenin (140), and β -catenin-deficient mice have no defects in memory T cell generation or function (139). Whether the structurally similar γ -catenin can compensate for the absence of β -catenin or there are other TCF-1 interacting intermediates is unknown. Nevertheless, during T cell priming in vitro, activation of the Wnt pathway using Wnt3a or an inhibitor of β -catenin degradation generates long-lived memory cells with high proliferative capacity and effector functions, and the capability to reconstitute immunodeficient hosts (118, 119). These T_{SCM}, described in the paragraph concerning memory cell markers are CD8⁺ CD44^{low} CD62L^{high} Sca-1⁺ IL-7R⁺ IL-2/IL15R β ⁺ Bcl-2⁺ in mice and CD45RA⁺ CCR7⁺, CD62L⁺ CD95⁺, IL-7R⁺ IL-2/IL15R β ⁺ Bcl-2⁺ in humans (118, 119) (Fig.2). How the Wnt pathway drives the acquisition of self renewal capabilities is unclear; however it has also been proposed to confer stem cell like properties to TH17 cells (141). These TH17 cells, displaying the phenotype of terminally differentiated effector memory cells, (141-143) were expected to be short-lived (142). However, associated with high levels of TCF7 and β -catenin (141), they show in vivo long

survival (141, 144) and increased proliferation (143). These findings are clinically relevant, because TH17 cells can exhibit a potent anti-tumor effect (141, 143) while, on the other hand, they contribute to the development of a variety of autoimmune diseases (141, 143, 144).

The balance between T-bet (favouring short-lived KLGR-1^{high} effector cells) and Eomes (favouring long-lived KLGR-1^{low} memory precursor cells) is also regulated by the transcription factor FoxO1. FoxO1 can directly activate the Eomes promoter (145). As in HSC, in memory T cells FoxO1 activity is counteracted by PI3K and mTOR pathways. Phosphorylation of FoxO1 leads to its nuclear export and degradation and is dependent on AKT and mTORC2 kinase (72). mTORC1 kinase potentiates mTORC2 activity, degrading FoxO1, turning off Eomes and promoting T-bet mediated differentiation in short-lived effector cells (145). This supports previous results showing that inhibition of mTORC1 by rapamycin during the contraction phase accelerates the differentiation into KLGR-1^{low} memory precursor cells (146). In vivo, a sustained AKT activity has been recently found to be associated with a reduction of CD8⁺ antigen specific T_{CM}, associated with phosphorylation of FoxO1 and mTOR kinase, along with elevated levels of T bet and decline of Eomes (147). Importantly, a reduced level of TCF-1 expression is observed in this setting, thus, AKT may be an upstream mediator of both mTORC1 kinase and Wnt proteins (147).

Control of survival by E proteins and their inhibitory Id proteins profoundly affects memory cell generation Id2 promoting T_{EM}, whereas Id3 promotes T_{CM}. In early studies it was found that Id2-deficient mice exhibited normal spleen and CD4/CD8 T cell ratio, but lymph nodes were absent (148). In these mice, following normal priming and proliferation, there was a defect in the formation of the T_{EM} subset, due to an increased rate of apoptosis during the phase of contraction (149). This was associated with increased expression of BimEL and decreased expression of Bcl-2 and Serpinb9 (also termed “Spi6”) (149). It is now clear, from elegant in vivo studies, that Id2 and Id3 control the survival of distinct memory CD8 T cell subsets. CD8⁺ T cells with high expression of Id3 generate KLGR-1^{low} long-lived memory precursor cells, (149) whereas CD8⁺ Id3 deficient cells, although able to proliferate in response to antigenic stimulation, undergo increased contraction and fail to generate them (149, 150). Comparison of cells with high and low expression of Id3 has revealed that the set of genes regulated by Id3 overlaps with genes with known E2A occupancy in T cells, (149).

