Chapter 2
Generation and Regeneration of T Cells

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Abstract T cells contain a variety of lineages, each of which is formed by passing through a number of strictly regulated steps. In this article we aim to clarify essential factors and critical developmental steps during T cell development. First, we will describe the process of T cell development that occurs *in vivo*. Then, by presenting recent approaches in synthetic biology, we will show that, in the murine case, a feeder-free culture system using a combination of cytokines and Notch ligand is sufficient to support T cell development starting from multipotent hematopoietic progenitors to the TCR-expressing $CD4^+CD8^+$ stage. Finally, in the human case, we will show that mature functional $CD8^+$ killer T cells can be produced *in vitro* starting as early as from the ES/iPS cell stage using feeder cells. These studies may help clarify "minimal requirements" for T cell development.

Abbreviations

HSC	hematopoietic stem cell
TCR	T cell receptor
MLP	myelo-lymphoid progenitor
LMPP	lymphoid-primed multipotent progenitor
DLL	delta-like ligand

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TEC	thymic epithelial cell
iLS cells	induced leukocyte stem cell
iPSC	induced pluripotent stem cell
CTL	cytotoxic T lymphocyte
TIL	tumor-infiltrating lymphocyte

Introduction

During early development, multipotent hematopoietic stem cells (HSCs) are stepwisely restricted to eventually become unipotent T cell progenitors. T cell progenitors then rearrange their T cell receptor genes and are subsequently strictly selected in the thymus based on T cell receptor (TCR) quality before maturation. In the physiological situation, developing progenitors sequentially encounter different environments, whereby they receive appropriate developmental cues *in situ*. It seems almost impossible to faithfully recapitulate the complexity of events *in vitro*. However, not all environmental factors are necessarily indispensable, and there is a possibility that T cells can be differentiated in a more simplified fashion. This idea can be straightforwardly tested in *in vitro* culture systems, and by doing so, factors that are essential for T cell development should be revealed.

Here, we first explain the process of lineage restriction from HSCs to T cell progenitors, based mainly on our previous work. Then, we focus on the steps that occur during early intrathymic T cell development. Finally, we discuss to what extent it is possible to recapitulate T cell development *in vitro* for both mouse and human cells.

Developmental Pathway from HSCs to T Cell Progenitors

Proposal of Myeloid Based Model

Most hematology and immunology textbooks describe that the first branch point from HSCs produces two progenitors, namely one for myelo-erythroid cells and another one for lymphoid cells including T and B cells (Fig. 2.1a). This model is based on the concept that the blood cell family can be subdivided into two major lineages, a myelo-erythroid lineage and a lymphoid lineage. In 2001, we proposed an alternative model in which myeloid potential is retained at an early stage of branching toward erythroid, T and B cell lineages (Fig. 2.1b) (Katsura and Kawamoto 2001), which we later named myeloid-based model in 2006 (Kawamoto 2006).

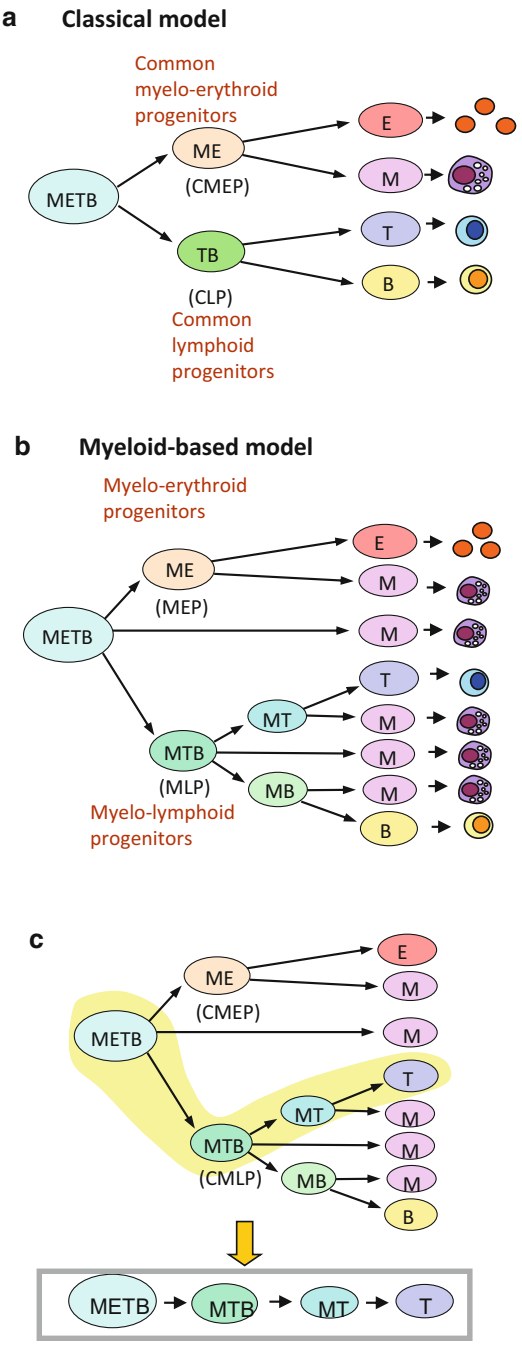

Fig. 2.1 Representative models of hematopoiesis (**a**) The classical model. This model proposes that the HSC first diverges into a CMEP and a CLP. It should be noted that CMEPs are sometimes referred to as common myeloid progenitors (CMPs). E, M, T, and B indicate the progenitor potential for erythroid, myeloid, T, and B cells, respectively. *CMEP* common myelo-erythorid progenitor, *CLP* common lymphocyte progenitor (**b**) Myeloid-based model. In this model, the first branch point generates MEPs and MLPs and the myeloid potential persists in the T and B cell branches even after these lineages have diverged. *MEP* myelo-erythorid progenitor; *MLP* myelo-lymphoid progenitor (**c**) Stepwise lineage restriction from multipotent progenitor toward T cell progenitor

Myeloid Based Model as a Map of Developmental Potentials

Cell differentiation models have two different aspects: they serve as a map of developmental potential and as a cell fate map. In other words, these two are expressed as "a map for lineage restriction" and "a map for physiological production routes". We argue that a map of developmental potential is first and foremost essential for the study of molecular mechanisms of lineage commitment.

In this article, we focus specifically on the route from HSCs to T cell progenitors (Fig. 2.1c). In the myeloid-based model, multipotent progenitors (M-E-T-B progenitors) are initially restricted to become myelo-lymphoid progenitors (MLPs) (or M-T-B progenitors). Subsequently, MLPs terminate B cell potential to form myeloid-T progenitors (M-T progenitors), and finally myeloid-T progenitors shut off myeloid potential to become T cell progenitors.

Myelo-Lymphoid Progenitor Stage (M-T-B Progenitor Status)

MLPs were first identified at a single cell level in fetal liver by our group in 2002 (Lu et al. 2002), and in bone marrow by Adolfsson et al. (2005). These cells are also called LMPPs (lymphoid-primed multipotent progenitors). The existence of this stage in murine and human hematopoiesis has been confirmed by many groups (Yoshida et al. 2006; Pronk et al. 2007; Doulatov et al. 2010). Further study showed that MLPs retain the potential to produce neutrophils, while myelo-erythroid progenitors are generating eosinophils and basophils (Gorgens et al. 2013). Although the contribution of MLPs to myeloid cell production *in vivo* is still a matter of debate, the presence of a commitment status having M-T-B potential seems indisputable.

