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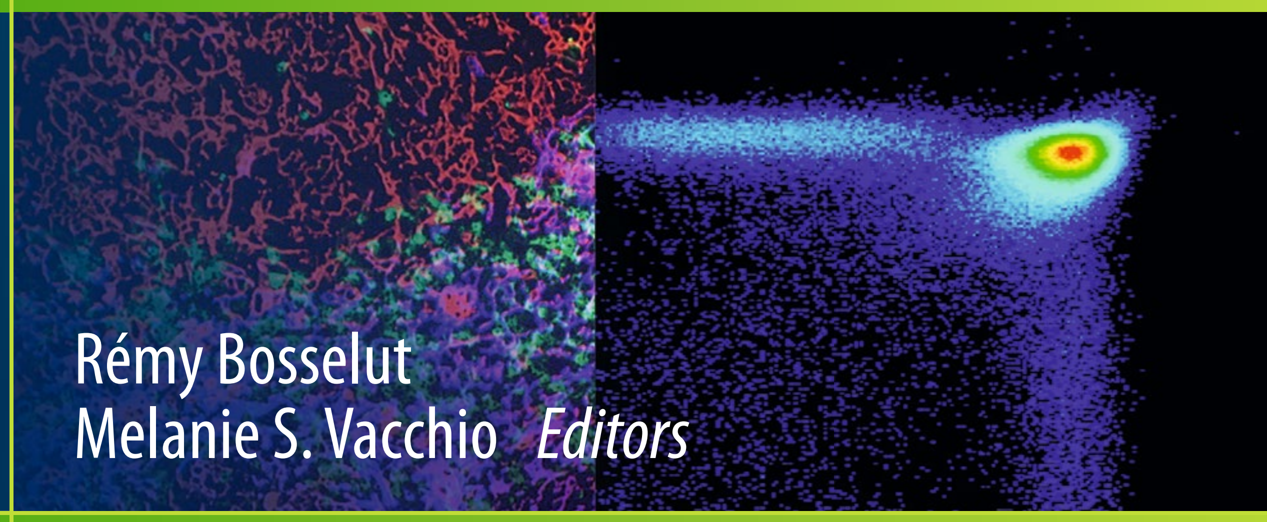

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T-Cell Development

Methods and Protocols

Methods in Molecular Biology

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T-Cell Development

Methods and Protocols

Edited by

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-2808-8 ISBN 978-1-4939-2809-5 (eBook)
DOI 10.1007/978-1-4939-2809-5

Library of Congress Control Number: 201506151

Springer New York Heidelberg Dordrecht London
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Preface

Fifty and a few years since the function of the thymus was discovered, the study of T cell development remains a fascinating part of immunology. This is certainly because T cells, essential components of immune responses, develop in the thymus. Interactions in the thymus shape the reactivity and function of T cell precursors and are essential to establish a functional and tolerant immune system. Thus, identifying intrathymic interactions and understanding their mechanisms are intrinsic parts of T cell immunology. In addition, the study of T cell (and more broadly lymphocyte) development has exerted broad appeal beyond the immunological realm. Indeed, throughout their thymic journey, T cell precursors recapitulate key processes involved in metazoan development, including proliferation, differentiation, death-survival, and migration decisions. Studies of T cell development have brought decisive insight into critical concepts of modern biology, including somatic DNA recombination, DNA repair, programmed cell death, and epigenetic gene silencing.

For all its accomplishments and potential, the study of T cell development enjoys the not-so-enviable reputation of being "complicated." While this is in part due to the sheer complexity of T cell development, it certainly also has to do with difficulties in experimental approaches. The objective of the present volume of Methods in Molecular Biology is to overcome such practical obstacles, by giving simple and accessible experiment protocols to explore thymus biology. This book is organized in three parts. The first two chapters offer short reviews on T cell development, for readers with little or no familiarity with the topic. Both provide basic concepts as a preparation to parsing the protocol chapters, and references for further study. The second part discusses analysis strategies and presents basic protocols for cell preparation, flow cytometry analyses, and the study of T cell responses. The last part includes state-of-the-art protocols to explore multiple aspects of thymocyte biology.

In building this selection, we have tried to put emphasis on three themes. The first is the emergence and refinement, over the last few years, of a remarkable panoply of in vitro approaches to study T cell development. While in vivo studies, harnessing the power of mouse genetics, remain the standard of proof, new strategies offer unprecedented possibilities to investigate thymic biology in a dish. Such approaches, using derivatives of the OP9 stromal line expressing Notch ligands, have truly revolutionized the study of early thymocyte development. In addition, continued refinement of organ culture systems has increased their power to address later stages of αβ T cell development, including mechanisms of selection. The present volume includes detailed protocols for three of these approaches: fetal thymic organ culture, reconstituted organ culture, and culture of thymic "slices." Future progress can be expected from attempts at generating thymic epithelial cells in vitro, including using "reprogramming" approaches. An important asset of organ culture is to open the door to live imaging techniques, as described in the chapter on thymic slices, which incorporates time and space resolution into the study of thymocyte differentiation. Last but not least, because in vitro techniques can combine T cell precursors and stromal cells of distinct genotypes, they offer useful alternatives to complex breeding strategies.

The second point we wanted to emphasize is the plasticity of thymocytes, which can fine-tune intracellular signaling and gene expression to specific environmental conditions. This is critical during the building of the TCR repertoire: by shifting the repertoire of TCR specificities being selected, thymocyte plasticity offers the thymic organ a major potential to compensate signaling or transcriptional changes at the single-cell level. This emphasizes the importance of genetic approaches analyzing the development of cells carrying a defined specificity, together with bone marrow chimera techniques allowing the study of cell development in a competitive environment. More broadly, even though there are few truly single-cell assays for the study of T cell development, such cell plasticity should be taken into consideration when designing experimental approaches.

Last, we have tried to extend the range of protocols presented in this volume beyond the "workhorse" laboratory mouse. The power of mouse genetics has been instrumental to T cell immunology, including in defining such essential concepts as MHC restriction and positive selection. The last decade has seen the realization that thymic functions are present early on during evolution, actually predating the emergence of the "modern" lymphoid system of jawed vertebrates. This strengthens the rationale to study T cell development in other organisms, to bolster concepts generated from mouse studies, especially if they offer unique opportunities for genetic intervention. The zebrafish embryo is one such powerful experimental model, allowing for oligonucleotide-directed "knock-down" of gene function and offering quick and powerful approaches for the study of T cell development. On the other hand, it is becoming possible, and increasingly important, to use the conceptual frameworks generated in mouse models as platforms to investigate human T cell development, with the objective of understanding how repertoire formation and selection drives T cell responsiveness in physiological and pathological situations. Three chapters address these issues, providing approaches to study human thymocytes and to model T cell development in humanized mice. New methods for in vivo gene recombination, including improvements of the bacteria-derived CRISPR system, should increase the power of such studies in the years to come.

It is our hope that the present volume will be useful to readers whose scientific curiosity brings them to interrogate the thymus, whether because of its role in shaping the T cell repertoire or of its ability to encapsulate, in a simple and accessible organ, most of the basic processes at work in developmental biology. We are grateful to the many authors who shared their unique expertise to make this volume possible.

Bethesda, MD, USA *Rémy Bosselut*
Melanie S. Vacchio

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Part I

Background Information

Chapter 1

200 Million Thymocytes and I: A Beginner's Survival Guide to T Cell Development

Melanie S. Vacchio, Thomas Ciucci, and Rémy Bosselut

Abstract

T lymphocytes (T cells) are essential for proper adaptive immune responses. They perform a variety of functions in defenses against pathogens, and notably control, positively or negatively, other cells involved in immune responses. T cells develop in the thymus from bone marrow-derived precursors. These precursors (thymocytes) proliferate, rearrange the genes encoding subunits of the T cell antigen receptor, which endow them with their unique antigen specificity, and undergo various degrees of pre-programming for their functions in immune responses. Thus, analyzing T cell development in the thymus is essential for understanding their functions in immune responses. In addition, the thymus constitutes an attractive experimental model to analyze mechanisms of cell proliferation, differentiation and survival, all of which are involved in thymocyte development. This chapter presents a quick overview of the key events characterizing intrathymic T cell development, as an introduction for readers entering this field of study.

Key words T cells, T cell development, T cell receptor, TCR gene rearrangement, Positive selection, Negative selection

1 T Cells and T Cell Antigen Recognition

T lymphocytes (T cells) are critical components of the adaptive immune system and are essential for responses to foreign organisms. Key aspects of T cell responsiveness are set during their development in the thymus [1, 2]; thus, studying the development of T cells provides invaluable insight into their functions, and has attracted much interest. The following chapter is intended as a primer for those entering this field of study, unfolding key events, highlighting checkpoints, and introducing experimental tools to explore the developmental sequence. We have directed the reader to in-depth reviews and a few original papers for more information of each of the points addressed in this overview. While key concepts discussed here are thought valid for both human and mouse T cell development, the description of specific stages, signals and

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_1, © Springer Science+Business Media New York 2016

transcription factors refer to mouse T cell development. Issues specific to human T cell development are discussed in Chapter 19.

T cells recognize antigens through a receptor (T cell antigen receptor, TCR) composed of two distinct polypeptide chains [3]. Two possible pairs of chains have been identified: TCRα and TCRβ, or TCRγ and TCRδ, defining αβ and γδ T cells, respectively. None of these chains has signaling activity per se. Rather, they are expressed at the cell surface as a complex with signaling chains (TCRζ and CD3 complex) [4–6], which generates intracellular signals upon antigen recognition (Fig. 1a). Expression of the genes encoding the signaling CD3 chains is a defining attribute of the T lineage. Signaling by TCR complexes has been extensively investigated, mostly in αβ T cells (but is thought to operate along the same principles in γδ T cells). It is triggered, upon antigen binding, by the phosphorylation of tyrosine residues in TCRζ and CD3 chains, which itself recruits kinases that propagates phosphorylation

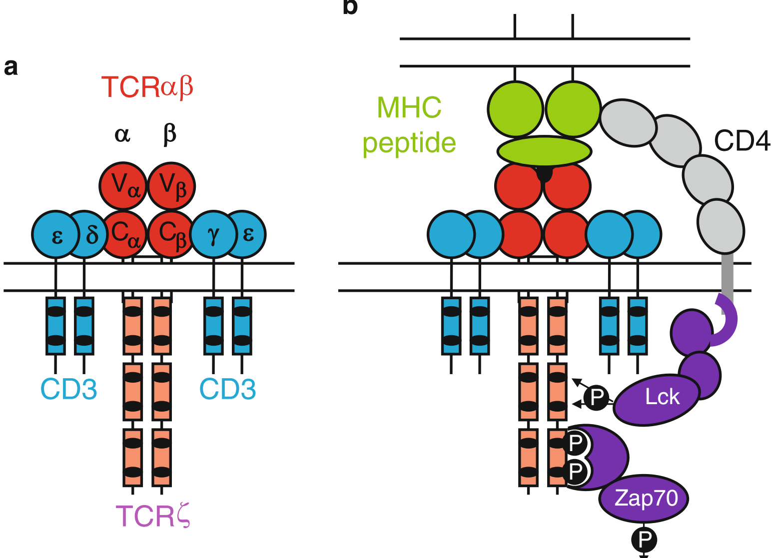

Fig. 1 T cell receptor structure and signaling. (**a**) Schematic representation of TCRαβ complexes, showing each subunit. *Rectangular boxes* in signaling chains depict Tyrosine (*black ovals*)-based ITAM motifs. While there is evidence that CD3δε interact with TCRα and TCRγε with TCRβ, the architecture of the complex as represented is speculative. V and C respectively indicate variable and constant domains of each TCR chain. (**b**) Schematic depiction of TCR and coreceptor (CD4) interactions with MHC-II-peptide (*filled black circle*) complexes, and of early TCR signaling. Co-engagement of TCR and CD4 by MHC-II (or of TCR and CD8 by MHC-I, not represented) results in the juxtaposition of Lck molecules and CD3 and TCRζ signaling chains, phosphorylation of tyrosine residues, recruitment and activation of the Zap70 kinase, which phosphorylates and activates downstream targets

and intracellular signaling to substrates not physically linked to TCR complexes (Fig. 1b).

The vast majority of αβ T cells recognize peptide antigens presented by "classical" class I (MHC-I) or class II (MHC-II) major histocompatibility complex molecules, and are referred to as "conventional" T cells [5]. MHC molecules are expressed at the cell surface in association with peptides generated through mechanisms that differ between MHC-I and MHC-II. MHC-I molecules, which are ubiquitously expressed, bind peptides resulting from the proteasome-mediated degradation of proteins synthesized in the cell; in contrast, MHC-II molecules are expressed mostly on professional antigen-presenting cells and bind peptides resulting from the lysosomal degradation of endocytosed proteins. Antigen recognition by αβ T cells involves interactions of TCRαβ chains with both peptide and MHC amino acid residues [7]. An important implication of this binding mode is that recognition of self (amino acids from MHC molecules) is an inherent component of αβ T cell antigen reactivity because it contributes to recognition of foreign antigens. As discussed below, this has critical consequences for αβ T cell development in the thymus.

Conventional T cells recognizing MHC-I- or MHC-II-associated peptides form the vast majority of T cells in peripheral lymphoid organs and at most effector sites [5]. Each of them expresses monospecific ("clonotypic") TCR complexes (i.e. each receptor complex in a given cell harbors the same uniquely rearranged TCRα and TCRβ sequence); upon recognition of a specific ("cognate") MHC-peptide complex, such T cells proliferate and acquire effector properties (e.g. cytokine production or expression of specific surface molecules). Even though each T cell carries one clonotypic TCR species, the multiple specificities they express as a population underpins the breadth of T cell antigen recognition. Conventional T cells are distributed into two subsets that differ by their function and their expression of CD4 or CD8, two surface glycoproteins that contribute to recognition of MHC molecules and signaling by T cells (Fig. 1b) [8]. MHC-I-specific cells express CD8 but not CD4, and typically differentiate into cytotoxic effectors upon activation. MHC-II-specific cells express CD4 but not CD8, and have a broader functional potential (often designated as "helper"); in fact, their effector differentiation is to a large extent determined upon antigen activation by the surrounding cytokine environment. Unlike in mature T cells, expression of CD4 and CD8 on thymocytes depends on their developmental stage, and distinguishes the conventional $CD4^{-}CD8^{-}$ "double negative" (DN), $CD4^{+}CD8^{+}$ "double positive" (DP) and $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ "single positive" (SP) cell subsets, the latter three being characteristic of the αβ lineage [2].

In addition to conventional T cells, many "non-conventional" αβ T cell subsets have been identified, often expressing neither

CD4 nor CD8. Among the best characterized are natural killer (NK) T cells, of which most recognize CD1d-bound lipids, mucosal-associated invariant T cells (MAIT), which recognize MR1-bound riboflavin derivatives, and intraepithelial lymphocytes (IELs) [9–12]. The organization of the γδ T cell population is less well understood (*see* Chapter 2). It includes multiple subsets differing by antigen receptor chain usage, functional properties, and unique developmental attributes (including their appearance at specific embryonic or fetal developmental stages). Most such cells express neither CD4 nor CD8.

2 An Overview of T Cell Development

2.1 A Brief Thymic Tour

The "objective" of intrathymic T cell development is twofold. It first must generate the variety of T cells necessary for immune responses. This implies a massive expansion of the small population of hematopoietic precursors that enter the T lineage, and the building, by somatic recombination of genes encoding TCR chains, of a large repertoire of antigen-specific receptors. Second, for αβ T cells, selection steps (death-survival decisions) ensure that this repertoire is appropriate to function with antigen-presenting molecules expressed by each individual. Whether and how the γδ repertoire is subject to selection is not yet fully understood (*see* Chapter 2).

Meeting these "objectives" is achieved through a complex developmental sequence associating differentiation, selection, and proliferation episodes (Fig. 2). Such events take place in the thymus, a thoracic organ made of bone marrow-derived hematopoietic-derived cells (including multiple cell types in addition to T cell precursors) and epithelial cells derived from the third pharyngeal pouch [13]. The thymus appears during early embryonic development, is most active during childhood and involutes after puberty. Thus, the T cell repertoire itself is mostly constituted during the early years of life and starts declining in early adulthood. Although this review focuses on the T cell component of the thymus, other cell types are essential to its function, perhaps most prominently thymic epithelial cells [14]. The distribution of T cell precursors and epithelial cells in the thymus is not homogenous, and its compartmentalization is critical to thymic function. The most salient, almost macroscopic, feature of the thymic architecture is the distinction between the thymic cortex, in which early T cell development takes place, and the medulla, where thymocytes complete their maturation. Each compartment is characterized by distinct subtypes of epithelial cells, although it is now clear that both medullary and cortical thymic epithelial cells derive from a common precursor [15, 16]. This will be discussed again near the end of this overview.

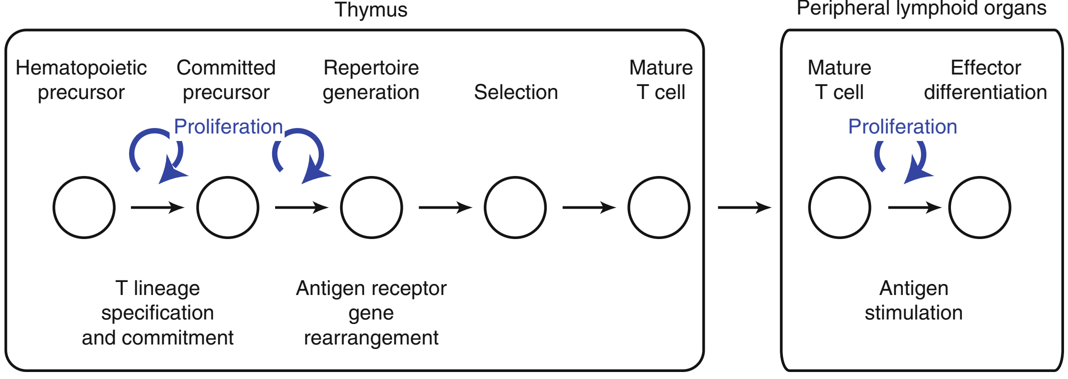

Fig. 2 Schematic outline of T cell development. T cell development can be conceptually separated into four steps: (i) the T lineage commitment of recent thymic immigrants, which enter the thymus as uncommitted precursors, (ii) the rearrangement of TCR genes (dependent on Rag gene expression), itself a multistep process, of which a critical outcome is the distribution of thymocytes into the αβ or γδ lineage, and (iii) the MHC-based selection of thymocytes expressing αβ TCRs, during which only cells with appropriate avidity for self antigens survive and complete their intrathymic differentiation. Most of these cells exit the thymus as "naïve," and acquire effector ("helper" or cytotoxic) functions during antigen-induced proliferation in peripheral lymphoid organs (*see* text for details). However, most γδ thymocytes, and a small fraction of αβ lineage cells, acquire such effector functions in the thymus. Note that there is "chronological" overlap between these conceptual steps during the developmental sequence. This is most striking in the αβ lineage, in which commitment to the αβ lineage follows the rearrangement of TCRβ genes but precedes that of TCRα, and termination of the latter is mechanistically coupled to MHC-based selection

2.2 Early T Cell Development: The Common Core

The earliest steps of intrathymic development, thought to be common to all T cells, lead bone marrow-derived hematopoietic precursors to enter the thymus, commit to the T cell lineage and initiate the rearrangement of genes that encode antigen receptor chains [17–19]. The migration of such precursors into the thymus requires their egress from the bone marrow, which we will not discuss here, and their leaving the circulation in blood vessels that irrigate the thymic cortico-medullary junction [20]. The latter process is controlled both by adhesion molecules and chemokines, including the production by the thymic epithelium of CCL19 and CCL21, the ligands for CCR7 expressed by colonizing precursors. As a result of their thymic entry, precursors are exposed to ligands for the Notch1 receptor, specifically Delta-like 4 expressed on thymic epithelial cells [21, 22]. Both gain and loss-of-function studies have shown that signaling by Notch1 in T cell precursors is required for their early development and notably for T-lineage commitment [23, 24]. Recent studies have identified transcription factors that mediate T lineage specification or commitment [25]. These include HES1 and TCF1, which appear directly downstream of Notch1, and other transcription factors, such as Gata3 and Bcl11b [26–28].

In addition to Notch1 signals, early T cell development requires the cytokine IL-7, which supports the massive proliferation that takes place prior to rearrangement of TCR genes, and promotes thymocyte survival in part at least by affecting the balance between pro- and anti-apoptotic molecules of the Bcl2 family [29].

TCR chain gene rearrangement is the critical event defining the T lineage. It occurs in distinct steps spreading over several stages of T cell development. Since the mechanisms of TCR gene rearrangement [30] are discussed in Chapter 16, only a few basic principles will be highlighted here. In the germline, non-rearranged, configuration, each TCR locus contains multiple copies of the DNA segments [referred to as variable (V), Joining (J) and (for TCRβ and TCRδ) Diversity (D)] encoding the variable domain of each TCR chain (Fig. 1a). Such copies, which differ by their sequence, are arranged in V, D and J clusters within each TCR locus. Rearrangement involves the cis-joining of such gene segments within the TCR chain-encoding locus, and joins a given member of one cluster to a member of another one (i.e. V to J, or V to D to J). This occurs through excision, mediated by Rag1 and Rag2 recombinase molecules, of large intervening DNA fragments, followed by ligation of the free chromosomal DNA extremities. In contrast to Rag1 and Rag2, which are specifically expressed in lymphocyte precursors, the joining of DNA breaks involves an ubiquitous DNA repair machinery.

Gene rearrangement generates TCR diversity by two processes: (1) combinatorial diversity, as the rearranged allele includes a single copy of each V, D or J segment, out of the many present in the germline, and (2) "junctional" diversity, resulting from nucleotide deletions and non-templated additions made during the joining process to the original DNA breaks generated by the Rag-mediated cleavage. Proper gene rearrangement requires the stage-specific targeting of the Rag-mediated excision machinery to the appropriate TCR gene locus. For αβ lineage cells, TCRβ is rearranged in DN cells and TCRα at the DP stage. Whether specific signals direct thymocytes to rearrange TCRβ vs. TCRγ and TCRδ, or whether they "randomly" attempt to rearrange genes encoding TCRβ or TCRγ and adopt an αβ or γδ fate if either rearrangement is productive, remains to be determined.

2.3 Emergence of the αβ Lineage

Upon successful completion of TCRβ gene rearrangement, the resulting TCRβ polypeptides associate with a surrogate TCRα chain, pre-Tα (instead of TCRα) and CD3 chains to form pre-TCR complexes [31]. Signaling by such complexes allows thymocytes to clear the checkpoint for TCRβ gene rearrangement, a processed referred to as β-selection, and causes them to adopt an αβ fate [32]. Because rearrangement of TCRβ or of TCRγ and TCRδ occur during the same developmental window, and because pre-TCR and TCR complexes use common signaling components,

the question arises how developing thymocytes distinguish between signaling by the pre-TCR, which drives the cells toward an αβ fate, vs. TCRγδ, which causes them to adopt a γδ fate. The current perspective, discussed in more detail in Chapter 2 is that there is no lineage-instruction signal delivered by either type of receptor. Rather, there is evidence that intracellular signals elicited by the pre-TCR are of lower magnitude than those triggered by TCRγδ complexes, and that developing thymocytes convert such differences in signaling intensities into αβ or γδ fate commitment signals [33–35].

Signaling by the pre-TCR fixes αβ lineage commitment by terminating TCRβ gene rearrangement [32]. It also causes the proliferation of αβ precursors and the expression of genes encoding CD4 and CD8 coreceptors, as well as multiple other gene expression changes [36]. Each cell going through β-selection gives rise to multiple daughter DP cells expressing the same TCRβ polypeptide and actively rearranging TCRα genes, thereby adding to the combinatorial diversity generated by antigen gene rearrangement and maximizing the potential for use of each TCRβ rearrangement. These cells, which form the bulk of the thymus, are characterized by low-level expression of surface TCRαβ (characteristically less than mature αβ T cells). Among these changes are increased expression of CD27 and CD28 surface antigens, which both can be used to identify the earliest precursors that have undergone β-selection, before the onset of CD4 or CD8 expression [37, 38] (Fig. 4). Several transcription factors, including Gata3, Runx1, E-proteins E2A and HEB (*see* below) and RORγt, and multiple signaling pathways are important for β-selection [2]. Of note, the differentiation of αβ lineage progenitors at the β-selection step retains a significant dependence on Notch signals [35].

2.4 Selection of *αβ* T Cells: Basic Principles

The selection and differentiation of DP thymocytes into mature CD4+ and CD8+ T cells has attracted much interest because of the critical importance of αβ cells in immune responses and their pathogenic role in autoimmune disease. DP thymocytes, which reside in the thymic cortex, do not proliferate; thus, even though the thymus is characterized by rapid cell renewal, it is mainly populated (85–90 % of thymocytes) by non-dividing cells, a paradox explained by the short life of DP thymocytes. The fate of these cells is primarily dictated by the reactivity of their αβ TCR to self-peptides bound to MHC molecules expressed on thymic epithelial cells [39]. Specifically, DP thymocytes whose TCR fails to interact with self MHC-peptide die in the cortex (by "neglect") within 3 days of their generation. In contrast, cells which interact with self MHC-peptide are rescued from cell death ("positive selection") and differentiate further. Thymocytes are selected on the basis of their reactivity to MHC because mature T cells recognize a complex of MHC and peptide determinants. Because of the high

polymorphism of MHC molecules (i.e. the large number of allelic variants at loci encoding MHC-I and MHC-II molecules), TCR specificities generated by Rag-mediated rearrangement must, at the species level, be able to interact with a broad diversity of MHC molecules. Consequently, at the individual level, most TCRs bind poorly, if at all, to the particular MHC molecules expressed by the thymic cortex, and are therefore "useless." The function of positive selection is to select those TCRs with sufficient affinity for self MHC. Of note, for conventional thymocytes, neither positive selection per se, nor the differentiation events that accompany or follow it, are associated with cell proliferation. Thus, these thymocytes undergo no intrathymic division after the β-selection-associated proliferative boost.

On the other side of the spectrum, a fraction of TCRs will strongly bind self MHC-peptide complexes, such that their expression on mature T cells would potentially lead to autoimmune disease. The "objective" of thymic selection is to eliminate these two extremes, and only allow cells in the middle of the range to differentiate into mature T cells [39, 40].

2.5 Signaling Positive Selection

Positive selection, strictly speaking the rescue from programmed cell death, results from TCR signaling and involves increased expression of anti-apoptotic molecules Bcl2 and Mcl1 [2]. It is associated with differentiation events that quickly follow or coincide with rescue from cell death. These first include the cessation of TCRα gene rearrangement, at least in part by silencing expression of Rag1 and Rag2 genes. Positive selection is also accompanied by the up-regulation of CCR7, a receptor for CCL19 and CCL21 chemokines, both expressed in the thymic medulla but not in the cortex. Together with the converse change in the expression of CXCR4, whose ligand (CXCL12, also called SDF1) is preferentially expressed in the cortex, CCR7 expression results in the migration of thymocytes from the cortex to the medulla [41]. A more distant consequence of TCR signaling is the expression or IL-7Rα, an event essential to T cell differentiation because it restores IL-7 responsiveness, which is essential for long-term survival of mature T cells [42]. In addition, IL-7 is important for the proper development of $CD8^+$-lineage T cells [43, 44].

How such changes in gene expression are related to positively selecting TCR signals is not yet fully understood [45]. In thymocytes as in T cells, TCR signaling activates a variety of intracellular signal transduction cascades, including the Ras-Erk kinase and calcium pathway, both important for positive selection. Nuclear targets of TCR signals include members of the Egr, AP-1, Ets and NFAT families, as well as inhibitors (Id2 and I3) of E-box binding proteins [46]. Because of genetic redundancy, the role of many of these factors and pathways remains to be fully elucidated, and it is likely that other, yet to be discovered, factors contribute to positive selection.

2.6 Central Tolerance and Negative Selection

The other side of thymic selection is the elimination (or inactivation) of thymocytes carrying TCRs with high avidity for self ligands. There is indeed strong genetic evidence for a thymic role in establishing immune tolerance [47]. Such "central" tolerance (i.e. established in the thymus) is now understood as having two components. The first prevents self-reactive cells from differentiating into mature T cells, by causing their TCR-induced cell death in the thymus (a deletion process referred to "negative selection"). The second causes self-reactive cells to adopt a "regulatory" (Treg) fate, characterized by and requiring expression of the transcription factor Foxp3, which endows the cells with suppressive functions [48, 49]. Both mechanisms appear important for central tolerance, although their respective contribution is not yet fully understood [50]. Analyses in mice suggest that deletion mechanisms do not result in complete elimination of self-reactive cells [51], although such studies have so far been carried only in experimental models with clonal or reduced-diversity repertoires, and future studies will be needed to fully measure the contribution of deletion mechanisms to central tolerance. The genetic evidence for the importance of Treg cells is compelling, both in mice, in which disruption of Foxp3 causes a severe autoimmune and inflammatory disease, and in humans in which Foxp3 mutations are the cause for a rare but severe autoimmune disease of infancy and childhood called IPEX syndrome (Immunodysregulation, Polyendocrinopathy, and Enteropathy, X-linked) [52]. The importance of Treg cell generation in the thymus is independently underscored by mouse neonatal thymectomy experiments; when performed before the appearance of Treg cells (i.e. by fourth day of age), thymectomy results in multiple autoimmune manifestations that are prevented by adoptive transfer of Treg cells [48].

Interactions between thymocytes and medullary epithelial cells are essential for both arms of central tolerance. That is at least in part due to the expression, by medullary epithelial cells, of tissue-specific antigens (epitomized by insulin, whose expression is otherwise limited to pancreatic islets) [40]. Expression of tissue-specific antigens in medullary epithelial cells requires the transcriptional regulator Aire (autoimmune regulator) [47], a multifunctional protein whose dysfunction causes an autoimmune disease targeting the endocrine system (Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy, APECED).

2.7 Positive vs. Negative Selection Signals and Mechanisms

Because both positive and negative selection depend on TCR signaling, perhaps no question has haunted the field of T cell development more than what distinguishes signals determining either outcome. While there is no definitive or simple answer to this question, we have tried to summarize below current concepts and perspectives.

MHC-peptide complexes that engage the TCR with high avidity in the thymus promote negative (i.e. cell death) instead of positive selection [39]. Experiments in a specific TCR transgenic system (*see* Chapter 3) suggest that intrinsic attributes of the TCR–ligand interaction (i.e. independent from the density of each partner) are the critical factor determining the positive vs. negative selection outcome; in other words, even minute amounts of a high-affinity ligand will trigger deletion. However, within a very narrow intermediate-affinity range, increasing ligand density can switch the outcome of signaling from positive to negative selection [53]. There is also evidence that high-avidity ligands promote Treg differentiation [54]. Deciphering the signals that drive a developing thymocyte toward deletion vs. Treg differentiation is an area of intense investigation.

Multiple studies have examined the consequences of such avidity differences on thymocyte signaling, and observed differences in the activation of Erk and Erk-related MAP kinases, whether in the nature of the kinases being activated, or in the kinetics or topography of their activation [45, 55]. In particular, there is evidence that the conventional MAP kinases Erk1 and Erk2 are required for positive but not negative selection [56]. Among possible nuclear effectors of negative selection, recent findings provide new evidence for the involvement of transcription factors of the Nur77 family [57, 58], and suggest that the transcription factor Schnurri-2 contributes to distinguish positive to negative selection signals [59]. Death effector mechanisms that drive thymocyte deletion involve the pro-apoptotic Bcl2-family protein Bim, whose activity is in part redundant with that of the related protein Puma [60]. Inactivation of both genes impairs negative selection and causes autoimmune manifestations, consistent with the idea of impaired central tolerance.

Whether negative selection happens in a specific thymic compartment has long been debated. It was initially envisioned that, unlike positive selection which occurs in the cortex, negative selection takes place in the medulla. This idea is in line with the fact that medullary epithelial cells express tissue-specific self antigens which contribute to negative selection. However, recent studies have highlighted the possibility of cortical deletion of DP thymocytes. The key observation is that inactivation of the pro-apoptotic molecule Bim causes the appearance of large numbers of DP cells with high expression of genes involved in negative selection (including Nur77) [61]; this suggests that such cells are normally targeted for deletion after undergoing strong TCR signaling at the DP stage (i.e. in the cortex) and rescued from cell death upon Bim disruption.

2.8 Differentiation of $\alpha\beta$ Lineage Cells and Thymic Egress

The selection of conventional TCRαβ thymocytes is accompanied by, and possibly mechanistically coupled to the differentiation into either $CD4^+$ or $CD8^+$ lineages, and pre-programming for cytotoxic

or helper differentiation [62, 63]. Recent work has identified transcription factors important for such lineage differentiation, including the zinc finger transcription factor Thpok for the CD4 lineage, and the Runx protein Runx3 for the CD8 lineage. These factors appear to work in a dual negative regulatory loop that ensures their mutually exclusive expression and commits developing cells to express either CD4 or CD8 but not both. Other transcription factors, including Gata3, Tox and E-box binding proteins E2A and HEB, are required for the differentiation of $CD4^+$ T cells, whereas IRF1, the cytokine signal transducer Stat5 (redundantly with the related protein Stat6) and Ets1 are important for the differentiation of $CD8^+$ T cells [63, 64]. Which signals dictate expression of these transcription factors, and therefore determines lineage differentiation, is not yet fully understood.

The last checkpoint in the development of αβ T cell precursors controls their egress from the thymus. Sphingosine 1 phosphate (S1P) and its receptor S1pr1 are essential for thymocytes to leave the thymus for the bloodstream [65]. The transcription factors Klf2 and Foxo1 are important for expression of S1pr1 and other markers of mature thymocytes [66, 67]; how their expression is determined by intrathymic ligands remains to be fully understood.