The comparison of Id3 positive versus negative cells has shown that the genes regulated by Id3 are implicated in DNA replication and repair, and differ from those involved in Id2-mediated control of survival (150). Most importantly, in this study Id3 is identified as one of the targets of the transcription factor Blimp-1 (150). As mentioned in the introduction, the transcriptional repressor Bcl-6 in germinal center B cells prevents the expression of Blimp-1, thus inhibiting plasma cell terminal differentiation (3). Bcl-6 is expressed by activated CD8⁺ T cells (151) and expression of Blimp-1 is inducible by TCR stimulation and cytokines (9). Since Bcl-6 and Blimp-1 mutually repress each other in B and in T cells (9), they were both hypothesized to regulate the generation of memory cells. In keeping with this hypothesis, in Bcl-6-deficient mice the percentage of splenic CD8⁺CD44⁺ IL-2/IL15Rβ⁺ T cells is decreased while it is increased in Bcl-6 transgenic mice (reviewed in 9). Blimp-1 plays the opposing role: murine Blimp-1-deficient CD8⁺ cells primarily develop as KLGR1^{low}

IL-7R^{high} memory precursor cells whereas the number of KLRG1^{high} IL-7R^{low} short-lived effector cells is severely reduced. (152, 153). The results showing that Blimp-1 directly binds the promoter of Id3 in CD8⁺ effector T cells and represses its activity (150) suggest that the phenotype observed in Blimp-1 deficient mice is due the unrestricted activity of Id3.

Cytokines influence the transition from effectors to memory cells regulating the expression of transcription factors and /or protecting cells from apoptosis (109, 149, 150, 154-156), and, as previously discussed, IL-7 and IL-15 are both required to generate human T_{SCM} (120).

IL-10 has been shown to promote HSC self renewal. The first two studies investigating whether IL-10 affected the generation of memory cells showed contrasting results (157, 158). Recent work, however, shows that in mice lacking both IL-10 and IL-21 (but not IL-10 or IL-21 alone) the ability to clear a primary infection is normal, but the formation of KLRG-1^{low} memory precursor cells is severely reduced, (159). Both IL-10 and IL-21 activate Stat-3, and Stat-3-deficient mice display a phenotype similar to that of IL-10/IL-21 double deficient mice. Moreover, Stat-3-deficient CD8⁺ cells have reduced expansion and life span, and reduced Eomes, Bcl-6 and Blimp-1 expression (159). Therefore Stat 3 integrates different extrinsic signals and regulates the activity of several members of a transcription factor network.

3.4 Homeostatic vs antigen-driven proliferation of memory T cells: IL-7, IL-15 and Notch

In addition to the rapid proliferation in response to recall antigens, memory T cells, like naïve T cells, undergo a periodic cell division termed “homeostatic proliferation” (HP). This type of proliferation contributes to maintaining a stable cell number. HP of naïve T cells requires contact of TCR with self peptides/MHC and the presence of IL-7 (102, 107). Naïve cells have an extremely low rate of HP (107, 120). For naïve T cells, T_{EM} and T_{CM} the rates of proliferation are approximately 0.2%, 4.7%, and 1.5% per day, with an intermitotic time of 1 year, 15 and 48 days, respectively (107). HP of CD4⁺ and CD8⁺ memory cells does not require TCR/self peptide /MHC contact, but IL-7 and IL-15 are essential both for proliferation and survival (102, 121).

In lymphopenia, CD8⁺ naïve T cells markedly increase the rate of HP and a significant proportion of them acquire the phenotype of memory cells although they have not recognized a foreign antigen. This process of HP-induced expansion (102, 160) generates cells called “memory-like” (161) or “memory phenotype” cells (160). These HP-induced memory-like cells are effective against infections, although less than antigen-induced memory cells (162). It has been recently found that HP-induced expansion requires the IL-7 dependent activation of the mTOR kinase, which is in turn associated with increased T-bet expression (162). This is followed, as previously mentioned, by sensitization to IL-15 due to increased expression of IL-2/IL-15R β . Interestingly, mTOR kinase silencing blocks T-bet expression but not the generation of memory phenotype T cells since it enhances the expression of Eomes. (162).