Prethymic Pathway of T Cell Development

T cells are produced in thymus from progenitors that immigrate from hematopoietic organs, specifically fetal liver during the fetal period and bone marrow after birth. The nature of thymus-seeding progenitors has long been disputed. Regarding the earliest thymic immigrants, we have shown that they are biased toward the T cell lineage. In 1997, we developed a clonal assay system able to detect T, B and myeloid lineage potential of individual progenitors (Kawamoto et al. 1997). By using this assay, a progenitor that generated only T cells was determined as "p-T (progenitor-T)". Such p-Ts were subsequently detected in the aorta-gonad-mesonephros (AGM) region (Ohmura et al. 1999), and fetal liver (Kawamoto et al. 1997, 1998). However, p-Ts determined by a "retrospective" method do not necessarily represent T cell lineage restricted progenitors, since the tested progenitors do not

always express their full potential in culture. Nevertheless, later we provided convincing evidence for the presence of T cell progenitors at the prethymic stage by showing that p-Ts can be highly enriched in the $Lin^{-}c\text{-}kit^{+}IL\text{-}7R^{+}$ population of fetal liver (Kawamoto et al. 2000) and fetal blood (Ikawa et al. 2004a). We and another group also showed that progenitors that are closely juxtaposed to the thymic epithelial primordium were T cell lineage restricted (Masuda et al. 2005a; Harman et al. 2005), validating that the earliest thymic immigrants are T cell lineage restricted. We further disclosed that these prethymic T cell progenitors specifically express paired immunoglobulin-like receptors (PIR) (Fig. 2.2) (Masuda et al. 2005b). The expression of PIR is immediately down-regulated upon entrance to the fetal thymus, substantiating the prethymic stage as a distinct stage. While we had referred to these prethymic progenitors as "T cell lineage restricted" progenitors, in our later studies we came to know that such prethymic progenitors retain significant myeloid potential and also residual B cell potential (Kawamoto 2006). We may thus call them "T-biased MLPs" at present.

Early studies have demonstrated that thymic migration takes place in two waves during fetal development (Jotereau et al. 1987; Dunon et al. 1999). Recently, it has been shown that the second wave-progenitors colonizing thymus just before birth retain robust B cell potential in addition to myeloid potential (Luc et al. 2012; Ramond et al. 2014). It is therefore probable that the second wave-progenitors are close to MLPs. On the other hand, characteristics of thymus-seeding progenitors in adult thymus still remain unclear.

Myeloid-T Progenitors

We then investigated whether myeloid or B cell potential is retained longer during the process of T cell development in adult thymus. This question was resolved by analyzing whether or not early thymic T cell progenitors retain myeloid potential,

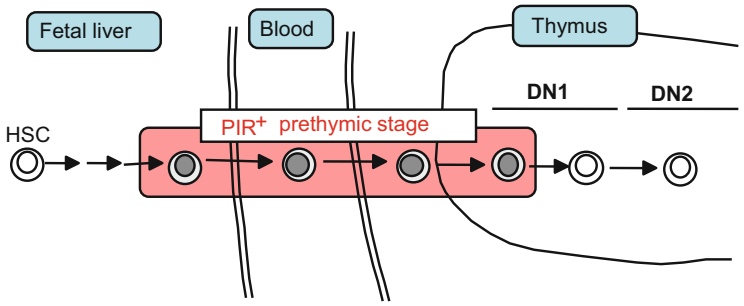

Fig. 2.2 Prethymic pathway of T cell development can be defined by the expression of PIR $Lin^{-}c\text{-}kit^{+}IL\text{-}7R^{+}PIR^{+}$ cells are present in fetal liver, fetal blood and fetal thymus at an early stage during the fetal period. These cells represent myeloid-T progenitors, while they retain residual B cell potential. *HSC* hematopoietic stem cell, *DN* double negative

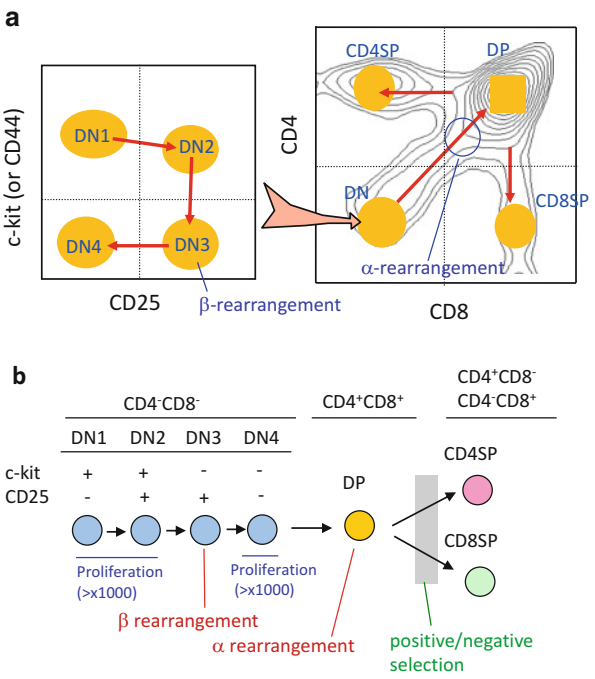

Fig. 2.3 Intrathymic T cell development
(**a**) The sequence of intrathymic developmental stages, which are defined by flow cytometric profiles, is shown
(**b**) Schematic illustration of intrathymic developmental stages

because earlier we and other groups had demonstrated that T cell progenitors in the earliest population in adult thymus have mostly lost B cell potential (Porritt et al. 2004; Balciunaite et al. 2005; Lu et al. 2005).

Figure 2.3a, b explain the definition of developmental stages of murine thymocytes. The early $CD4^{-}CD8^{-}$ (DN: double negative) stage of thymocytes can be further subdivided into DN1, DN2, DN3, and DN4 stages, based on c-kit vs CD25 expression profiles, with the DN1 stage representing the earliest stage. DN cells then differentiate into the $CD4^{+}CD8^{+}$ (DP: double positive) stage, where thymocytes come to express αβTCR. DP cells undergo positive and negative selection to become mature $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ (SP: single positive) cells.

By analyzing individual DN1 cells in a culture system which can support generation of both T and myeloid cells, we found that a substantial proportion of thymic T cell progenitors produced macrophages in addition to T cells (Wada et al. 2008) (Fig. 2.4a, b). A similar finding was reported by Bell and Bhandoola (2008). These results demonstrated that T cell progenitors retain myeloid potential after terminating B cell potential (Fig. 2.4c). Therefore, it became clear that a commitment status producing myeloid cells and T cells, but not B cells, exists on the developmental pathway from HSCs to T cell progenitors, strongly arguing against the classical model of hematopoiesis.

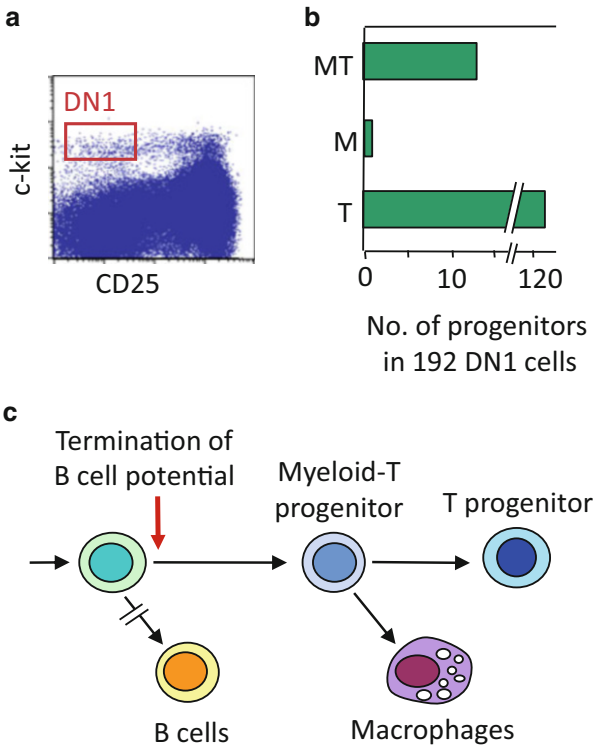

Fig. 2.4 T cell progenitors in adult thymus that have lost B cell potential retain macrophage potential (**a**) c-kit vs CD25 profile of the $CD3^{-}CD4^{-}CD8^{-}$ fraction of adult thymus cells from 8-week-old mice (**b**) A total of 192 individual DN1 cells were cultured with TSt-4/DLL1 that can support generation of T cells and myeloid cells. Seeded cells were retrospectively classified according to the cells generated in each clonal culture: progenitors that generated both macrophages and T (MT), only macrophages (M) and only T cells (T) (**c**) Early T cell progenitors in the adult thymus that have lost B cell potential still retain a significant ability to generate macrophages