2.9 High-Avidity Cells and Acquisition of Effector Properties

Even though CD4 and CD8-lineage conventional thymocytes are functionally "pre-programmed" for helper or cytotoxic differentiation, they exit the thymus as resting cells, i.e. lacking effector functions. They acquire such functions (e.g. expression of cytotoxic enzymes or cytokines) only upon antigen stimulation in peripheral lymphoid organs. In contrast, a small subset of cells become effectors in the thymus [54]. This, to some extent, is the case of Treg precursors displaying high reactivity against MHC-peptide complexes, which start expressing the key effector transcription factor Foxp3 in the thymus. However, specific thymocyte subsets acquire actual effector properties, including NK T cells, characterized by high-level expression of cytokines or cytotoxic enzymes, in response to thymic ligands. As in the case of effector differentiation in the periphery, acquisition of effector functions by thymocytes appears to be associated with their proliferation. Unlike for conventional thymocytes, which are selected by MHC-peptide complexes expressed by the thymic epithelium, selection of and acquisition of effector properties by such "innate immune" thymocytes involve ligands (MHC-like molecules and costimulatory molecules) expressed on DP thymocytes themselves [68].

2.10 Thymic Architecture

Of all the non-T cell types of the thymus (collectively referred to as the stroma), the epithelial component has attracted the most attention [13, 14]. Its critical role is demonstrated by the thymic aplasia and massive disruption of T cell development in the *nude* mouse, caused by a mutation in the gene encoding the transcription factor

Foxn1 that intrinsically disrupts thymic epithelial cell development. Other transcription factors, including HoxA3, have since been shown to be important for the development of the thymic epithelial cells.

Medullary and cortical epithelial cells are functionally distinct and express unique phenotypic markers. We previously mentioned the role of the medullary epithelium in establishing central tolerance; indeed, impairing thymocyte migration to the medulla (by disrupting the gene encoding CCR7) results in defective central tolerance and autoimmune manifestations [69]. The last few years have assigned key functions to the cortical epithelium. Its high-level expression of Notch ligands is essential to the development of early T cell precursors. Cortical epithelial cells express specific proteases or proteasome components which differ from those produced by professional (bone marrow-derived) antigen-presenting cells, generating unique peptide sets essential to the generation of a proper T cell repertoire [70–72]. Last, chemokines secreted by cortical epithelial cells are needed to attract circulating bone marrow precursors to the thymus [20]. The thymus cortex has a highly organized architecture, with specific location for DN thymocytes at distinct stages of their differentiation, and a complex network of interactions between DP thymocytes and cortical cells. New approaches, especially the use of intravital microscopy on thymic "slices" (*see* Chapter 11) have started to unveil the underappreciated dynamics of interactions between thymocytes and the thymic stroma, and the correlations between thymocyte motion and signaling events.

Although their role is not as well characterized, hematopoietic cells residing in the thymus are important for T cell development. In particular, cortical macrophages are thought to be critical to eliminate dead DP thymocytes, whereas medullary dendritic cells contribute to negative selection.

Mirroring the thymocyte dependence from the thymic epithelium, the development of the thymic epithelium itself requires signals from thymocytes. The mechanistic bases for such "cross-talk" have been elucidated in the differentiation of medullary epithelial cells, whose survival and expression of Aire require ligation of TNF-family receptors (RANK and TRAIL) by ligands expressed by CD4 SP thymocytes [41]. This requirement explains the original observations that the thymic medulla fails to develop in mice carrying mutations that prevent the development of mature thymocytes [73, 74]. More recent work has hinted at the possibility that the differentiation of the cortical epithelium be dependent on DP thymocytes, through an indirect mechanism that would involve IL-22 production by epithelial cells [75]. Deciphering the signals that control the development and homeostasis of thymic epithelial cells, and the extent of their "cross-talk" with the thymocyte compartment, are essential goals for strategies aiming a restoring

thymic function in the elderly or in patients whose immune system has been compromised by cancer radiation or chemotherapy.

3 Tracking Developing Thymocytes

Investigating T cell development typically involves a variety of approaches, many of them discussed in this book. Among these, flow cytometry occupies a special place: because of its irreplaceable contribution to define thymic cell subsets and thymocyte developmental stages, flow cytometric analysis is typically an indispensable part of any experimental strategy. Thus, the following pages include a summary of flow-based approaches to explore thymocyte development; the reader is referred to Chapters 4 and 20 (for human cells) for detailed procedures. The simplest and by far most widely used relies on expression of CD4 and CD8 molecules and distinguishes DN, DP, and CD4 and CD8 SP cells. This approach is useful to assess the overall balance of αβ lineage populations (most of which express CD4, CD8 or both), but has limited resolution to dissect selection events, and is not useful to evaluate early differentiation or γδ T cells development.

3.1 Early T Cell Development

Although early thymocytes express neither CD4 nor CD8, it is important to realize that they only form a fraction of DN thymocytes. Thus, identifying early progenitor cells requires additional gating strategies to eliminate the large number of "other" DN cells (e.g. γδ T cells, iNK T cells, B cells, etc.). This is easily done by combining antibodies reacting against each specific lineage (e.g. TCRγδ, CD19 as a B cell marker) labeled with the same fluorochrome, so that all cells expressing any of these markers can be electronically excluded from further analyses. This strategy is of broad applicability to analyze small size subsets as it excludes aggregates that can form during cell preparation.

DN thymocytes are conventionally subdivided into four subsets (DN1-4) based on the expression of CD44 and CD25 (Figs. 2 and 3), although CD117 (cKit) seems to be a more specific marker than CD44. Various strategies have been proposed to dissect the earliest (DN1) subset and identify early T progenitors (ETP) within the so-called DN1a compartment ($CD24^{-}$ $CD117^{hi}$) [18]. There is only imperfect matching between such flow-cytometry "staging" and developmental checkpoints: notably, the T lineage commitment checkpoint occurs as cells down-regulate CD117. Similarly, β-selection occurs in DN3 cells, and thymocytes that have undergone β-selection can usually be identified on the basis of their greater size, expression of intracellular TCRβ molecules, or expression of surface CD27 or CD28 (Fig. 3).

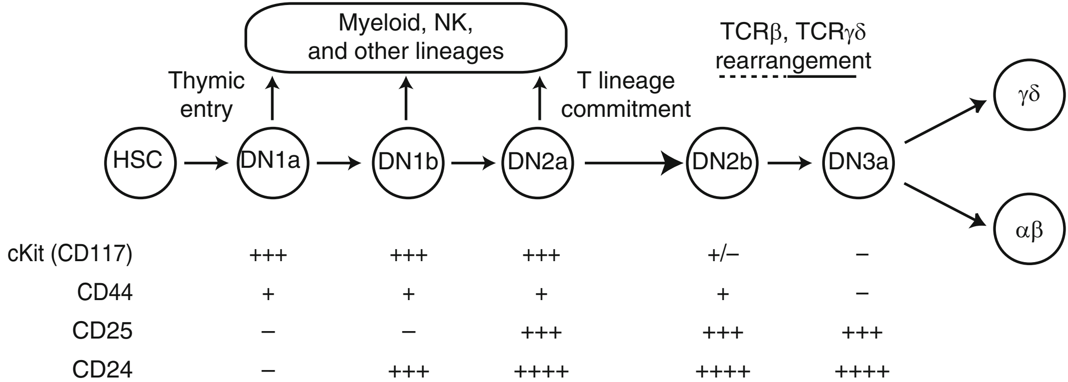

Fig. 3 Early T cell development. Early T cell development stages are schematically depicted, using expression of CD25 and CD44 to define DN1 ($CD25^{-}CD44^{+}$), DN2 ($CD25^{+}CD44^{+}$) and DN3 ($CD25^{+}CD44^{-}$) stages. The DN2 stage is further divided into pre- and post-commitment DN2a and DN2b stages, which can be distinguished by the reduction in CD117 (cKit) expression. At the DN3 stage, successful completion of TCRβ or TCRγ rearrangement distinguishes DN3b cells (not depicted), which can be distinguished from DN3a cells by several markers (*see* Fig. 4)

3.2 αβ T Cell Development

Surface markers useful to analyze the development of conventional αβ lineage cells are summarized in Figs. 3 and 4 and in Table 1. Although the differentiation of $CD4^{+}$ and $CD8^{+}$ lineages is a key outcome of αβ lineage development, expression of CD4 and CD8 molecules is paradoxically of limited interest to distinguish developmental intermediates during the selection of αβ T cells. This is because intrathymic TCR signaling, whether MHC-I or MHC-II induced, can repress expression of *Cd8* genes in thymocytes [76]. While such repression is transient only in MHC-I-specific cells (which eventually re-express CD8 and become CD8 T cells), it results in both MHC-I and MHC-II-restricted thymocytes acquiring a $CD4^{+}CD8^{int}$ "transitional" surface phenotype. Three surface markers, TCR complexes (assessed by staining for TCRβ or CD3ε), CD69 and CD24, provide the best strategies to distinguish selection intermediates. TCRβ expression is the most reliable surface marker for selection, whereas CD24 assesses cell maturation. In most circumstances, CD69 expression provides a faithful readout of TCR signaling in thymocytes (so that $CD69^{hi}$ cells are those undergoing TCR signaling). Consequently, a staining protocol associating CD4, CD8, TCRβ, CD69 and CD24 distinguishes key cell subsets undergoing selection. It has the additional advantage of separating an immature CD8 SP subset, which in most mouse strains contains cells transitioning from the DN4 to DP stage, and appear as TCR^{lo} $CD69^{-}$ $CD24^{hi}$. Other markers can be used to distinguish subsets of SP cells (Table 1). Note that none of these strategies reliably distinguishes MHC-I from MHC-II-signaled thymocytes before they have terminated expression of CD4 or

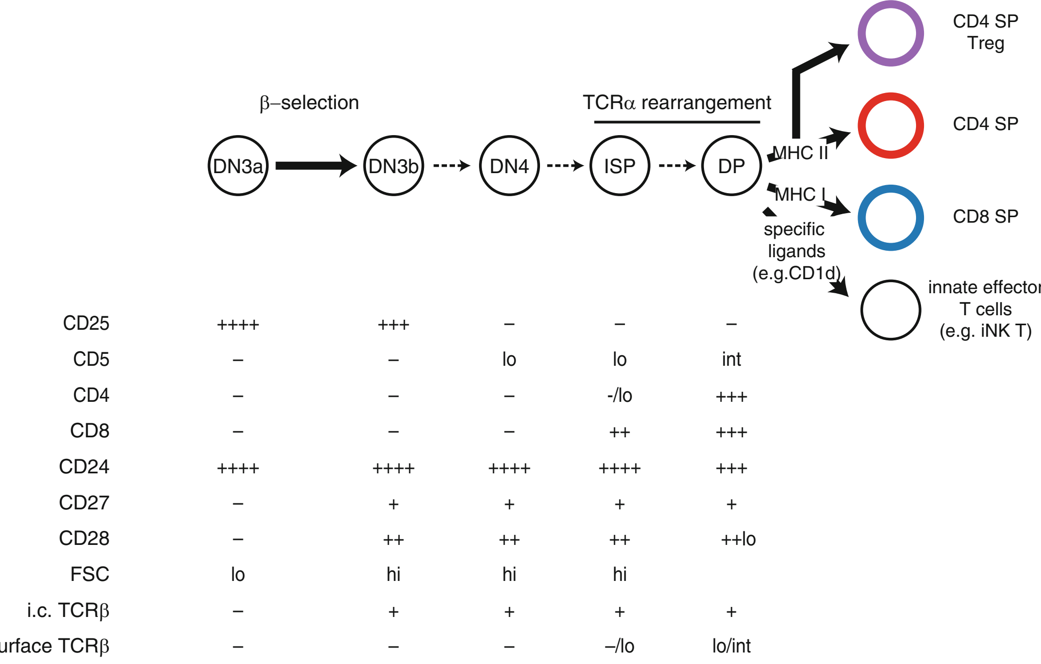

Fig. 4 αβ T cell development. The key developmental stages of the αβ lineage are schematically depicted. Commitment to the αβ lineage occurs during β-selection, following successful rearrangement of TCRβ in DN3 cells, and is accompanied by increased cell size (evaluated by flow cytometry on Forward light Scatter [FSC]), and changes in expression of CD27 and CD28. Subsequent developmental stages and their defining markers are schematically depicted

Table 1
Surface markers in the study of positive selection

Marker	Pre-selection DP	Signaled DP	Selected SP	Mature SP	Comments
CD5	+/++	+++	++++	++++	
CD69	–	++	+++	–/+	
TCRβ, CD3ε	–/+	+++	+++	+++	
IL-7Rα	–	–	+/–	+/++	
CCR7	–	–/+	++	++	
CXCR4	++	+	–	–	
CD4	+++	+++	variable	+++ or –	Depending on MHC restriction
CD8	++++	++++	variable	++++ or –	Depending on MHC restriction
MHC-I	++	–	–	++	
Qa-2	–	–	–	++	Strain-specific

CD8 (as mentioned, the TCR^{hi} CD69^{hi} CD4^{+}CD8^{int} subset includes both MHC-I and MHC-II-restricted cells, and therefore both CD4 and CD8 T cell precursors) or identifies cells targeted for negative selection.

4 Concluding Remarks

This brief overview highlights the tremendous progress made over the past 30 years in elucidating the functions of the thymus and its role in generating and shaping the T cell repertoire. The combination of improved investigative approaches, an ever increasing availability of marker-specific antibodies and the extraordinary versatility of mouse genetics has helped the field build a detailed map to T cell development, delineated essential cell–cell interaction and intracellular signaling pathways. Efforts are underway to fill the many remaining gaps, and to decipher transcriptional circuits directing cell differentiation. Future investigations will build on these advances, and tackle key challenges lying ahead, including understanding the basis for thymic involution (and elaborating strategies for thymic regeneration), and further analyses of repertoire generation.

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Chapter 2

Development of γδ T Cells, the Special-Force Soldiers of the Immune System

David L. Wiest

Abstract

While the functions of αβ T cells in host resistance to pathogen infection are understood in far more detail than those of γδ lineage T cells, γδ T cells perform critical, essential functions during immune responses that cannot be compensated by αβ T cells. Accordingly, it is essential to understand how the development of γδ T cells is controlled so that their generation and function might be manipulated in future for therapeutic benefit. This introductory chapter will cover the basic processes that underlie γδ T cell development in the thymus, as well as the current understanding of how they are controlled.

Key words Gamma-delta T cells, Gamma-delta TCR, V gamma elements, Lineage commitment, Effector fate

1 Introduction

There are two major T lineages marked by the T cell antigen receptor (TCR) complexes they express, αβ and γδ. By analogy to the military, αβ lineage T cells are like conventional soldiers found primarily in lymphoid organs, which can be thought of as military bases where the staging of military operations occur. This is consistent with the 7-day delay required for αβ T cells to mount a primary response to an infection and travel to the site of the battle. In contrast, γδ T cells make up a small proportion of T cells in the peripheral lymphoid organs, and instead predominate in the epithelial tissues that form the inner and outer surfaces of the body [1–3]. Accordingly, γδ cells are much more like special-force soldiers found primarily in the "field," patrolling epithelial barriers and possessing rapid-strike capabilities that do not require priming. Indeed, γδ cells have been implicated in stress-surveillance by rapidly responding to stress-induced proteins on epithelial cells (e.g., Rae1 and H60), which is more consistent with an innate-like mode of function [3]. Nevertheless, γδ T cells are also capable of staging delayed, more classical adaptive-type responses following immunization, including the mounting of recall responses consistent

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_2, © Springer Science+Business Media New York 2016

with the generation of memory [4]. As such, γδ T cells combine attributes of adaptive immunity, encoded in their TCR complexes, with rapid, innate-like capabilities linked to the initiation phase of immune responses. Although the precise role of γδ T cells in immune responses remains unclear at present, these cells perform functions that are at least partially distinct from those of αβ T cells. Indeed, certain bacterial infections (e.g., *Nocardia asteroides*) that are normally cleared in wild type mice are rapidly fatal in mice lacking γδ T cells [5]. Likewise, resistance of neonates to parasitic infection is critically dependent upon γδ T cell function [6]. γδ T cells have also been implicated in the preservation of epithelial barriers and in eradication of cutaneous malignancies [7–9]. Efforts to exploit the function of γδ T cells therapeutically in human disease are now under investigation, as clinical grade agonists for the human Vγ9Vδ2 γδ T cells are currently being tested for various infectious diseases and in cancer [10, 11]. In contrast to their beneficial effects, γδ T cells have also been found to contribute to pathologies. IL-17-producing γδ T cells have been implicated in the pathology of psoriasis [12]. IL-17-producing γδ T cells have also been found at the borders between colon cancer lesions and surrounding normal tissue and have been implicated in disease progression through the recruitment of myeloid-derived suppressor cells [13]. Nevertheless, despite the growing appreciation of the importance of γδ T cell function in disease resistance and pathogenesis, many important questions regarding their generation and function remain unanswered.

2 Antigen Recognition by γδ T Cells

While αβ T cells recognize and respond to proteolytically derived peptide ligands in the context of MHC class I and II, γδ T cells do not recognize ligand in an MHC-restricted manner and instead recognize a far more diverse collection of intact, unprocessed ligands [14]. These include non-classical MHC molecules, heat shock proteins, lipids and stress-induced molecules. In their recognition of unprocessed, intact ligands, γδ T cells are quite similar to immunoglobulins (Ig). Consistent with this notion, as is true for Ig, the lengths of the CDR3 regions of the Vγ and Vδ subunits are quite diverse, as is expected for a structure that is not constrained in recognizing cargo presented by a specific presenting element [15]. The ability to recognize intact antigens confers upon γδ T cells greater flexibility of function.

3 Links Between Vγ Usage and Behavior

γδ T cell development is initiated at the time of seeding of the fetal thymus by progenitors from fetal liver at embryonic day 13 (E13). γδ cells represent about 1–2 % of thymocytes (~1000 γδ TCR+

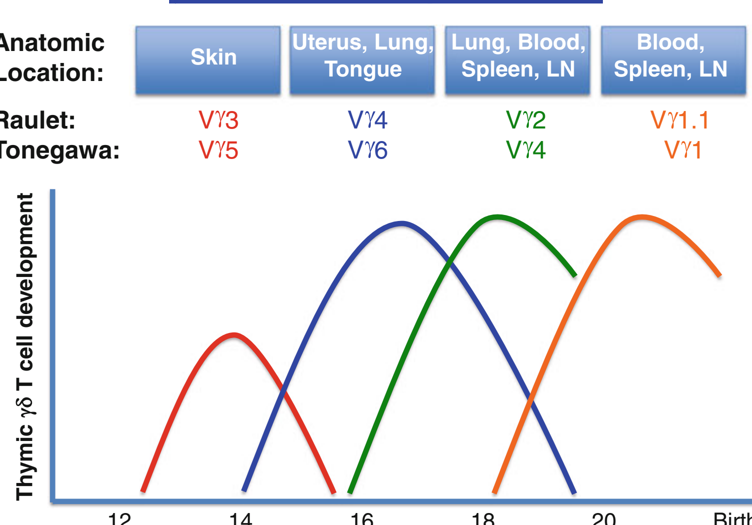

Fig. 1 Timing of developmental waves of Vγ subsets. Schematic of waves of development of Vγ subsets and the anatomical locations to which they home. The Tonegawa and Raulet nomenclature systems for the Vγ are indicated. Development of the Vg7 (Tonegawa)/Vg5 (Raulet) subset, which homes to the gut, is thought to occur extrathymically

cells per thymic lobe) at E14. Their frequency increases to approximately 10 % at E16, following which their representation begins to fall due to the rapid expansion of αβ lineage CD4+CD8+ progenitors. The absolute number of γδ TCR-expressing cells continues to rise during fetal development, reaching more than 30,000 cells per lobe at E18 and beyond [16]. γδ development during fetal life is characterized by waves of progenitors defined by their Vγ usage (Fig. 1). There are two Vγ nomenclature systems that are in use at present, one developed by the Raulet lab and the other by the Tonegawa lab (Fig. 1) [2, 17]. In this chapter, we will be employing the Raulet nomenclature. The first wave of γδ progenitors from E13 to 15 is characterized by Vγ3 usage, and is followed successively by waves defined by Vγ4, Vγ2, and Vγ1.1 (Fig. 1). These waves are significant as there is a strong correlation between these waves of developing γδ T cells defined by Vγ usage and their residence in particular anatomical sites [18, 19] (Fig. 1); however, the molecular basis for this linkage remains poorly understood. One exception is the homing of Vγ3+ dendritic epidermal T cells (DETC) to the epidermis. During selection in the thymus, DETC progenitors upregulate the chemokine receptor, CCR10, which is

Table 1
Commercially available anti-V region antibodies to study mouse γδ T cell subsets

TCR subunit	Ab clone	Source
Vγ1.1	2.11	Biolegend
Vγ1.1 + Vγ1.2	4B2.9	Biolegend
Vγ2	UC3-10A6	Biolegend/Becton Dickinson
Vγ3	536	Biolegend/Becton Dickinson
Vδ4	GL2	Ebiosciences/Becton Dickinson
Vδ6.3	8F4H7B7	Becton Dickinson

required for their trafficking to the epidermis [20]. Consequently, it is possible that selection events in the thymus are responsible for the induction of trafficking molecules that mediate homing of each wave of γδ progenitors, expressing a particular Vγ subunit, to the intended anatomical location (e.g., Vγ4 to the genital tract). Nevertheless, this has not been established for any Vγ subset other than the Vγ3+ DETC. An alternative explanation is that each particular Vγ subset expresses γδ TCR complexes with specificity for ligands expressed in the anatomic location where they reside, and it is this interaction that is responsible for their homing and retention. A number of commercially available anti-Vγ and Vδ antibody reagents have been generated that can be used to monitor the behavior of the aforementioned subsets (Table 1).

4 Development

Phenotypic characterization of γδ cells during fetal ontogeny: Despite substantial effort, the only truly unique identifier of γδ T cell progenitors is the γδ TCR itself. γδ lineage progenitors typically remain CD4−CD8−, although some do acquire expression of CD8 [21]. Immature γδ lineage progenitors are characterized by being CD24+ and as they mature they increase expression of CD45RB and downregulate the expression of CD24 [22, 23]. Upon downregulation of CD24, many γδ T cells in the thymus have acquired the ability to secrete cytokines including interleukin-17 (IL-17) or interferon-γ (IFN-γ) [24]. Cells with the capability of producing IFN-γ are typically CD27+, while those that produce IL-17 are CD27− [25]. An impediment to studying γδ T cell development is that some CD4−CD8− γδ TCR-expressing

progenitors have not irreversibly committed to the γδ fate, and can fate switch to the αβ lineage and develop to the CD4+CD8+ stage upon removal from the selecting milieu in the thymus [26]. It should be noted that γδ T cells do not develop through a CD4+CD8+ intermediate. Consequently, a critical need in the field is a phenotypic marker that distinguishes CD4−CD8− γδ TCR-expressing progenitors that have committed to the γδ fate, from those yet to do so. Indeed, we have recently identified such a marker, CD73. CD73-expressing CD4−CD8− γδTCR+ progenitors remain CD4−CD8− and committed to the γδ fate even upon removal from the selecting milieu, whereas CD73− progenitors retain the ability to switch to the αβ fate [24]. Other markers have been proposed that do not appear to mark γδ lineage commitment. Indeed, through Serial Analysis of Gene Expression (SAGE) performed by the Hayday lab, a γδ-biased gene signature was established; however, while this profile is linked to γδ function it does not mark lineage commitment [27]. Likewise, the transcription factor Sox13 has been reported to be highly enriched in some γδ T cells. Nevertheless, it is now clear that this factor marks only a subset of γδ lineage cells [28].

Cross-talk between αβ and γδ progenitors: γδ T cells influence the development of αβ T cells and vice versa. γδ T cell development precedes that of αβ T cell progenitors by a few days, as the first αβ progenitors competent to undergo intrathymic selection do not appear until ~E16. This delay is critical, as prior to this time, the thymic medulla compartment has not yet emerged in a form capable of supporting negative selection of autoreactive αβ progenitors. Importantly, γδ T cell progenitors play an important role in establishing this capability. Indeed, development of the Vγ3+ DETC subset begins around E14 and these progenitors promote the generation of medullary thymic epithelial cells (mTEC) that express AIRE, and are thus capable of ectopic expression of peripheral antigens [29]. The ability of DETC progenitors to induce AIRE expressing mTEC is mediated by the expression of Rank Ligand on their surface. Lymphoid tissue inducer cells possess the same capability and also contribute to induction of AIRE expression by mTEC. Conversely, αβ lineage progenitors also influence the development of γδ T cells. CD4+8+ αβ lineage thymocytes transcondition developing γδ progenitors by presenting cell surface-bound lymphotoxin β. This is not responsible for γδ lineage commitment, but has been reported to influence their functional competence [27, 30].

Control of αβ/γδ lineage commitment: Compelling evidence exists indicating that the αβ and γδ T cell fates arise from a common progenitor in the thymus, raising the question of how lineage commitment is controlled [31, 32]. While the TCR complexes of these progenitors, the pre-T cell receptor (pre-TCR) and γδ TCR for the

αβ and γδ lineages, respectively, certainly influence lineage choice, the way that they do so remains somewhat controversial. Attempts to explain the role of the TCR in αβ/γδ lineage commitment have been distilled into two basic models, stochastic and instructional. The stochastic model predicts that lineage fate is specified independently of TCR expression and that TCR signals serve only to rescue viability of already committed progenitors, provided the TCR isotype matches the preordained lineage fate [33]. Conversely, the instructional model proposes that TCR signals direct uncommitted precursors to adopt either the αβ or γδ fate [34]. That is, the signals transduced through the pre-TCR or γδTCR actively specify the αβ and γδ fates, respectively. These models share the basic idea that the pre-TCR or γδTCR complexes transduce unique signals inextricably linked to specification of the αβ and γδ fates, respectively. However, these models are not adequate to explain the status of TCR gene rearrangements in αβ and γδ lineage cells, nor do they appropriately explain the lineage infidelity observed in TCR transgenic and gene-targeted mice [21]. To address these inconsistencies, a signal strength model was proposed which posits that strong signaling through a TCR promotes adoption of the γδ lineage, while weaker signals lead to adoption of the αβ lineage, irrespective of the isotype of the TCR complex from which those signals originate [35]. Compelling support for the signal strength model was provided by the demonstration that thymocytes expressing a single γδTCR transgene could adopt either the αβ or γδ fate upon manipulating the γδTCR to transduce weak or strong TCR signals, respectively [36, 37].

While the signal strength model is now widely regarded as providing the best explanation for the role of the TCR complex in lineage commitment, it remains unclear how the γδTCR complex transduces the stronger signals required for adoption of the γδ fate. Ligand stimulation remains a possible mechanism by which the γδ TCR could produce those stronger signals. The role of ligand stimulation in the intrathymic selection of αβ T cell progenitors is well established; however, the role of ligand in regulating the γδTCR signals that specify the γδ fate remains controversial [38, 39]. Based on the restriction of the chain usage and CDR3 sequences of the γδTCR complex that characterizes the DETC subset of γδ T cells, it is likely that their development is ligand-dependent [40]. DETC development requires expression of Skint1, although whether Skint1 functions as a selecting ligand remains to be fully established [41]. Analysis of γδTCR Tg model reactive with the T-10/22 selecting ligand has provided very clear evidence in support of a role for ligand in their selection [37, 42], but it is not clear whether the involvement of ligand is a general phenomenon or only involved in a select few cases. This issue can only be addressed by either identifying the ligand specificities of a large number of γδ progenitors, or, alternatively, through the use of a surrogate for ligand engagement. Interestingly, we have recently

identified a surrogate for ligand engagement, CD73 induction. CD73 is a TCR-ligand inducible surface protein that is expressed on a large fraction of thymic γδ T cells, and nearly all peripheral γδ T cells, indicating that a substantial fraction of developing γδ T cells have encountered ligand [24].

Acquisition of effector fate during development in the thymus. Unlike αβ lineage T cells, which exit the thymus in a naïve state and acquire functional competence in the periphery, many γδ lineage progenitors acquire functional competence prior to exit from the thymus [18]. γδ T cells have been subdivided into effector classes based on the cytokines they produce [43]. γδ cells can adopt an IL-17-producing effector fate linked to the expression of the transcription factor RORγt, the IFN-γ-producing effector fate linked to the expression of the transcription factor Egr3, or an innate effector fate characterized by the expression of the PLZF transcription factor and the simultaneous production of both IFN-γ and IL-4 [44]. The molecular processes underlying specification of these effector fates remain poorly understood, but there are two models attempting to explain how this is controlled and how TCR signaling is involved in the specification process. One model suggests that effector fate is predetermined and linked to Vγ usage but not influenced by TCR signaling, while the other model suggests that effector fate is influenced by TCR signaling (Fig. 2). Supporting evidence for both models can be found. Indeed, a

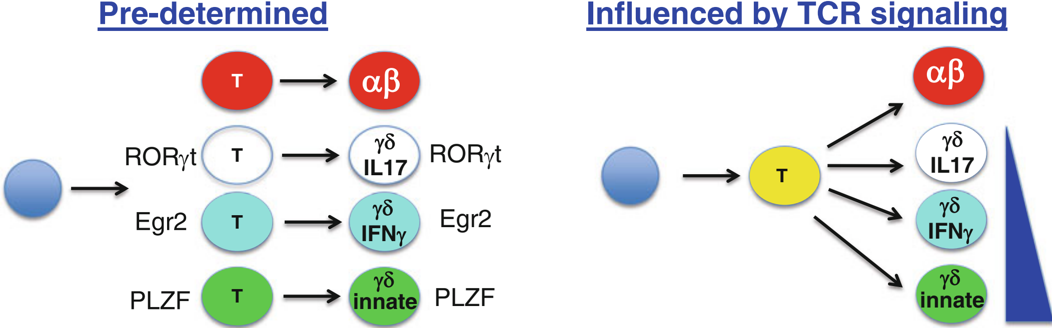

Fig. 2 Models describing the basis for linkage of effector fate to Vγ usage. Many γδ T cells acquire effector function during development in the thymus. Two models have been advanced to explain how this occurs. The first is the "pre-determination model," which suggests that effector fate is pre-programmed and is linked to Vγ usage by virtue of their developmental timing, but is not influenced by TCR signaling. The second suggests that effector fate is influenced by TCR signal strength with a gradation of signaling (schematized by *blue triangle*) ranging from weakest, which specify the IL-17 producing effector fate to the strongest, which specify the PLZF-expressing innate effector fate associated with co-production of IL-4 and IFNγ

recent report indicated that immature CD24 high γδ progenitors exhibited evidence of predetermined effector fate, because these immature cells expressed elevated levels of the effector fate-specifying transcription factors listed above, and this was linked to particular Vγ [45]. For example, Vγ2+ cells, which are associated with IL-17 production, exhibited elevated RORγt levels, while Vγ1.1+ cells, which are linked to IFN-γ production, exhibited elevated Egr3 levels [45]. Predetermination predicts that this link between the effector fate-specifying transcription factor and the Vγ region should be retained throughout maturation. Nevertheless, our recent analysis suggests that this linkage is severed during maturation, which is inconsistent with predetermination, and suggests that TCR signaling influences fate [24]. There is additional evidence suggesting that TCR signal strength can influence effector fate. For example, the γδ subset reactive with the non-classical MHC-I molecule, H-2T10/22 become IFNγ producers when exposed to ligand (stronger signal) and IL-17 producers in the absence of ligand (weaker signal) [46]. Similarly, the DETC subset of Vγ3-expressing γδ cells adopt the IFNγ-producing effector fate and home to the skin in response to exposure to presumptive ligand, Skint1, but become IL-17 producers and are diverted to the uterus in the absence of Skint1 [44]. Thus, while the role of γδ TCR signaling in adoption of γ effector fate in the thymus remains unclear, there is evidence that TCR signaling is able to exert some influence over this process.

5 Conclusion

For many years, γδ T cells were considered to be few in number and of questionable importance to host defense; however, in the last 10–15 years, the importance of γδ T cell function in immune responses has become abundantly clear, justifying the efforts to gain insight into how γδ development and specification of effector fate are controlled. As a result of those efforts an understanding is beginning to emerge that specification of at least some γδ populations is dependent on more intense and/or prolonged TCR signals and that these signals also influence effector fate specification. Nevertheless, many important questions remain to be addressed. How is the linkage between Vγ usage and effector fate accomplished? Why do particular Vγ home to characteristic anatomic locations? And, finally, it is becoming clear that some γδ T cells emerge from the thymus capable of rapid innate-like responses, while others require prior stimulation to acquire effector function [47]. It will be important to understand the origins of these modes of function as they almost certainly make distinct contributions to host defense.

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Part II

Core Approaches and Strategies

Chapter 3

Genetic Tools to Study T Cell Development

Thomas Ciucci, Melanie S. Vacchio, and Rémy Bosselut

Abstract

Genetics tools, and especially the ability to enforce, by transgenesis, or disrupt, by homologous recombination, gene expression in a cell-specific manner, have revolutionized the study of immunology and propelled the laboratory mouse as the main model to study immune responses. Perhaps more than any other aspect of immunology, the study of T cell development has benefited from these technologies. This brief chapter summarizes genetic tools specific to T cell development studies, focusing on mouse strains with lineage- and stage-specific expression of the Cre recombinase, or expressing unique antigen receptor specificities. It ends with a broader discussion of strategies to enforce ectopic lineage and stage-specific gene expression.

Key words Cre recombinase, Genetic strategies, Lineage-specific gene disruption, TCR transgenic mice, Deletion reporter genes

1 Introduction

Perhaps more than any other aspect of immunology, the study of T cell development is intimately connected to mouse genetics. Early discoveries elucidating the genetic bases for lymphocyte responsiveness and tumor rejection proved instrumental for the emergence of key concepts of T cell biology, including MHC restriction and T cell selection. The advent of directed homologous gene recombination has paved the way to the study of mechanisms of antigen receptor rearrangement and has been essential to decipher signaling and differentiation in developing thymocytes. Much is expected from new approaches to genetic manipulation, including those using the bacteria-derived CRISPR-Cas9 system [1, 2]. This brief chapter provides specific information on genetic tools and strategies currently available to study mouse T cell development; the reader is referred to Chapter 1 for information on specific developmental stages.