TCR-dependent proliferation of memory cells is dependent on Bcl-6. In Bcl-6-deficient mice CD8⁺ memory T cell proliferation in vivo, following antigen stimulation, is lower than in

wild type mice (reviewed in 9). Similar results have been observed studying CD4⁺ memory cells (163). As mentioned in the previous paragraph, Stat-3 may be one of the upstream molecules in this pathway (159).

The PcG protein Bmi-1, essential for HSC proliferation, also promotes proliferation of naïve and memory T cells (164, 165). Bmi-1 deficient mice were known to display an altered T cell development and reduced T cell number (6). It was subsequently demonstrated that stimulation with OVA antigen increased Bmi-1 expression by transgenic OVA specific naïve CD8⁺ cells and that silencing or overexpression of Bmi-1 in T cells caused reduction and increased proliferation, respectively (164). Among CD8⁺ CD44⁺ T cells KLRG1⁻, but not KLRG⁺, cells increased Bmi-1 expression after TCR stimulation. Following a polyclonal stimulus KLRG1⁺ cells proliferated poorly, and this was associated with an increased level of mRNA encoding the cell cycle inhibitors p16 and p19. Since Bmi-1 represses p16 and p19 expression, its low expression in KLRG1⁺ cells was proposed to account for their poor proliferation. (164). In a different setting these results have been recently confirmed (165).

The effect of Notch ligands Jagged 1 and Delta-Like 4 has been studied on effector and memory cells in mice responding to antigens generating Th1 or Th2 responses. Jagged 1 increases proliferation of effector cells in both Th1 and Th2 conditions. However, Delta-like 4 increases Th2 but not Th1 driven proliferation of memory cells and, interestingly, two observations have suggested that Notch proliferative effects are mediated by Bmi-1: 1) the expression of Bmi-1 in naïve T cells stimulated in the presence of Delta-like 4 is higher than in its absence 2) the transcriptional factor RBPJ, acting downstream Notch ligands, binds Bmi-1 promoter and its binding increases when TCR stimulation occurred in the presence of Delta-like 4 (165).

3.5 Pathways regulating quiescence: there is one more reason for T cells to maintain quiescence

T cell “exhaustion” is a progressive functional unresponsiveness which occurs in chronic infections. In severe infections exhausted cells can also undergo physical deletion, mainly by apoptosis (166). Although T cell exhaustion is observed under conditions of antigen persistence it is not yet clear whether it can be considered a loss of quiescence caused by excessive proliferation, as it is for HSC exhaustion. X-linked lymphoproliferative (XLP) syndrome results from mutations in the SLAM associated protein SAP, the adaptor which partly mediates the signalling of SLAM family members (24). In XLP the excessive early proliferation of CD8⁺ cells following EBV infection has been hypothesized to cause a subsequent marked decrease in the replicative capacity (167).

For T lymphocytes there is another reason to be quiescent: prevention of autoimmunity. The abrogation of T cell quiescence is observed in mice harbouring CD4⁺ cells deficient for the transcription factor Runx-1, who develop a fatal lung autoimmune disease (168) and also in patients at high risk for multiple sclerosis (169).