Myeloid Potential of T Cell Progenitors is Terminated in the Midst of the DN2 Stage

We also examined at which step the myeloid potential of M-T progenitors is terminated to become fully T cell lineage restricted. We showed that in plckGFP Tg mice, where GFP expression is driven by the proximal *lck* promoter, DN2 cells can be subdivided into GFP^{-} and GFP^{+} cells (Masuda et al. 2007) (Fig. 2.5a). We found that GFP^{-}DN2 cells retain the potential to produce non-T lineage cells, including macrophages, dendritic cells, and natural killer cells, while GFP^{+}DN2 cells failed to produce such non-T cells (Wada et al. 2008; Masuda et al. 2007). We recently designated these two stages DN2mt (myeloid-T) and DN2t (T-lineage determined) (Fig. 2.5b) and termed the step between these stages the DN2-determination step (Kawamoto et al. 2010). Rothenberg et al. also paid attention to this step and proposed to subdivide the DN2 stage into DN2a and DN2b stages based on c-kit expression levels (DN2a: $c\text{-}kit^{high}CD25^{high}$, DN2b: $c\text{-}kit^{mid}CD25^{high}$) (Rothenberg et al. 2008). We presumed that the DN2-determination step should be a critical developmental checkpoint for the production of the T cell lineage.

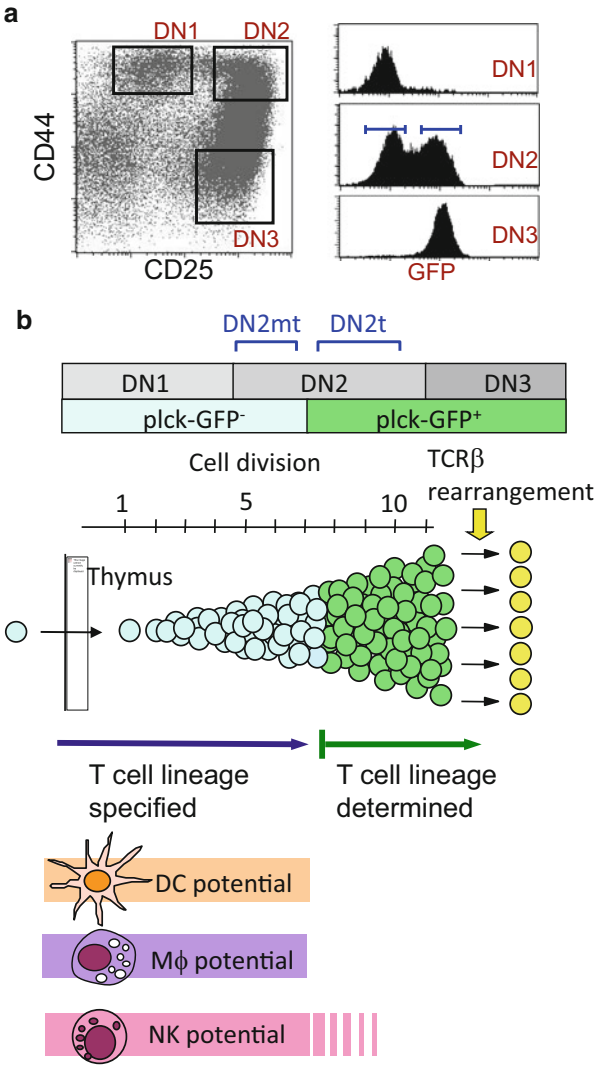

Fig. 2.5 Myeloid potential is terminated in the midst of the DN2 stage
(**a**) CD44 vs CD25 profile of the Lin$^-$ fraction of thymocytes from 15 dpc (days post coitum) fetuses of plckGFP Tg mice is shown in the left panel. The right panels show GFP expression by cells in the gates denoted in the left panel
(**b**) Schematic illustration of the early differentiation/proliferation of thymocytes. A single early thymic progenitor undergoes more than 10 cell divisions during the DN1 and DN2 stages to generate more than 1000 DN3 cells. The shut-off of non-T lineage potential, including that for dendritic cells (DC), macrophages (Mϕ), and natural killer (NK) cells, occurs during the transition from the GFP$^-$DN2 stage to GFP$^+$DN2 stage, and subsequently the T cell lineage determined progenitors undergo several cell divisions before they enter the DN3 stage to initiate TCRβ gene rearrangement. We designate GFP$^-$DN2 and GFP$^+$DN2 stages as DN2mt (myeloid-T) and DN2t (T-lineage determined) stages, respectively

Identification of a Developmental Checkpoint for Production of the T Cell Lineage

A reliable way to substantiate that a presumed step is critical for the development of a certain cell lineage is to demonstrate that developmental arrest occurs at the stage prior to that step under defined conditions. If the arrested cells begin self-renewal, then the existence of a critical step becomes clear.

To visualize such critical step, we used a feeder-free culture system that had been modified from the one originally developed by Bernstein's group (Varnum-Finney et al. 2003), in which murine hematopoietic progenitors were cultured on immobilized Notch ligand DLL4 molecules in the presence of a cocktail of cytokines including IL-7 (Fig. 2.6a). We found that progenitors developing toward T cells were arrested at the DN2mt stage (Fig. 2.6b), and that the arrested cells entered a self-renewal cycle maintaining non-T lineage potentials (Ikawa et al. 2010). A similar arrest and self-renewal of progenitors was observed in thymocytes of mice deficient in the transcription factor Bcl11b (Fig. 2.6c), indicating that Bcl11b plays a critical role in the DN2-determination step. The importance of Bcl11b in the T cell lineage determination step was also demonstrated by other groups (Li et al. 2010a, b). Fig. 2.6d summarizes the T cell lineage determination step that occurs at the DN2mt-DN2t transition step, which is Bcl11b-dependent.

Previously, it has been reported that Gata3 is absolutely indispensable for the production of T cells, and a developmental block was thought to take place at the prethymic stage (Ting et al. 1996). However, to date, it remains unclear at which stage the developmental arrest occurs. Tcf-1, together with Lef-1 that exhibits functional redundancy with Tcf-1, has also been known as a critical regulator of T cell development (Okamura et al. 1998). Recent studies showed that Tcf-1 starts to play a role at the DN1 stage (Weber et al. 2011); however, it may not be critical for T cell lineage determination, since Tcf1$^{-/-}$ Lef-1$^{-/-}$ thymocytes were shown to develop into the DN4 stage (Yu et al. 2012).