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_3, © Springer Science+Business Media New York 2016

2 Lineage- and Stage-Specific Gene Disruption

The development of mouse strains expressing the Cre recombinase at distinct stages of T cell development, using expression cassettes specifically active in the T lineage, has considerably facilitated genetic studies of T cell development. A selection of strains useful to target Cre activity to specific developmental stages is presented in Table 1. Several considerations affect the choice of a "deleter" strain. First is the developmental timing of expression of the Cre recombinase, schematically indicated in Table 1. It is important to verify the kinetics of gene inactivation on a case-by-case basis. The actual timing of loss-of-function depends not only on the gene "accessibility" to Cre, which controls actual DNA excision, but also on downstream parameters, such as mRNA and protein half-life if the gene is expressed at the time of deletion. Deletion "reporters" (e.g. Rosa26 derivatives, available from commercial repositories) [21] are useful to identify cells having undergone Cre activity. However, there is often imperfect matching between reporter expression and deletion of the gene of interest, especially at the developmental time where Cre expression is initiated. Thus, assessing actual expression of the gene of interest, which can be done by staining (including intracellular staining) and flow cytometry for an ever greater number of protein-encoding genes, or by western blotting, remains the standard to evaluate gene deletion efficiency.

Because of the possibility of off-target effects of Cre, the frequency of which appears to be correlated to the level of Cre protein expression, breeding strategies should include the generation of Cre-expressing controls that do not carry the homozygous floxed allele [9, 22].

The intensive cell proliferation characteristic of early thymocyte development raises issues specific of this stage. If inactivation of the target gene results in a survival or proliferation disadvantage, heterogeneity in the timing of deletion may result in compensatory proliferation of cells ("escapees") that have not undergone deletion, which in turn masks the phenotypic consequences of deletion. The most appropriate remedy to this situation is to place mutant cells in competition with wild-type thymocytes, which can be done in vivo in mixed bone marrow chimeras (*see* Chapter 9). In these conditions, the wild-type cells will efficiently compete for proliferation-driving ligands (e.g. IL-7) and keep in check the compensatory expansion of the "escapee" population.

3 Fixing TCR Specificity to Analyze Selection Events

The diversity of TCR specificities expressed in αβ lineage thymocytes (typically as many as there are thymocytes at steady state, i.e. >10^8 in a typical laboratory mouse) poses daunting challenges for

Table 1
Cre-expressing strains

Promoter or enhancer	Reference	Cre expression		Commercial[a]	Comments
		Range	Onset		
Vav1	[3]	All hematopoietic cells	HSC	Jax	
Tie2	[4]	All hematopoietic cells	HSC	Jax	Also endothelial cells
Rag1	[5]	Rag1-expressing B and T cell precursors			
Il7r (IL-7Rα)	[6]	B and T cells	BM lymphoid progenitors		
Lck, proximal promoter[b] (Wilson)	[7]	T lineage cells[c]	DN2	Taconic	
Lck, proximal promoter[b] (Marth)	[8]	T lineage cells[c]	DN3	Jax	Cre expression, lower and delayed relative to Wilson strain [9]. Potential for incomplete deletion
CD2	[3]	T and B cells[c]	DN2	Jax	
Ptcra (Pre-Tα)	[10]	T lineage cells	DN2a		
Cd4	[7]	αβ lineage only[c]	DN3 to DP, therefore deleting in all αβ T cell precursors	Taconic	
Cd8 E(III)	[11]	αβ lineage only	ISP-DP		
Cd8 E(I)	[12, 13]	CD8⁺ T cells only	CD24^lo CD8 SP thymocytes		
Lck (distal)	[14]	T cells	T cells		Higher in SP thymocytes and T cells
CD2	[15]	T cells	Mature thymocytes to Naïve T cells		Expression pattern unrelated to other *CD2*-based constructs. Incomplete in CD4⁺ T cells

(continued)

Table 1 (continued)

Promoter or enhancer	Reference	Cre expression		Commercial[a]	Comments
		Range	Onset		
Cd4-Thpok	[16]	$CD4^+$ T cells			Not active in DP thymocytes; strongly specific for $CD4^+$ T cells, but variegated expression
Ox40 (*Tnfrsf4*)	[17, 18]	Activated $CD4^+$ T cells		Jax	
Gzmb	[19]	Activated $CD8^+$ T cells		Jax	
Cd4 (ERt2 Cre)	[20]	$CD4^+$ T cells			Tamoxifen-inducible Cre

[a]Jax: Jackson laboratories; Taconic: Taconic Farms

[b]Deletion with both *Lck*-Cre strains, notably that developed by Hennet et al. [8], tends to remain incomplete until the DP stage, facilitating compensatory expansion of cells that have escaped deletion of genes involved in survival or proliferation [9]

[c]See comparison in the study by Shi and Petrie [9]

Abbreviations: *DN*: $CD4^-CD8^-$ thymocytes; *DP*: $CD4^+CD8^+$ thymocytes; *HSC*: hematopoietic stem cells

analyses of T cell differentiation. Despite major progress in mapping the T cell repertoire of naïve T cells (i.e. those that have not yet expanded in response to antigenic stimulation) [23], it is not yet possible to track the development of antigen-specific thymocytes in a truly wild-type thymus. In addition, distinct TCR gene rearrangement events can give rise to receptors recognizing the same antigen, often with distinct avidities. The latter property has important consequences for investigations of gene function in developing thymocytes. Indeed, compensatory changes in the selected repertoire can mask the cell-intrinsic impact of genes involved in TCR-induced signaling or differentiation (e.g. selection of αβ T cells).

To overcome these limitations, it is possible to force thymocytes to use a defined TCR specificity. This is traditionally performed by using mice expressing a transgenic TCR, initially obtained by microinjecting cDNA for TCRαβ or TCRγδ chains with known specificity, driven by appropriate T cell-specific promoters [24]. Multiple such lines have been developed over the last 25 years. Specific information on a few lines that have been extensively used in the study of intrathymic T cell selection is provided in Tables 2 and 3. However,

Table 2
MHC-I-restricted TCR transgenic strains

Transgene	Restriction	Antigen	Chains	Comments	References
OT-I	H-2K^b	Ovalbumin 257–264 (SIINFEKL)	Vα2, Vβ5	Extensively studied for analyses of thymocyte responsiveness to MHC-peptide ligands [25, 26]	[25, 27]
HY	H-2D^b	Smcy (*Kdm5d*) 738–746 (KCSRNRQYL) [28]	Vβ8	Recognized by clonotype-specific antibody T3.70 [29]	[30]
2C	H-2K^b	Discussed in ref. 31	Vα3, Vβ8	H-2L^d alloreactive. Recognized by clonotype-specific antibody 1B2 In-depth structural studies of binding to MHC-peptide [31]	[32, 33]
P14	H-2D^b	LCMV gp33 33–41 (KAVYNFATC)	Vα2, Vβ8	Extensively used for studies of CD8 T cell effector responses.	[34]
F5	H-2D^b	Influenza Virus Nucleoprotein 366–374 (ASNENMDTM)	Vα4, Vβ11		[35]

many more TCR transgenic lines have been generated and used in a variety of studies of T cell function during physiological and pathological responses (e.g. refs. 45–47).

An important condition to the success of this strategy is that cells expressing the transgenic TCR do not express other TCR chains generated by rearrangement of endogenous loci. That is only partly the case. Expression of a rearranged TCRβ chain (whether endogenous or transgenic) prevents further rearrangement at the TCRβ loci (a phenomenon called allelic exclusion) [48] and at the TCRγ loci (isotypic exclusion) [49]. However, TCRβ allelic exclusion is not perfect; as a result, in many TCR transgenic strains, small fractions of thymocytes express both the transgenic and an endogenous TCRβ chain. In addition, there is no allelic exclusion at the TCRα locus, so that expression of a transgenic TCRαβ does not guarantee absence of endogenous TCRα gene rearrangement, which has potential implications for

Table 3
MHC-II-restricted TCR transgenic strains

Transgene	Restriction	Antigen	Chains	Comments	References
OT-II	I-A^b	Ovalbumin 323–339 (ISQAVHAAHAEINEAGR)	Vα2, Vβ5	Core recognition ovalbumin sequence at residues 329–337 [36]	[37]
DO11.10	I-A^d	Ovalbumin 323–339 (ISQAVHAAHAEINEAGR)	Vα13, Vβ8	Recognized by clonotype-specific antibody KJ126. Core recognition ovalbumin sequence at residues 329–337 [36]. Promotes positive selection in H-2^b mice (selected by I-A^b).	[38]
AND	I-E^k	Pigeon Cytochrome c 88–104 (KAERADLIAYLKQATAK)	Vα11, Vβ3	TCRα and TCRβ chain originated from two distinct T cell clones (TCRβ chain identical to 5C.C7 TCR). Promotes positive selection in H-2^b mice (selected by I-A^b), and more efficiently in mice carrying one allele of I-A^b and I-E^k (H-2^{bxk}). Promotes negative selection in mice expressing I-A^s, and incompletely in mice homozygous for I-E^k [39].	[40]
5C.C7	I-E^k	Pigeon Cytochrome c 88–104 (KAERADLIAYLKQATAK)	Vα11, Vβ3	TCRβ chain identical to AND TCR. Promotes positive selection in mice expressing I-E^k and negative selection in mice expressing I-A^s. Not selected by I-A^b [39].	[41, 42]
3A9	I-A^k	Hen Egg Lysozyme (HEL) 46–61 (NTDGSTDYGILQINSR)	Vα3, Vβ8	Used in negative selection studies [43]	[44]

negative selection [50]. The frequency of such "secondary" TCRα and TCRβ rearrangements depends on the specific TCR transgenic strain, and can only be evaluated on a case-by-case basis. Introducing mutations in the *Rag1* or *Rag2* genes [by intercrossing with lines carrying such mutations [51, 52], which are available from commercial repositories] prevents any endogenous TCR gene rearrangement and eliminates the potential for development of such cells.

While the choice of a specific model is dictated by the question being examined, one specific approach deserves mention here because of it usefulness to study $CD4^{+}CD8^{+}$ ("double positive," DP) thymocyte signaling [25, 26]. For MHC I-induced selection, it is possible to manipulate MHC-I peptide binding in fetal thymic organ cultures (FTOC, *see* Chapter 12) when epithelial cells lack β2 microglobulin molecules or the TAP transporters needed for MHC-I peptide loading; in such circumstances, addition of peptides and exogenous β2 microglobulin to the FTOC allow partial reconstitution of MHC-I-peptide complexes and intrathymic signaling. Many studies have used this approach with the OT-I TCR (*see* Table 2). Derivatives of this peptide have been generated that promote positive or negative selection, making this system very useful to study relationships between intrathymic signaling and developmental outcomes.

The conventional approach to TCR transgenesis has important limitations. It is tedious and costly to introduce a TCR transgene on an otherwise compound mutant background. Because they are not expressed from the endogenous corresponding loci, most TCR transgenes are expressed prematurely, resulting in multiple unwanted effects. Last, the broad expression of transgenic TCRs in thymocytes disrupts the physiological signaling balance in the thymus, by generating an artificial situation in which all thymocytes share the same MHC-peptide ligands, which then become limiting [53]. The latter issue can be dealt with by reducing the frequency of transgenic precursors using a mixed bone marrow chimera approach. Strategies to address the first two limitations are summarized below.

Retroviral transduction can avoid costly and lengthy breeding of TCR transgenic strains. It involves transducing bone marrow precursors with a retroviral vector expressing the TCR chains and a fluorescent marker to identify transduced cells. Vectors expressing both chains of a TCR dimer from a monocistronic mRNA are particularly useful [54]. Retroviral transduction strategies do not avoid premature TCR expression (see below). Of note, using recipient mice lacking their own T cells (e.g. deficient for Rag genes) has the potential to result in spurious developmental effects that should be carefully considered during experiment design [55].

Premature TCR expression has been one of the most vexing limitations of TCR transgenes, especially for the study of negative

selection. Indeed, TCRαβ engagement by high-avidity ligands in early thymocytes results in their lineage redirection toward a γδ-like fate (even though they continue expressing the transgenic αβ TCR), and therefore hampers the study of negative selection itself [56, 57]. More physiological approaches to negative selection have been developed, based on the Aire-dependent activity of tissue-specific promoters in medullary epithelial cells. One specific strategy uses two transgenes to express antigen and TCR: a transgenic TCR specific for a known antigen, and a transgene expressing this antigen under the control of the rat insulin promoter (RIP), which in the thymus targets expression of specific antigens to medullary epithelial cells [43, 45, 58]. In such settings, premature expression of the TCR transgene has a lesser impact on T cell development as transgenic thymocytes do not encounter negatively selecting ligands until they reach the medulla. However, the outcome of these experiments varies with the level of expression of antigen in the specific system being used [59].

In addition, strategies have been devised to improve the timing of TCR expression. The most successful so far relies on the expression of two separate transgenes, one encoding the TCRβ chain, and a "conditional" transgene encoding the TCRα chain, whose expression is normally prevented by a strong "floxed" transcription termination signal located upstream of the coding sequence [60]. Removing the floxed sequence, using a Cre deleter active in DP thymocytes (e.g. *Cd4*-Cre), allows a timely expression of the TCRα chain, and therefore of TCRαβ complexes. Similar strategies have been developed for retroviral vectors [61].

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Chapter 4

Assessment of T Cell Development by Flow Cytometry

Jan Y.M. Lee and Paul E. Love

Abstract

T cell development is a complex multistep process that requires the coordinated activation of distinct signaling responses and the regulated progression of developing cells (thymocytes) through key stages of maturation. Although sophisticated techniques such as fetal thymus organ culture, in vitro thymocyte culture, and multi-parameter flow cytometric analysis are now widely employed to evaluate thymocyte maturation by experienced laboratories, defects in T cell development can usually be identified with more simplified screening methods. Here, we provide a basic protocol for assessment of T cell development that will enable laboratories with access to a four parameter flow cytometer to screen mouse strains, including those generated from embryonic stem cells with targeted gene mutations, for thymocyte maturation defects.

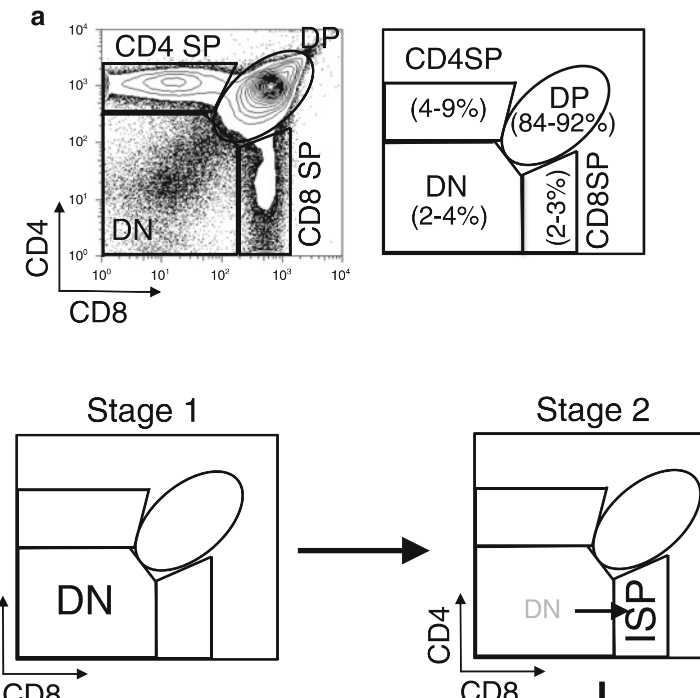
Key words T cell development, Thymocytes, T lymphocytes, Flow cytometry, Antibody staining, Intracellular staining, αβ T cells, γδ T cells, NKT cells, Regulatory T cells

1 Introduction

T cell development takes place throughout life, beginning during late embryogenesis and continuing into adulthood. T cell progenitors originate in the fetal liver or in the adult bone marrow then migrate to the thymus where they mature, acquire functional competence, and eventually leave the thymus and populate peripheral lymphoid organs [1–3].

Once T progenitor cells reach the thymus they progress through several discrete stages of development (Fig. 1). The maturation status of the major subset of T cells in humans and mice [designated αβ T Cell Receptor (αβ TCR)$^{+}$ T cells] can be generally assessed by staining for two cell surface molecules (CD4 and CD8). The most immature thymocytes do not express CD4 or CD8 and are designated double negative (DN). DN thymocytes develop into CD4^{+}CD8^{+} (double positive, DP) thymocytes which comprise the majority (approximately 80–90 %) of cells in the thymus after going through a transitional CD4^{-}CD8^{+} Single Positive

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_4, © Springer Science+Business Media New York 2016



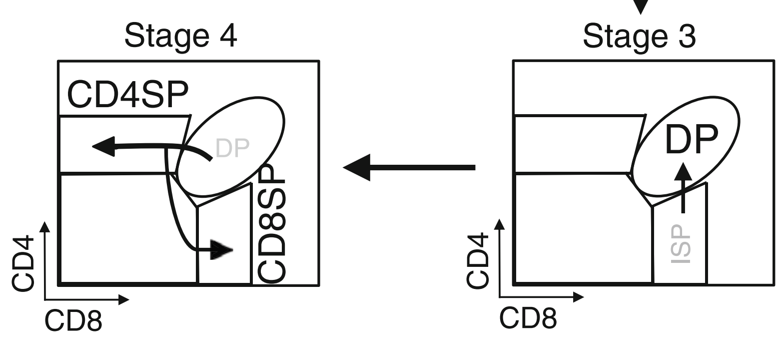
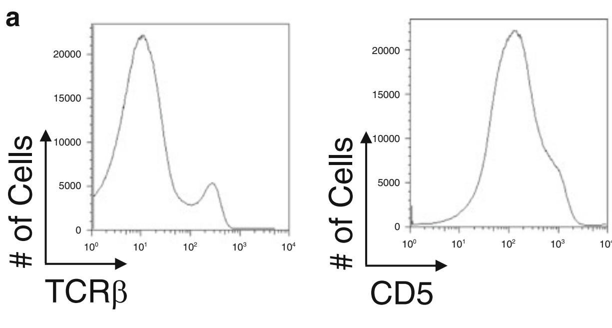


Fig. 1 Major stages of thymocyte development. Thymocytes are stained with anti-CD4 and anti-CD8α. (**a**) The classic "bird" profile of thymocytes stained with anti-CD4 and anti-CD8α, showing the four major populations (*DN* double negative, *DP* double positive, *CD4 SP* CD4 single positive, and *CD8 SP* CD8 single positive). Also shown is the expected percentage of each population for a wild-type mouse thymus. (**b**) Stage 1: $CD4^{-}CD8^{-}$ (*DN* double negative); Stage 2: $CD4^{-}CD8^{+}TCR\beta^{low/-}$ (*ISP* immature CD8 single positive); Stage 3: $CD4^{+}CD8^{+}$ (*DP* double positive); Stage 4: $CD4^{+}CD8^{-}$ (*CD4 SP* CD4 single positive) or $CD4^{-}CD8^{+}$ (*CD8 SP* CD8 single positive). Note that DP thymocytes transition through a $CD4^{+}CD8^{low}$ intermediate stage before becoming either CD4 SP or CD8 SP



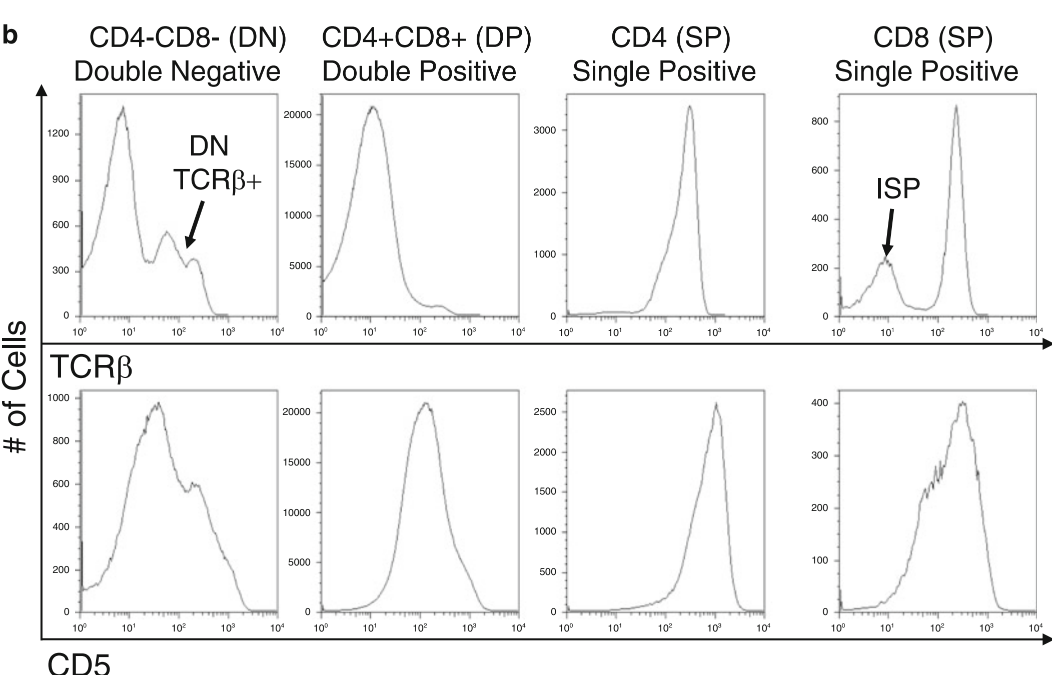


Fig. 2 TCRβ and CD5 surface expression on thymocytes. Thymocytes are stained with anti-CD4, anti-CD8α, anti-TCRβ, and anti-CD5. (**a**) TCRβ and CD5 levels on total thymocytes. (**b**) TCRβ and CD5 levels on DN, DP, CD4 SP, and CD8 SP thymocyte populations. The TCRβ$^{low/-}$ cells in the CD8 SP population are ISPs

(ISP) stage. The final stage of development results in downregulation of either CD4 or CD8 resulting in the generation of "helper" CD4$^+$CD8$^-$ (CD4 Single Positive, CD4 SP) or "cytotoxic" CD4$^-$CD8$^+$ (CD8 Single Positive, CD8 SP) T cells. Two other markers that are commonly used to evaluate thymocyte maturation are TCRβ and CD5 (Fig. 2).

DN thymocytes are typically divided into four sub-groups on the basis of CD25 and CD44 surface expression that represent progressive stages of maturation: CD44$^+$CD25$^-$ (DN1), CD44$^+$CD25$^+$ (DN2), CD44$^-$CD25$^+$ (DN3), and CD44$^-$CD25$^-$ (DN4) [4] (Fig. 3). At the earliest DN1 stage, thymocytes contain

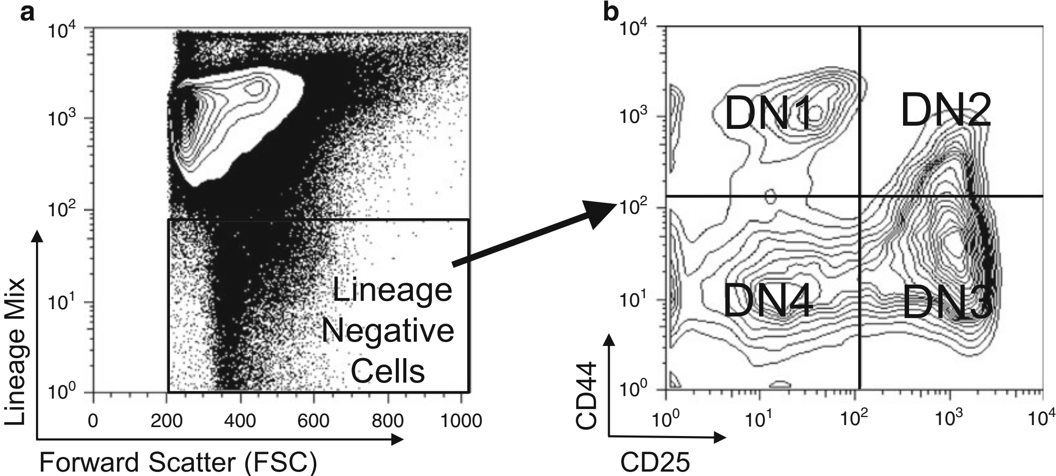


Fig. 3 Stages of double negative (DN) thymocyte development. Thymocytes are stained with anti-CD25, anti-CD44, and a lineage mix containing the following markers: anti-CD4, anti-CD8α, anti-TCRβ, anti-TCRγδ, anti-CD19, anti-NK1.1, anti-DX5, anti-Gr1 (Ly6C/G), anti-CD11b, and anti-Ter119 to facilitate gating on DN cells (*see* **Note 1**). All the antibodies used in the lineage mix are conjugated to the same fluorochrome. (**a**) Shown is a Lineage Mix vs. Forward Scatter (FSC) plot of total thymocytes and the gate used to analyze DN cells. (**b**) Gating on thymocytes that are negative for the lineage mix, the DN subsets are revealed by the CD44 vs. CD25 plot

clonotypic α and β TCR genes in the germline configuration [5]. Rearrangement of the TCRβ locus begins at the DN2 stage and continues in DN3 cells. Productive (V-(D)-J) rearrangement of TCRβ results in the surface expression of a precursor form of the mature αβTCR (designated the pre-TCR) which consists of TCRβ chain paired with the invariant pre-Tα chain in addition to the CD3 signal transducing subunits. Expression of and signaling through the pre-TCR triggers DN3 cells to transition first to the DN4 stage and then through an transitional Immature ($TCR\beta^{low/-}CD24^{hi}$) CD8 Single Positive (ISP) stage on their way to becoming DP thymocytes (Fig. 4) [6, 7]. TCRα locus rearrangement begins in DP thymocytes and the productive rearrangement of TCRα results in surface expression of the mature αβTCR complex. DP thymocytes are subjected to a “selection” process on the basis of the specificity of their TCR for self-ligands that promotes the survival and differentiation of functionally competent cells (positive selection) and triggers the death of non-self-reactive cells (non-selection) as well as overtly auto-reactive cells (negative selection) [8, 9]. Signaling through the TCR complex on DP thymocytes regulates selection and results in upregulation of the TCR as well as the surface proteins CD69 and CD5 which can be used to monitor thymocyte activation and maturation at the DP stage (Fig. 5). Newly generated CD4 SP and CD8 SP thymocytes are functionally immature and express high levels of CD24 (Fig. 6). The end product of the αβ differentiation pathway is the generation

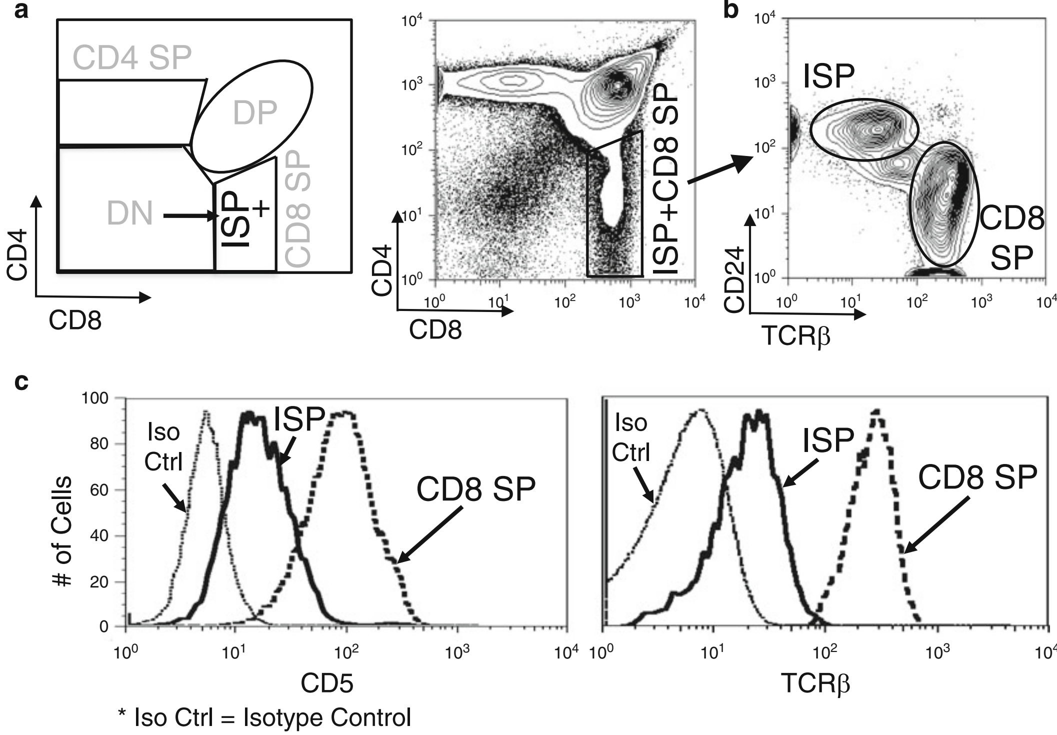


Fig. 4 Immature CD8 single positive thymocytes (ISPs) are intermediates between the DN and DP stages. Thymocytes are stained with anti-CD4, anti-CD8α, anti-TCRβ, and anti-CD24 (HSA) or anti-CD5. (**a**) Focusing on the ISP/CD8 SP gate of the thymus "bird" profile. (**b**) ISPs can be distinguished from more mature CD8 SP thymocytes by staining for CD24 and TCRβ. ISPs are $CD24^{hi}TCR\beta^{low/-}$ whereas CD8 SP cells are $CD24^{int/low}TCR\beta^{hi}$ (Gated on CD8 SP). (**c**) ISPs express low surface levels of CD5 and TCRβ compared to more mature CD8 SP thymocytes

of mature functional $TCR\beta^{hi}CD24^{low}$ CD4 SP and CD8 SP thymocytes that are exported to the periphery (Fig. 7).

Peripheral CD4 SP and CD8 SP that have not encountered antigen are referred to as "naïve" and can be identified by their $CD44^{low}$ $CD62L^{hi}$ phenotype. After activation by antigen stimulation, peripheral T cells acquire either a central memory ($CD44^{hi}$ $CD62L^{hi}$) or effector memory ($CD44^{hi}$ $CD62L^{low}$) phenotype [10] (Fig. 8).

In addition to "conventional" CD4 SP and CD8 SP T cells, three less abundant specialized groups of T cells also develop in the thymus. Natural Killer T (NKT) cells express a restricted αβTCR that recognizes self or foreign lipids/glycolipids bound to the non-polymorphic CD1d molecule [11]. NKT cells can be either CD4 SP or DN and are detected by staining with α-galactosylceramide loaded CD1d tetramers (Fig. 9). Regulatory T cells (Tregs) are suppressive CD4 SP T cells that maintain tolerance to self antigens [12]. Tregs in the thymus and periphery can be detected by CD25

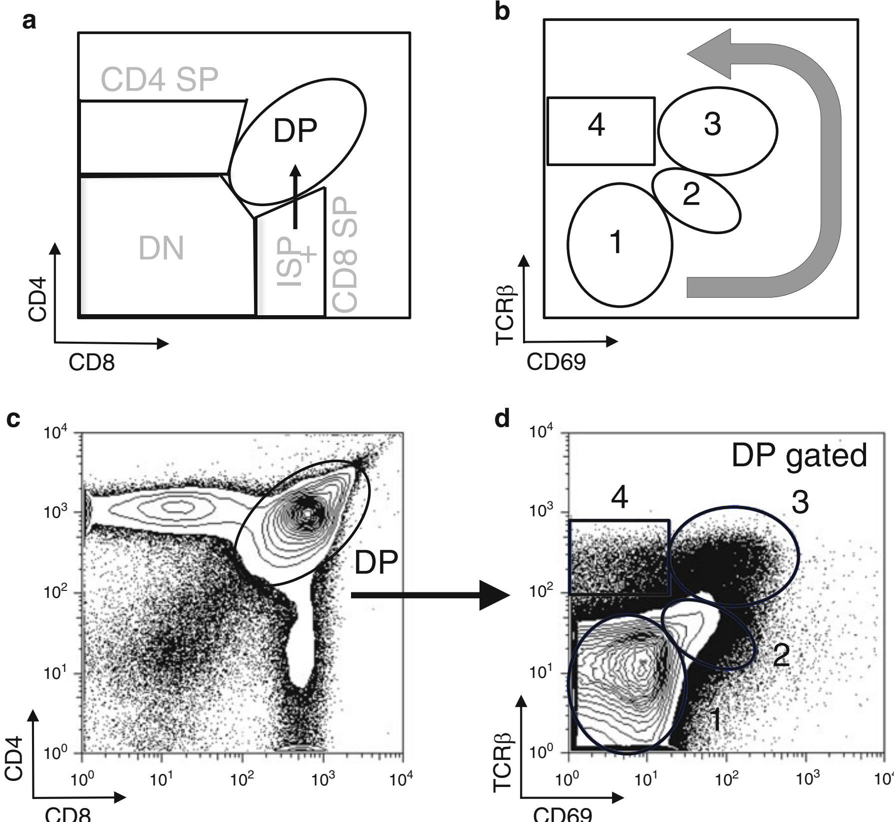


Fig. 5 Assessment of Double Positive (DP) thymocyte development. Thymocytes are stained with anti-CD4, anti-CD8α, anti-TCRβ, and anti-CD69. (**a**) Focusing on the DP population in the thymus "bird" profile. (**b**) Gating on the DP population, the four stages of DP development can be visualized by TCRβ vs. CD69 expression: Stage 1, $TCR\beta^{low/-}CD69^{low/-}$; Stage 2, $TCR\beta^{low}CD69^{low}$; Stage 3, $TCR\beta^{hi}CD69^{hi}$; Stage 4, $TCR\beta^{hi}$ $CD69^{low}$. (**c**) CD4 vs. CD8 plot of total thymocytes as depicted in (**a**). (**d**) TCRβ vs. CD69 plot of DP thymocytes as depicted in (**b**)

surface expression and by intracellular staining for FoxP3, a transcription factor essential for their development (Fig. 10). Finally, γδ T cells arise from immature DN thymocytes that have productively rearranged the TCRγ and TCRδ loci and that express a distinct γδTCR complex in lieu of the αβTCR [13]. Most γδ T cells remain $CD4^{-}CD8^{-}$ and make up a small percentage of peripheral T cells (Fig. 11).