Information about quiescence of T cells is scanty. Recently, the status of chromatin condensation (170), Fox factors and mTORC1 kinase activity, have been shown regulate quiescence of naïve T cells (171). How quiescence is maintained in memory T cells is still

unknown. However, a mechanism underlying their readiness to undergo proliferation upon a secondary challenge has been elucidated. The interaction between CD27, a TNF receptor family member expressed by T cells, and its ligand CD70 on dendritic cells maintains memory T cells in the G1 phase of the cell cycle, so that deprivation of dendritic cells reverts memory T cells from the G1 to the G0 phase (172). As already mentioned, activation of the PI3 kinase-Akt pathway leads to phosphorylation of FoxO1, its release from DNA, and translocation to the cytoplasm and degradation, and this is followed by cell cycle progression (71, 72). T cell stimulation with an antibody recognizing CD27 results in Akt phosphorylation and in phosphorylation and translocation of FoxO1 from the nucleus to the cytoplasm (172). In keeping with these results, in CD27-deficient mice the majority of memory T cells after a recall viral infection are in G0 while in wild type mice they are in G1 (172). This state of “readiness” may enable a fast secondary response to pathogens. We observed that CD44⁺ cells from lymph nodes of mice treated with IL-7 produced high levels of IFN- γ (173). If the cells were sorted as CD3⁺ CD44⁺ cells, however, IFN- γ was not detected, suggesting that CD44⁺CD3⁻ cells were dendritic cells, necessary to sustain IFN- γ production. (Aiello FB, Durum S unpublished results).

It has been recently proposed that after infections undergo resolution the remaining low doses of antigens induce a state of dormancy in CD4⁺ memory T cells. Whether this could be considered as an induction of quiescence has yet to be demonstrated (174).

3.6 Memory T cell protection from apoptosis by IL-7 and IL-15: Bcl-2 family, Spi, IEX-1, Bmi-1

After differentiation memory cells surviving the phase of contraction are protected for long-term survival by IL-7 and IL-15 (102, 121), that are essential for the generation and persistence of human T_{SCM} (120). These γ -c cytokines regulate the mitochondrial pathway of apoptosis regulating the expression and the activity of anti-and pro-apoptotic Bcl-2 family members. Relevant anti-apoptotic members include Bcl-2 itself, Mcl-1 and Bcl-x_L. Pro-apoptotic members include Bim, Bax, Bad, Puma and Noxa.

T cells with Mcl-1 conditional deletion show marked spontaneous and activation-induced apoptosis (175), and Mcl-1 is required for survival of CD4 and CD8 naïve and memory T cells (176). IL-7 upregulates Bcl-2, Mcl-1 and Bcl-x_L expression in memory T cells (176, 177). The upregulation of Bcl-2 is mediated by the transcriptional activity of phosphorylated Stat-5 (121, 178), and constitutive activation of Stat-5 enhances survival and homeostatic proliferation of CD8⁺ effector and memory T cells (179). In addition, it has been recently shown that IL-7R-deficient thymocytes undergo telomere erosion, which leads to DNA damages responses, p53-dependent apoptosis and chromosome abnormalities (180). Perhaps the protection of genomic stability may partly explain the IL-7-mediated preservation of the T_{SCM} phenotype (120). IL-15 upregulates Bcl-2 expression in memory T cells in vivo (181) and is a potent inducer of Mcl-1 expression in T cells (176). In addition, it inhibits the expression of the pro-apoptotic protein Bim (181) which is important for memory cell survival (182).

Suppression of Bim by IL-15 in NK cells was shown to be mediated by the PI3-kinase pathway, which leads to the inactivation of FoxO3 (183). A recent study demonstrates that in

FoxO3-deficient mice, after LCMV infection, virus specific CD8⁺ cells accumulate in the spleen, and the number of both KLRG-1^{high} short-lived effectors and KLGR^{low} long-lived memory precursor cells increases due to reduced apoptosis. FoxO3-deficient memory cells show reduced expression of Bim and do not undergo apoptosis when deprived of IL-15 (184). Thus, as in HSC, specific influences on different Bcl-2 family members might explain why more cytokines, even belonging to the same family, are necessary for survival. Besides Bim, FoxO3 controls the expression of additional genes regulating apoptosis, such as Fas-ligand and TRAIL, and cell cycle, such as p27, Cyclin B and others (185). Noteworthy, in CD4⁺ T_{CM} the level of FoxO3 phosphorylation is more than two fold higher than in TEM, and this correlates well with their better survival and with a higher expression of FoxO3 target genes in TEM. (186).