Environmental Factors That Support T Cell Development

Factors Required for Thymic Colonization

For the earliest thymic immigrants, three chemokines, namely CCL21, CCL25, and CXCL12, are essential to colonizing the not yet vascularized thymic anlage (Liu et al. 2006; Calderon and Boehm 2011). Interestingly, CCL21 is expressed in the parathyroid anlage (Liu et al. 2006), which is not separated from the thymic anlage at this stage (12 days post coitum). This finding is in line with previous observations showing that colonizing progenitors are attracted close to the fetal thymic anlage of nude mouse, in which parathyroid anlage is normally formed (Itoi et al. 2001). These three molecules may be also important for thymic immigration via blood

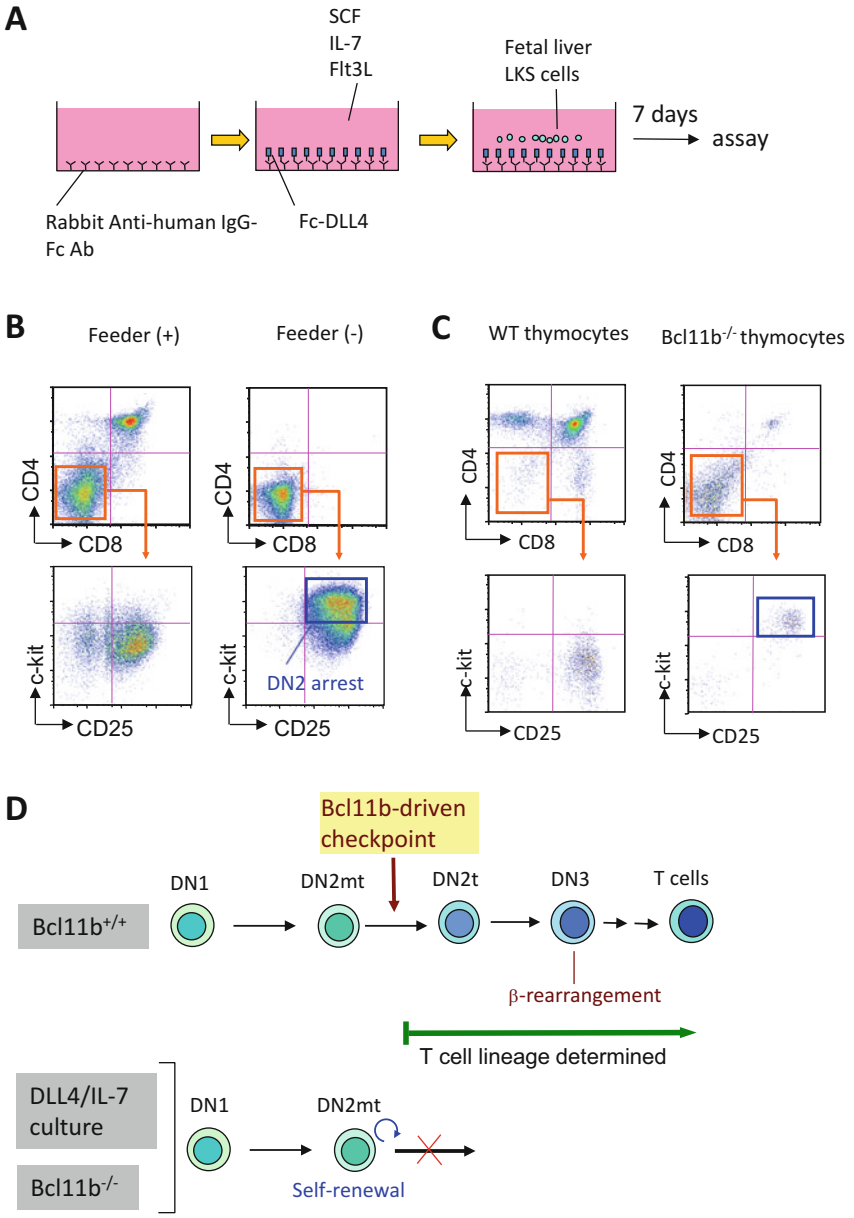

Fig. 2.6 The step for the termination of myeloid potential serves as a critical developmental checkpoint in T cell development

(**a**) Schematic illustration of the feeder-free culture system. A 96-well plate was coated with rabbit anti-human IgG-Fc antibody. Then, Fc-DLL4 fusion proteins were added and immobilized to the dish. $Lin^{-}ckit^{+}Sca1^{+}$ (LKS) cells (200 cells) from murine fetal liver were cultured with immobilized Fc-DLL4 in the presence of SCF, IL-7 and Flt3L

(**b**) In the feeder-free condition, cells are developmentally arrested at the DN2 stage. Left lanes show the cells generated using feeder cells. LKS cells from murine fetal liver mice were cultured

vessels, since thymocyte numbers are drastically reduced in fetuses deficient in CCR7, CCR9 and CXCR4 (receptors for CCL21, CCL25, and CXCL12, respectively) even several days after vascularization (Calderon and Boehm 2011). It was also demonstrated that P-selectin and its ligand PSGL-1 are important for thymic colonization in adult thymus (Rossi et al. 2005).

Notch Signal is Essential for T Cell Development

Early T cell development has been shown to be dependent on IL-7 and stem cell factor (Peschon et al. 1994; von Freeden-Jeffry et al. 1995; Rodewald et al. 1997). However, expression of these factors is not specific for thymus. Thus, the exclusive production of T cells in thymus has long been enigmatic, and researchers have been seeking for key environmental cues that induce exclusive T cell production in thymus.

In 1999, Notch was revealed to be such a key molecule. Conditional knock out of Notch1 resulted in a complete loss of T cells in thymus (Radtke et al. 1999), while enforced expression of a constitutively active form of Notch1 in hematopoietic progenitors brought about production of T lineage cells in bone marrow (Pui et al. 1999). Later, it was shown that a major ligand for Notch1 in thymus is Delta like 4 (DLL4) (Hozumi et al. 2008; Koch et al. 2008).

Expression of Unique Peptides in Cortical Thymic Epithelial Cells (cTECs) is Required for Positive Selection

DP cells have to pass positive selection in order to differentiate into SP cells. Positive selection is thought to select T cells that can recognize the complex of MHC and exogenous antigen, whereas negative selection is thought to deplete harmful autoreactive T cells. As mechanisms for these selections, it has been believed that T cells receiving a moderate signal by the MHC-self-peptide complex are positively selected, while those receiving a strong signal by the MHC-self-

Fig. 2.6 (continued) with TSt-4/DLL4 cells for 7 days. Right lanes show cells generated on day 7 in the feeder-free condition indicated in (**a**)
(**c**) Bcl11b is essential for T cell lineage determination. Fetal liver cells from $Bcl11b^{+/+}$ or $Bcl11b^{-/-}$ mice (Ly5.2) were transferred into lethally irradiated mice (Ly5.1). Flow cytometric profiles of reconstituted thymocytes of recipient mice 8 weeks after transfer are shown. In the lower panels, c-kit vs CD25 profiles of cells gated on the $CD3^{-}CD4^{-}CD8^{-}$ fraction are shown
(**d**) A scheme of T cell lineage determination. Non-T lineage potential of T cell progenitors is terminated during the DN2 stage, subdividing this stage into DN2mt and DN2t stages (see also Fig. 2.5). Bcl11b is required for the DN2mt uncommitted cells to pass through this checkpoint

peptide complex are deleted. Such difference in TCR signal strength has been thought to result from a differential reactivity of TCR that is formed by random gene recombination.

However, recent findings seem to require revision of the above concept. "Cathepsine L" is a lysosomal cysteine protease specifically expressed in cortical thymic epithelial cells and thus is thought to play a role in the presentation of cTEC-specific peptide on MHC-class II. In 2002, it was shown that CD4SP cells are reduced in cathepsine L deficient mice (Honey et al. 2002). Later, another type of cTEC-specific protease, TSSP (thymus-specific serine protease), has also been demonstrated to be involved in processing peptide for positive selection of CD4 T cells (Viret et al. 2011).

A similar finding was reported for CD8 T cells. In 2007, a cTEC-specific proteasome component, β5t, was found, and its deletion was shown to result in marked reduction of CD8 T cells (Murata et al. 2007). A very recent study has shown that OTI-TCR expressing T cells generated in β5t-deficient thymus were functionally inferior to those generated in wild-type thymus, indicating that the positive selection process not only allows survival of T cells but also determines functional maturation of T cells (Takada et al. 2015).