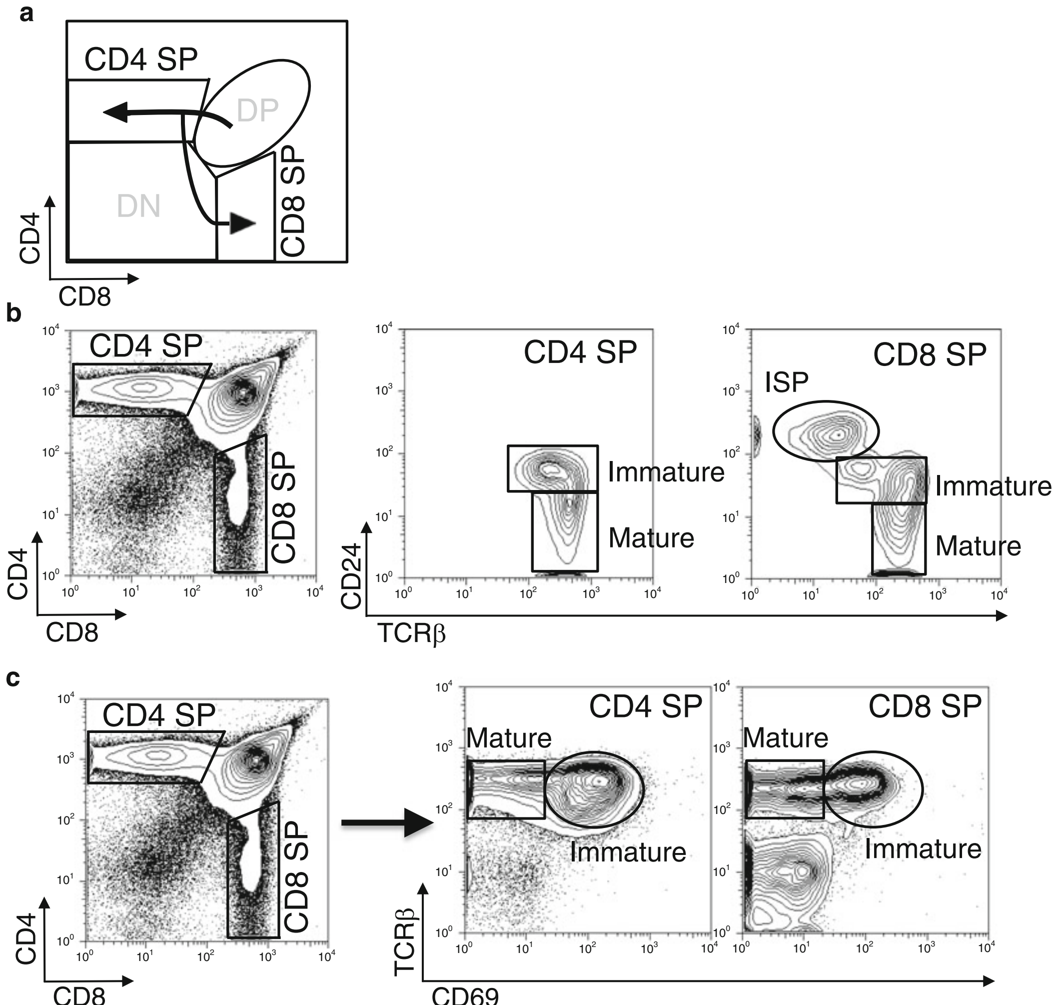


Fig. 6 Assessment of Single Positive (SP) thymocyte maturation. Thymocytes are stained with anti-CD4, anti-CD8α, anti-TCRβ, and anti-CD24. (**a**) Focusing on the CD4 SP and CD8 SP thymocyte populations. (**b**) Gating on either CD4 SP or CD8 SP population, one can differentiate between immature (TCRβ^{hi}CD24^{hi}) or mature (TCRβ^{hi}CD24^{low}) SP thymocytes. Note that in the case of CD8 SP, the ISPs are also visible as TCRβ$^{low/-}$CD24^{hi} cells. (**c**) CD69 can also be used as a maturation marker. Gating on CD4 SP or CD8 SP population, immature SP cells are TCRβ^{hi}CD69^{hi} while mature SP cells are TCRβ^{hi}CD69$^{low/-}$

2 Materials

2.1 Removal and Preparation of Lymphoid Organs for Flow Cytometry

1. Refrigerated centrifuge capable of 450×*g* (e.g., Sorvall® Legend RT).
2. Hemocytometer (for cell counting).
3. Light microscope.
4. Scissors and forceps for dissection.

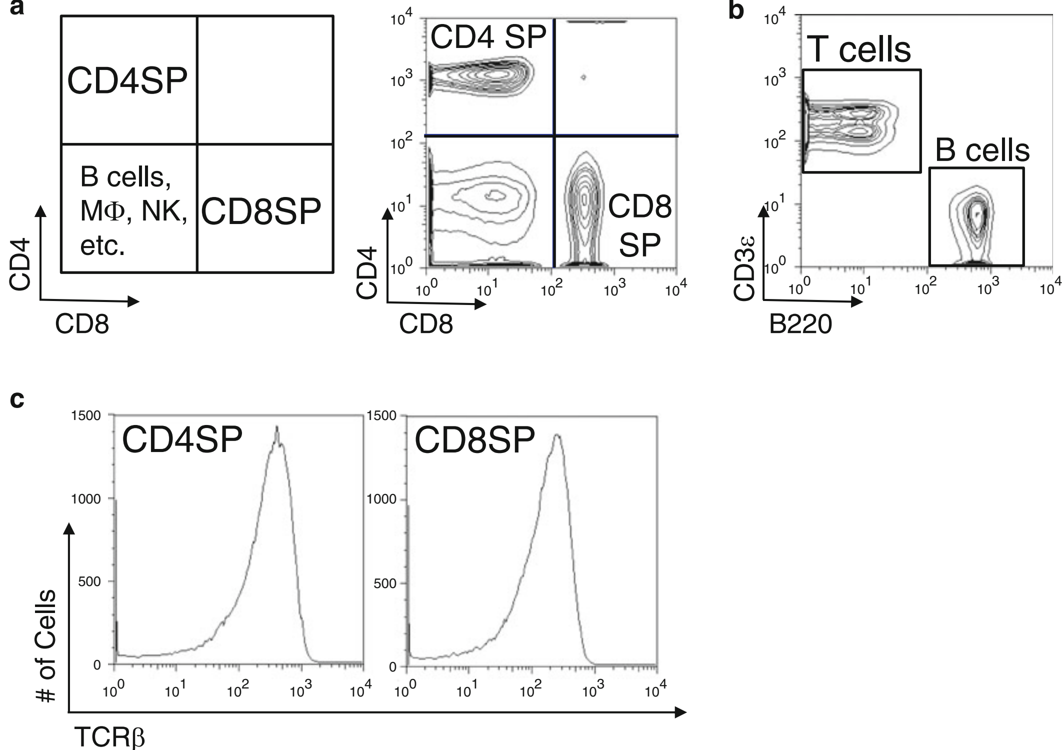


Fig. 7 T cell population in the periphery. Lymph node cells are stained with anti-CD4, anti-CD8α, anti-CD3ε, and anti-TCRβ or anti-B220 (CD45R). (**a**) CD4 SP, CD8 SP, and CD4$^-$CD8$^-$ cells in the lymph nodes. (**b**) Peripherial T and B cells can be differentiated by the expression of CD3ε and B220, respectively. The majority of CD4$^-$CD8$^-$ cells in the lymph nodes are B cells with a very small population of NKT, macrophages, etc. (**c**) TCRβ levels are high on CD4 SP and CD8 SP cell populations

5. Cell Staining Buffer: 1× PBS or 1× HBSS, 0.1 % bovine serum albumin (Fraction V), 0.01 % Sodium Azide (optional), 4 °C.
6. RPMI 1640 medium, 4 °C.
7. 60 mm × 15 mm tissue culture dishes.
8. 80–100 μm nylon mesh, 1″ × 1″ squares.
9. 15 mL conical tubes.
10. 3 mL syringes, single use only.

2.2 Cell-Surface or Intracellular Staining with Fluorochrome-Conjugated Antibodies

1. FcγIII Receptor (CD16) clone 2.4G2 purified.
2. Various antibodies to detect lymphocyte markers (e.g., anti CD4, anti-CD8, anti-TCRb, anti-CD24, anti-CD69, anti-CD5) or other molecules of interest conjugated with different fluorochromes appropriate for use on your flow cytometer. Commonly used fluorochromes include fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein

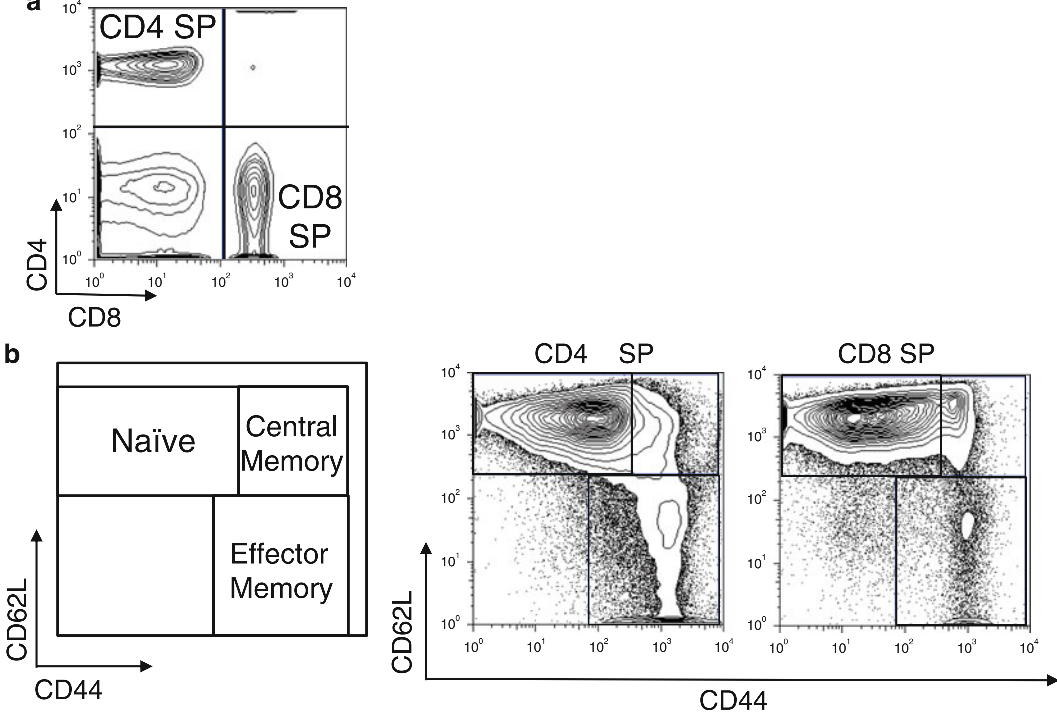


Fig. 8 Naïve and memory CD4 SP and CD8 SP cells in the periphery. Lymph node cells are stained with anti-CD4, anti-CD8α, anti-CD62L, and anti-CD44. (**a**) Focusing on CD4 SP and CD8 SP cells in the lymph nodes. (**b**) Gating on either CD4 SP or CD8 SP lymph node T cells, the naïve and memory (central and effector, respectively) phenotypes can be differentiated by CD62L vs. CD44

complex (PerCP or PerCy-Cy5.5 tandem dye), and allophycocyanin (APC) although others are also available.

3. 7-aminoactinomycin-D (7AAD) (*see* **Note 3**).
4. α-galactosylceramide or PBS-57 complexed to CD1d tetramer (NIH Tetramer Core Facility).
5. anti-FoxP3 (clone FJK-16s) multiple fluorochrome conjugations.
6. Flow Cytometer (e.g., BD Biosciences FACSCalibur™) that detects Forward Scatter (FSC), Side Scatter (SSC), and a minimum of four different fluorochromes.
7. Cell Staining Buffer: 1× PBS or 1× HBSS, 0.1 % bovine serum albumin (Fraction V), 0.01 % Sodium Azide (optional), 4 °C.
8. FoxP3/Transcription Factor Staining Buffer Set (eBioscience #00-5523-00).
9. Sample tubes, 5 mL polystyrene round bottom (Falcon #352052) (*see* **Note 10**).
10. 7AAD staining buffer: 0.14 M NaCl, 2.5 mM $CaCl_2$, and 0.01 M HEPES in 1× HBSS or 1× PBS.

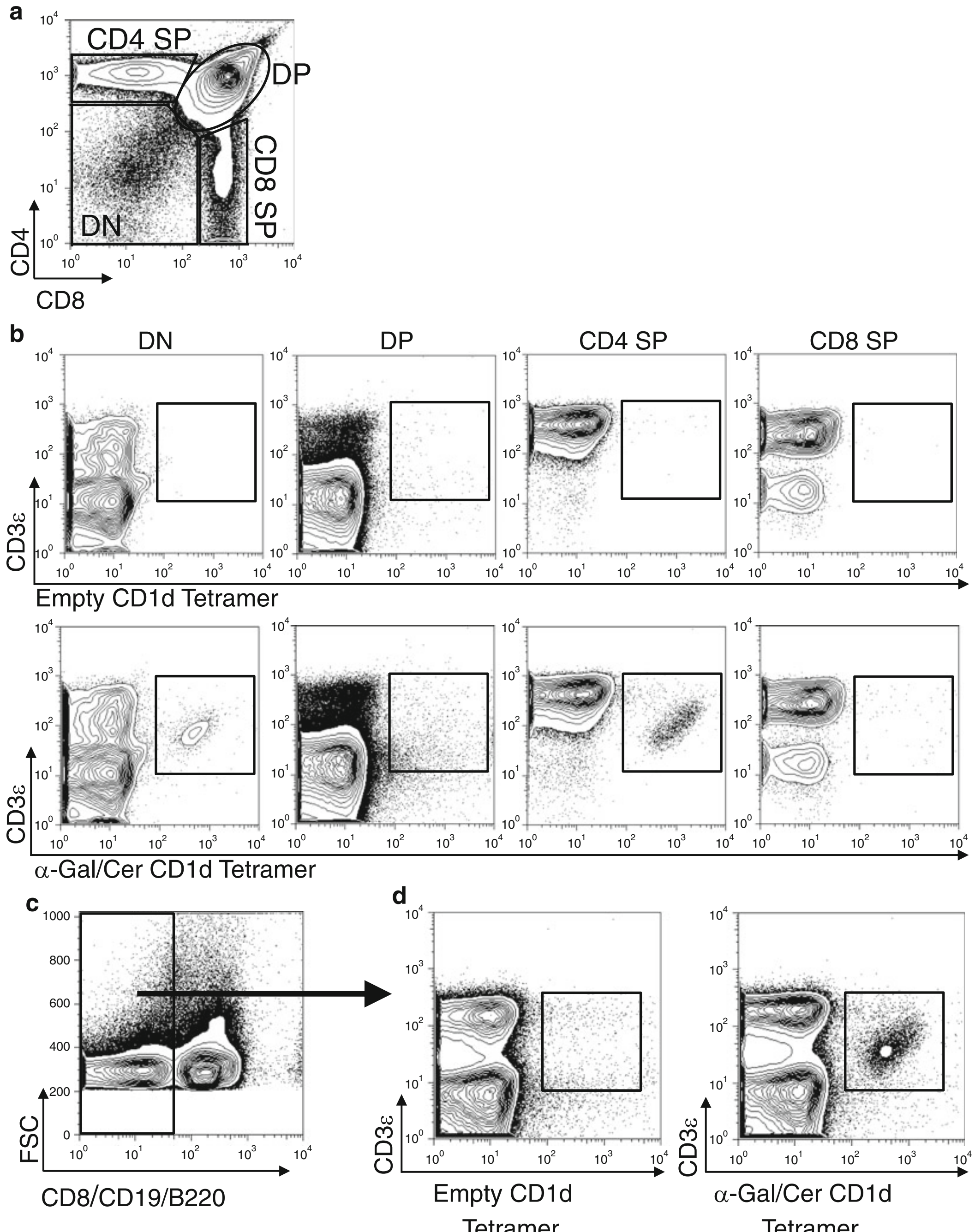


Fig. 9 Detection of Natural Killer T cells (NKT) in the thymus and periphery. Thymocytes are stained with anti-CD4, anti-CD8α, anti-CD3ε, and α-Gal/Cer CD1d tetramer or "empty" CD1d tetramer. Splenocytes are stained with anti-CD4, anti-CD3ε, α-Gal/Cer CD1d tetramer or "empty" CD1d tetramer, and an antibody mixture of anti-CD8α + anti-CD19 + anti-B220. (**a**) Focusing on all populations of the thymus "bird" profile. (**b**) CD3ε vs. α-Gal/Cer CD1d (or "empty" CD1d) tetramer surface expression on DN, DP, CD4 SP, and CD8 SP populations in the thymus. (**c**) Gating on CD8, CD19, and B220 negative cells in the spleen. (**d**) CD3ε vs. α-Gal/Cer CD1d (or empty CD1d) tetramer surface expression on splenocytes that are CD8/CD19/B220 negative. Expected % of CD1d+ cells in a C57BL/6 mouse thymus: <1 % in DP and CD8 SP and ~2–3 % in DN and CD4 SP, respectively. Expected % of CD1d+ cells in the spleen is ~3 %

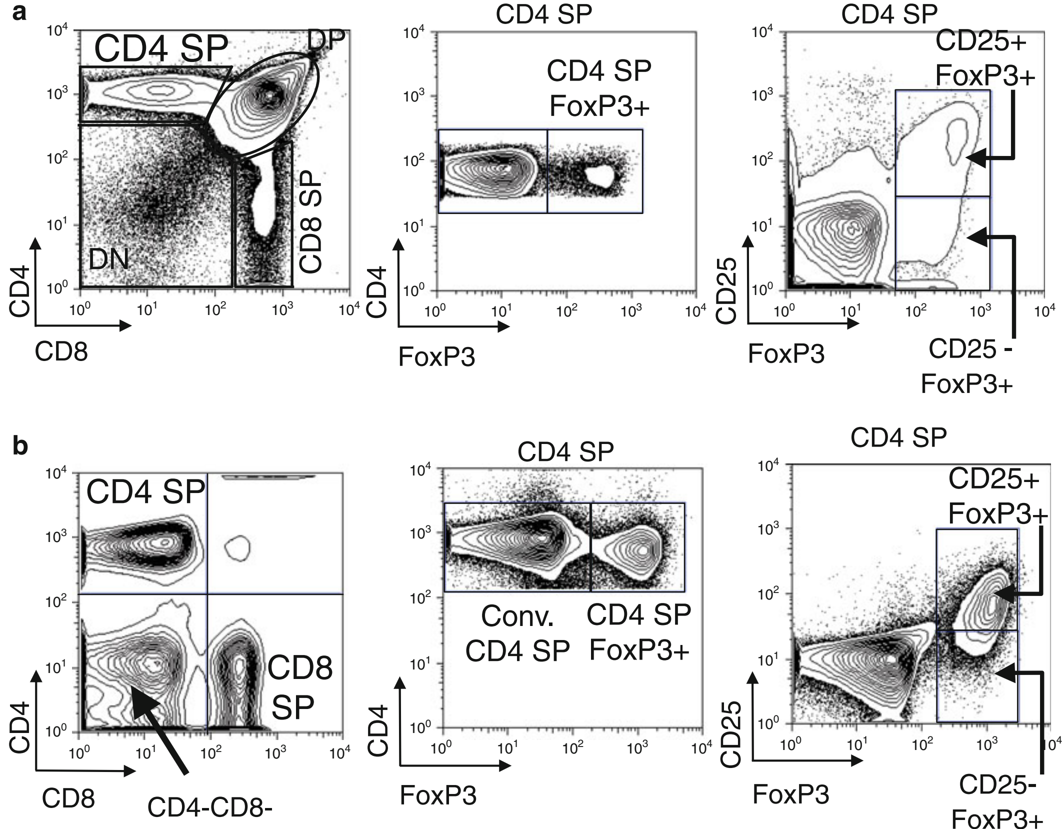


Fig. 10 Regulatory T cells (Treg) in the thymus and periphery. Thymocytes and lymph node cells are surface stained with anti-CD4, anti-CD8α, anti-CD25, and then permeabilized and stained intracellularly with anti-FoxP3 (*see* **Note 2**). (**a**) Focusing on the CD4 SP population on the thymus "bird" profile, FoxP3 expression on CD4 SP and FoxP3 vs. CD25 expression gated on the CD4 SP population. (**b**) Focusing on the CD4 SP population in peripheral (lymph node) cells, FoxP3 expression on CD4 SP and FoxP3 vs. CD25 expression gated on the CD4 SP population. Expected % of FoxP3+ cells in the CD4 SP population of a C57BL/6 mouse thymus and lymph nodes are ~5 % and ~12–18 %, respectively

3 Methods

3.1 Removal of Lymphoid Organs and Preparation of Cells for Antibody-Fluorochrome Staining

1. Euthanize mouse according to institutional guidelines and remove desired lymphoid organs: thymus, spleen and/or lymph nodes. Please refer to J.P. Reeves and P.A. Reeves, Removal of Lymphoid Organs, *Current Protocols in Immunology* (*1991*) *1.9.1–1.9.3* for additional guidance.
2. Place the thymus, spleen, or lymph nodes (LNs) between two squares of nylon mesh in separate tissue culture dishes that contain 1 mL of RPMI 1640 medium. For LNs, combine all

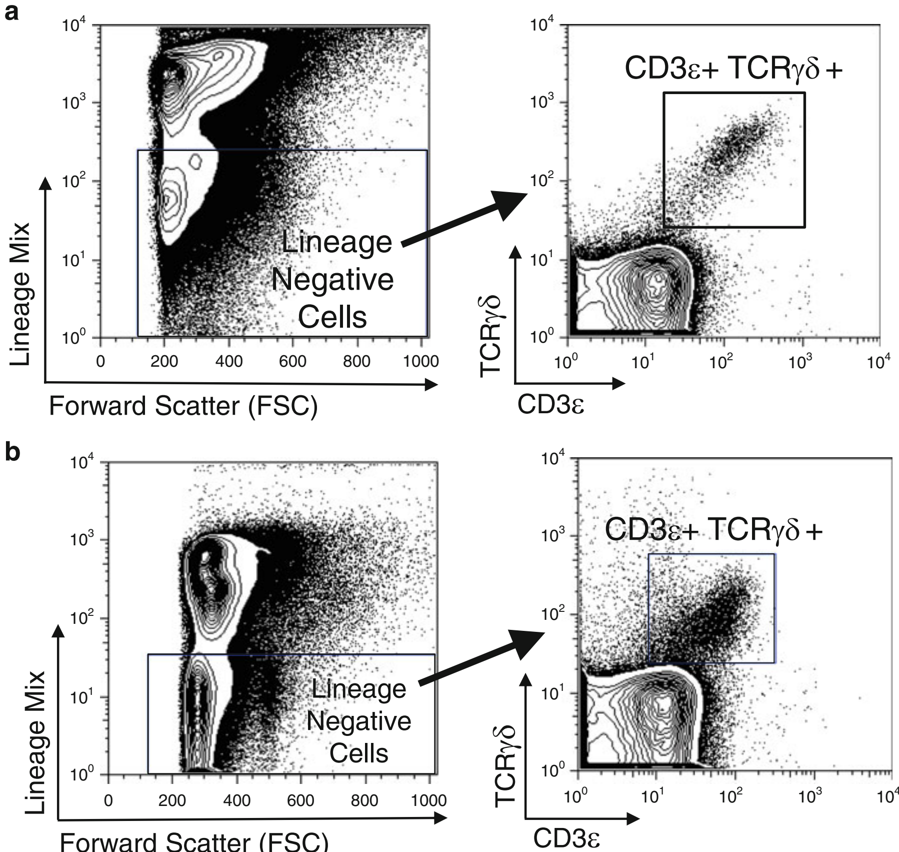


Fig. 11 γδ T cells in the thymus and periphery. Thymocytes are stained with anti-CD3ε, anti-TCRγδ, and lineage mix that contains anti-CD4, anti-CD8α, anti-TCRβ, anti-CD19, anti-NK1.1, anti-DX5, anti-Gr1 (Ly6C/G), anti-CD11b, and anti-Ter119. Lymph node cells are stained with anti-CD3ε, anti-TCRγδ, and a lineage mix that contains anti-CD4, anti-CD8α, and anti-TCRβ. (**a**) Gating on thymocytes that are lineage negative, $TCR\gamma\delta^+$ cells can be detected as $CD3\varepsilon^+TCR\gamma\delta^+$. Because of their low numbers, it is advantageous to use a lineage mix and acquire multiple events on the flow cytometer. (**b**) Gating on lymph node cells that are lineage negative, $TCR\gamma\delta^+$ lymphocytes can be detected as $CD3\varepsilon^+TCR\gamma\delta^+$. Expected % of $TCR\gamma\delta^+$ cells in a C57BL/6 mouse: ~1–2 % in the thymus and ~3 % in the lymph nodes

eight individual LNs (two each of inguinal, brachial, axillary, and superficial cervical) in one tissue culture dish.

3. Gently tease the cells out of the organs using the flat end of curved forceps or the plunger end of a 3 mL syringe. (Recommend forceps for the thymus and 3 mL syringe for the LNs and spleen). The nylon mesh will trap most of the debris.
4. Wash the dish by adding 5 mL of RPMI 1640 medium and gently pipet up and down. Dispense the medium containing the cells into a 15 mL conical tube.

5. Rinse the dish of any remaining cells with another 5 mL of RPMI 1640 medium. Pipet the medium into the same 15 mL conical tube for a total of 11 mL.
6. Centrifuge for 5 min at 450 × *g* at 4 °C.
7. Aspirate or decant excess medium, taking care not to disturb the cell pellet at the bottom of the tube.
8. Wash cells again with 10 mL of RPMI 1640 medium, centrifuge, and aspirate medium.
9. Resuspend the cell pellet in 1 mL of RPMI 1640 medium.
10. Count cells and store at 4 °C for staining.
11. Expected cell numbers for lymphoid organs from one C57BL/6 mouse (~6–8 weeks old): ~100 million thymocytes, ~80–100 million splenocytes, and ~20 million lymphocytes (LNs).

3.2 Cell Surface Staining with Fluorochrome Conjugated Antibodies

1. Add one million cells in a sample tube (*see* **Note 4**).
2. Add 2 mL of cell staining buffer to the tube and centrifuge for 5 min at 450 × *g*, 4 °C.
3. Vacuum-aspirate excess buffer, leaving cell pellet undisturbed at the bottom of the tube. Excess buffer can also be decanted.
4. Stain with antibody mix* (see below) for 30 min at 4 °C in the dark (*see* **Notes 5** and **6**). Taking into account residue buffer from the cell pellet, final volume of cells AND antibody mix should be between 50 and 100 μL.

   *Composition of Antibody Mix:

   10 μL of FcγIII Receptor (CD16) clone 2.4G2 (titered)

   10 μL of FITC labeled antibody (titered)

   10 μL of PE labeled antibody (titered)

   10 μL of PerCp-Cy5.5 antibody (titered)

   10 μL of APC antibody (titered)

   50 μL Total volume

5. Add 2 mL of cell staining buffer to the tube and centrifuge for 5 min at 450 × *g*, 4 °C.
6. Vacuum-aspirate excess buffer, leaving cell pellet undisturbed at the bottom of the tube. Excess buffer can also be decanted.
7. Repeat **steps 5** and **6**.
8. Resuspend cells in 300 μL of cell staining buffer.
9. Cover with aluminum foil to protect from light and store at 4 °C until ready to run on the flow cytometer (*see* **Notes 7–9**).
10. If using 7AAD to exclude dead cells from a sample, start by washing ~1 million cells with 2 ml of 1× PBS (4 °C).

Centrifuge for 5 min at 450 × *g* and vacuum-aspirate excess buffer. Repeat for a total of two washes. Resuspend cell pellet in 100 μL of 7AAD staining buffer. Add 0.25–1 μg of 7AAD per sample and incubate for 15 min at RT, protecting the sample from light. At the end of the incubation, add 400 μL of the 7AAD staining buffer directly into the sample tube. Do not wash 7AAD out of the sample tube. Run samples within 1 hr. Samples should be kept cold during acquisition. Since 7AAD is detected in the PerCp/PerCp-Cy5.5 (FL3) channel, leave that channel "open" when surface staining.

11. If running CD1d tetramer in a sample follow cell-staining instructions **steps 1**–**9**, substituting CD1d tetramer for one of the fluorochrome conjugated antibodies.

3.3 Intracellular Staining for the FoxP3 Transcriptional Factor

1. Perform surface staining (Subheading 3.2, **steps 1**–7).
2. To the cell pellet, add 100 μL of 1× Fixation buffer (eBioscience #00-5523-00). Pipet up and down to resuspend the cell pellet in buffer. Incubate samples for 30 min at room temperature in the dark (*see* **Note 11**).
3. Add 1 mL of 1× permeabilization buffer to the tube, pipet up and down once to mix, and pellet the cells for 5 min at 450 × *g*, 4 °C. Aspirate excess buffer. The cells will be translucent.
4. Repeat **step 3**.
5. Add 1 μg of fluorochrome-conjugated anti-FoxP3 antibody (eBioscience) to the cells in a total volume of 100 μL of permeabilization buffer. Incubate for 30 min at room temperature in the dark.
6. Add 1 mL of 1× permeabilization buffer to the tube and centrifuge for 5 min at 450 × *g*, 4 °C.
7. Aspirate excess buffer off, taking care not to disturb the cell pellet on the bottom of the tube.
8. Resuspend cells in 300 μL of cell staining buffer.
9. Cover with aluminum foil to protect from light and store at 4 °C until ready to run on the flow cytometer (*see* **Notes 7–9**).

4 Notes

1. It is highly recommended to include a lineage mix (Fig. 3) when analyzing the DN population in the thymus. This allows for the removal of all lineage positive cells, leaving the lineage negative cells more visible. This and the acquisition of multiple events on the flow cytometer allows for the visualization of the DN1–DN4 profiles. The negative cells are also slightly larger

in size (larger FSC profile) than conventional thymocytes. This becomes important when setting "live cell" and collection gates (Fig. 12).

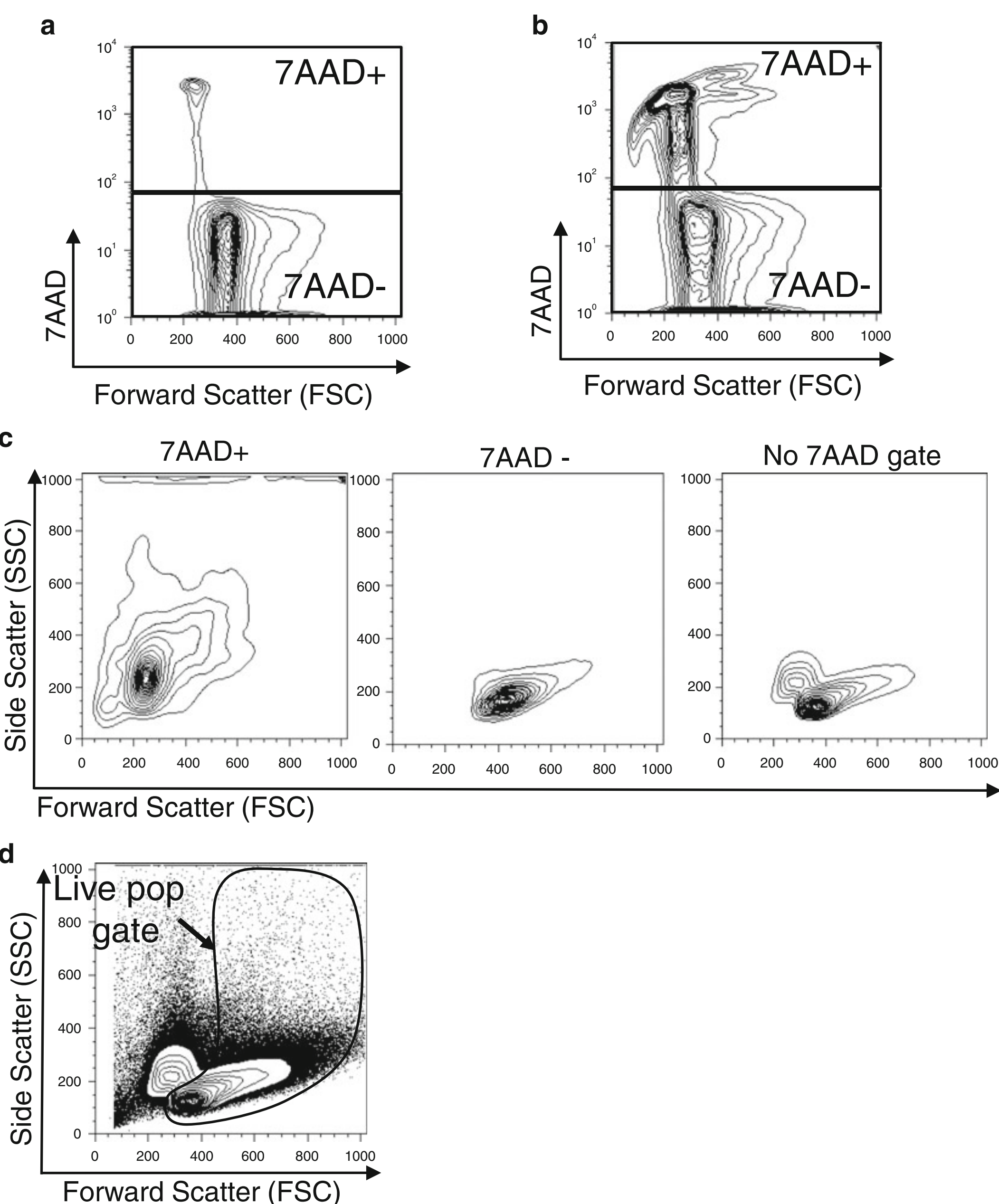


Fig. 12 7AAD and live gating. Thymocytes are stained with 7AAD. (**a**) Thymocytes stored at 4 °C, measuring 7AAD expression ex vivo. (**b**) Thymocytes stored at room temperature for 48 h ex vivo, showing 7AAD expression. (**c**) 7AAD positive ("dead") cells are small (low SSC, low FSC profile). 7AAD negative ("live") cells are larger (larger FSC profile). Cells that do not have a 7AAD gate applied show a dual population of "dead" cells (lower FSC) and "live" cells (larger FSC). These thymocytes are stained with 7AAD ex vivo and stored at 4 °C. (**d**) The application of a "live" gate on SSC vs. FSC (on the No 7AAD profile from (**c**))

2. Refer to Subheading 3.3 for intracellular staining of the FoxP3 transcriptional factor. CD25 surface expression can be used as a substitute marker for regulatory T cells even though not all CD25 positive cells are FoxP3 positive.