The intracellular serine protease inhibitors Serpins (Spi), also expressed by HSC, are important for memory cell survival at multiple levels. The expression of Spi2A is inducible by IL-7 and IL-15 (87). Spi2A-deficient mice after LCMV infection show a normal number of T_{CM} immediately after infection, but there is an impairment in the long-term maintenance of memory cells which is worsened by reinfections. This is associated with decreased homeostatic proliferation and survival (87). Importantly, the number of virus specific memory cells can be restored by cathepsin B deficiency, since Spi2A inhibits cathepsin B driven lysosomal permeabilization (87). Spi6 also protects CD8₊ cytotoxic T cell from apoptosis by inactivating Granzyme B (86). Spi6-deficient mice show a normal number of memory cells, however the number of memory cells following a secondary challenge is markedly decreased, suggesting the occurrence of self inflicted apoptosis (187).

The role of the immediate early response gene X 1 (IEX-1), expressed by long-term HSC and memory T lymphocytes (8) is still poorly understood. IEX-1 is inducible by stress and cytokines (TNF, TPO), and in different conditions it can either induce or inhibit survival (188). In murine T cells, activation increases its expression, and its decline correlates with susceptibility to apoptosis (189). IEX-1 transgenic mice accumulate T cells with an altered phenotype in the spleen and in lymph nodes, and develop a lupus-like autoimmune disease (189). IEX-1-deficient mice display a decreased survival of CD4⁺ TH-1 cells, associated, however, with an increased number of TH17 cells and severe arthritis after collagen immunization. (190). High levels of ROS can cause mitochondrial membrane disruption and apoptosis (191), and one of the mechanisms underlying IEX-1 anti-apoptotic activity is the reduction of mitochondrial ROS production (192). In addition, IEX-1 interacts with Mcl-1 (193), and controls its nuclear localization when Mcl-1 participates to the DNA damage response (194). In 293T cells IEX-1 overexpression induces apoptosis, and this requires the presence of Bim (193). It is unclear how IEX-1 acts in memory T cells. It is also unclear whether IEX-1 modulates HSC survival, although CD34⁺ bone marrow cells from patients affected by myelodysplastic syndrome frequently show rearrangements and altered expression of IEX-1 (195, 196).

The Polycomb group protein Bmi-1 is important for survival of CD4⁺ memory T cells (197). In its absence TH1 and TH2 effectors develop normally. However, when Bmi-1-deficient-OVA-specific transgenic cells are adoptively transferred into nu/nu mice there is a dramatic enhancement of apoptosis during the secondary response and a severe decrease in the

number of TH2 memory cells. Bmi-1-deficient TH2 effector and memory cells show increased expression of p16Ink4A and p19ARF mRNA, and of mRNAs encoding for Bax, Puma, Noxa and other pro-apoptotic molecules (197). Thus, in T cells as in HSC, Bmi-1 suppresses the Ink4A locus, and in T cells the suppression of Noxa expression is responsible for protection from apoptosis.

Bmi-1 suppression of Noxa expression in T cells has been linked to histone modifications that regulate the functional activity of chromatin. Methylation of specific lysine residues in histones at regulatory regions such as promoters and enhancers can maintain chromatin domains as constitutive heterochromatin, or control whether genes can be activated or silenced (198). In particular, histone H3 methylation of lysine 9 and 27 is associated with gene silencing whereas methylation of lysine 4, which can be observed in combination with acetylation of lysine 9 and 14, is associated with active chromatin domains (198). The CpG methylation of DNA is a chromatin modification well known to promote gene silencing (198). In TH1 and TH2 effectors the binding of Bmi-1 to the Noxa gene is associated with tri-methylation of histone 3 at lysine 27 (H3-K27) resulting in silencing. In addition, a DNA methyltransferase methylates Noxa DNA at CpG islands, in a Bmi1-dependent manner (198). Thus Noxa is inhibited by both histone and DNA modifications.