These findings indicate that cTECs have to express unique peptides for positive selection of thymocytes. It is thus difficult to explain above findings in the light of a classical selection model that presumes that the difference in TCR-MHC signal strength is exclusively attributable to TCR diversity. Regarding the role of such unique peptides proper to cTECs, two models have been proposed. In model (i), cTEC-specific peptides preferentially generate moderate signals to TCR. In model (ii), cTECs merely induce positive selection for reactive T cells, while medullary TECs (mTECs) and medullary dendritic cells induce negative selection for reactive cells. In model (ii), a certain number of cells survive because cTECs express a set of peptides different from that of mTECs. Using the latter model, the phenotype of cathepsine L- or β5t-deficient mice can be explained by the assumption that when both cTECs and mTECs express the same repertoire of peptides, only few T cells can survive. In contrast, recently reported findings that peptides processed by the β5t-containing proteasome tend to exhibit low affinity for TCR tend to support the former model (Sasaki et al. 2015). Notwithstanding the possibility of either model, the basic mechanisms regulating positive selection still remain to be elucidated.

Approaches Based on the Concept of Synthetic Biology

In Vivo *Recapitulation of the Phylogeny of Thymus*

In nude mouse, thymus fails to form. Foxn1 has been shown to be the molecule responsible for this athymic phenotype (Nehls et al. 1994). However, even in nude mice, thymic anlage is formed in the correct location (Itoi et al. 2001). Recently,

T. Boehm's group genetically modified Foxn1-deficient mice so that TECs come to express a specified number of molecules (Calderon and Boehm 2012). Among them, two major chemokines, CCL25 and CXCL12 that were shown to work as thymic attractants (Liu et al. 2006; Calderon and Boehm 2011), were selected in addition to SCF and DLL4. IL-7 was not used in this experiment, since Foxn1-deficient TECs express IL-7. None of these four molecules solely reconstituted thymopoiesis, although CCL25 or CXCL12 attracted hematopoietic progenitors and induced myeloid cell generation in thymus. Combination of chemokines and SCF lead to B cell generation in thymus. Addition of DLL4 then recapitulated generation of T lineage cells. Despite the fact that cellularity is much lower in such reconstituted thymus DP cells were formed. Although these findings may provide some insight into the events that occurred during thymus evolution, it seems that the findings themselves were mostly within expectations based on the results obtained in *in vitro* culture systems.

In Vitro *Recapitulation of T Cell Development*

Fetal Thymic Organ Culture

Thymic epithelial cells support T cell development in thymus. Then, is it possible to culture thymic epithelial cells *in vitro* and use them as feeder cells for T cells? It is indeed possible to culture thymic epithelial cells in a monolayer fashion. However, such monolayer-cultured thymic epithelial cells immediately lose their ability to support T cell development (our unpublished findings).

To circumvent the loss of TEC function in monolayer culture, fetal thymic lobes have been used in order to experimentally induce T cell development *in vitro*. In this method, fetal thymic lobes are first incubated with deoxyguanosine (dGuo), a treatment that depletes developing thymocytes but keeps stromal components alive (Jenkinson et al. 1982). Then, dGuo is removed by washing and hematopoietic progenitors are cultured together with the lobe. This method is very powerful because T cell development is fully supported (Ueno et al. 2005).

Co-Culture with Stromal Cells

Mesenchymal stromal cells derived from bone marrow are able to support the development of B cells and myeloid cells (Nishikawa et al. 1988; Nakano et al. 1994), but they are not capable of supporting T cell development. As mentioned earlier, Notch signal was found to be essential for T cell development (Radtke et al. 1999; Pui et al. 1999). Based on this finding, a method to culture T cells *in vitro* was developed in 2002 (Schmitt and Zuniga-Pflucker 2002). In this method, hematopoietic progenitors are cultured with OP-9 cells that were genetically engineered to over-express DLL1. Using this setup, it is possible to induce DP

cells from hematopoietic progenitors. This co-culture system has been extensively used to study T cell development.

Besides its usefulness for the study of murine T cells, this co-culture system can equally well support human T cell development. It was shown that DP cells can be induced from cord blood $CD34^+$ cells (La Motte-Mohs et al. 2005). Some groups have proposed to use this culture system for the expansion of T cell progenitors that are to be transferred to humans in the clinical setting (Awong et al. 2009; Meek et al. 2010). For example, patients who undergo bone marrow transplantation often face the problem that reconstitution of T cells from donor-derived stem/progenitor cells tends to be delayed, and that such delayed T cell production may lead to an immune-compromised status. In this case, it is expected that *in vitro* cultured T cell progenitors may efficiently colonize the thymus and thus accelerate reconstitution of the recipient's immune function.

Developmental Arrest-Restart Controlling Culture System

Here, we show several cases where we used a stromal co-culture system in order to elucidate mechanisms of cell fate decisions during hematopoiesis. We thought that, if we can recapitulate differentiation of progenitors *in vitro* in a "synchronous" manner, time course sampling of these cells would give us a lot of information regarding gene expression and epigenetic profiles.

To this aim, we first tried to develop a culture system in which we can manipulate arrest and restart of progenitor development. Recently, E. Rotherberg's group performed a comprehensive gene expression analysis and epigenetic profiling along with DN1-DN2a-DN2b-DN3-DP stage analysis and revealed counteraction of Gata3 vs PU.1 during lineage specification in terms of T vs myeloid lineage program (Zhang et al. 2012). We then decided to focus in more detail on the DN1 stage, where MLPs colonizing the thymus immediately terminate B cell potential and initiate the program toward the DN2 stage upon encountering thymic environments. We then paid attention to our previous finding that hematopoietic progenitors deficient in E2A, one of the E-protein family transcription factors essential for B cell development, give rise to self-renewing MLPs when they are cultured under B cell inducing conditions (Ikawa et al. 2004b). Progenitors deficient in PAX5 and EBF1 have been shown to form self-renewing MLPs as well (Nutt et al. 1999; Pongubala et al. 2008).

Based on these findings, we came to think that the simple blockade of differentiation should be sufficient to generate stem cells, i.e., to endow MLPs with self-renewing capability. In line with this idea, we have recently shown that overexpression of Id3, which dominant-negatively inhibits E protein activity, in murine multipotent hematopoietic progenitors leads to the generation of stem cells with T, B and myeloid potentials (Fig. 2.7) (Ikawa et al. 2015). We call these progenitors induced leukocyte stem (iLS) cells. We also showed that this blockade can be done in a more inducible way by using ER-Id3 transgene. There, iLS cells are produced by adding Tamoxifen to the culture medium. Then, by removing it

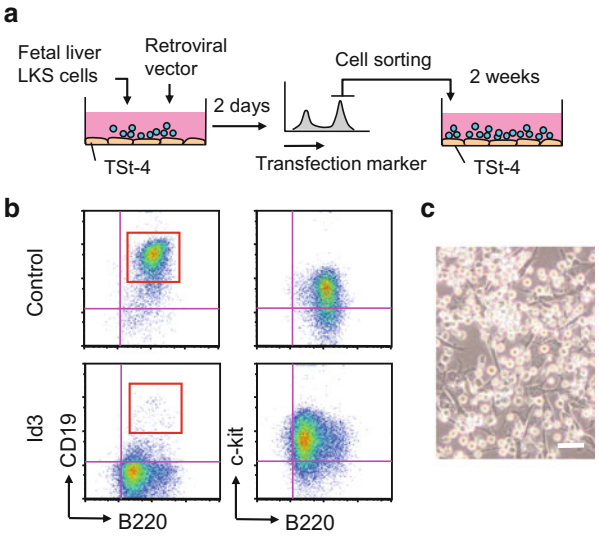

Fig. 2.7 Production of induced leukocyte stem cells
(**a**) Schematic representation of Id3-induced hematopoietic progenitor cell generation. Lin$^-$ckit$^+$ Sca1$^+$ (LKS) cells from murine fetal liver were transfected with retrovirus containing the Id3 gene, and transfected cells were further cultured with TSt-4 cells
(**b**) Flow cytometric analysis of control (empty vector) and Id3-overexpressing fetal liver progenitor cells. These Id3-overexpressing cells are developmentally arrested and enter a self-renewal cycle, retaining T, B and myeloid potentials. We term them induced leukocyte stem (iLS) cells
(**c**) Photomicrograph of iLS cells. The white bar indicates 10 μm

again, cells restart differentiation toward B cells. In addition, this method can be used for the arrest-restart system controlling the step from MLPs toward B cell lineage.