3. 7-amino-actinomycin (7AAD) is a fluorescent reagent used to exclude nonviable cells that incorporate 7AAD into the GC-rich regions of double stranded DNA through compromised plasma membranes. 7AAD is most commonly excited by the 488 nm laser. 7AAD is detected in the FL3 channel (using a 670 nm long pass filter) and can be compensated out of the FL2 (PE) channel allowing for multi-color flow cytometry.

   There are other alternatives to 7AAD. Live/dead or viability stains/dyes are commercially available now in multiple fluorochromes, allowing for multi-color customization and are useful with cells that will be fixed and permeabilized. DAPI (4′,6-diamidino-2-phenylindole) and PI (Propidium Iodide) are two popular "dyes" used to assess cell viability. DAPI is a fluorescent stain that can be detected by an ultraviolet (355 nm) laser when bound to the A-T regions in DNA. Propidium Iodide also intercolates into the DNA, but nonspecifically. One advantage to PI is that it can be detected by the blue (488 nm) laser, which is more commonly found on flow cytometers compared to an ultraviolet laser. Please also note that both DAPI and PI can also be used for cell cycling analysis. For further information, please refer to Z. Darzynkiewicz, G. Juan and E. Bedner, Determining Cell Cycle Stages by Flow Cytometry, *Current Protocols in Cell Biology* (*1999*) *8.4.1–8.4.18*.

   In some cases when 7AAD cannot be included in the antibody staining, one can use a "live" gate method where the majority of "dead" cells are excluded in the Side Scatter (SSC) vs. Forward Scatter (FSC) profile. This "live" gate is used only with cells that are stained *ex vivo* and stored at 4 °C (Fig. 12).

4. To stain more than one million cells in a single sample tube, scale up the amount of antibody mix as needed. For example, if staining two million cells, double the antibody mix and so on. This is very useful when collecting rare cell populations.

5. Titer all fluorochrome conjugated antibodies used for cell staining. Most antibodies that are commercially obtained can be used at lower concentrations than recommended by the vendor. Using less will limit background fluorescence and save money/reagents in the long run.

6. Because fluorochromes are light sensitive, it is recommended that all fluorochrome conjugated antibodies are stored in the dark at 4 °C. Cells labeled with fluorochrome conjugated antibodies should be covered with aluminum foil at every incubation step. Fluorochromes, especially tandam dyes, can lose intensity or even dissociate when exposed to light.

7. Unstained, single color, and FMO (fluorescence minus one) compensation controls should be used to set up the flow cytometer. This will help with issues pertaining to background and auto-fluorescence, compensation, negative/positive gate settings, and expose any issues with the actual antibody staining procedure. If cells are stained intracellularly (e.g., for FoxP3), the set of compensation controls should also undergo the same fixation and permeabilization procedure as the experimental samples. Cells that are fixed/permeabilized exhibit a different forward scatter (FSC) vs. side scatter (SSC) profile compared to non-fixed/non-permeabilized cells.
8. To prevent clogging in the flow cytometer by cell samples, filter cells with the pre-cut nylon mesh right before event acquisition. Alternatively, a 5 mL sample tube that has a "cell-strainer" cap can also be used to filter out cell clumps (e.g., Falcon #352235).
9. Fluorochrome conjugated antibody stained cell samples that are in cell staining buffer can be stored overnight for analysis by flow cytometry the next day. Although not optimal, this is sometimes necessary especially when faced with multiple cell groups and long antibody staining procedures. Sample tubes should be covered with aluminum foil and stored at 4 °C in the dark. If samples must be held longer than overnight, samples can be fixed with a commercial fixation reagent or 2 % paraformaldehyde.
10. Falcon (#352052) 5 ml tubes fit the majority of flow cytometers from BD Biosciences. If using other flow cytometers, please refer to their specifications for plastic disposable wear.
11. It is essential to use the fixation/permeabilization kit from eBioscience formulated for the specific staining of FoxP3. Using other fix/perm methods might result in sub-optimal or no staining of FoxP3. Please also note that this fix/perm kit for FoxP3 can also be used for detection of other nuclear transcription factors. Which nuclear transcription factors will require testing on the user's part.

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Chapter 5

Flow Cytometry Analysis of Thymic Epithelial Cells and Their Subpopulations

Izumi Ohigashi and Yousuke Takahama

Abstract

The parenchyma of the thymus is compartmentalized into the cortex and the medulla, which are constructed by cortical thymic epithelial cells (cortical TECs, cTECs) and medullary thymic epithelial cells (mTECs), respectively. cTECs and mTECs essentially and differentially regulate the development and repertoire selection of T cells. Consequently, the biology of T cell development and selection includes the study of TECs in addition to the study of developing T cells and other hematopoietic cells including dendritic cells. In this chapter, we describe the methods for flow cytometric analysis and sorting of TECs and their subpopulations, including cTECs and mTECs.

Key words Thymic epithelial cell, mTEC, cTEC, Aire, β5t, Flow cytometry, Cell sorting

1 Introduction

The thymic parenchyma provides a three-dimensional thymic microenvironment that supports the development of T cells. Cortical thymic epithelial cells (cTECs) are the major stromal component of the thymic cortex and support the early stages of T cell development as well as the positive selection of T cells. On the other hand, medullary thymic epithelial cells (mTECs) are the major stromal component of the thymic medulla and support the negative selection of autoreactive T cells as well as the generation of regulatory T cells [1–3].

To better understand how T cells are generated and selected in the thymus, studies that focus on thymic epithelial cells (TECs) are as important as studies on developing T cells and other hematopoietic cells, including dendritic cells. It has been revealed that a number of molecules expressed in TECs play essential roles in T cell development and selection. Those molecules include IL-7 [4, 5], DLL-4 [6, 7], β5t-containing thymoproteasome [8, 9], Aire [10, 11], CCR7 ligand chemokines [12, 13], and self-peptide-presenting MHC molecules [14].

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_5,

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In order to further understand the molecular and cellular characteristics of TECs and their subpopulations at the single-cell level, flow cytometry analysis and cell sorting have been adopted [15, 16]. In this chapter, we will describe methods for the dissociation of thymic stromal cells (Subheading 3.1). Flow cytometric analysis of TECs, including mTECs and cTECs, as well as their intracellular molecules will be discussed in Subheadings 3.2 and 3.3, respectively, and methods for sorting TECs will be described in Subheading 3.4.

2 Materials

2.1 Dissociation of Stromal Cells from Thymus

1. Euthanasia chamber.
2. Scissors and forceps.
3. PBS, pH 7.2.
4. RPMI 1640 medium.
5. Collagenase solution: RPMI 1640 supplemented with 0.125 % collagenase D and 0.01 % DNase I (*see* **Note 1**).
6. PBS + EDTA buffer: PBS supplemented with 2 mM EDTA and 2 % fetal calf serum (FCS).
7. 30-mm dish.
8. 1.5-ml microtubes.
9. 1-ml syringes.
10. 26-gauge needles.
11. 100-μm nylon mesh.
12. Water bath or block incubator.

2.2 Cell Surface Staining for Flow Cytometric Analysis

1. 1.5-ml microtubes.
2. Fluorochrome-unlabeled anti-FcγR antibody (clone 2.4G2).
3. Fluorochrome-conjugated antibodies specific for CD45, CD205, and CD326 (*see* **Note 2**).
4. Biotinylated *Ulex europaeus* agglutinin-1 (UEA-1) (*see* **Note 2**).
5. FACS buffer: PBS supplemented with 0.2 % BSA and 0.1 % NaN_3.
6. FACS-PI buffer: FACS buffer supplemented with 0.1 μg/ml propidium iodide.
7. 48-μm nylon mesh.
8. Flow cytometer.

2.3 Intracellular Staining for Flow Cytometric Analysis

1. Antibodies specific for β5t and Aire (*see* **Note 3**).
2. FACS buffer (*see* Subheading 2.2).
3. 2 % Paraformaldehyde: PBS supplemented with 2 % paraformaldehyde.

4. Saponin buffer: PBS supplemented with 0.1 % saponin.
5. 48-μm nylon mesh.
6. Flow cytometer.

2.4 Sorting of Thymic Epithelial Cells

1. Anti-CD45-conjugated microbeads (Miltenyi Biotec).
2. MACS buffer: PBS supplemented with 0.5 % BSA and 2 mM EDTA.
3. MACS LS column (Miltenyi Biotec).
4. MACS separation unit (Miltenyi Biotec).
5. MACS multistand (Miltenyi Biotec).
6. FACS buffer (*see* Subheading 2.2).
7. 48-μm nylon mesh.
8. Cell sorter.

3 Methods

3.1 Dissociation of Stromal Cells from Thymus

1. Prepare a 30-mm dish containing 1 ml of PBS.
2. Euthanize mouse according to relevant institutional guidelines (*see* **Note 4**).
3. Wipe the chest of the mouse with 70 % ethanol, open the chest, and remove the thymus using a set of scissors and forceps.
4. Transfer the thymus into the 30-mm dish containing PBS. Where needed, transfer the thymus to another PBS-containing dish to remove blood.
5. Mince the capsules of the thymus with scissors.
6. Transfer the minced thymus to a 1.5-ml microtube containing 1-ml of RPMI medium.
7. Pipette gently with a wide-bore tip to mechanically release thymocytes (*see* **Note 5**).
8. Remove the medium containing thymocytes released from the thymus.
9. Add 1 ml of RPMI medium.
10. Repeat the gentle pipetting with the wide-bore tip.
11. Remove the medium.
12. Add 1 ml of collagenase solution.
13. Incubate at 37 °C for 15 min. Gently pipette every 5 min during the incubation (*see* **Note 6**).
14. Collect the released cells, add 1 ml of PBS + EDTA buffer, and keep on ice.
15. Repeat the collagenase treatment as specified in **steps 12–14** (*see* **Note 7**).

16. Mix the cells collected at **steps 14** and **15**.
17. Centrifuge at 200 × *g* for 5 min at 4 °C and remove the supernatant.
18. Resuspend cells in 1 ml of PBS + EDTA buffer.
19. Filter the cells through a 100-μm nylon mesh.

3.2 Cell Surface Staining for Flow Cytometric Analysis

1. Prepare cell suspension as in Subheading 3.1.
2. Centrifuge at 200 × *g* for 5 min at 4 °C and remove supernatant.
3. Resuspend cells in 1 ml of FACS buffer.
4. Perform a cell count and transfer 1×10^7 cells into a 1.5-ml microtube.
5. Centrifuge at 200 × *g* for 5 min at 4 °C and remove supernatant.
6. Incubate cells with unlabeled anti-FcγR antibody on ice for 5 min.
7. Add antibodies specific for CD45 and CD326. Where needed, UEA-1 and anti-CD205 antibody are also added. Incubate the mixture on ice for 30 min.
8. Add 1 ml of FACS buffer.
9. Centrifuge at 200 × *g* for 5 min at 4 °C and remove the supernatant.
10. Where needed, further stain the cells with additional antibodies or secondary antibodies on ice for 30 min.
11. Add 1 ml of FACS buffer.
12. Centrifuge at 200 × *g* for 5 min at 4 °C and remove supernatant.
13. Resuspend cells in 300 μl of FACS-PI buffer.
14. Filter the cells through a 48-μm nylon mesh.
15. Analyze the cells with a flow cytometer. Representative results are shown in Fig. 1.

3.3 Intracellular Staining for Flow Cytometric Analysis

1. Prepare cell suspension as in Subheading 3.1.
2. Perform cell surface staining as in Subheading 3.2 (**steps 1–12**).
3. Resuspend cells in 200 μl of 2 % paraformaldehyde.
4. Incubate at room temperature for 30 min.
5. Add 1 ml of FACS buffer.
6. Centrifuge at 200 × *g* for 5 min at 4 °C and remove supernatant (*see* **Note 8**).
7. Resuspend cells in 1 ml of saponin buffer.

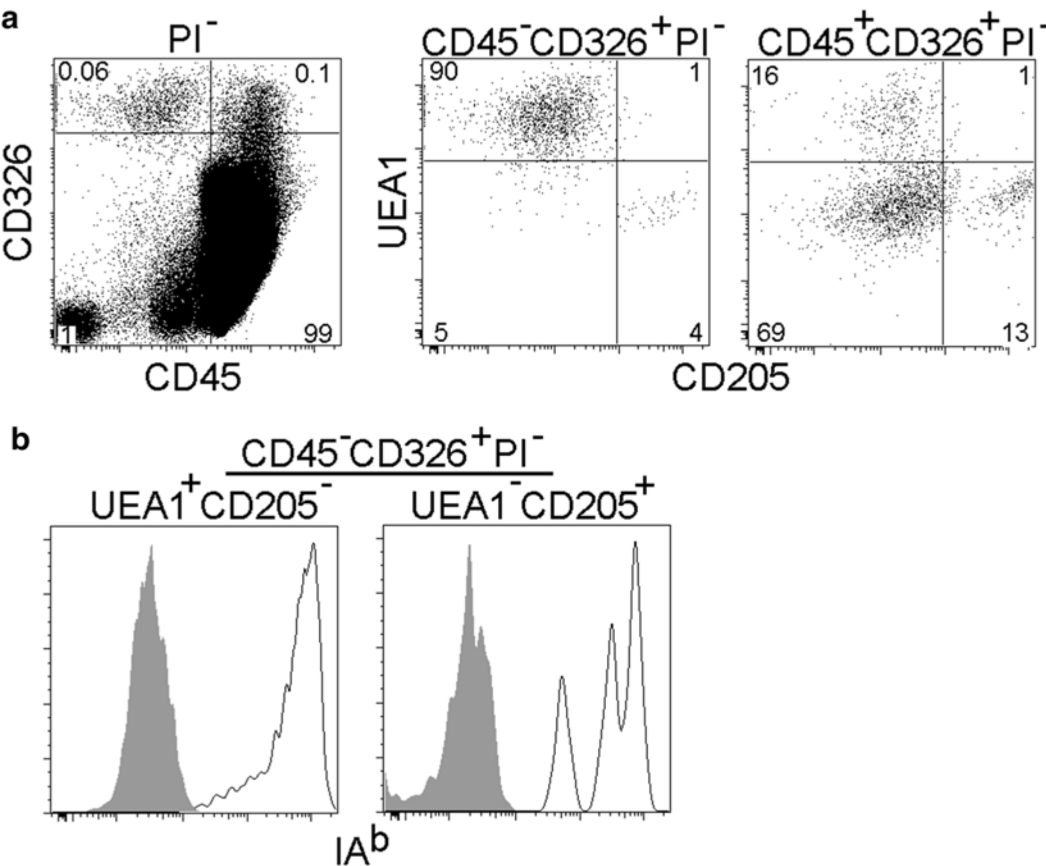

Fig. 1 Representative flow cytometry profiles of TECs. Collagenase-digested thymus cells from 3-week-old C57BL/6 mice (as in Subheading 3.1) were stained with antibodies specific for CD326 (PECy7), CD45 (FITC), and CD205 (APC), as well as with UEA-1 (APCeFluor780) and propidium iodide (PI) (as in Subheading 3.2). (**a**) Profiles show CD45 and CD326 expression in PI^- total viable cells (*left*) and CD205 and UEA-1 expression in $CD45^-CD326^+$ PI^- viable cells (*middle*) and $CD45^+CD326^+$ PI^- viable cells (*right*) (*see* **Note 11**). *Numbers* in dot plots indicate frequency within indicated area. Most $CD45^-CD326^+$ PI^- viable cells consisted of either $UEA1^-CD205^+$ cTECs or $UEA1^+CD205^-$ mTECs. $CD45^+CD326^+$ PI^- viable cells consisted of thymocyte-associated cTECs and thymocyte-associated mTECs (*see* **Note 11**) as well as many $UEA1^-CD205^-$ cells. (**b**) Profiles show class II MHC (I-A^b) expression (*solid lines*) and isotype control (*shaded histograms*) in $CD205^-UEA\text{-}1^+$ $UEA1^+CD205^-$ PI^- viable mTECs (*left*) and $UEA1^-CD205^+$ $CD45^-CD326^+$ PI^- viable cTECs (*right*). The class II MHC expression indicates that the cells indeed represent TEC subpopulations

8. Centrifuge at 200 × *g* for 5 min and remove supernatant.
9. Stain cells with antibody specific for the intracellular molecule on ice for 30 min.
10. Add 1 ml of saponin buffer.
11. Centrifuge at 200 × *g* for 5 min and remove supernatant.
12. Repeat washing as described in **steps 10** and **11** (*see* **Note 9**).

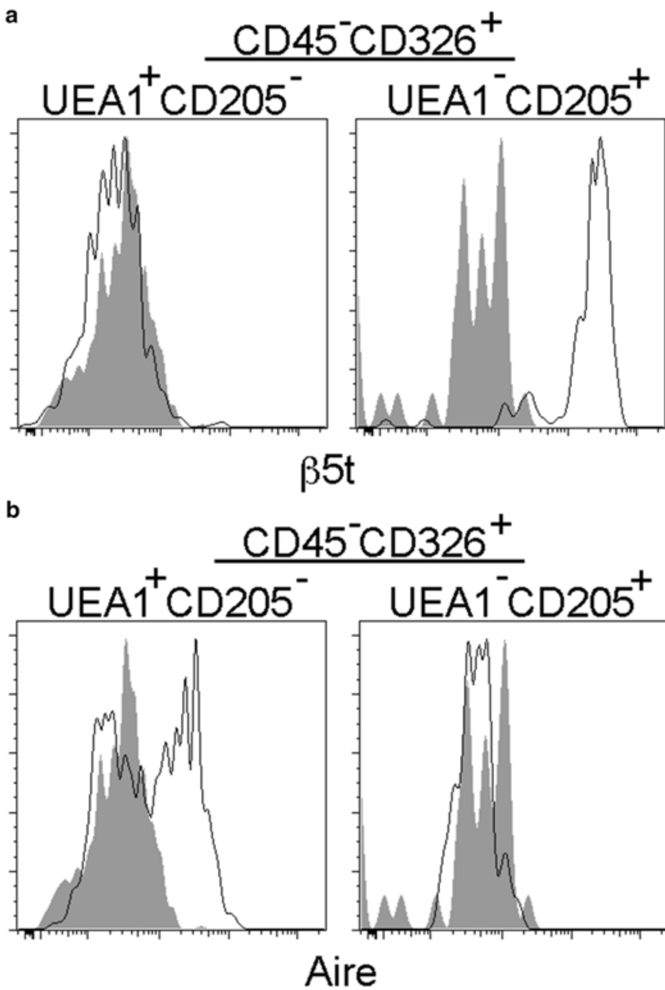

Fig. 2 Representative flow cytometry profiles of intracellular molecules expressed in TECs. The profiles show β5t expression (*solid lines* in (**a**)) and Aire expression (*solid lines* in (**b**)) in UEA1$^+$CD205$^-$ CD45$^-$CD326$^+$ mTECs (*left panels*) and UEA1$^-$CD205$^+$ CD45$^-$CD326$^+$ cTECs (*right panels*). *Shaded histograms* indicate profiles obtained by staining with isotype control antibodies

13. Filter the cells through a 48-μm nylon mesh.
14. Analyze the cells with a flow cytometer. Representative results are shown in Fig. 2.

3.4 Sorting of Thymic Epithelial Cells

1. Prepare cell suspension as in Subheading 3.1.
2. Centrifuge at 200 × *g* for 5 min at 4 °C and remove supernatant.
3. Resuspend cell pellet in 90 μl of MACS buffer and 10 μl of anti-CD45-microbeads per 1×10^7 cells. Incubate for 30 min at 4 °C.

4. Add 1 ml of MACS buffer.
5. Centrifuge at 200 × *g* for 5 min at 4 °C and remove supernatant.
6. Repeat **steps 4** and **5**.
7. Resuspend cell pellet in 1 ml of MACS buffer.
8. Place the MACS separation unit on the MACS multistand. Expose the MACS LS column to a magnetic field.
9. Rinse the column with 3 ml of MACS buffer.
10. Apply cell suspension onto the column. Collect cells that pass through the column.
11. Add 3 ml of MACS buffer onto the column and collect cells that pass through the column.
12. Centrifuge at 200 × *g* for 5 min at 4 °C and remove supernatant.
13. Resuspend cell pellet in 1 ml of FACS buffer.
14. Perform cell surface staining as in Subheading 3.2 (**steps 5–14**).
15. Sort cells with a cell sorter (*see* **Note 10**).

4 Notes

1. Collagenase/dispase (Roche, working concentration at 0.125 %) or liberase TM (Roche, working concentration at 1 unit/ml) may be used instead of Collagenase D. Collagenase/dispase can dissociate thymic stromal cells more effectively than Collagenase D. However, the inclusion of these enzymes weakens subsequent antibody-mediated staining for several surface molecules, such as CD205.
2. Anti-CD45 and anti-CD326 (EpCAM) antibodies are used for the detection of leukocytes and epithelial cells, respectively. Among the TECs, cTECs can be detected by the expression of CD205 or CD249 (Ly51), whereas mTECs can be detected by the expression of CD80 or the binding to lectin UEA-1. PECy5-labeled anti-CD45 antibody, FITC-labeled anti-CD45 antibody, PECy7-labeled anti-CD326 antibody, APC-labeled anti-CD205 antibody, FITC-labeled CD249 antibody, and FITC-labeled anti-CD80 antibody can be purchased from BioLegend. Biotinylated UEA-1 can be purchased from Vector Laboratories.
3. Anti-β5t and anti-Aire antibodies can be purchased from MBL and eBioscience, respectively. Saponin buffer should be used to prepare the working solution.

4. In the collagenase-digested thymus, the frequency of cTECs exceeds that of mTECs in mice at 2-weeks-old or younger age but remarkably drops at 3-weeks-old or older age. Therefore, it is easiest to use mice at 2-weeks-old or younger for the sorting of cTECs.
5. Wide-bore pipette tips (at 1–2 mm diameter) can be made by cutting 1-ml plastic pipette tips with scissors. The bore size should not be too large in order to efficiently release thymocytes from the minced thymus.
6. Start pipetting with a wide-bore tip. When the minced thymus becomes small, change to a regular tip and then a 26G-needle-attached syringe.
7. Repeat the collagenase treatment until thymus clumps become invisible. For adult thymus, repeat the treatment two or three times. For newborn thymus, repeat it once or twice.
8. Cells tend to float after the fixation. When needed, the cells may be spun at a speed of up to $500 \times g$.
9. Where needed, cells may be further stained with the secondary antibodies (as specified in Subheading 3.3, **steps 9–11**).
10. 100-μm-nozzle should be used for the sorting of TECs. Collection medium is RPMI supplemented with 30 % FCS. Before collecting the sorted cells, coat the wall of collection tube with RPMI 1640 supplemented with 30 % FCS.collection medium.
11. Even after the collagenase treatment, many CD326$^+$ TECs are still associated with CD45$^+$ thymocytes (Fig. 1a). This tight association with thymocytes is more prominent in cTECs than mTECs, reflecting the strong adhesiveness of cTECs to thymocytes [17]. A fraction of cTECs form large multicellular complexes termed thymic nurse cells (TNCs), which represent cTECs that completely enclose multiple thymocytes [17]. For the detection of TNCs by flow cytometry, voltage for the forward scatter channel should be reduced [17]. It should also be noted that because the majority of cTECs are openly associated with thymocytes, the depletion of CD45$^+$ cells during the procedure for the enrichment of TECs (Subheading 3.4) actually removes the majority of cTECs [17].

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Chapter 6

Identifying the Spatial Relationships of Thymic Stromal and Thymocyte Subsets by Immunofluorescence Analysis

Virginia Bain and Ellen R. Richie

Abstract

Immunofluorescence analysis of thymic tissue sections is an indispensable technique for visualizing spatial relationships among thymocyte and stromal cell subsets. The thymus is organized into distinct microenvironmental zones in which particular thymic epithelial cell (TEC) subsets support specific stages of thymocyte maturation. Conversely, thymocytes and lymphoid tissue inducer cells support functional maturation of TECs. The composition and organization of TECs change during ontogeny to generate a maximally functional organ in the young adult. Deterioration of thymic architecture and stromal organization occurs with age as the thymus undergoes involution. Such changes can be monitored by immunofluorescent staining of thymic sections obtained at different ages throughout the life-span. Here we describe methods to generate frozen or paraffin-embedded thymic tissue sections for multicolor immunofluorescence staining using antibodies to surface and/or cytoplasmic antigens.

Key words Immunofluorescence, Thymus, Thymocytes, Thymic epithelial cells, Thymic microenvironment

1 Introduction

The thymus is composed of two primary cell types: thymocytes and stromal cells that form a unique and indispensable microenvironment required for T cell development. Thymic epithelial cells (TECs) comprise the major type of thymic stromal cells and provide growth, differentiation, and survival signals to thymocytes. Reciprocally, thymocyte-derived signals are required for TEC development. Thus, cross talk between thymocytes and TECs is essential for development of both cell types (reviewed in [1–4]).

The thymus is spatially organized into thymocyte-dense cortical regions, thymocyte-sparse medullary regions, the corticomedullary junction (CMJ), and a subcapsular region. Bone marrow hematopoietic progenitors enter the thymus via blood vessels at the CMJ. As thymocytes differentiate from CD4−CD8− precursors

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_6, © Springer Science+Business Media New York 2016

to the CD4+CD8+ stage, they migrate through the cortex to the subcapsular region, then reverse course, and traverse the cortex again where cortical TECs (cTECs) present peptide in the context of self-MHC to positively select thymocytes with self-MHC-restricted T cell receptors (TCRs). Positively selected CD4+CD8− and CD4−CD8+ thymocytes migrate into the medulla where they encounter medullary TECs (mTECs) that express a diverse array of tissue-restricted antigens required for deletion of T cells expressing high-avidity autoreactive TCRs. This chemokine-directed migration pattern brings thymocytes at successive stages of maturation into close contact with phenotypically and functionally distinct subsets of cTECs and mTECs required for development of self-restricted and self-tolerant T cells [5, 6].

Thymic architecture, organization, and spatial relationships among thymocytes, TECs, and non-epithelial stromal cells can be visualized by immunofluorescence analysis of thymic tissue sections. This chapter outlines thymus embedding and immunofluorescence antibody (IFA) staining techniques that identify TEC and thymocyte subsets in thymus sections. IFA analysis is an efficient way to define mutant thymic phenotypes and to assess changes in the developing and aging thymus. There are three general methods to prepare the thymus for IFA staining: (1) snap freeze tissue (referred to as fresh frozen); (2) briefly fix tissue in 4 % PFA, dehydrate, and then freeze (referred to as fixed frozen); and (3) fix tissue in 4 % PFA for a longer duration, dehydrate the tissue, infiltrate with xylenes, and paraffin embed the tissue (referred to as paraffin embedded). The method of choice is dictated by several factors, including the objective of the analysis. Better discrimination of cytoplasmic and surface proteins is obtained using fresh-frozen thymi as opposed to fixed tissues (Fig. 1). However, for morphological studies especially hematoxylin and eosin stains, paraffin-embedded sections provide better discrimination of the structural characteristics of different cell types than can be obtained using fresh-frozen tissue. Another important consideration is the sensitivity of different antigenic epitopes to various fixation techniques. For example, the vascular marker CD31 is extremely sensitive to PFA fixation, such that IFA analysis is suboptimal using PFA-fixed tissue. In contrast, the nuclear transcription factor FOXN1 requires PFA fixation for optimal staining results (Fig. 2).

2 Materials

All reagents can be stored at room temperature unless indicated otherwise. Solutions are made using ddH_2O. Reagents requiring autoclaving or a fume hood are noted. Please follow proper disposal procedures for waste containing PFA, ethanol, or xylene.

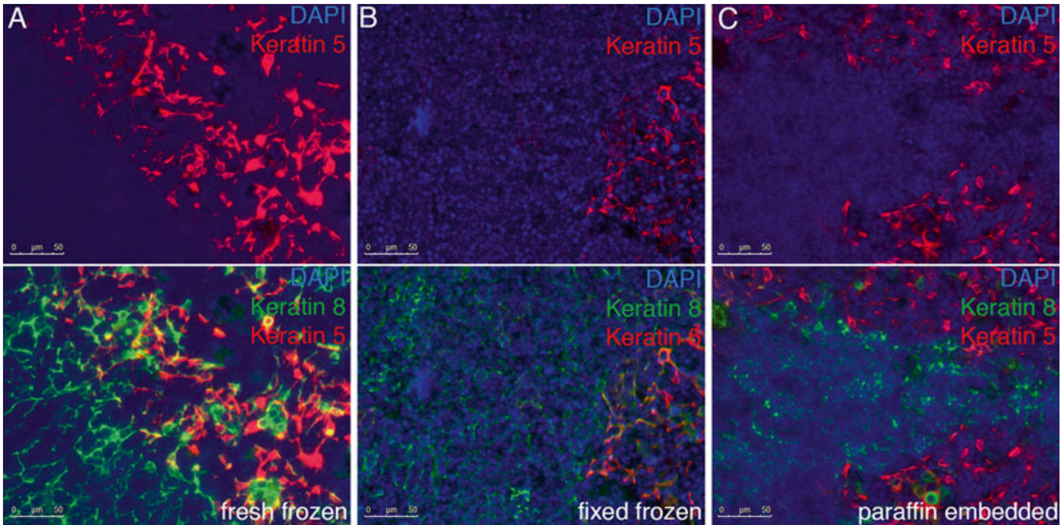

Fig. 1 Tissue morphology varies with fixation. Keratin 5 and keratin 8 staining of 4-week thymi. (**a**) Fresh-frozen tissue is optimal for cytoplasmic markers such as keratin 5 and keratin 8 but is difficult to distinguish nuclear morphology. (**b**) Fixed-frozen tissue gives better morphology but has less optimal cytoplasmic stain and more tissue defects when sectioning. (**c**) Paraffin-embedded tissue gives clear nuclear morphology and fewer tissue defects but requires antigen retrieval and has less optimal cytoplasmic or cell surface stain. Scale bars indicate 50 μm

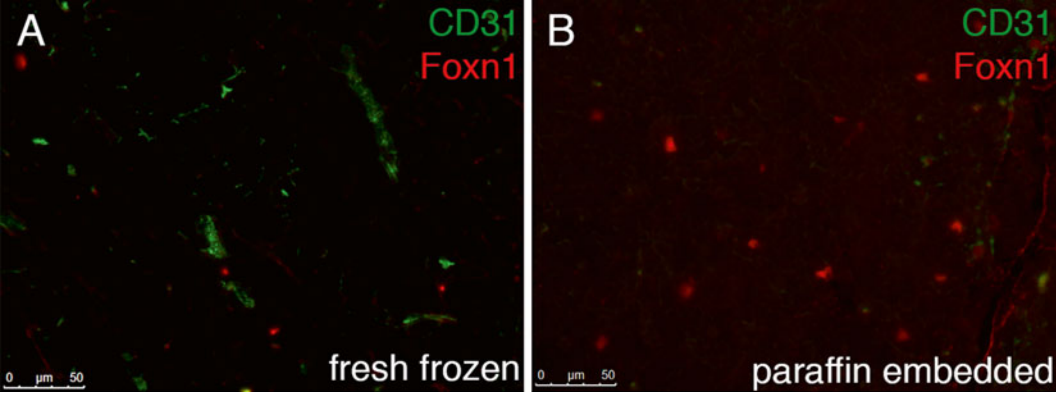

Fig. 2 Antibodies may require different tissue preparation. (**a**) Fresh-frozen tissue is required by some cell surface markers such as the vascular marker CD31. (**b**) PFA fixation is required by some nuclear markers such as the TEC-specific transcription factor Foxn1. Scale bars indicate 50 μm and tissue used is 4-week thymi

2.1 Frozen Tissue

1. Kimwipes.
2. Cryomolds (e.g., disposable base molds 15 × 15 × 5 mm).
3. Embedding medium (e.g., OCT—optimal cutting temperature compound).
4. 10× PBS pH 7.2.

5. 4 % PFA: 4 % paraformaldehyde by weight dissolved in PBS. Heat to dissolve. Work in a fume hood (*see* **Note 1**).
6. 5 % Sucrose: 5 % sucrose by weight dissolved in PBS. Add 0.5 % sodium azide to prevent bacterial growth. Autoclave.
7. 20 % Sucrose: 20 % sucrose by weight dissolved in PBS. Add 0.5 % sodium azide. Autoclave.
8. Acetone.