3.7 Pathways that maintain the identity of memory T cells: IL-4, Gata-3, Trithorax and Pgc

HSC need to live “forever” remaining undifferentiated. In contrast, memory T cells need to maintain a differentiated phenotype, useful to fight specific pathogens. Therefore, the pathways utilized by HSC and memory T cells to maintain their identity could be reasonably expected to be different. Nevertheless, molecules utilized by HSC in various pathways of self renewal are utilized by T cells to keep their functional identity.

The development of different identities in effector cells (TH1, TH2, TH17 etc.) after the first encounter with an antigen has been thoroughly investigated, but only a few studies have been performed about the maintenance of subset identity in memory cells. These mainly concern TH2 cells. TH2 cells, involved in humoral immunity and allergic reactions, produce IL-4, IL-5 and IL-13. TCR engagement in the continuous presence of IL-4 is required for their differentiation and this is defined as a TH2-skewing condition (102). IL-4 induces the activation of Stat-6, which upregulates the transcription factor GATA3 (102), required for differentiation (199, 200).

As previously mentioned, the hyperacetylation of histones is known to characterize an open chromatin status, with increased accessibility of promoters and enhancers to transcription factors. A week after TCR stimulation in the presence of IL-4 a selective hyperacetylation of histone H3 and H4 at the IL-4 promoter and enhancer is observed (201). This is Stat6 dependent, as it does not occur in Stat6-deficient cells (201). Other studies have subsequently found that GATA3 is required for hyperacetylation of histone H3 (H3K9-14) at the IL-5 promoter and for maintaining unmethylated CpG sites in IL-4 intron 2 DNA (202). In addition, they identify, at previously examined regulatory regions of IL-4 and IL-13 loci, a pattern of histone H3 methylation in lysine 4, which in combination with the acetylation of lysine 9 and 14 is associated with active chromatin domains (202, 203).

Effector and memory cells display similar levels of H3 hyperacetylation and lysine 4 methylation at the regulatory regions of the IL-4, -5 and -13 genes. However in memory cells, TCR engagement by a recall antigen no longer requires IL-4 to maintain the chromatin status to produce IL-4, IL-5 and IL-13, and to express a high level of GATA3 mRNA. (203). Which mechanism accounts for this difference? The answer to this question has been obtained studying the long-term maintenance of GATA3 expression in memory T cells. As mentioned in the first part of the review in HSC PcG and Trithorax group proteins regulate the chromatin status, and exert opposing effects on the transcription of Hox genes (204).

MLL is a member of the Trithorax protein family with histone methyltransferase activity and it specifically methylates lysine 4 in histone H3 (204). Moreover, MLL can form a protein complex containing RNA Polymerase II (Pol II), and specific sites in IL-4 and IL-13 promoters were previously found to bind Pol II (203). GATA3 locus expression is regulated by two promoters, one distal (1a) and one proximal (1b) to the coding exons (205). Onodera et al have demonstrated that in naive T cells a PcG complex containing Bmi-1 is bound upstream to 1b, concomitantly with a pattern of histone H3 lysine 27 methylation (associated with silencing) (206). In contrast, a Trithorax complex, formed by MLL and the protein Menin, is bound downstream of this promoter up to exon 2, concomitantly with H3 methylation in lysine 4 (206). Menin is the product of the tumor suppressor gene *Men1*, and is essential for DNA binding of the complex (207). Following TCR stimulation in the presence of IL-4 the PcG complex dissociates from DNA and this is accompanied by decreased lysine 27 histone H3 methylation whereas the Trithorax complex now binds the whole GATA 3 locus, and this is associated with increased lysine 4 histone H3 methylation (Fig. 3) (207). Remarkably, the same group demonstrates that the Trithorax complex is not necessary for the development of normal effector cells, but is essential for the maintenance of the TH2 phenotype in memory cells. Since MLL-deficient mice are embryonic lethal, they utilize MLL^{+/-} TH2 memory cells, which in contrast to MLL^{+/+} cells, following TCR stimulations in the absence of IL-4 are not able to produce TH2 cytokines, to maintain the chromatin status at IL-4 and IL-13 gene regulatory regions, and to upregulate GATA3 mRNA (208).