These findings may also give us new fundamental insights about stem cells. Self-renewal potential and multipotency are hallmarks of stem cells, and it is generally thought that the acquisition of such "stemness" requires rejuvenation of somatic cells through reprogramming of their genetic and epigenetic status. In contrast to this, our results propose that a simple blockade of cell differentiation is sufficient to induce and maintain stem cells.

Recapitulation of DN1 Stage Development Using the Arrest-Restart Controlling Culture System

We then tried to use self-renewing multipotent progenitors as a source of starting cells for synchronized differentiation culture toward T cells. For this purpose, E2A-deficient progenitors are not suitable, because E2A is also required for T cell development. We chose EBF1-deficient progenitors rather than PAX5-deficient progenitors, as EBF1-deficient progenitors are thought to be more immature.

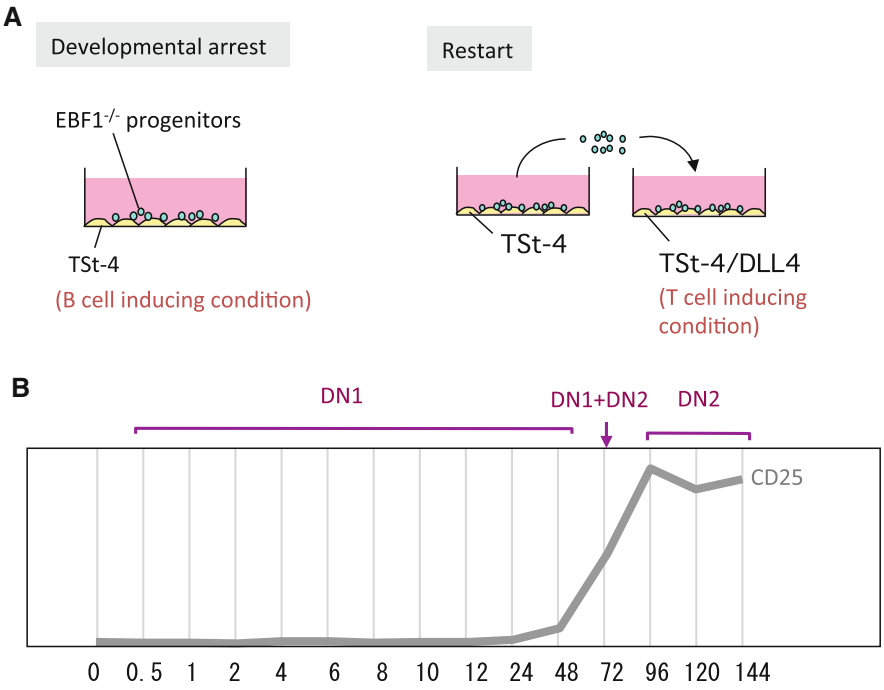

Fig. 2.8 Recapitulation of DN1 stage development in an *in vitro* culture system
(**a**) When EBF-1-deficient hematopoietic progenitors are maintained in co-culture with TSt-4 cells, cells are developmentally arrested and enter a self-renewal cycle, retaining the potential to produce T, B, and myeloid lineages. To induce differentiation toward the T cell lineage, these EBF-1-deficient multipotent progenitors were transferred to a co-culture with TSt-4/DLL1 cells (**b**) Transferred cells come to express CD25 on day 3 (72 h), and fully become DN2 cells on day 4. Time course samples were provided to the RIKEN FANTOM 5 project, and data of CAGE analysis for these samples are available online (http://fantom.gsc.riken.jp/zenbu/)

Using EBF1-deficient progenitors, we developed a culture system that recapitulated DN1–DN2 stages. EBF1-deficient progenitors can be maintained as self-renewing MLPs in a co-culture with TSt-4 cells optimal for B cell development (Fig. 2.8a, left panel). We then transferred EBF1-deficient progenitors to a co-culture with TSt-4/DLL1 cells, that is, TSt-4 cells that overexpress the Notch ligand DLL1 (Fig. 2.8a, right panel). On day 3 after transfer, cells came to express CD25, and on day 4 the entire cells became c-kit^{+}CD25^{+} DN2 cells. Thus, transferred cells undergo differentiation in a very synchronous manner, and the first 2 days of culture are thought to recapitulate the DN1 stage where thymus-seeding progenitors terminate B cell potential upon encountering Notch ligand. In collaboration with the RIKEN FANTOM 5 project we prepared and analyzed a total of 15 time point RNA samples. FANTOM 5 performed CAGE analysis, which sequences the first 20 nucleic acids of cDNA in a quantitative manner by next generation sequencing, making it possible to quantify gene transcription levels and determine whole genome transcriptional starting sites. In line with flow cytometric analysis, CD25 started to be expressed on day 3 (Fig. 2.8b). The data have been

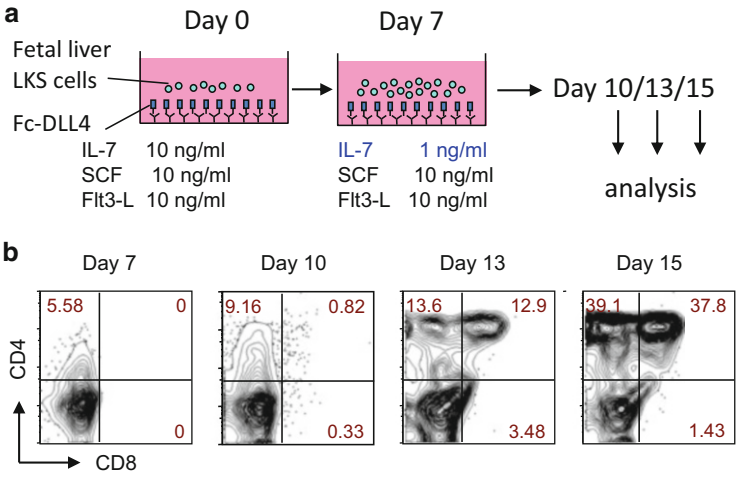

Fig. 2.9 Reduction of IL-7 concentration induces DP cell generation
(**a**) LKS cells sorted from 13 dpc fetal liver were cultured with immobilized DLL4 in the presence of SCF, IL-7 and Flt3L (10 ng/ml). The concentration of IL-7 was reduced to 1 ng/ml, and the cells were further cultured
(**b**) Kinetics of T cell generation in the immobilized Fc-DLL4 cultures

published (Arner et al. 2015) and are now available on the RIKEN FANTOM 5 "zenbu" site (http://fantom.gsc.riken.jp/zenbu/).

Feeder-Free Culture System

Stromal cells produce a myriad of undefined factors. Therefore, it is preferable to develop a culture system that does not use feeder cells. In 2003, it was reported that immobilized DLL1 and cytokines including IL-7 can support T cell development to the $CD25^+$ stage (Varnum-Finney et al. 2003), but it seemed difficult to induce further development.