2.2 Paraffin-Embedded Tissue

1. Ethanol: 70–90 % ethanol should be mixed by volume with ddH_2O (*see* **Note 2**).
2. Xylene: Work in a fume hood (*see* **Note 3**).
3. Cassettes for embedding (e.g., biopsy cassettes).
4. Paraffin (e.g., tissue embedding medium, melting point 56 °C).
5. Molds for paraffin embedding (e.g., peel-a-way disposable embedding mold).
6. AR buffer: 10 mM sodium citrate, 0.05 % Tween 20, HCl to pH 6 (*see* **Note 4**).

2.3 Staining

1. Slides (*see* **Note 5**).
2. Slide boxes.
3. Slide mailers (*see* **Note 6**).
4. Parafilm M: 2 in. × 250 ft cut into 8 cm strips.
5. 1 % Triton-X: 1 % Triton-X by volume dissolved in ddH_2O.
6. Donkey serum.
7. Tyramide Signal Amplification kit.
8. 4′,6-Diamidino-2-phenylindole (DAPI).
9. Mounting media (*see* **Note 7**).
10. Cover slips (*see* **Note 8**).

2.4 Antibodies

1. Primary antibodies: Antibody selection depends on the cell type of interest (*see* **Note 9**).
2. Secondary antibodies: This will depend on the filter sets for your microscope and the host of the primary antibody. A standard four-color fluorescent microscope will have filters for blue (Alexa350), green (Alexa488), red (Alexa594), and far-red (Alexa647) (*see* **Note 10**).

3 Methods

For the purpose of immunostaining, tissue may be fresh frozen, fixed frozen, or fixed paraffin embedded. Tissue preparation depends on desired quality of morphology and antibodies to be used.

3.1 Tissue Preparation for Fresh-Frozen Tissue

Dissected thymi from embryos or adult mice as well as whole embryos can be flash frozen for cryosectioning. Embryos and adult mice should be euthanized according to institutional guidelines.

1. Dab tissue gently on a Kimwipe to remove excess PBS.
2. Place the tissue in a cryomold and cover in OCT. Rotate the tissue to make certain that it is completely surrounded by OCT with no pockets of PBS or air.
3. Use forceps to hold the cryomold in liquid nitrogen. Remove the mold as soon as the entire block has frozen to avoid cracking (*see* **Note 11**).
4. Store frozen blocks at −80 °C until ready to cryosection.
5. Move frozen blocks to the cryostat at least 30 min before cutting to allow the blocks to thaw.
6. Trim the block with a single-edged razor blade to reduce cutting area.
7. Mount the blocks to specimen discs using enough OCT to surround the base of the block on all sides and allow the additional OCT to completely freeze before sectioning.
8. Section thawed and trimmed blocks at desired thickness (*see* **Note 12**).
9. Allow frozen sections to dry completely at room temperature for 15 min after the final section on the slide is cut.
10. Store sectioned tissue at −80 °C until ready to stain. Avoid repeated thawing of the slides.
11. When ready to stain, first allow slides to thaw for 15 min on the bench top.
12. Fix slides in ice-cold acetone for 10 min (*see* **Note 13**).
13. Proceed to Subheading 3.4.

3.2 Tissue Preparation for Fixed-Frozen Tissue

Tissue fixed for frozen embedding receives a short PFA fix and does not require antigen retrieval.

1. Collect thymi or embryos in PBS and store on ice until ready to fix.
2. Fix tissue in cold 4 % PFA with duration depending on size of embryo or thymi (*see* **Note 14**).
3. Wash fixed tissue three times for 5 min each in PBS (*see* **Note 15**).
4. Dehydrate tissue in 5 % sucrose/PBS for 1 h at 4 °C. Replace 5 % sucrose/PBS with 15 % sucrose/PBS and leave at 4 °C overnight.
5. Rinse tissue in OCT (*see* **Note 16**).
6. Place tissue in a cryomold and cover with OCT. Freeze on dry ice (*see* **Note 17**).

7. Store at −80 °C until ready to cut. See cryostat-cutting instructions in Subheading 3.1, **steps 5–10**.
8. Proceed to Subheading 3.4.

3.3 Tissue Preparation for Paraffin-Embedded Fixed Tissue

Thymi or embryos collected for paraffin embedding require a longer PFA fixation, must be thoroughly dehydrated, and require antigen retrieval.

1. Collect thymi or embryos in PBS and store on ice until ready to fix.
2. Fix tissue in cold 4 % PFA with duration depending on the size of embryo or thymi (*see* **Note 18**).
3. Wash fixed tissue three times for 5 min each.
4. Dehydrate tissue in 70 % ethanol, 90 % ethanol, 95 % ethanol, and three 100 % ethanol washes at 4 °C. Dehydration time will vary with size (*see* **Note 19**). Tissue can be stored at −20 °C in the third 100 % ethanol wash.
5. When ready to embed, tissue must be cleared in xylenes. This will remove fat and allow wax to permeate the tissue. Xylene washes are also size dependent (*see* **Note 20**). Once the tissue is translucent remove it from xylene, and place it in an embedding cassette (*see* **Note 21**).
6. Cassettes containing cleared thymi or embryos are incubated in three paraffin washes at 65 °C (*see* **Note 22**).
7. Place your specimen in a paraffin-embedding mold, fill with warm paraffin, orient appropriately, and allow the block to cool. Blocks may be stored at 4 °C.
8. Allow cold blocks to warm to room temperature. Cut off excess wax from the block to form a square to allow for ribboning during sectioning.
9. Mount the block in the base of a cassette and clamp it to the microtome for sectioning.
10. Because paraffin-embedded tissue is thoroughly dehydrated, cells are condensed and thinner sections should be cut. Do not exceed 8 μm of paraffin-embedded tissue for immunofluorescence.
11. Cut sections may be kept in ribbons and floated on a warm water bath (do not exceed 45 °C).
12. Slides must be thoroughly dried (*see* **Note 23**).
13. Dry slides can be stored in slide boxes at 4 °C indefinitely.
14. Allow slides to warm to RT. Use two sets of mailers: one set used for dewaxing and rehydration and another set used for antigen retrieval.
15. Fill one set of mailers 2/3rd full with AR buffer and place in a 300 mL beaker filled with 150 mL of water. Heat the water

and buffer to 95 °C. Cover the beaker with foil to avoid excessive evaporation (*see* **Note 24**).

16. Place slides in clean mailers and wash twice for 5 min each in xylene to remove the paraffin.
17. Remove excess xylene from the tissue by washing twice for 2 min in 100 % ethanol.
18. Rehydrate tissue by 2-min washes in 90, 70, and 30 % ethanol. Leave slides in a dH_2O wash for at least 5 min.
19. Transfer slides from rehydration mailers to AR mailers (*see* **Note 25**).
20. Tissue is antigen retrieved for 30 min at 95 °C. Keep the water level in the beaker at the level of the slide labels (*see* **Note 26**).
21. Allow mailers of slides in hot AR buffer to cool on your bench for 20 min with the lid open.

3.4 Staining

1. Wash slides in PBS for 5 min.
2. Prepare primary antibody master mix. Dilute antibodies at the appropriate concentration in 5 % serum of the secondary host (e.g., donkey serum) and 0.05 % Triton-X in PBS (*see* **Note 27**).
3. Pour off the PBS and lay slides flat in a humid chamber (*see* **Note 28**). Incubate each slide in 100 μL of primary mix. Gently cover slides in parafilm to help spread antibody evenly and prevent evaporation.
4. Depending on the antibody incubate overnight at 4 °C or 1 h at RT in a humid chamber (*see* **Note 29**).
5. Gently remove the parafilm. Return slides to mailers and wash three times in PBS.
6. OPTIONAL STEP: When working with a low-affinity or low-titer antibody, it is possible to boost the signal with either a biotin intermediate step or TSA amplification. In the case of the biotin intermediate, the primary antibody is removed followed by an amplification step in which 100 μL of a secondary biotinylated anti-primary Ig antibody diluted at 1:200 is added for 30 min. The secondary biotinylated reagent is detected using a streptavidin reagent conjugated to the fluorochrome of choice. Briefly wash slides in PBS (*see* **Note 30**).
7. Prepare secondary antibody master mix. Dilute secondary Abs in PBS—1:400 (*see* **Note 31**).
8. Incubate with secondary antibody. Apply 100 μL of secondary antibody mix per slide. Cover in parafilm and incubate in a humid chamber in the dark for 30 min to 1 h at RT (*see* **Note 32**).
9. Wash slides three times in PBS for at least 1 min per wash (*see* **Note 33**).

10. Lay slides flat on a Kimwipe and add at least 100 μL of mounting media to the bottom edge of each slide (*see* **Note 34**).
11. Coverslip each slide and blot the edges of the slide to remove excess liquid (*see* **Note 35**).
12. Slides may be stored in the dark at 4 °C for up to 2 weeks to preserve fluorescence.

4 Notes

1. PFA will dissolve at temperatures as low as 65 °C; however PFA heated to boiling will still work for fixation. Dissolved PFA has a short shelf life—a week at 4 °C or several months at −20 °C. Dissolved PFA can be filtered using Whatman paper.
2. Ethanol can be reused, although 100 % ethanol steps are best done with fresh ethanol.
3. For best results use fresh xylene every time when embedding. Previously used xylene can be used for removing paraffin from slides.
4. pH is very important. Take note of how much HCl you add the first time and add a similar amount every time you make this buffer. Replace AR buffer every month.
5. Pre-prepared silanized slides are commercially available and will prevent tissue from falling off the slide. Check to make certain that the coating does not autofluoresce as quality of pre-prepared slides is variable.
6. Slide mailers come in many shapes and sizes. The optimal mailer to use for antigen retrieval is the 5-slide mailer with an end-opening flip-top lid.
7. Mounting media must include an anti-fade reagent. Commercially available mounting medias differ in price and duration of signal preservation.
8. Cover slips with dimensions of 24 × 50 mm will cover the non-labeled portion of the slide. Cover slip thickness is indicated on the microscope objectives (most objectives indicate 0.17 mm). Because mounting media adds to the thickness of the cover slip, #1 thickness is used for 0.17 mm even though the #1 range is 0.13–0.16 mm.
9. Antibodies should be titrated and tested for optimal fixation conditions. Cytoplasmic and cell surface markers favor fresh tissue while nuclear markers often require PFA-fixed tissue. 1:200–1:50 is a good starting point for titrations.
10. *Microscope filter sets or lasers for confocal microscopy vary.* Determine what wavelengths are detected by the filter sets for your microscope. In choosing fluorochrome-conjugated

reagents, avoid spectral overlap. For instance if your far-red filter detects 594 nm, use secondary fluorochrome reagents that emit at 549 nm in the red channel to avoid nonspecific spillover. The green and red channels give the strongest signals and should be reserved for antibodies that give weaker signals. The blue and far-red channels require a longer exposure and are best used for antibodies that give strong signals.

11. Alternatively the cryomold can be placed on a weigh boat on top of the liquid nitrogen to allow for a more gentle freeze to avoid cracking. When embedding embryos make certain to orient all embryos the same way.
12. Thickness will vary. I prefer 10 μm sections for immunostaining, but have cut sections as thin as 6 μm when using the same tissue for multiple stains. Alternating slides while cutting is a handy trick to allow for multiple sets of stains on serial sections (Fig. 3). Handle slides with gloves to avoid getting oil on the slides that will cause sections to wash off later.
13. I prefer to fix slides laid flat in the humidity chamber to avoid loss of tissue that can occur when fixing slides vertically in a mailer. Add 1 mL of acetone to cover each slide. Additional acetone must be added several times due to evaporation.
14. Fixation time is determined by trial and error. A good starting time for whole embryos is 15 min for embryonic day 11.5

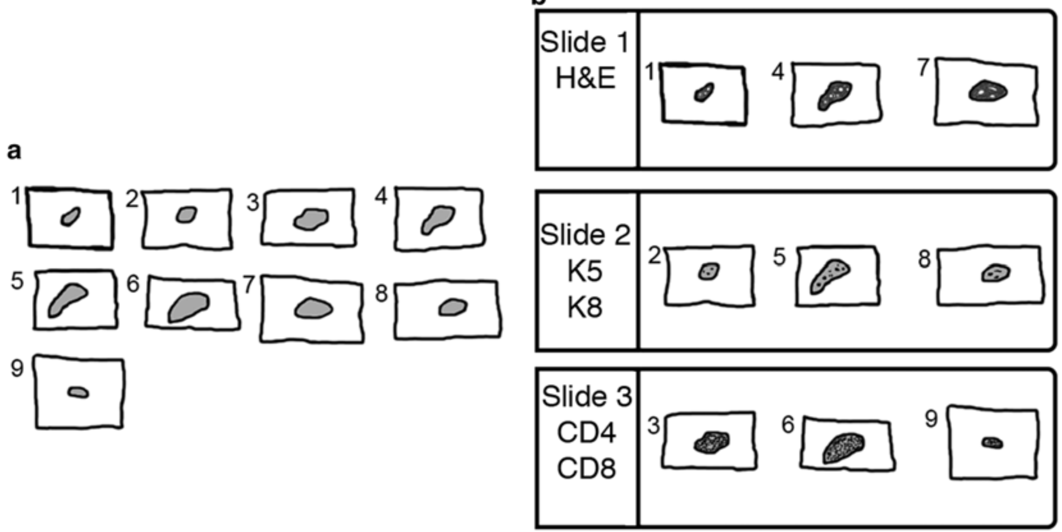

Fig. 3 Alternating slides allows for staining the same tissue with many markers. (**a**) When cutting through tissue, adjacent sections are similar while further apart sections may have distinct features. (**b**) Consecutive sections are placed on alternating slides to allow multiple stains to be performed on the same specimen while still analyzing many parts of the specimen

(E11.5) or 30 min for E15.5. Adult thymi can be fixed for 30 min and dissected embryonic thymi require shorter fixation times of 5 min for E12.5–E15.5 and 10 min for E16.5–E18.5. Tissue which is insufficiently fixed will fail to stain for antibodies requiring fixation while tissue which is over-fixed will autofluoresce.

15. Ten rapid successive PBS washes can be used instead of three 5-min washes to reduce autofluorescence.
16. Place a small amount of OCT in a Petri dish and coat the tissue thoroughly to remove excess PBS. This will help to reduce cracks when sectioning.
17. PFA-fixed tissue will crack when snap frozen in liquid nitrogen. Use dry ice instead.
18. Aim for the shortest fixation time that allows the tissue to survive the embedding process. A good starting time for embryos is 30 min for E11.5 or 2 h for E15.5. PFA will not permeate the skin of embryos older than E15.5 and it must be removed. Fix adult thymi for 45 min. Tissue that is under-fixed will fall apart during the embedding process or fail to stain for antibodies requiring fixation while tissue that is over-fixed will autofluoresce.
19. 20-min washes are sufficient for E11.5 embryos or similar sized tissue. Older embryos will require longer washes. Adult thymi should be kept at each stage for at least 1 h. Longer dehydration washes do not harm the tissue.
20. Adult thymi require two 10-min washes, whereas fetal thymi require two to three 5-min washes. It is best to check the tissue frequently while clearing it by holding it to the light and looking for translucency.
21. This is a critical step. Excess time in xylene can lead to brittle tissue that causes the sections to crumble.
22. Paraffin permeabilization is size dependent. Find the time that exposes your tissue to the least amount of heat. For E11.5 embryos three 20-min washes are sufficient. For adult thymi three 2-h washes are necessary.
23. If you are in a hurry, slides may be sufficiently dry as soon as 4 h after sectioning. Spacing sections apart from one another on the slide will expedite this process. It is best to dry slides overnight to make certain that no water is left on the slide as any water under the tissue will cause it to fall off the slide in future steps.
24. This limits you to 20 slides—four mailers maximum. Processing more than 20 slides can result in less than ideal antigen retrieval.
25. It is best to pour off the water before transfer. Do this step quickly to avoid lowering the temperature of the AR buffer.
26. Both time and temperature are critical for antigen retrieval. This is the most important step in paraffin embedding.

Make certain that the beaker stays full of 95–100 °C water and remove slides from the water bath at *exactly* 30 min.

27. Make your antibody mix at $N+1$. (If you have 20 slides make enough mix for 21 slides—2.1 mL of mix.)
28. Your chamber should contain the same liquid you incubate in—in this case PBS. Cover the base of the slide box with folded paper towels. Pour at least 25 mL of PBS in each side.
29. Antibodies that give low signal but little background give the best results with an overnight incubation at 4 °C, while antibodies with higher signal and background give better results when incubated at RT for a shorter duration.
30. Tyramide streptavidin amplification will substantially boost the signal but has the potential to significantly increase the background. If you use a TSA kit to amplify a primary antibody, use a streptavidin secondary reagent for staining, **step 7**.
31. Good results can be obtained with highly absorbed secondary antibodies conjugated with DyLight or Alexa Fluor fluorochromes. Cy3 and Cy5 also give bright signals. FITC and Texas Red conjugates give weaker signals due in part to the rapid photobleaching and relatively higher background staining, respectively.
32. Longer incubations especially at 4 °C give higher background and rarely amplify the signal.
33. DAPI can be added to the second PBS wash. DAPI concentration is determined by user preference (I use 1:15,000). Do not add DAPI if you use an AMCA- or Alexa350-conjugated secondary reagent.
34. Do not exceed five slides at a time.
35. Lower the cover slip gradually from bottom to top to spread the mounting media without introducing bubbles on the slide. Alternatively, place the edge of the cover slip at the bottom of the slide and allow adhesive forces to move the cover slip. Avoid bubbles as attempts to remove them may damage your tissue.

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Chapter 7

Purification of Thymocyte and T Cell Subsets

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Abstract

Many analytical or cell culture procedures require homogeneous starting cell populations that cannot be obtained directly from organ dissection. Here, we describe two enrichment procedures to achieve this goal and discuss their respective advantages in specific experimental contexts. Notes in this chapter include some tips on how to determine the appropriate level of purity (*see* **Note 1**).

Key words Single-cell suspension, T cell purification, Magnetic bead depletion, Flow cytometric sorting

1 Introduction

This chapter describes two procedures for preparing homogeneous thymocyte and T cell suspensions from organs that contain a heterogeneous mixture of cell types. Both techniques rely on the availability of appropriate antibodies against surface molecules differentially expressed on desired and unwanted cells.

The first procedure uses antibody-coated magnetic beads to retain ("positive selection") or discard ("negative selection") cells expressing specific surface molecules. The positive selection approach is of broad applicability, as one only needs a single cell-specific surface antigen, but has the significant disadvantage of leaving the purified cells coated with beads. This has the potential to interfere with cell activation or differentiation in culture (antibodies that bind the T cell receptor, for example, are detrimental for certain procedures as they can activate T cells). In addition, the presence of cell aggregates tends to limit cell purity, as one desired cell in the aggregate will positively select the whole aggregate. The negative selection approach is more complex, because it requires antibodies against all other cell types present in the starting population. However it yields cells "untouched" (i.e., without attached

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_7, © Springer Science+Business Media New York 2016

antibodies or beads), which is of particular importance if the cells are to be cultured or tested functionally in subsequent steps.

The second procedure uses flow cytometric sorting of cells stained with appropriate fluorescent antibodies. As with the bead purification procedure, antibodies can be used in flow cytometric sorting to positively or negatively mark cells, and in the latter case leaves cells untouched. In contrast, positively marked cells carry fluorescent antibodies that have to be taken into account when subsequent staining steps are considered, and, as mentioned above, can contribute to activation or differentiation. The choice of the sorter is generally dictated by locally available equipment. Except for experienced users, professional operators generally perform sorts; prior consultation with operators is highly recommended to discuss staining and sorting strategies, including gate definitions.

Several experimental considerations guide the choice of one procedure over the other. The first regards biological consequences on purified cells. Especially with negative selection, bead purification induces only minimal cell change or damage. In contrast, hydrodynamic stress, electrical charging, and repeated centrifugations are unavoidable side effects of flow cytometric sorting and can result in reduced survival and functionality. Time is also an important consideration. Bead purification is generally faster, particularly when large cell numbers are desired, as processing time is not proportional to input size. In contrast, the time required for sorting is directly proportional to input size, potentially affecting cell viability for large inputs. Specific situations (e.g., purification of extremely rare subsets) will require pre-purification steps. Cell yields are often higher with bead purification than with flow cytometric sorting. Last but not least, cost is a major consideration, and in many cases tips the scale in favor of bead purification when it can reach purities consistent with the study objectives. This is particularly true in applications requiring high cell numbers and repeated measurements.

However, flow cytometric sorting has decisive advantages. It offers unequaled flexibility for defining purification criteria. It is irreplaceable when gradient expression levels (e.g., $CD44^{lo}$ vs. $CD44^{hi}$) or co-expression of two marks (e.g., $CD4^+CD8^+$ from $CD4^-CD8^+$ or $CD4^+CD8^-$ thymocytes) defines desired populations. Despite major progress in bead purification approaches, cell purity is a second key asset of flow sorting. Although purities greater than 95 % can be achieved by bead purification with appropriate antibodies, they are routinely much lower when the frequency of the target population falls below 10 % of the starting cell suspension. In contrast, flow cytometric sorting can provide highly purified cells (>99 %) even when they are at a very low frequency in the starting population.

Regardless of the procedure chosen, it is important to carefully delineate the experimental plan well in advance. Estimate the size

of the starting population (and when applicable the number of experimental animals) by calculating frequency in the target population and expected yield. When working with small cell numbers or precious samples, it is highly recommended to perform pilot studies with similar cells that are more easily replaceable. Slower flow cytometric sorting speeds may be required for infrequent target populations to increase purity and yield.

Initially a 95–99 % yield may seem ideal; however it is useful to know the nature of contaminants. After flow sorting, the operator should "rerun" a small portion of the purified sample, although in some cases additional stains may be needed. For bead-purified cells, purity checks are often done by staining an aliquot of the purified cells and analyzing them by flow cytometry. Antibodies used for purification, whether positive or negative, can potentially impede binding of those used for a purity check. Thus, depending on each specific situation, purities should be verified with antibodies that do not cross-block those used in the purification step.

In this chapter, we provide detailed procedures to prepare purified T cell subsets (from thymus or peripheral organs) using either depletion ("negative selection") or flow cytometry sorting. These procedures can easily be adapted to specific requirements and reagents. Protocols for bead purification by positive selection depend heavily on the specific reagents being used and will therefore not be described in detail here. As with every antibody-mediated experimental procedure, pilot analyses titrating the amount of antibodies (and related reagents such as beads) are highly recommended to optimize yield and purity prior to proceeding with the actual experiment.

2 Materials

All necessary efforts should be made to keep organism contaminants out. If the end procedure is sterile, all reagents in purifying T cells should also be sterile (and the flow cytometry operator should be notified in advance that the cells must remain sterile during the sort). Medium should be made just before the experiment. If the sterility of the starting reagents is in question, sterile filter (≤45 nm pore size) the final buffer or media before use.

1. Heat-inactivated fetal bovine serum (FBS) (*see* **Note 2**).
2. Isolation buffer: 500 mL PBS pH 7.4 (no Ca^{2+}, no Mg^{2+}), 0.1 % FBS (or bovine serum albumin), 2 mM EDTA pH 8.0.
3. Complete medium: RPMI 1640 medium, 10 % FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.292 mg/mL l-glutamine, 20 mM HEPES (*see* **Note 3**).

4. Red cell lysis buffer: 8290 mg/L ammonium chloride, 1 g/L potassium bicarbonate, 37 mg/L EDTA pH 8.0.
5. Nylon tissue filters (pore size 100 μm, cut to 2 or 4 cm^2).
6. 1 or 3 mL syringe pestles.
7. 60 mm petri dishes.
8. 5, 15, and 50 mL polypropylene conical tubes.
9. 5 mL polystyrene round-bottom tubes 12×75 mm style (flow-sorting procedure).
10. 50 μm filters (flow-sorting procedure).
11. Appropriate fluorochrome-conjugated antibodies.
12. Magnetic beads compatible with appropriate purified antibodies.
13. Rocker.
14. Two curved forceps.
15. Scissors appropriate for dissection.
16. 37 °C incubator with 5 % CO_2.
17. Vertical magnet holding 15 or 50 mL conical tube (DynaMag 15, 50, or equivalent).

3 Methods

3.1 Obtaining Single-Cell Suspensions

Minimizing total purification time is critical in this procedure. Often a trial run with cells is advisable, not only to practice the procedure, but also to get an idea of the efficiency for the specific application. Depending on the desired final purity and the fragility of the cell type to be isolated, two to five times as many target cells should be in the starting population.

1. Prepare the workspace before euthanizing the animal(s). For each organ, set up one 60 mm petri dish on ice with 3 mL complete medium and a 2 cm^2 piece of nylon filter on the bottom of the dish. Prepare one syringe pestle (1 or 3 mL syringe) (excluding thymus), plus one 4 cm^2 100 μm filter per organ, and keep them in a clean container. For the thymus, no syringe pestle is needed.
2. Remove thymus, spleen, and/or lymph nodes (LN)s from animal(s) (*see* **Note 4** on LN selection). Before adding the thymus to the petri dish with medium, roll it on gauze or clean tissue paper to remove any blood, and carefully dissect it from surrounding connective or lymphoid tissue (which tends to stick to the gauze or paper). Do not pull tissue from the thymus and make sure not to damage the thymus or allow it to dry. When removing lymph nodes, carefully remove all fat.

3. For the thymus, take two curved forceps and gently tease apart the organ.
4. For the LNs and spleen, use the rubber end of the syringe pestle to dissociate the cells from the organ tissue, and ensure that you have dissociated any observable clumps as well. It is helpful to use the lid of the dish to store 5 mL additional medium for **step 5**. At this point, wash the used end of the syringe pestle in this medium, by swirling it in the medium; then discard the pestle.
5. Tilt the dish containing the cells at a 30° angle, draw up the medium with the free cells, and wash this medium back over the dish. Gently pipet up the medium from the dish again and run it through the 4 cm^2 100 μm filter into a 15 mL conical tube. Before passing the medium through the filter, use the pipet tip to push the filter half way into the top of the tube, and then pull the pipet tip a few mm back from the filter. Pulling the pipet back prevents the medium from directly hitting the filter and splashing out of the tube. Leave the filter balanced in the top of the conical tube. Next, use the additional 5 mL medium to wash the dish and pass that through the same filter into the conical tube. The dish and filter can now be discarded. Pellet the cells at $150\times g$, for 5 min at 4 °C, and decant the supernatant.
6. For spleen cell preparation, lyse red blood cells by resuspending the cell pellet in 2 mL red cell lysing buffer for 2 min on ice. Then immediately, dilute out the red cell lysing buffer by adding 8 mL complete medium. Pellet the cells at $150\times g$, for 5 min at 4 °C, and decant the supernatant.
7. Resuspend the organs at $10–20\times10^6$ cells/mL in complete medium (*see* **Note 5** on expected yields).
8. Count the cells using a dye that distinguishes dead cells (for example: trypan blue). Calculate the cell concentration and total number. Set aside an aliquot (10^6 cells is ideal) from each starting cell suspension for flow cytometric analysis.
9. In unmanipulated mice, spleen and LN cells can be combined to maximize the size of the starting population. For T cells, when large cell numbers are not needed, LN may be a preferable starting point because of the greater frequency of T cells in LN than in spleen.

3.2 Magnetic Bead Purification of T Cell Subsets by Depletion

Several commercial kits are available to purify target populations (e.g., CD4 or CD8 T cells), most of them using proprietary reagents and materials (magnets). The following procedure uses Dynabeads (LifeTech) to negatively select the desired population. Provided the beads carry the appropriate secondary reagent, Dynabeads can also be used with user-provided antibodies (*see* **Note 6**). Volumes and bead numbers are given for 10^7 starting

cells. They should be scaled up proportionally with higher cell numbers. Up to 5×10^7 cells can be processed in a 15 mL conical tube; use several tubes or scale up to 50 mL tubes if processing more cells.

1. Pellet cells to be purified by spinning at $150 \times g$, for 5 min, at 4 °C.
2. Resuspend cells in 100 μL isolation buffer. If any cell clumping is observed, refilter before proceeding.
3. Add 20 μL FBS and 20 μL kit antibody.
4. Incubate for 20 min at 2–8 °C (wash beads during this incubation and keep on ice until needed; *see* **steps 5** and **6**).
5. Wash the beads (to eliminate free antibody that might have been released during storage): first resuspend beads in the vial by vortexing for 30 s. Pipet 200 μL beads into a new 15 mL tube and resuspend in equal volume or at least 1 mL isolation buffer.
6. Put the tube without the lid on magnet for 1 min, remove buffer by pipetting, and discard. Remove tube from magnet and resuspend beads in the same volume of isolation buffer as the original bead volume.
7. After antibody incubation, wash cells by adding 2 mL isolation buffer. Pellet the cells at $150 \times g$, for 5 min, at 4 °C and decant the supernatant.
8. Resuspend the cells in 800 μL isolation buffer and add prewashed beads.
9. Incubate for 15 min at 25 °C with tilt and rotation on a rocker at a speed sufficient to keep the beads in suspension.
10. Add 1 mL isolation buffer, and gently pipet up and down five times with a large-bore pipet (5 mL or similar).
11. Put tube without lid on magnet for 2 min. Beads and bead-attached cells will be pulled to the tube wall on the magnet side.
12. Being careful not to dislodge the beads, transfer supernatant containing non-adherent cells to a new tube. For applications that require special media or buffers, wash cells with that medium or buffer as soon as possible after transferring. Discard beads at this point.
13. Count purified cells to determine recovery and take an aliquot (if possible, at least 10^5 cells) to quantify purity by flow cytometric analysis (*see* **Note 7**).
14. Purity from magnetic bead isolation depends on the application (routinely >85 % for CD4 or CD8 T cell purification from LN).

3.3 Magnetic Bead Enhancement Pre-flow Cytometric Sorting

A similar procedure used in Subheading 3.2 can be used to increase the frequency of rare target cells prior to flow cytometric sorting. This is especially useful when starting from high cell numbers (>10^8) for which sorting times quickly become prohibitive. In this case, because the objective is to remove most of the nontarget cells rather than achieving high purity, significant savings can be achieved by lowering bead numbers and antibody concentrations. While pilot experiments are needed to titer down these reagents, a good starting point is to use 1/8 of the recommended amounts of beads and antibodies while keeping cell concentration intact and all incubations at 4 °C. This "light" procedure routinely achieves >40 % cell purity with very little loss of desired cells.

1. Pellet cells to be run through kit by spinning at 150×*g*, for 5 min, at 4 °C.
2. Resuspend cells in 100 μL isolation buffer per 10^7 cells. If any cell clumping is observed, refilter before proceeding.
3. Add 20 μL FBS cells and 2.5 μL kit antibody.
4. Incubate for 20 min at 2–8 °C (wash beads during this incubation and keep on ice until needed; *see* **steps 5** and **6**).
5. To wash the beads: first resuspend beads in the vial by vortexing for 30 s. Pipet 25 μL beads into a new 15 mL tube and resuspend in equal volume or at least 1 mL isolation buffer.
6. Put the tube without the lid on magnet (DynaMag 15, 50, or equivalent) for 1 min, remove buffer by pipetting, and discard. Remove tube from magnet and resuspend beads in the same volume of isolation buffer as the original bead volume.
7. After antibody incubation, wash cells by adding 2 mL isolation buffer. Pellet the cells at 150×*g*, for 5 min, at 4 °C and decant the supernatant.
8. Resuspend cells in 975 μL isolation buffer per 10^7 cells and add pre-washed beads.
9. Incubate for 15 min at 4 °C with tilt and rotation on a rocker at a speed sufficient to keep the beads in suspension. (The 4 °C incubation reduces efficiency, but better preserves cell viability in advance of flow cytometric sorting.)
10. Add 1 mL isolation buffer per 10^7 cells, and gently pipet up and down five times with a large-bore pipet (5 mL or similar).
11. Put tube without lid on magnet for 2 min.
12. Transfer supernatant containing enhanced homogeneity to a new tube, and wash with complete medium as soon as possible after transferring.
13. Count cells to determine recovery and take an aliquot of 10^5 cells to quantify purity by flow cytometric analysis (*see* **Notes 7** and **8**).
14. Purity will be >40 % with very little loss of desired cells.

3.4 Flow Cytometric Sorting

Before sorting cells, consider doing a pre-depletion with beads (*see* Subheading 3.3), especially when preparing rare cells (<5 % of the input). Although this increases the pre-sort preparation time, it often reduces the total cell manipulation time before downstream procedures. When the target frequency is under 1 %, the pre-depletion strategy should seriously be considered as the sorter error rates can substantially decrease purity in this situation (also *see* Subheading 3.4.2). The procedure outlined below assumes basic understanding of flow cytometry procedures and does not address operation of the cell sorter itself, which is generally left to a professional operator.

3.4.1 Flow Cytometric Sorting: General Procedure

1. *Plan ahead*. Consult the flow cytometry facility and inquire about specifics of available equipment and procedures. Discuss the choice of fluorochromes and evaluate the time needed for cell purification and to verify purities. It is also important to choose a sorting nozzle of appropriate diameter, as a general rule at least five times as large as the cells in the starting population. For lymphocytes, a 70 μm nozzle provides adequate speed without damaging the cells. However, a 100 μm nozzle may be better suited when sorting larger cells or when using the double-sort procedure as described in Subheading 3.4.2. For most applications, cells should be kept cold, and it is important to verify that the sorter has a chillable (4 °C) sample holder.
2. Resuspend cells to be stained for flow sorting at 2×10^7 cells/mL complete medium (use 15 mL tube for <6.0×10^7 cells, 50 mL for >6.0×10^7 cells). Before staining for sort, a titration of the antibodies is advisable; 0.25 μg antibody per 10^7 cells is a good starting point. If using the protocol in Subheading 3.3 prior to sorting, ensure that the antibodies used for flow cytometric sorting were not blocked by the antibodies in the bead kit (*see* **Notes 7–9**).
3. Do not forget to prepare cell samples for flow cytometer setup if needed. Typically set aside 0.5×10^6 cells per compensation tube. These will be the single-color tubes used to set up the flow cytometric sorter or analyzer for purity checks.
4. Add appropriate antibodies, mix well, and incubate at 4 °C for 45 min in the dark.
5. Wash with complete medium using four times staining volume. Pellet the cells at $150 \times g$, for 5 min, at 4 °C and decant the supernatant.
6. Resuspend cells to be flow sorted at 2×10^7 cells/mL complete medium in 5 mL round-bottom polystyrene tubes with caps.