More recently, Nakata et al have identified two other components in the Trithorax complex, clarifying another important issue (209). GATA3 is known to activate its own transcription leading to increased IL-4 production. Thus, it exerts an important positive feed-back (210). In developing thymocytes the transcription factor cMyb binds GATA3 (211). Nakata et al have detected cMyb and GATA3 itself within the Trithorax complex and found that they act cooperatively to activate the GATA3 promoter (209). In addition they find Menin to be essential for cMyb-GATA3 binding and cMyb to be important to obtain the expected chromatin modifications at the GATA3 locus (209). These data clarify the mechanism of GATA3 autoactivation. Since lysine 4 methylation by MLL is required for recruitment of Pol II (212) a temporal sequence for the transcriptional initiation may be hypothesized (Fig. 3).

The role of PcG complexes in the maintenance of a memory phenotype is less clear than Trithorax components. *Mel-18*-deficient mice die within 6 weeks after birth (213). Mice with a partial deficiency of *Mel-18* display an impaired IL-4 production by TH2 cells associated with a low degree of demethylation at the IL-4 locus and low GATA3 mRNA

expression (214). It has been recently demonstrated that during the development of TH1 and TH2 effector cells PcG complex proteins (Mel-18, Ezh2, Eed, Ring1A) bind the cytokine signature genes (IL-4 promoter in TH2, IFN- γ promoter in TH1 cells) and enhance their transcription (215). This is also observed in memory TH cells. These data were unexpected, since PcG complexes are considered repressors. Moreover, Mel-18 did not increase IFN- γ transcription through chromatin modification, but rather by facilitating binding of the transcription factors T-bet and NFAT1 to the IFN- γ promoter. An important observation is that all these PcG proteins repress Hoxa7 gene expression in both TH1 and TH2 cells (215). Hox genes play a crucial role in HSC self renewal. Stimulation with PHA upregulates Hox B gene expression in naive human T cells, and treatment with antisense oligonucleotides for Hox B2 or B4 decreases PHA induced proliferation (216). The expression of a number of Hox genes belonging to all 4 groups (A-D) has been assessed in unstimulated and polyclonally stimulated naïve CD4⁺ and CD8⁺ T cells (217). Whether Hox genes play a role in memory T cell self renewal, however, is at presently unknown.

In Table 2 we have summarized the molecules involved in the different functions contributing to memory T cell self renewal and the related references.

4 Conclusions

“Stemness” results from the integration of several functions, *proliferation, quiescence and survival*, thus we have attempted to compare pathways involved in HSC and memory T cell self renewal on a functional basis. *T cell subset identity* is an additional property that distinguishes memory T cells from HSC. Proliferation is controlled by several pathways in HSC and memory T cells, and many are similar, perhaps underscoring the importance of regulating this function at multiple levels.

Quiescence mechanisms in memory T cells are unclear, but the available observations suggest that they might be similar to those of HSC. Survival (anti-apoptotic) mechanisms of memory T cells and HSC are similar in terms of downstream mechanism, although different upstream factors control them. T cell subset identity appears to be maintained in memory cells by reciprocal interactions between transcription factors acting concomitantly with epigenetic modifications. These identity mechanisms are cell type specific, and many questions, including the biochemical distinction between temporary and permanent repressive mechanisms are still open (218). Although there are more publications concerning CD8⁺ than CD4⁺ memory T cells, the mechanisms maintaining cell identity have been mostly studied in CD4⁺ TH2 cells, thus how the identity of the other helper subsets including Treg, Th17 and the newly identified subsets of innate lymphocytes that are important cytokine producers remain to be investigated.