As we have mentioned earlier, in our hand cells were also arrested at the DN2 stage in feeder-free culture initially (Fig. 2.6a, b). However, when we systematically optimized the feeder-free culture system, we found that arrested cells initiate differentiation when the concentration of IL-7 is reduced on day 7 of culture (10 ng/ml to 1 ng/ml) (Ikawa et al. 2010) (Fig. 2.9a). Notably, cells in these cultures developed up to the αβTCR-expressing DP stage (Fig. 2.9b). These results demonstrate that $\alpha\beta TCR^+$ cells can be generated from multipotent hematopoietic progenitors in a "feeder-free" culture system, merely using cytokines and Notch ligand. Therefore, any cellular interactions are dispensable for T cell development, at least until the DP stage. Of interest was the finding that once cells reached the DN3 stage and started TCRβ chain rearrangement, DP cells were generated without changing culture conditions. This finding indicates that so called TCRβ-selection, which is thought to serve as a critical checkpoint for preTCR formation, does not require additional environmental factors in this feeder-free culture system.

Toward Clinical Application

Currently Ongoing Efficient Strategies in Cancer Immunotherapy

Recently, our group is trying to apply our *in vitro* culture methods to cancer immunotherapy. Over the past several years, cancer immunotherapy has remarkably progressed. One of the leading achievements is the strategy to block inhibitory signals in the immune system. CTLA-4 and PD-1 are expressed on activated T cells as inhibitory receptors. By blocking these receptors with specific monoclonal antibodies (mAbs), it is possible to augment the immune response. Indeed, anti-PD1 mAb as well as anti-CTLA4 mAb alone or in combination have been clinically shown to be effective for some types of cancers, including melanoma, lung cancer, and kidney cancer, even at the advanced stage (Hodi et al. 2010; Topalian et al. 2012; Wolchok et al. 2013). The approach using immune checkpoint blockade is thus thought to be an epoch-making breakthrough in cancer therapy. Nevertheless, several problems remain to be solved. One issue is that autoimmune reactions take place at a certain frequency since this strategy activate the immune system in a non-antigen-specific manner. Another issue is that, in most cases, the effect is limited to ~20 % of patients.

Another effective method in cancer immunotherapy is to directly use CTLs. S. A. Rosenberg's group has been taking the strategy in which tumor-infiltrating lymphocytes (TILs) are collected from the patient, activated *in vitro*, and then returned to the patient (Rosenberg et al. 1988). Recently, the group is taking a more aggressive strategy. In this strategy, the patient firstly undergoes chemotherapy and lethal irradiation to deplete pre-existing lymphocytes, followed by autologous HSC transplantation, and then TILs are transferred back to the patient (Rosenberg 2011). An obvious effect was seen in 70 % of metastatic melanoma patients, 40 % of whom survived more than 5 years.

TCR gene transfer is another effective approach, where peripheral T cells of a patient are transduced with a defined TCR gene using a retrovirus or a lentivirus vector system. For example, in clinical trail of MART-1-specific TCR gene against melanoma, 10–30 % of patients showed tumor regression (Morgan et al. 2006; Johnson et al. 2009). In another trial, T cells transduced with NY-ESO1-specific TCR have been shown to be effective in 60 % of synovial cell sarcoma patients and 40 % of melanoma patients (Robbins et al. 2011).

Cloning of Antigen-Specific T Cells by Using iPSC Technology

Although the checkpoint-blocking mAbs are effective in various types of cancer, autoimmune reactions inevitably occur at a certain frequency, as mentioned earlier. Direct use of CTLs has been also shown to be effective, but invasive pretreatment

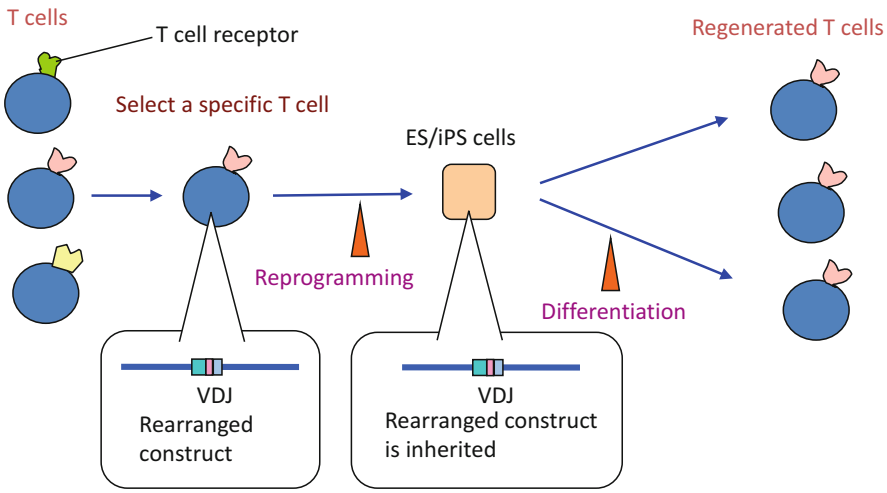

Fig. 2.10 Reprogramming of T cells with certain antigen specificities into iPSCs serves as a method of cloning
iPSCs produced by reprogramming of a T cell having a defined antigen specificity inherit rearranged genomic constructs of TCR genes of the original T cell. All T cells regenerated from such iPSCs are expected to express the same TCR as the original T cell

or gene therapy is required for the method to work well. The question may arise why one does not simply expand antigen-specific T cells *in vitro* by using tumor antigens and antigen presenting cells. Actually, antigen-specific CTLs can be expanded *in vitro*, but it has been extremely difficult to get a sufficient number of cells, since *in vitro* expanded CTLs tend to become easily exhausted after a certain period of culture.

To overcome this problem, we came to think of the idea to utilize induced pluripotent stem cell (iPSC) technology for the cloning and *in vitro* expansion of CTLs. Essentially iPSCs are established from mature antigen-specific T cells. As T cell receptors are formed from rearranged TCR genes, iPSCs derived from a T cell (T-iPSCs) should inherit those rearranged genomic structures (Fig. 2.10). All T cells regenerated from T-iPSCs are thus expected to express the same TCR on their surface. Since iPSCs can be expanded almost unlimitedly, it is possible to obtain as many fresh CTLs as needed.

Production of iPSCs from Tumor Antigen-Specific CTLs

Pursuing this idea, we have recently succeeded in establishing iPSCs from melanoma antigen MART1-specific CTLs originally derived from a melanoma patient,

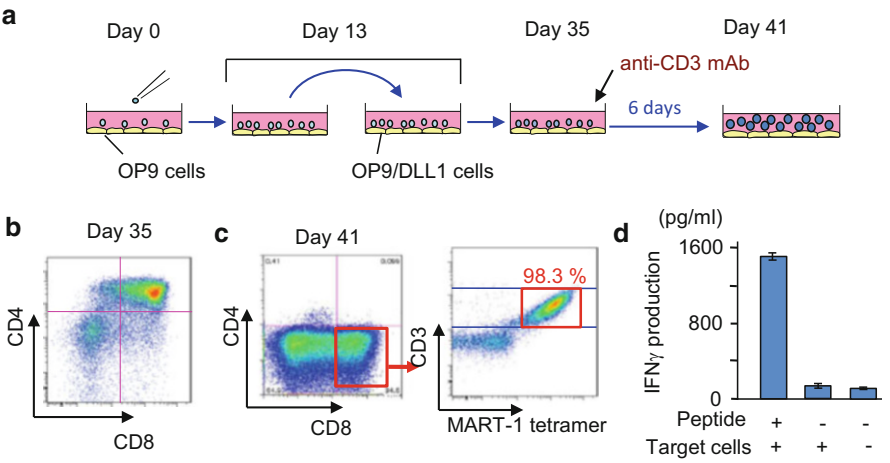

Fig. 2.11 Regeneration of MART-1-specific CTLs from MART1-T-iPSCs
(**a**) MART1-T-iPSCs were sequentially cultured with two types of feeder cells, OP9 and OP9/DLL1. On day 35 of cultivation, anti-CD3 mAb was added to induce the generation of mature T cells
(**b**) CD4/CD8 double positive cells were generated on day 35 of cultivation
(**c**) A large number of CD8 single positive cells were generated 6 days after the stimulation with anti-CD3 mAb. Virtually all of them expressed a TCR specific for the MART-1 antigen
(**d**) Production of IFNγ by regenerated $CD8^+$ T cells upon antigen-specific stimulation. IFNγ secretion by $CD8^+$ T cells was measured by ELISA using the supernatant after co-culturing $1x10^5$ regenerated $CD8^+$ T cells for 24 h with $1x10^4$ HLA-A*02:01-positive EBV-lymphoblastoid cells (CIRA0201) pulsed or not pulsed with MART-1 peptide (EAAGIGILTV). Mean ± SE of triplicates is shown

and in regenerating MART1-specific CTLs from these iPSCs (Vizcardo et al. 2013).