7. Filter the starting population immediately before sorting.
8. Define sorting gates in collaboration with the operator (*see* **Note 10**).
9. Use polypropylene collection tubes that are large enough to hold the volume collected. For example two to three million cells will fit in a 5 mL polypropylene round-bottom tube; however more than that will need a polypropylene 15 mL conical tube (*see* **Note 11** on plastics in flow cytometric sorting). Add a "cushion" of 50 % FBS and 50 % complete medium to the collection tubes, a total of 1 mL for 5 mL tubes and 2 mL for 15 mL tubes.
10. Upon finishing each sorted sample, immediately fill the collection tube containing the purified cells with complete medium. If the sorted cells have filled the tube, transfer its contents as soon as possible to a larger tube and then fill that tube with complete medium. Diluting the sheath fluid with complete medium improves cell viability.
11. Take a small sample of purified cells and run in on the sorter to verify purity. Depending on the target population, it may be necessary to stain with other markers (e.g., for intracellular proteins, *see* **Notes 7–9** on staining post-sort).
12. Spin cells at 65 × *g*, for 30 min, at 4 °C. Resuspend in an appropriate volume and count cells (*see* **Note 12**).
13. Cells are ready for downstream applications.

3.4.2 Secondary Protocol for Sorting Populations ≥0.01 %

This procedure uses two consecutive sorts to isolate good purity (>95 %) populations from very rare cells (less than 0.01 % of the starting population) if bead isolation procedures are unavailable or inadequate for pre-sort enrichment (*see* Subheading 3.3).

1. Perform the primary sort following the procedure defined in Subheading 3.4.1 through **step 10** with the following changes:
   (a) Only use a 100 μm nozzle.
   (b) In **step 8**, use a wide gate to select the entire desired population and maximize yields. Unwanted cells gated in error will be removed in the second sort.
2. After **step 10**, pellet the cells at 150 × *g*, for 7 min, at 4 °C and decant the supernatant.
3. Resuspend the cells in a small volume, ≥300 μL complete medium.
4. Perform the secondary sort, using a tight gate that is slightly inside the edges of the desired population. This will increase purity to acceptable levels (>95 %).
5. Follow **steps 10–13** in Subheading 3.4.1.

4 Notes

1. It is important to define objectives for target cell purity. High cell purity (99 % or more) is often necessary when purified cells will undergo multiple rounds of cell division (e.g., activating T cells). In this case, preferential expansion of contaminating cells has the potential of significantly altering experimental outcomes. Although high cell purity is always desirable, realistic objectives should be defined based on the properties of expected contaminants and on the dynamic range of assays to be run on purified cells.
2. FBS should be heat inactivated by incubation at 56 °C for 50 min.
3. HEPES can be used to buffer media outside of a CO_2 incubator.
4. Because LNs from different body areas are to be exposed to different antigenic environments, care should be exercised in deciding whether to pool populations obtained from distinct anatomic sites. Typically, in unmanipulated laboratory mice housed in specific pathogen-free facilities, the major site of microbial exposure is the gut. Consequently, the frequency of activated cells is greater in mesenteric LNs that drain intestinal tissues, and these should generally be processed separately. In contrast, peripheral LNs (including popliteal, inguinal, axillary, and cervical) have few germinal-center and activated T cells, and it is legitimate to pool these populations.
5. Typical cell yields from organs are as follows for a 6–8-week-old female mouse: thymus, $150–250 \times 10^6$; spleen $50–80 \times 10^6$; and LNs $30–40 \times 10^6$.
6. Although commercial bead kits typically include specific antibody mixes designed for purification of homogeneous cell populations, it is possible to use beads with user-prepared antibodies. In such cases, it is paramount to verify that the bead-bound secondary reagent (e.g., streptavidin or anti-IgG) binds the primary antibodies used for purification. In addition, pilot experiments should titer each antibody for its ability to remove undesired cells.
7. When verifying purity of "negative" bead purification, it is essential to avoid staining with the same antibodies as those used for purification (or antibodies that cross-block each other). Otherwise, unwanted cells may escape detection by staining if the epitope is masked by the purifying antibody. Commercial kits often use near-saturating levels of anti-CD8α antibody to purify $CD4^+$ T cells and of anti-CD4 to purify $CD8^+$ T cells. In such situations, cell purity can be verified

using anti-CD8β, a-non cross-blocking anti-CD4, anti-TCRβ (H57), anti-MHC class II, an Fc blocking antibody, and a live/dead discriminating dye (DAPI for example, *see* **Note 8**). Three of the four commonly used anti-CD4 monoclonal antibodies (GK1.5, H129, and RM4-5) cross-block, whereas the fourth one (RM4-4) does not.

8. Live/dead dyes like DAPI remain in sorted cell suspensions and could be detrimental. Instead of these membrane-permeable dyes, use LiveDead fixable dyes that only bind dead cells and are washed away with the excess antibody. Also consider sorting live cells based on FSC and SSC and then testing viability post-sort on a small sample used only for purity check.
9. The fluorochromes (especially PE) used for flow sorting can remain associated with sorted cells for an extended time, unless they are diluted by proliferation. If additional stains are needed, make sure to leave open fluorochromes for these stains.
10. Gating strategies obviously depend on each application. In general, it is advisable to tightly gate the target population. This slightly reduces the yield but minimizes contaminations. It is also recommended, when possible, to exclude cells with unwanted markers by negative gating. Several markers staining with the same fluorochrome can be combined to exclude multiple cell types. This also contributes to the exclusion of cell aggregates (which should be primarily excluded by light scatter-based doublet discrimination).
11. Polystyrene collection tubes have the potential to build up static electricity, which can interfere with sorting, and should be avoided for cell collection.
12. Flow sorters often misreport cell counts, and there is unavoidable cell loss during the centrifugation of sorted cells. If possible, manually recount sorted cells after centrifugation with a live/dead discriminating dye.

Chapter 8

Retroviral Transduction of T Cells and T Cell Precursors

Amie Simmons and José Alberola-Ila

Abstract

Transduction of lymphoid progenitors with retroviral or lentiviral vectors is a powerful experimental strategy to tease out the role of a gene or pathway in T cell development via gain-of-function or loss-of-function strategies. Here we discuss different approaches to use this powerful technology, and present some protocols that we use to transduce murine HSCs, thymocytes, and lymphoid cell lines with these viral vectors.

Key words Retrovirus, Hematopoietic stem cell, Thymocytes, Bone-marrow chimera, Development

1 Introduction

Transduction of hematopoietic cells with retroviral vectors is an efficient way to manipulate gene expression during development of the immune system. Retroviral vectors are easy to manipulate in the laboratory and provide stable, long-term gene expression in the infected cells and their progeny because they stably integrate into the genome. Their major limitation is that they can only efficiently infect and integrate in cycling cells.

Most popular vectors are derived from the murine stem cell virus (MSCV) [1], a derivative of the Moloney murine leukemia virus (MoMLV) which can maintain long-term expression in infected cells and their progeny. While the genomes of these viruses are typically 7–12 kb in size and code for three structural genes, *gag*, *pol*, and *env*, the vectors consist only of the essential *cis*-acting elements of the viruses. *gag*, *pol*, and *env* are provided in *trans*, either in a stably transfected cell line (*see* ref. 2) or, as we will discuss in this review, cotransfected with the viral vector in independent plasmids. The separation of the structural elements in different plasmids increases the safety of the vectors, since the virus produced is replication defective, and provides space in the viral backbone to clone the gene of interest and/or markers to follow the infected cells.

The host range of a retrovirus is determined by its envelope glycoprotein (Env). The most widely used are the Env proteins of

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_8, © Springer Science+Business Media New York 2016

ecotropic MLVs. A major advantage (and a limitation) of ecotropic viruses is that they cannot infect human cells, and therefore can be used safely, with most inserts, under normal BSL2 laboratory conditions. For infection of human cells, the retrovirus can be packaged with an MLV amphotropic Env. Alternatively, Env proteins from a different virus can be used to package the retroviral vector. This process is called pseudotyping, and the most common heterologous Env used is the vesicular stomatitis virus glycoprotein G (VSV-G). Besides the extension in host range, VSV-G envelope protein forms very stable viral particles, which can be concentrated by ultracentrifugation [3]. The use of these modifications significantly increases the biosafety concerns (*see* **Note 1**).

An alternative to MSCV-based retroviruses is HIV-1- or FIV-based lentiviral vectors. Their major theoretical advantage over conventional MSCV-based retroviral vectors is their ability to transduce nondividing cells, which would make them better to infect HSCs. While this seems to be the case with human HSCs, the results with murine HSCs are not so clear [4–6] and it seems that murine HSCs need to be in G1 for an effective transduction to occur, and therefore they need to be stimulated in vitro with cytokines, as is the case for transduction with retroviruses. In our hands, lentiviral vectors have not been consistently superior to MSCV-based retroviral vectors for HSC transduction, and tend to be harder to manipulate in the laboratory.

One limitation of conventional retroviruses and lentiviruses in some experimental situations is that they express the transgene at high levels throughout development. If this early expression results in developmental phenotypes that predate one's stage of interest, the approach is not useful. There have been many different retroviral designs that try to overcome this limitation. These include tetracycline-inducible viruses [7], viruses engineered so that the transgene is turned on by Cre (either by inserting a stop sequence before the transgene [8, 9], or by inserting the floxed transgene in an antisense orientation that is irreversibly inverted [10]), or by the generation of self-inactivating viruses (SIN), where the endogenous LTR is disrupted upon integration, and the transgene is then expressed by a tissue-specific promoter (*see* refs. 11–14 for some examples relevant to T lymphocyte development). The SIN approach has been performed mostly with lentiviral backbones, because conventional retroviruses engineered this way suffered from very low titers. Recent modifications seem to have alleviated this problem [15, 16] (*see* **Note 2**).

For the study of T cell development, the cells that are normally targeted are hematopoietic stem cells, derived from either bone marrow or fetal liver, immature thymocytes, and even early DP thymocytes. In all these cases, retroviral infection provides a fast and efficient method to induce or inhibit (via shRNA or dominant negative approaches) the function of a gene of interest in vivo

(via bone marrow chimeras, or intrathymic injection of infected cells) or in vitro (via rFTOCs, or using the OP9Δ system [17]).

Infection of hematopoietic stem cells, and adoptive transfer into irradiated hosts, is a very powerful approach to rapidly test the function of a gene of interest in development, to perform in vivo structure-function analysis, or genetic rescue experiments. The main limitation of this approach is the ability to infect a sufficient number of long-term reconstituting HSCs (since a very small proportion of them are normally in cycle). Therefore most approaches involve enrichment of LT-HSCs, and treatments to make them enter cell cycle, so that they can be infected by the retroviral vector while not losing their functionality. One approach is to treat the mice with fluorouracil, which depletes fast-replicating cells in the bone marrow. This has two effects; it increases the percentage of LT-HSC in the marrow, and promotes cell cycle entry, as these cells try to replenish the multipotent progenitors [18, 19]. Then cells are cultured in media with cytokines and infected (*see* **Note 3**). Another approach is to enrich for HSCs in vitro, using magnetic depletion with commercially available lineage marker cocktails (*see* **Note 4**), and then stimulate them with the same cytokine cocktail in vitro before infection. Below we detail our current protocols to infect HSCs and thymocytes.

2 Materials

2.1 Production of Retrovirus: Transient Transfection

1. 1.5 ml microfuge tubes.
2. 5 and 14 ml polypropylene tubes.
3. *293T growth media*: Dulbecco's modified Eagle's medium (DMEM), 10 % fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin.
4. *HEK 293T cells* (ATCC, CRL-11268) (*see* **Note 5**).
5. *2× HBS*: 50 mM Hepes, 10 mM KCl, 12 mM dextrose, 1.5 mM NaH_2PO_4, 280 mM NaCl, pH 7.05 ± 0.05.

   Prepare three or four independent 100 ml batches, filter sterilize with a 0.2 μm filter, and aliquot in 10 ml/15 ml conical tubes. Test the four batches with viral vector and packaging stocks that have worked well previously, and discard those 2× HBS batches that do not work well (*see* **Note 6**).
6. *2 M CaCl2*: Prepare a 2 M solution (29.4 g in 100 ml), filter through a 0.2 μm filter, and store aliquots at −20 °C.
7. *Packaging plasmid(s)* containing retroviral gag, pol, and env genes: We routinely use pCL-Eco [20] for retroviral constructs (e.g., Addgene #12371). Alternatively, gag/pol, and another envelope such as VSV-G (e.g., Addgene #8454 or #12259), can be used. For lentivirus we use psPAX2 (e.g., Addgene #12260) to provide the missing structural elements.

8. *Vector plasmid*: There are many different vectors that can be used. As discussed above, for lymphoid cells, and long-term stable expression, backbones derived from MSCV are preferred. We use MIGR1, originally described in [21] for overexpression, and pBanshee [22] for shRNA expression [23]. For lentivirus, we normally use FUGW [13] or derivatives.

Prepare plasmid stocks using standard molecular biology techniques (*see* **Note 7**), and determine plasmid DNA concentration. Our laboratory routinely uses PEG purification [24], but other methods, or commercial kits, also produce good DNA.

2.2 Retroviral Transduction

1. *Bone marrow growth media* (BMGM): RPMI, 10 % fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml SCF (*see* **Note 8**).
2. 2 % BSA in PBS (sterile).
3. *Retronectin-coated plates*: Retronectin (*see* **Note 9**). 1 mg/ml in sterile PBS.

   Non-tissue culture-treated plates (*see* **Note 10**). Dilute retronectin stock to 12 μg/ml and add 250 μl/well for 24-well plates, or 1 ml/well for 6-well plates. Incubate for 2 h at room temperature, or wrap the plates and incubate overnight at 4 °C. Remove the retronectin solution and block the plates with 2 % BSA (in sterile PBS) for 30 min at room temperature. Wash twice with PBS. Plates can be stored with PBS at 4 °C.
4. Polybrene.
5. Lipofectamine.

3 Procedures

3.1 Transient Transfection

1. Twenty-four hours before transfection plate $0.4–0.8 \times 10^6$ exponentially growing 293T cells per 6 cm Petri dish in a 4 ml volume of 293T growth media; or $1–2 \times 10^6$ cells per 10 cm dish in 8 ml (*see* **Note 11**).
2. On the day of the transfection: have 2× HBS, 2 M $CaCl_2$ solution, and sterile water at room temperature. Use polypropylene tubes (microfuge, 4 or 15 ml):

   For 10 cm dish:

   (A) Microfuge tube: In a final volume of 500 μl H_2O, mix, 7 μg retroviral construct (*see* **Note 12**), 7 μg pCL/Eco (*see* **Note 13**), and 62 μl 2 M $CaCl_2$.

   (B) 4 or 15 ml tube: 500 μl 2× HBS.

   For 6 cm dish:

   (A) Microfuge tube: In a final volume of 300 μl H_2O, mix 4 μg retroviral construct, 4 μg pCL/Eco, and 37 μl 2 M $CaCl_2$.

(B) 4 or 15 ml tube: 300 μl 2× HBS.

Take the Petri dish with the 293T cells to be transfected out of the incubator into the tissue culture hood. Take one plate out at a time. It is very important to keep the pH of the media buffered.

Mix (A) and add dropwise to (B) while vortexing (B). Alternatively, instead of vortexing (B), blow air into (B) using a Pasteur pipette while adding (A) dropwise. Keep vortexing or blowing air into (B) for 30 s or so. Immediately add the mix to the 293Ts distributing it dropwise throughout the plate, and then swirl dish gently; do not eject liquid into dish because 293T cells will be stripped off. Put dish back into incubator. Transfect next dish.

3. Incubate cells with transfection mix for 16–20 h, after which the plates are rinsed once with PBS or media and fed with 3 ml of fresh media. At this stage the cells are slightly detached and rinses should be done gently to avoid stripping them off.
4. Supernatant is collected 24 and 48 h after the rinse. Filter through a 0.45 μm syringe filter to remove cells. It is ideal to prepare fresh supernatant for every experiment, but we routinely snap freeze the supernatant in aliquots (keep at least at −70 ° C). The freezing causes a 50 % reduction in viral titer.

   GFP expression can be seen 16 h after transfection and is maximal at 24–48 h. Check transfection efficiency of the 293T cells by flow cytometry. If the transfection efficiency is not high (>60 %), you can assume that the virus titer will probably not be good (*see* **Note 14**).

3.2 Transduction

In general, the method involves plating cells in 24-well plates with 2 ml of fresh warm viral supernatant and 4 μg/ml lipofectamine or 5 μg/ml polybrene, centrifuging cells at 20 °C for 1–1.5 h at $460 \times g$, culturing cells for 1 h at 37 °C, and then replacing the viral supernatant with media (*see* **Note 15**); additional details are provided below. These approaches yield good efficiency of infection when coupled with a good virus, e.g., >90 % with lymphoid lines, up to 60 % with fetal liver and early thymocytes, 5–20 % with DP thymocytes, and 15–30 % with enriched bone marrow cells.

3.2.1 Retroviral Transduction Hematopoietic Cells

1. Day 0: Cells are plated in BMGM, and cultured for 24–36 h at 2×10^6 cells/ml (the yield is approximately $2–5 \times 10^6$ cells per 5-FU treated mouse).
2. Day 1: Use freshly produced virus, or thaw on ice an aliquot of previously frozen virus. Calculate how much virus you will need, depending on how many cells are going to be infected. For a 6-well plate we use 2 ml viral supernatant and 2.5×10^6 cells/well. For a 24-well plate, we use 0.5 ml virus and 0.5×10^6 cells/well.

3. Add virus to previously prepared retronectin-coated plates. Incubate at 37 °C for 2 h or centrifuge at 2000 × *g* for 2 h at 22 °C and then wash with PBS (*see* **Note 16**).
4. Add the cells in BMGM and centrifuge at 2000 × *g* for 5 min, to facilitate adhesion to the fibronectin. Incubate at 37 °C overnight.
5. Day 2: Collect the cells (*see* **Note 17**). Wash, resuspend in fresh BMGM, either culture for 48 h or repeat the infection on fresh virus-loaded retronectin plates (*see* **Note 18**), and then culture for 24–48 h.
6. Day 3: If your virus has a fluorescent marker, infection efficiency can be checked by flow cytometry.
7. Day 4: Adoptively transfer the infected cells into irradiated hosts following standard bone marrow chimera protocols (*see* **Note 19**).
8. Analyze the bone marrow chimeras 6–12 weeks after transfer for your phenotype.

3.2.2 Retroviral Transduction of Lymphoid Cell Lines

1. Resuspend cells from a culture in exponential growth at 5×10^6/ml of fresh media. Mix 100 μl of the cell suspension with 2 ml of fresh viral S/N, and polybrene at 5 μg/ml, and dispense in one well, in 24-well plates.
2. Centrifuge plate for 1 h at RT, 450 × *g*.
3. Incubate cells for 1 h in the incubator (37 °C 5 % CO_2). Replace 1.5 ml of media in each well with fresh media. Afterwards, cells are fed and split as necessary.

3.2.3 Retroviral Transduction of Primary Thymocytes

1. Plate 1.5–2×10^6 cells/well in 24-well plates, in 2 ml of retroviral supernatant, plus Lipofectamine at 4 μg/ml. Spin for 1–1.5 h, at 450 × *g*, at RT (*see* **Note 20**).
2. Wash cells by aspirating most of the supernatant without disturbing the layer of cells and replace with fresh media.
3. Expression of the transgene (at least when monitored by GFP expression can be detected after 18–24 h). The thymocytes can be now used in your model (rFTOC, OP9Δ differentiation, etc.) (*see* **Note 21**).

4 Notes

1. Using oncogenic inserts increases the biosafety requirements. In any case, approval from the Institutional Biosafety Committee is required for any work with these vectors, and different institutions have slightly different requirements.
2. When considering all these alternatives it is important to keep in mind that retroviral and lentiviral vectors have a limited cargo

capacity, and that the bigger the final construct is, the lower the titer of the virus we will be able to produce [25]. Retroviruses bigger than 8 kb, or lentiviruses bigger than 10 kb, are very hard to work with. Keep this in mind when thinking about SIN lentiviruses with a tissue-specific promoter, your gene of interest, and IRES-GFP to follow infection. Also, systems that work well with one transgene may not work with other transgenes.

3. Mice are injected I.P. with 250 μg/g body weight of 5-fluorouracil dissolved in PBS (10 mg/ml), and the bone marrow cells are harvested 4–5 days later.

4. We use the BD Biosciences Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set and magnetic depletion, but there are other equally good commercial alternatives.

5. 293T cells are grown in a 37 °C degree incubator containing 5 % CO_2. The cells should not be allowed to become overconfluent, and should not be split more than 1:5. 293T cells easily detach from the tissue culture dishes after approximately 30 s of treatment with trypsin at room temperature (0.05 % trypsin/0.53 mM EDTA). It is important to freeze multiple (50–100) vials of the 293T cells after first receiving and expanding them. This will ensure a ready supply of backup vials to allow for uniform virus production over several years. Go back to an early freeze and expand again when running low on backup vials.

6. This is the most finicky reagent in the process. It normally takes two or three batches of 2× HBS before finding one that works well for transfections. The ability of the 2× HBS solution to produce working $CaPO_4$ precipitates deteriorates after 6 months to 1 year, even when the 2× HBS solution is stored at −20 °C. Prepare and test a fresh batch before the old one is finished or too old! Despite this, in our experience no commercial reagent works as well as a good homemade batch.

7. It is recommended to grow the viral backbone in RecA-negative strains, and at 30 °C, to minimize recombination.

8. Other cytokines may also be added to this cocktail such as Flt3 (50 ng/ml) and TPO (25 ng/ml).

9. Matrix proteins such as fibronectin mediate colocalization of target cells and vector [26]. The highest gene transfer is obtained with the recombinant protein CH-296 (RetroNectin).

10. This is important. Retronectin binds much better to non-tissue culture-treated plates [27].

11. Be careful with your 293T cells. Do not use cells that have been kept in culture for a long time, or your transfection efficiency will be reduced. It is extremely important that the cells are not overly clumped and are at the correct density. It is essential that the cells are extremely healthy prior to

plating. It is recommended to count the cells rather than estimating the split.

12. The amount of DNA may be increased up to certain limits, when it starts to be toxic. This is our default start amount.
13. We normally use pCL/eco as the packaging vector. This can be substituted by other combinations. Please note that if you pseudotype the retrovirus with VSV-G, as discussed, it is important to titer the amount of VSV-G env plasmid in the transfection. Too much results in toxicity, cell fusion, and death, and low virus titers.
14. For most applications that require infection of primary cells, it is important to obtain a high titer virus. As a general rule, the bigger the final vector, the lower the titer, but there are other factors that affect this process that are not well defined. If your viral backbone expresses GFP or another surface marker, virus titer is easy to estimate by infecting a receptive, exponentially growing cell line. In our laboratory we routinely use 16610D9, a murine thymoma cell line [28], to test our viral preps.
15. Most primary hematopoietic and lymphoid cells show reduced viability when cultured for long periods of time with retroviral supernatant and other infection reagents such as lipids or polybrene. Therefore, we use either spin infection or incubation on virus-loaded retronectin-coated plates as our preferred method for retroviral transduction because it minimizes the time cells are exposed to these reagents.
16. Alternatively, cells can be mixed with the viral supernatant, incubated in the retronectin plates for 4 h, and then washed and replated with fresh media. Some viral supernatants can affect HSC survival and function, so we prefer to preabsorb the virus to retronectin.
17. They will be weakly adherent, so forceful pipeting or gentle scraping with a rubber policeman is recommended.
18. The second infection increases the infection efficiency, but also prolongs the amount of time the cells spend in culture, which may decrease their ability to reconstitute irradiated hosts. We tend not to do a second infection, unless the virus we are using is not very good.
19. If the infection efficiency is very low, infected cells can be enriched by sorting, and then injected alone (or with rescue bone marrow if their numbers are low). This approach can salvage some experiments. We prefer to inject both infected and uninfected cells, because the uninfected cells provide a good internal control in each reconstituted animal.
20. Use freshly isolated cells and never put on ice for best results.

21. We have used this approach to infect DP thymocytes, and analyze the role of different signal transduction pathways on GATA-3 induction [29]. In this case it is useful to culture the infected thymocytes on a OP9Δ layer, because it seems to improve their viability. For these experiments we use them in experiments 24–36 h after infection.

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Chapter 9

Bone Marrow and Fetal Liver Radiation Chimeras

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Abstract

Radiation chimeras are prepared by subjecting recipient mice to sublethal or lethal dose of irradiation and injecting them with hematopoietic stem cells (HSC) from untreated donor mice. HSC can be obtained from bone marrow or fetal liver. This technique is a powerful tool when coupled with gene targeting strategies to investigate function of HSCs, thymocyte development, and T cell function. This protocol describes how to produce bone marrow or fetal liver chimeras.

Key words Bone marrow, Transplantation, Hematopoietic stem cells, Immune reconstitution, T cell

1 Introduction

The presence of hematopoietic stem cells (HSCs) in bone marrow was inferred from experiments that arose from studies on the effects of irradiation on mammals early in the nuclear age [1]. HSCs are defined by their ability to support long-term reconstitution of all mature blood cell types. Bone marrow transplantation (BMT) has become a valuable research tool to study immune cell development and function especially in mouse gene knockout models. In cases where gene targeting results in late embryonic death, fetal liver cells can be used as a source of HSC for transplantation in wild-type adult mice to allow study of the effects of mutations specific to immune cell development and function. Based on the work done in animal models, HSC transplantation has evolved into an effective treatment for hematopoietic diseases and as an increasingly important component of anticancer therapy.

HSC transplantation can be used to differentiate between cell intrinsic and environmental effects on immune cell development and function. For example, a targeted mutation that causes a severe defect in T cell development can be traced to lymphoid cells if the phenotype is observed in wild-type mice transplanted with bone marrow from the mutated mouse. Conversely, a defect in thymic epithelial cell function would be evidenced by defective T cell development in

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_9, © Springer Science+Business Media New York 2016

spite of transfer of wild-type HSC into the mutant mouse. Competitive transplantation includes a mixture of HSC from different mouse strains to assess the ability of mutant cells to function in competition. This technique allows characterization of reduced function due to mutations that may not be evident in the absence of competition. In HCS transplantation experiments it is often critical to be able to differentiate host from donor cells. This can be done using antibodies that recognize different congenic alleles (such as CD45.1 and CD45.2), or the use of transgenic markers such as EGFP. These markers can be used to assess reconstitution using blood samples or harvested tissues at experimental endpoints. The timing for reconstitution varies but one should expect to be able to observe donor cell development in the thymus within 2–3 weeks and stable thymic reconstitution within 8–9 weeks.

2 Materials

Use sterile technique throughout and work in a laminar flow hood when possible or practical. Dissection of leg bones can be done outside of hood.

Water bath, ice, centrifuge, microscope, trypan blue, and hemocytometer or automated cell counter are used in the procedures listed below.

2.1 Host Mice (See Note 1)

1. C57BL/6 CD45.2 congenic mice 6–12 weeks old, weight at least 20 g (*see* **Notes 2** and **3**).
2. Drinking water acidified with hydrochloric acid to pH 2.5–3.0.
3. Antibiotics: Amoxicillin or other.

2.2 Bone Marrow Preparation

1. C57BL/6 CD45.1 congenic mice 6–12 weeks old.
2. 10 ml syringes tipped with 25 g needles.
3. Flushing media: RPMI 1640, 2 % fetal calf serum, 1 % penicillin/streptomycin, 1 % HEPES, and 1 % l-glutamine.
4. ACK lysing buffer.

2.3 Optional T Cell Depletion

1. Wash media: RPMI 1640, 1 % penicillin/streptomycin.
2. Low-Tox rabbit or guinea pig complement.
3. Antibodies (clone name): Anti-CD90.2 (HO-13-4), anti-CD8 (83-12-5), anti-CD4 (C3PO) (*see* **Note 4**).
4. DNase—2 mg/ml stock.

2.4 Fetal Liver Preparation

1. Flushing media (see above).
2. Sterilized dissecting tools (curved forceps, tweezers, small and large scissors), dissecting microscope.
3. Timed pregnant mice days E12.5–18.5 (*see* **Note 5**).

2.5 Analysis

1. Flow cytometer and software to analyze data.
2. 5 ml polystyrene tubes required for flow cytometer.
3. FACS buffer: 1× PBS, 0.5–1.0 % BSA, 0.1 % (W/V) sodium azide (*see* **Note 6**).
4. Antibodies (clone name): Anti-CD45.1-FITC (A20), anti-CD45.2-PE (104), anti-B220-APC (RA3-6B2), anti-CD90-APC (53-2.1), anti-CD16 (2.4G2) antibodies.
5. Commercial 1-step fix/lyse solution.

3 Methods

3.1 Prepare Host Mice

1. One week before irradiation, the recipient mice are given acidified drinking water (to prevent growth of *Pseudomonas* species) supplemented with antibiotics such as amoxicillin (0.5 mg/ml).

3.2 Prepare Bone Marrow Suspension from Donor Mice

1. Prepare 10 ml syringes with 10 ml of flushing media.
2. Sacrifice donor mice following institutional guidelines and soak fur with 70 % ethanol. Snip skin at the base of tail and peel over legs and up the body to contain hair inside the inverted skin. Dissect long bones (femur, tibia), and strip off tissue using sterile forceps, scissors, and gauze (*see* **Note 7**). Place stripped bones in Petri dishes containing ice-cold flushing media and keep on ice.
3. Snip both ends of bones with sharp scissor or bone clippers to allow easy entry of needle into one end. Use needle to ensure that bone is open on both ends if needed. The red marrow should be easily visible in the bone. Insert needle into one end of the bone and using some force, flush marrow cavity with 2–3 ml of flushing media into 50 ml conical tubes on ice. In most cases, the marrow comes out as an intact tube of red tissue.
4. Agitate gently, and then spin marrow cells at 1000 × *g* for 5 min at 4 °C.
5. Carefully remove supernatant from the loosely pelleted cells and resuspend in complete flushing medium using about 1 ml for each mouse used for marrow collection.
6. Process cells by pipetting up and down several times and then run through a cell strainer. Use a sterile forceps or 5 ml syringe plunger to mash marrow clumps and rinse the strainer with 3 ml complete flushing media. Place marrow cells on ice.
7. Remove 5 μl of the marrow cell suspension for counting and place in an Eppendorf tube. Add 95 μl of ACK lysis buffer to the 5 μl cell sample in the Eppendorf tube, mix well, and count the cells.

3.3 Optional T Cell Depletion of Marrow

T cell depletion of marrow eliminates resident T cells in the graft which allows analysis of T cell function of cells derived from engrafted HSC and prevents graft-versus-host disease (GVHD) if MHC mismatches are present (*see* **Note 2**).

1. Place counted bone marrow cells in 50 ml tube and fill with washing media, spin down cells, and remove media (*see* **Note 8**).
2. Prepare bone marrow cell suspension at 2.0×10^7 cells/ml in RPMI/PSG without FCS.
3. Add antibodies at predetermined concentrations and mix gently (*see* **Note 4**).
4. Place on ice for 15 min.
5. Wash bone marrow cells with RPMI/PSG without FCS. Do not resuspend pellet.
6. Add diluted guinea pig complement to bring cell number to 2.0×10^7/ml (*see* **Note 9**). Add DNase to 40 μg/ml of cell suspension. Mix well.
7. Place in 37 °C water bath × 40 min.
8. Wash with RPMI/PSG and resuspend cells in 50 % volume used in **step 1**. Count cells and resuspend at 5.0×10^7/ml in sterile PBS (*see* **Note 10**).

3.4 Prepare Liver Cell Suspension

1. Harvest E14–E15 embryos and place in flushing media in a 15 cm tissue culture dish on ice (*see* **Note 11**).
2. Once cooled, rinse embryo in flushing media, place on a piece of gauze, and euthanize the following institutional guidelines. Remove a piece of tissue (tail, foot) and freeze for genotyping if needed (*see* **Note 12**).
3. Dissect liver and place in sterile Eppendorf tube with 1 ml flushing media. Be sure that liver is numbered the same as genotyping biopsy.
4. Prepare cell suspension by mashing and pipetting the liver up and down first with a P1000 tip and then with a P200 tip.
5. Allow cells to settle for 5 min on ice. Transfer cell suspension to a new sterile Eppendorf tube taking care to avoid debris that has settled to the bottom of the tube (*see* **Note 13**).
6. Strain cells with a 40 μM filter. Count viable cells and adjust to $0.25–1 \times 10^7$ cells/ml in sterile PBS for injection.

3.5 Inject HSC into Mice

1. At least 4–6 h before injection, irradiate recipient mice using 9.5 Gy (*see* **Note 14**). It is advisable to wait ~2 h after the last irradiation dose before injecting cells.
2. Inject 1.0×10^7 depleted BM cells in 200 μl sterile PBS in the tail vein of the irradiated recipient mouse. Alternatively inject $0.5–2 \times 10^6$ fetal liver cells in 200 μl sterile PBS (*see* **Note 15**).

3. Keep mice on acidified water containing antibiotics and in autoclaved cages for 2 weeks after injection of stem cell inoculum.
4. Wait for 1 month before testing for T cell chimerism. Peripheral T cell reconstitution is complete after approximately 8 weeks.

3.6 Analysis of Chimera Reconstitution

Immune reconstitution can be monitored using blood samples. If bone marrow was T cell-depleted, the presence of donor B cells can be used to quickly determine if transplant was successful.