Adoptive T cell therapy based on the transfer of specific anti-viral or anti-tumor T cells into affected patients has shown efficacy and safety, and is increasingly exploited.

Transfer of T_{SCM} or of anti-tumor specific TH17 cells with stem cell properties displaying the highest degree of proliferation and survival may markedly improve the results of adoptive cell therapy in clinical trials.

Thus, a comprehensive understanding of memory T cell self renewal may shape future vaccination protocols and tailored therapeutic strategies for infectious diseases as well as suggesting therapeutic strategies for autoimmunity and immunodeficiency.

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Fig. 1. Markers of murine and human hematopoietic stem cells.

Specific markers permit the purification of long-term hematopoietic stem cells which represent less than 0.5% of bone marrow cells. The presence of CD150 and CD38 on murine cells greatly facilitates their purification.

Fig. 2. Markers of murine and human memory T cells characterized by high self renewal capacity.

This illustration describes cells defined as “T memory stem cells” according to References [118], [119]. These cells are generated by activating the Wnt pathway during the priming phase. They are long-lived, display robust proliferative and effector capacities. Murine cells are able to reconstitute an effective immune response in immunodeficient hosts. They express low levels of CD44 in mice and high levels of CD45RA in humans.

Fig. 3. Maintenance of GATA3 mRNA transcription in TH2 memory cells.

Schematic representation of the transcriptional regulation of the GATA 3 gene in memory TH2 cells according to References 206 and 209. After the development of TH2 cells, the Trithorax complex binds to the whole GATA3 gene, inducing an open chromatin status that maintains high expression of GATA3 mRNA in the absence of IL-4.

Table 1.

Self-renewal regulators for hematopoietic stem cells

FUNCTION	SELF-RENEWAL REGULATOR ^I	REFERENCE
Proliferation	Hox family	10, 29, 30
	Bmi-1	10, 31, 32
	Mel-18 *	35
	MLL	38-40
	Notch family members	12, 41, 93
	Wnt3A	46, 48, 52
	SCF	55, 56
	Stat3, Stat5	55-57
	IL-10	58
Quiescence	MLL	39, 40
	p-21	61, 62
	Gfi-1	61
	E47	61, 64
	Id-1	66-68
	Wnt	52, 69
	AKT*	70
	pten	61, 74
	FoxO3a	73
	p38MAPK*	73
Survival	mTORC1*	75
	SCF	56, 79, 80
	IL-3	79, 82, 83
	IL-6	81
	Flt3-ligand	81, 82
	Bcl-2 family	77-78
	Bim*	83, 84
	Serpins	85
	Bmi-1	89
Maintenance of identity	Notch	94, 96, 97, 98-100

^I Asterisks indicate negative regulation

Table 2.

Self-renewal regulators for memory T lymphocytes

FUNCTION	SELF-RENEWAL REGULATOR ^I	REFERENCE
Proliferation	IL-7, IL-15	102, 120, 121, 160-161
	mTOR	162
	Bcl-6	9, 159, 163
	Stat3	159
	Stat5	179
	Bmi-1	164, 165
Quiescence	SLAM	167
	FoxO1	172
Survival	IL-7, IL-15	121, 176, 180, 181
	Bcl-2 family members	121, 175, 176, 181
	Stat5	121, 178, 179
	Bim*	182-184
	FoxO3	185, 186
	Serpins (Spi2A, Spi6)	86, 87, 187
	Iex-1	189, 190
	Bmi-1	197
	GATA3	202, 203, 206, 209
Maintenance of identity	MLL	203, 204, 208, 204
	Menin	206, 209
	cMyb	209
	Polycomb family	213-215

^I Asterisks indicate negative regulation.