As a cell source we used JKF6 cells (Yang et al. 2011), which are specific for the melanoma antigen MART-1. JKF6 cells are long-term cultured TILs originally derived from a melanoma patient. Some previous studies reported that iPSCs can be produced from human peripheral T cells by using Yamanaka factors (Seki et al. 2010; Loh et al. 2010). However, in our case we also used SV40 (Park et al. 2008) to increase reprogramming efficiency. For transduction, we used the Sendai virus system (Fusaki et al. 2009). We thus succeeded in making iPSCs from MART1-specific CTLs (MART1-T-iPSCs).

Regeneration of Tumor Antigen-Specific CTLs from iPSCs

We then induced differentiation of T cells from MART1-T-iPSCs (Fig. 2.11a) by modification of a previously published method (Timmermans et al. 2009). After 35 days of cultivation, $CD4^+CD8^+$ DP cells were generated (Fig. 2.11b). However,

these *in vitro* culture systems have been unable to support the efficient generation of SP cells beyond the DP stage.

To induce further differentiation, we simply added anti-CD3 mAb to the culture on day 35. The proportion of $CD8^+$ T cells clearly increased during a period of 6 days following TCR stimulation (Fig. 2.11c, left panel), and cells expanded by 300-fold (data not shown). Importantly, the resulting $CD8^+$ T cells were almost exclusively specific for the MART-1 antigen (Fig. 2.11c, right panel). Thus, in this culture system positive selection of $CD8^+$ T cells can be recapitulated *in vitro*.

To examine whether these $CD8^+$ T cells can be activated in an antigen-specific manner, regenerated $CD8^+$ T cells were co-cultured with target cells (human EBV-lymphoblastoid cell line) with or without MART-1 peptide. $CD8^+$ T cells produced a substantial amount of IFNγ only in the presence of MART-1 peptide (Fig. 2.11d). Collectively, this T-iPSC approach appears to be efficient in regenerating functional antigen-specific CTLs.

We recently found that DP cells expressing antigen-specific TCR can be induced to $CD8^+$ T cells by co-culturing with antigen presenting cells pulsed with agonist peptide (our unpublished observation). Such apparent easiness in positive selection of $CD8^+$ T cells seems to be in favor of model (ii), presented earlier in the section of positive selection, which assumes that positive selection is not induced by a moderate TCR signal but simply by a strong TCR signal given in thymic cortex.

Allogeneic Transfusion Setting is Preferable

The above method, which is based on the autologous transfusion setting, is bound to face some problems. Not only the production of iPSCs for each patient will be costly, it will also be difficult to ensure TCR affinity quality, and it will need a substantial amount of time (more than 6 months) to prepare regenerated CTLs. We will now discuss how an allogeneic transfusion strategy will solve these problems. In order to make such a strategy work, it will be required to establish a "T-iPSC bank".

In this scheme, T-iPSCs are produced from tumor antigen-specific CTLs collected from healthy donors (Fig. 2.12). Regenerated CTLs will be tested with regard to TCR affinity and ability to give rise to T cells, and if sufficient in quality, such T-iPSCs will be stored as frozen stocks in the bank. Regenerated CTLs themselves will also be frozen and pooled, and they will be immediately used when a HLA-matched patient develops cancer expressing the same tumor antigen. HLA haplotype-homozygous donors are desired as a source of T-iPSCs, since regenerated cells can be given to a patient having the same HLA haplotype allele as a heterozygote. It is also possible to make iPSCs equivalent to T-iPSCs by transducing non-T derived iPSCs with a defined TCR gene. In any cases, it should be tested *in vitro* whether regenerated CTLs happen to exhibit alloreactivity to

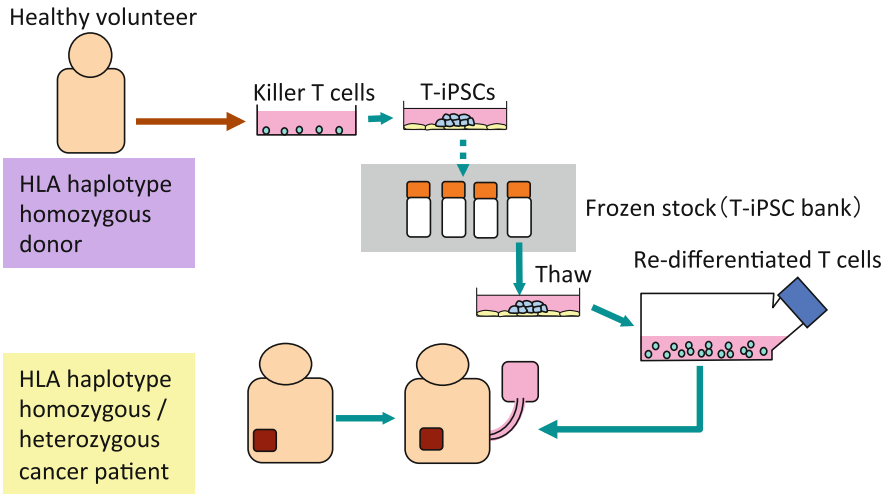

Fig. 2.12 Application of the method to the allogeneic transfusion setting
T-iPSCs are produced from tumor antigen-specific CTLs collected from healthy HLA haplotype-homozygous donors. Regenerated CTLs will be tested with regard to TCR affinity. If they pass quality testing T-iPSCs will be stored as frozen stocks in the T-iPSC bank. Frozen and pooled regenerated CTLs could then be immediately applied to a HLA-matched patient bearing cancer expressing the same tumor antigen

recipient cells, before the use in patients. In addition, there will be another big advantage: patients receiving regenerated CTLs will be free from malignant transformation of transferred CTLs. This is because allogeneic grafts will be eventually rejected based on mismatch of minor histocompatibility antigens even in the HLA-matched case.

Concluding Remarks

In this article, we have presented current knowledge on T cell development and elaborated on the question to what extent T cell development can be recapitulated *in vitro*. Apart from the fact that the studies mentioned in the latter part will be directly applicable in clinical settings, we argue that such *in vitro* studies can be principally regarded as a synthetic biology approach. In other words, when one can control the entire developmental process of a certain type of cell *in vitro* in a culture system using a limited number of defined factors, then it can be said that most of the essential parts regarding the development of that cell type have been clarified. In this respect, in the field of T cell development, we feel that the goal is near. In fact, generation of CD8 T cells starting from ES/iPS cells can be almost perfectly recapitulated *in vitro*, although feeder cells are required for efficient induction. We trust that in the near future it will become possible to induce other types of T cells, and also to do so in a completely defined xeno-free culture system. Thus, we strongly believe that our approach will help clarify essential issues governing the developmental biology of T cells.

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