1. Obtain 100 μl heparinized blood samples from transplanted mice and divide into two FACS tubes. For controls, obtain 150 μl blood samples from a CD45.2 mouse and divide into three FACS tubes (*see* **Note 16**). Obtain 50 μl blood from a CD45.1 mouse and place into one FACS tube. Add 0.5 μl anti-CD16/CD32 antibody to block antibody binding by the Fc receptor and incubate on ice for 10 min.
2. Add antibody mixes (CD45.1, CD45.2, and B220 or CD45.1, CD45.2, and CD90) to blood from transplanted mice at predetermined concentrations (0.25–1.0 μl/sample should work). Add single antibodies to the four control tubes. Incubate on ice for 15 min.
3. Add 1 ml 1-step fix/lyse solution to each FACS tube, mix gently, and incubate for 15 min minimum (*see* **Note 17**).
4. Spin down cells at 500×*g* for 5 min at room temperature. Remove supernatant and resuspend cells in 1 ml FACS buffer, spin down again, remove supernatant, and add 500 μl FACS buffer.
5. Use single-color controls to set up compensation on cytometer and run samples. If successful, a population of donor CD45.1-positive cells should be readily apparent. Gate on the donor cells to determine levels of B cell (B220) and T cell (CD90) chimerism. Depending on results, proceed to experimental endpoints (*see* **Note 18**).

4 Notes

1. Ensure that institutional guidelines are followed for all animal housing, handling, procedures, and euthanasia.
2. It is important to match MHC antigens of donor and host mice to avoid complications due to GVHD or host-versus-graft disease (HVGD) unless BMT is being used to study these syndromes in animal models [2].
3. Here we use allelic differences in CD45 to identify host and donor cells. Another option is the use of a transgenic marker, such as EGFP expression by donor mouse cells.

4. We use supernatants from hybridoma cultures as an antibody source. These are IgM antibodies to enhance complement lysis. Other antibodies and isotypes (IgG) can be used but all antibodies should be titrated beforehand to optimize efficiency. Titrations ranging from 0.25 to 5.0 μg/ml should be sufficient.
5. Timed pregnant mice are commercially available for commonly used strains. Alternatively, any strain of mice can be used to produce timed pregnant mice following procedures outlined by Mader et al. [3].
6. Fetal bovine serum can also be used at 3–10 % instead of BSA. Sodium azide is used as a preservative; its addition to the buffer is optional.
7. Bone marrow from humerus, hips, and vertebral bodies can also be used to increase the number of cells. Vertebral bodies and hips can be crushed and bone fragments filtered out.
8. Ensure that cells are thoroughly washed with at least 10 volumes to eliminate serum proteins.
9. Nonspecific cell loss is variable, but can be substantial using complement treatment. The following suggestions can help to minimize nonspecific cell lysis. Ensure that cells, centrifuge, and buffers are kept cool or on ice at all times. Use care to gently resuspend cell pellets, especially after washes. Antibodies should be titrated. New lots of complement should be prepared according to the specification sheet and dilutions tested for efficient lysis and minimal nonspecific killing. Typically a 1:5–1:20 dilution should be sufficient. Low-toxicity rabbit complement may work better if IgG antibodies are used, or may be used instead or in combination with guinea pig complement to improve depletion or reduce unwanted toxicity. There are several reliable commercially available alternative methods to effectively deplete defined cell populations. For example commercially available kits can be used to deplete various populations using antibodies coupled with a magnetic bead capture strategy. Although convenient (especially for small-scale depletions), these kits and the needed equipment can be costly. For reference, bone marrow of normal mice should contain about 5 % T cells and about 12–20 million marrow cells/mouse after T cell depletion.
10. Use flow cytometry (Subheading 3.6) to assess the efficiency of T cell depletion using anti-CD90 or similar antibodies. If needed, another cycle of complement depletion can be done. If using two cycles, results may be improved if different antibodies are used in each cycle.
11. Hematopoiesis peaks in liver at days E14–E15 making this an ideal time to harvest HSC. However, HSC can be harvested from fetal liver from days E12.5–E18.5 if needed [4].

12. Use care to avoid cross contamination between embryos and avoid contamination with mother's blood.
13. Fetal liver cells can be frozen at this point in FCS with 10 % DMSO and stored in liquid nitrogen. To prepare cells for injection, thaw cells, add 50 ml cold PBS, incubate for 10 min, spin down cells, and repeat cold PBS wash to eliminate DMSO. Filter cells, count, and adjust as described in procedure.
14. C57BL/6 mice are relatively resistant to radiation effects and can be given a single 9.5–10.0 Gy dose. Irradiation can be divided into two 4.75 Gy treatments 2–3 h apart to reduce potential radiation injury. Immunodeficient mice such as Rag2 knockout can be injected without irradiation or should only be given 6.0 Gy. BALB/c mice are radiation sensitive and should be given two 4.50 Gy doses.
15. Fewer cells can be injected, down to about 1×10^6 per mouse, but may delay reconstitution. Bone marrow cells can also be injected retro-orbitally into anesthetized mice. Newborn mice can be injected intraperitoneally.
16. This procedure works equally well with heparin- or EDTA-treated blood.
17. Cells can be stored for up to 48 h in the 1-step fix/lyse solution following the manufacturer's recommendations.
18. The choices for antibodies to stain cells are great and are dictated by experimental design. In general, consider using fluorochromes (such as Pacific-Blue) that have minimal overlap in other channels for gating on donor cells. Preliminary experiments should be done to optimize fluorochrome choices and antibody concentrations. Be sure to include appropriate controls to allow accurate gate setting for data analysis.

Acknowledgement

The Intramural Research Program of the National Cancer Institute at the National Institutes of Health supports the authors. The authors have no conflicts of interest to disclose.

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Chapter 10

In Vitro Analyses of T Cell Effector Differentiation

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Abstract

In vitro culture is an important complement, or substitute, to in vivo approaches in order to study T cell effector differentiation. Here, we describe culture conditions that generate specific effector cell types by exposing naïve T cells to appropriate cytokine signals.

Key words T cell differentiation, Tc, Th0, ThN, Th1, Th2, Th17, iTreg

1 Introduction

Effective T cell function is required for protection from invading pathogens. T cell effector differentiation is determined by several signals, notably from the innate immune cells, including (1) stimulation through the T cell receptor (TCR), (2) co-stimulation through the CD28 costimulatory molecule and (3) cytokine exposure that induces acquisition of specific T cell differentiation. Studies exploring how naïve T cells differentiate into fully functional effector cells often require assessing the effector potential of these T cells; protocols for such analyses are presented in this chapter.

During in vivo immune responses, effector T cell differentiation occurs in secondary lymphoid organs or tissues, and is driven by architecturally constrained interactions between T cells, antigen-presenting cells (APC)s and other immune cells. Early stages of an immune response involve small cell numbers of antigen-specific T cells, which may be difficult to identify and purify. Thus, it can be advantageous to reproduce the conditions that promote T cell effector differentiation in vitro. In vitro differentiation allows acquisition of a large number of cells, e.g., for biochemical or gene expression studies. In addition, in vitro studies allow for tighter control of cytokines and other stimuli offered to T cells. These in vitro cultures typically include two distinct stimuli. First, a ligand

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_10, © Springer Science+Business Media New York 2016

Table 1
Effector differentiation in vitro

Conditioning mix	Cytokines (2× concentrations)	Blocking antibodies (2× concentration)	Cytokines (final concentration)	Blocking antibodies (final concentration)
ThN	IL-2 (20 ng/ml)	–	IL-2 (10 ng/ml)	–
Tc	IL-2 (20 ng/ml)	–	IL-2 (10 ng/ml)	–
Th17	IL-6 (20 ng/ml) TGF-β (5 ng/ml)	Anti-IL-4 (20 μg/ml) Anti-IFNγ (20 μg/ml) Anti-IL-12 (20 μg/ml)	IL-6 (10 ng/ml) TGF-β (2.5 ng/ml)	Anti-IL-4 (10 μg/ml) Anti-IFNγ (10 μg/ml) Anti-IL-12 (10 μg/ml)
+Th0	IL-2 (20 ng/ml)	Anti-IL-12 (20 μg/ml) Anti-IFNγ (20 μg/ml) Anti-IL-4 (20 μg/ml)	IL-2 (10 ng/ml)	Anti-IL-12 (10 μg/ml) Anti-IFNγ (10 μg/ml) Anti-IL-4 (10 μg/ml)
Th1	IL-2 (20 ng/ml) IL-12 (20 ng/ml)	Anti-IL-4 (20 μg/ml)	IL-2 (10 ng/ml) IL-12 (10 ng/ml)	Anti-IL-4 (10 μg/ml)
Th2	IL-2 (20 ng/ml) IL-4 (20 ng/ml)	Anti-IL-12 (20 μg/ml) Anti-IFNγ (20 μg/ml)	IL-2 (10 ng/ml) IL-4 (10 ng/ml)	Anti-IL-12 (10 μg/ml) Anti-IFNγ (10 μg/ml)
iTreg	TGF-β (5 ng/ml) IL-2 (20 ng/ml)	Anti-IL-4 (20 μg/ml) Anti-IFNγ (20 μg/ml)	TGF-β (2.5 ng/ml) IL-2 (10 ng/ml)[a]	Anti-IL-4 (10 μg/ml) Anti-IFNγ (10 μg/ml)

[a]IL-2 is added at 48 h

for the T cell antigen receptor complex is required to promote expression of specific cytokine receptors (e.g., for IL-2) and cell proliferation. This can be a specific peptide antigen if using cells of defined specificity (e.g., carrying a TCR transgene); in many instances however, antibodies against TCR or CD3 are used to mimic antigen stimuli and trigger TCR signaling. In both cases, TCR stimulation needs to be accompanied by engagement of CD28 costimulatory molecules. The second series of ligands is intended to direct cytokine gene expression; it includes cytokines and anti-cytokine antibodies to neutralize the effect of unwanted cytokines. Appropriate combinations of these reagents typically "polarize" T cell differentiation into specific effector fates: for CD4 cells, these generally include Th1 [1], Th2 [1–3], Th17, and inducible (i) T regulatory (Treg); and for CD8^{+} cytotoxic T (Tc) cells (*see* Table 1 and refs. 2, 4, 5). While it is possible to activate highly purified T cells with the latter reagent combinations, using separately purified APCs as "feeder" cells for the differentiating effector T cells results in greater survival for most effector types [6].

During in vitro culture, antigenic stimulation (or its surrogate) and cytokines drive cell proliferation, which typically starts within 24–36 h after stimulation and continues for 3 or 4 days. Expression of cytokine genes, and cytokine production (evaluated by ELISA

Table 2
Effector differentiation in vivo

	Cytokine for differentiation	Transcription factor	Cytokine produced
Th1	IL-12	Tbet	IFNγ
Th2	IL-4	Gata3	IL-4 IL-5 IL-13
Th17	IL-6 TGF-β IL-1 IL-21	RORγt	IL-17A IL-17F IL-22
iTreg	TGF-β Retinoic acid IL-2	Foxp3	IL-10 TGF-β
Tc	IFNγ	Tbet	IFNγ

or intracellular cytokine staining) is detected within 3–5 days of stimulation, depending on the type of cytokine. Similar kinetics are observed for fate-determining transcription factors.

Although the choice of the starting T cell population is typically dictated by the specific application, special emphasis must be placed on separating naïve from antigen-experienced (generally referred to as "memory") cells obtained from peripheral lymphoid organs. In laboratory mice housed under specific pathogen-free conditions, most spleen and lymph node T cells are "naïve." That is, they are directly derived from thymic precursors without having encountered the antigen that their TCR specifically reacts against and therefore, they do not express effector (e.g., cytokine) genes. A simple conceptual example of a naive cell is a lymphocyte carrying a TCR directed against a virus-derived peptide in a host that has not been in contact with that particular virus. In contrast, upon infection or immunization with the cognate antigen, these newly "antigen-experienced" cells proliferate, and acquire effector properties or differentiate into memory cells. In unmanipulated laboratory mice, cells exhibiting marks of antigen experience are typically reactive against commensal and environmental antigens.

Regardless of whether they are effector or memory, antigen-experienced cells have two properties not shared by naïve cells. They are generally "preprogrammed" to produce specific cytokines (most antigen-experienced cells in a mouse spleen make IFNγ), and they produce these cytokines quickly, within hours of antigen receptor triggering. In contrast, naïve cells typically do not produce effector cytokines until they have undergone multiple rounds of proliferation, and their effector differentiation is heavily influenced by the surrounding cytokine milieu (Table 2). Thus, in activation

cultures, the cytokines produced by antigen-experienced cells have the potential to skew, often towards IFNγ production, the effector differentiation of the naïve cells. To avoid this potential bias, it is generally advisable to purify naïve cells for in vitro stimulation cultures. This is all the more necessary when such cells are prepared from inflammatory contexts, in which the frequency of effector or memory cells is much higher. For both $CD4^+$ and $CD8^+$ mouse T cells, CD44 is the most commonly used marker to distinguish naïve ($CD44^{lo}$) from antigen experienced ($CD44^{hi}$) subsets.

Here we delineate protocols designed to evaluate the effector potential of naïve T cells exposed to conditions that partially mimic in vivo antigen stimulation. These protocols are designed to differentiate naïve T cells into $CD4^+$ iTregs and $CD4^+$ and $CD8^+$ T effector cells. Because of the versatility of in vitro cultures, the choice of appropriate controls is crucial to establish sound conclusions. We have provided suggestions in Subheading 4 in this regard.

The protocols described below use antibody (anti-CD3)-mediated TCR triggering as a surrogate for antigen stimulation, APCs (either dendritic cells or T cell-depleted splenocytes), and mixes of cytokines and anti-cytokine antibodies appropriate for promoting the differentiation of Th1, Th2, or Th17 $CD4^+$ effectors, Tc $CD8^+$ effectors, or $CD4^+$ iTreg cells. We also provide an alternate protocol that eliminates the APCs.

2 Materials

2.1 Blocking Antibodies

1. Anti-CD3e (without azide, unlabeled), Clone 145-2C11.
2. Anti-CD28 (without azide, unlabeled), Clone 37.51.
3. Anti-IFN-γ (without azide, unlabeled), Clone XMG1.2.
4. Anti-IL-4 (without azide, unlabeled), Clone 11B11.
5. Anti-IL-12 p40/p70 (without azide, unlabeled), Clone C17.8.

2.2 Cytokines

1. Recombinant murine IL-2.
2. Recombinant murine IL-6.
3. Recombinant human TGF-β.
4. Recombinant murine IL-4.
5. Recombinant murine IL-12.

2.3 Beads

1. Mouse pan T (CD90.2) depletion magnetic beads (*see* **Note 1**).

2.4 Media Components

1. Fetal calf serum (FCS) (*see* **Note 2**).
2. Phosphate buffered saline (PBS, Ca^{2+} Mg^{2+} free) pH 7.4.
3. Iscove's Modified Dulbecco's Medium (IMDM) (*see* **Note 3**).

4. Culture medium: 10 % FCS, 100 U/ml of penicillin, 0.10 mg/ml of streptomycin, 0.292 mg/ml of l-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid) pH range 7.2–7.5 (*see* **Note 4**), 55 μM 2-Mercaptoethanol (*see* **Note 5**), 1 mM Sodium pyruvate (*see* **Note 6**), 1× MEM nonessential amino acids (NEAA) (*see* **Note 7**), in RPMI 1640.
5. Digestion medium: 100 U/ml of penicillin, 0.10 mg/ml of streptomycin, 0.292 mg/ml of l-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid) pH range 7.2–7.5 (*see* **Note 4**), 0.25 mg/ml of deoxyribonuclease I from bovine pancreas ≥85 % protein, and 0.5 mg/ml of Liberase TL (Roche), in RPMI 1640.
6. 500 mM ethylenediamine tetraacetate (EDTA) pH 8.0.
7. Staining medium: PBS ($Ca^{2+}Mg^{2+}$ free) pH 7.4, 0.1 % bovine serum albumin (BSA), 2 mM EDTA pH 8.0.
8. Isolation medium: PBS ($Ca^{2+}Mg^{2+}$ free) pH 7.4, 0.1 % BSA, 2 mM EDTA pH 8.0.
9. Red cell lysis buffer: 8290 mg/l ammonium chloride, 1 g/l potassium bicarbonate, 37 mg/l EDTA pH 8.0.

2.5 Hardware

1. 96-well flat bottom tissue culture treated plates.
2. 96-well round bottom tissue culture treated plates.
3. Cell scraper.
4. 70 μm cell strainers.
5. Nylon tissue filters (pore size 100 μm, cut to 2 or 4 cm^2).
6. 1 or 3 ml syringe pestles.
7. 60 mm petri dishes.
8. 15 and 50 ml conical tubes.
9. Magnetic beads compatible with appropriate purified antibodies.
10. Rocker.
11. Two curved forceps.
12. Scissors appropriate for dissection.
13. 37 °C incubator with 5 % CO_2.
14. Vertical magnet holding 15 or 50 ml conical tube (DynaMag 15, 50, or equivalent).
15. 5 ml tuberculin syringe.

2.6 Fluorescent Antibodies for Flow Cell Sorting (*See* Note 8)

1. Anti-CD4, Clone RM-4.5.
2. Anti-CD44, Clone 1M7.
3. Anti-CD25, Clone 7D4.
4. Anti-CD11c, Clone N418.
5. Anti-MHC Class II (*see* **Note 9**).

2.7 Animals (See Note 10)

1. Mice of interest for preparation of T cells.
2. Mice of interest for preparation of dendritic cells or T-depleted splenocytes (*see* **Note 11**).

3 Methods

Subheadings 3.1–3.3 describe a protocol to stimulate T cells in the presence of antigen presenting cells (APCs). The protocol involves three distinct steps: (1) prepare a suspension of purified APCs to which antibody and cytokines will be added to form the "conditioning mix"; (2) isolate naïve T cells; and (3) set up cultures by mixing the APC (in the conditioning mix) and T cell suspensions. Addition of antibody and cytokines to APCs, to form conditioning mixes, should be performed last, immediately before setting up the cultures and aliquoted into the culture wells promptly.

Subheading 3.4 presents an alternative protocol for T cell stimulation on antibody-coated plates, without APCs.

3.1 Preparation of APCs

Carry out all procedures at 4 °C unless otherwise specified. Completing the procedures outlined in the section below produces a suspension of APCs in 100 μl per 96 well to which the conditioning mixes are added. Adding the conditioning mixes to the APCs streamlines the procedure.

3.1.1 Isolation of Splenic CD11c+ DC

For naïve T cells conditioned in Th0 Th1, Th2, Th17, or Tc conditions: use a ratio of 25–50 T cells for every DC. iTregs Conditioning mix should include a 5–25 ratio of T cells for every DC cultured (*see* **Note 12**). The following procedure is for one spleen and volumes can be scaled proportionally as needed. All work should be performed under sterile conditions in a tissue culture cabinet.

1. Place 5 ml of Digestion medium into a 60 mm petri dish. Surgically remove the spleen from a euthanized mouse and place directly into Digestion medium.
2. While holding the spleen with forceps, inject spleen with the Digestion medium using a tuberculin syringe. Then, using scissors, cut spleen into five to ten smaller pieces.
3. Place in 37 °C incubator with 5 % CO_2 for 20 min.
4. Add a final concentration of 5 mM EDTA to the dish and incubate five additional minutes to stop the enzymic activity of the Digestion medium.
5. Transfer spleen pieces and medium to a 50 ml conical tube and pass through a 70 μm cell strainer. Use cell scraper to remove any adherent cells and rinse the petri dish thoroughly with 5 ml Staining medium.

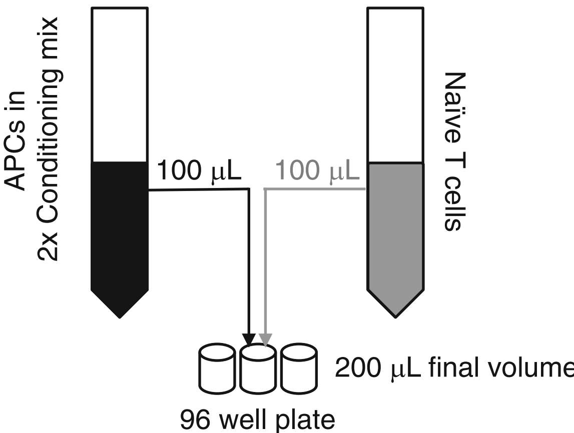

Fig. 1 Schematic of procedure

6. Lyse with 1 ml Red cell lysis buffer for 2 min on ice, wash by filling tube to 10 ml with Staining medium.
7. Resuspend in 5 ml Staining medium and count cells.
8. Refer to Chapter 7 on "Isolating T cell subsets" for staining cells with fluorescently labeled antibodies and sorting $CD11c^{+}MHC\ II^{+}$.
9. Prepare DC suspensions in Culture medium at a concentration of $1–2 \times 10^3$ cells/100 μl/well for Th0, Th1, Th2, Th17, or Tc cultures, or of $2–10 \times 10^3$ cells/100 μl/well for iTreg cultures (*see* introduction to this section and Fig. 1 for more information). Refrain from aliquoting cells into wells at this point as the Conditioning mix will be added to this master mix. Keep DCs on ice until ready to prepare Conditioning mixes.
10. *See* Subheading 3.3 for preparing Conditioning mixes.

3.1.2 Irradiated T Cell-Depleted Splenocytes

Carry out all procedures at 4 °C unless otherwise specified and under sterile conditions in a tissue culture cabinet. The ideal ratio for this type of APC is 5 for every one T cell.

1. *See* Chapter 7 on "Isolating T cell subsets" for procedure on isolating a single-cell suspension from a mouse spleen.
2. Immunomagnetic cell separation for depletion of pan T cells. The following protocol is for a 1×10^8 total splenocyte suspension, and can be scaled up or down as necessary. While this protocol utilizes immunomagnetic beads from Dynabead, users may employ immunomagnetic beads of their choice with the appropriate protocol.
3. Preparation of Dynabeads: Resuspend beads fully (either vortex whole vial or place on a tilt rotator for at least 5 min).

4. Remove 1×10^8 beads and wash in equal volume of Isolation buffer in a fresh tube. Use 1 ml of Isolation buffer to wash if bead volume is under 1 ml.
5. Place tube without lid in DynaMag-15 (or other magnet suitable for bead isolation procedures) for 1 min and discard the supernatant.
6. Remove tube from magnet and resuspend beads in the original starting volume of Isolation buffer.
7. Preparation of pan T cell-depleted splenocytes: Spin down 1×10^8 splenocytes in a separate tube and resuspend in 1 ml of Isolation buffer.
8. Add 1×10^8 pan T cell CD90.2 Dynabeads to cells and incubate for 30 min at 4 °C.
9. Place tube containing cell and bead mixture without lid onto a magnet for 2 min.
10. The T cells will bind to the beads and be removed from the supernatant. Move T-depleted supernatant in a fresh tube, wash with Culture medium, spin down and resuspend in Culture medium to count.
11. Irradiate T-depleted splenocytes with 3,000 RADS (*see* **Note 13** on irradiator use).
12. Make a master mix of 2.5×10^5 T cell-depleted splenocytes in 100 μl Culture medium per well (*see* introduction to this section and Fig. 1 for more information). Refrain from aliquoting cells into wells at this point as the Conditioning mix will be added to this master mix. Keep DCs on ice until ready to aliquot for culture.
13. *See* Subheading 3.3 for preparing Conditioning mixes.

3.2 T Cell Preparation

Carry out all procedures at 4 °C unless otherwise specified.

1. Isolate T cells from spleens and lymph nodes using either flow cytometry sorting or alternatively, an immunomagnetic bead selection approach (*see* Chapter 7 on "Isolating T cell subsets", *see* **Note 14** on caveats to using bead selection over flow sorting.)
2. Resuspend T cells at 5.0×10^4/100 μl in Culture medium. Keep this on ice until ready to aliquot for culture.

3.3 Conditioning Mixes

To the APCs prepared in Subheading 3.1, add the following reagents to prepare the corresponding 2× Conditioning mixes. These should be prepared immediately before setting up cultures and kept on ice until ready to aliquot for culture. Note that all concentrations given below refer to the concentration in the conditioning mix, therefore twice as high as in the final culture (*see* Table 1).

3.3.1 ThN or CD8+ Tc Conditioning Mix

1. Add: 2 μg/ml anti-CD3, 6 μg/ml anti-CD28, 20 ng/ml IL-2.

3.3.2 Th17 Conditioning Mix

Substituting IMDM for RPMI increases the frequency of IL-17 producing cells (*see* **Note 7**). The Th17 Conditioning mix does not contain IL-2.

1. Add: 2 μg/ml anti-CD3, 6 μg/ml anti-CD28, 20 ng/ml IL-6, 5.0 ng/ml TGF-β, 20 μg/ml anti-IL-4, 20 μg/ml anti-IFN-γ, 20 μg/ml anti-IL-12.

3.3.3 Th0 Conditioning Mix

1. Add: 2 μg/ml anti-CD3, 6 μg/ml anti-CD28, 20 ng/ml IL-2, 20 μg/ml anti-IL-12, 20 μg/ml anti-IFNγ, 20 μg/ml anti-IL-4.

3.3.4 Th1 Conditioning Mix

1. Add: 2 μg/ml anti-CD3, 6 μg/ml anti-CD28, 20 ng/ml IL-2, 20 ng/ml IL-12, 20 μg/ml anti-IL-4.

3.3.5 Th2 Conditioning Mix

1. Add: 2 μg/ml anti-CD3, 6 μg/ml anti-CD28, 20 ng/ml IL-2, 20 ng/ml IL-4, 20 μg/ml anti-IL-12, 20 μg/ml anti-IFNγ (*see* **Note 15**).

3.3.6 iTreg Conditioning Mix

1. Add: 2 μg/ml anti-CD3, 5.0 ng/ml TGF-β, 20 μg/ml anti-IL-4, 20 μg/ml anti-IFNγ (*see* **Note 16**). (Retinoic acid can be added to the culture to enhance the induction of Foxp3, *see* **Note 17**.)
2. At 48 h, add 10 ng/ml IL-2 (final concentration) and incubate for an additional 24 h. This is earlier than the day 3 culture split recommended for non-iTreg cultures (*see* Subheading 3.5).

3.3.7 Culture Set Up

1. Add 100 μl/well of each APC-containing conditioning mix to wells in a 96 well round bottom tissue culture plate.
2. Add 100 μl T cell suspension (5×10^4 cells) to each APC-containing well. The final volume should now be 200 μl per well.
3. Place in a 37 °C incubator with 5 % CO_2 for 3 days (*see* Subheading 3.5) (*see* **Note 18**).

3.4 Alternate Protocol: Plate-Bound Anti-CD3/CD28 Stimulation

Plate-bound anti-CD3/CD28 antibody stimulation is useful when examining direct effects on T cells without the complications of a different cell population. Note that anti-CD28 should be omitted for iTreg conditions (*see* **Note 16**). T cell preparation in this alternate protocol is performed as in Subheading 3.2. If using this protocol, additional anti-CD3/CD28 in the Conditioning mix is not recommended, as the plate-bound anti-CD3/CD28 is sufficient.

3.4.1 Preparation of Anti-CD3 and Anti-CD28 Coated Plates

1. Make a solution of 1 μg/ml of anti-CD3 antibody and 1 μg/ml anti-CD28 antibody in room temperature PBS.

2. Immediately aliquot 100 μl into each well.
3. Place at 4 °C overnight or 1 h at 37 °C.
4. Wash plate two times with PBS by gently adding to the side of the wells and aspirating off. Wash last time in Culture medium taking care to not let the wells dry out. If necessary, leave the last Culture medium wash in the well until the T cells are isolated and the Conditioning mixes are prepared (*see* Subheadings 3.2 and 3.3).

3.4.2 Preparation of APC-Free Conditioning Mixes

Prepare 2× cell-free conditioning mixes for each stimulation condition by supplementing APC-free Culture medium with cytokines and anti-cytokine antibodies (but not soluble anti-CD3 and soluble anti-CD28) using concentrations indicated in Subheading 3.3 above (*see* also Table 1).

3.4.3 Culture Set Up

1. Per well in a 96 well flat bottom tissue culture plate, add 100 μl/well of APC-free conditioning mix and 100 μl T cell suspension (5×10^4 cells, prepared as in Subheading 3.2), for a final volume of 200 μl per well.
2. Place in a 37 °C incubator with 5 % CO_2 for 72 h (*see* Subheading 3.5).

3.5 Split the Culture at Day 3

1. Incubate for 72 h and then split each 200 μl well into two 100 μl wells and add 100 μl of Culture medium to each well.
2. Incubate an additional 24 h.
3. Harvest cells for downstream applications.

4 Notes

1. Syngeneic mouse strains express either CD90.1 or CD90.2 allelic isoforms. Beads exist against each isoform and should be chosen according to the mouse strain used to prepare APCs.
2. Fetal calf serum should be heat inactivated by incubation at 56 °C for 50 min. Serum from different sources and even different lots can vary in efficacy. Each lot should be tested as it may differentially support T generation and cytokine production. Companies usually offer samples for this purpose.
3. Using IMDM in Th17 culture increases the efficacy of Th17 differentiation. This medium contains natural agonists for aryl hydrocarbon receptor that support differentiation of Th17 cells [7].
4. HEPES extends media stability outside of a CO_2 incubator.

5. 2-Mercaptoethanol reduces oxygen radicals in Culture medium. This chemical is not stable in solution and should be added immediately before use.
6. While not essential for cell growth, Sodium pyruvate is beneficial for most effector differentiation and especially recommended for iTreg induction cultures.
7. NEAA are not required but promote cell growth and viability.
8. The fluorochrome choice for antibodies for flow cytometric sorting of populations should be carefully considered during planning. These antibodies can remain on cells and interfere with secondary staining post culture.
9. Choose an antibody that will recognize MHC-II isoforms expressed in the mouse strain used as a source of $CD11c^{+}$ DCs.
10. Animals must be housed, handled, and used according to applicable guidelines and after securing required authorizations.
11. APCs (either dendritic cells or T cell-depleted splenocytes) should come from animals that are sex and strain matched to the experimental T cells.
12. Optimal ratio of T cells to APC (i.e., DCs or T-depleted spleen) should be determined for each specific situation. The ratios suggested here are starting points that may need to be optimized.
13. Secure required authorization training before irradiator use, and follow applicable guidelines.
14. Most bead isolation kits for $CD4^{+}$ or $CD8^{+}$ T do not remove $CD44^{hi}$ (memory or effector cells). Upon antigen stimulation (anti-CD3 exposure in culture), cytokines produced by $CD44^{hi}$ cells can bias cell differentiation independently from those included in the conditioning medium. It is therefore preferable to exclude $CD44^{hi}$ cells from in vitro cultures.
15. Overgrowing the cells will result in a loss of IL-4 production. Split them earlier if they are turning yellow, or start from lower cell numbers.
16. iTreg conditions should include polarizing cytokines and blocking antibodies, but no anti-CD28.
17. Retinoic Acid enhances induction of de novo Foxp3-expressing cells [8–10].
18. When plating cells, avoid wells at the edge of the plate—these wells will experience more evaporation. To the surrounding wells add equal volumes of your final culture volume of sterile PBS or water to help reduce evaporation over the course of your cultures.

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Part III

Specific Techniques

Chapter 11

Studying T Cell Development in Thymic Slices

Jenny O. Ross, Heather J. Melichar, Joanna Halkias, and Ellen A. Robey

Abstract

Recently, tissue slices have been adapted to study both mouse and human T cell development. Thymic slices combine and complement the strengths of existing organotypic culture systems to study thymocyte differentiation. Specifically, the thymic slice system allows for high throughput experiments and the ability to introduce homogenous developmental intermediate populations into an environment with a well-established cortex and medulla. These qualities make thymic slices a highly versatile and technically accessible model to study thymocyte development. Here we describe methods to prepare, embed, and slice thymic lobes to study T cell development in situ.

Key words Thymic slice, Organotypic culture, Vibratome, Thymus, Agarose-embedding

1 Introduction

Thymocytes develop within the highly organized, complex, three-dimensional microenvironment of the thymus, which many in vitro models fail to fully recapitulate. Although two-dimensional culture methods such as antibody cross-linking, peptide-MHC-tetramer stimulation, and stromal cell co-culture provide important information, they do not support efficient positive selection and differentiation. Thymic slices were initially developed to examine the potential of ex vivo cultured tissue for thymic transplantation in different clinical settings of T cell dysfunction [1], but are now being used to study T cell development [2–6]. Prior to the adaptation of the thymic slice system, organotypic culture systems that fully supported the processes of positive and negative selection were fetal thymic organ cultures (FTOC) or reaggregate thymic organ cultures (RTOC) [7, 8]. The development of the thymic slice model provides the added advantage of maintaining the structural integrity of mature thymic tissue with well-defined cortical and medullary microenvironments. Defined thymocyte populations can be overlaid atop the thymic slices, where they migrate to their correct anatomical location following entry into the slice [3].

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_11, © Springer Science+Business Media New York 2016

Furthermore, the short time required for the entry of different thymocyte populations after addition to the slice allows for analysis of thymocyte development within hours of culture establishment. Additionally, roughly 0.5–2 % of the cells in the thymic slices are derived from the added thymocytes several hours after overlay. This relatively low precursor frequency is a more physiological representation of the precursor frequency within the thymus compared to other developmental systems that support positive selection. In this way, development of a relatively synchronized cohort of purified thymic subsets can be followed over time, and the robust cell recovery is amenable to efficient analysis of T cell development by flow cytometry. Thymocytes can also be labeled with fluorescent dyes for direct observation by live-cell imaging to visualize cell migration, intracellular calcium changes, and cell death, for example.

The thymic slice model is a highly versatile system that is currently being used to study positive and negative selection of developing mouse thymocytes as well as the behavior of developing human thymic subsets. Owing to the relative ease of thymocyte entry, the diverse cell types that can be introduced, and the abundance of tissue, this system has the potential to be further expanded to examine other aspects of thymic development. Here, we outline the process of generating thymic slices (including thymic harvest, embedding lobes in agarose, and vibratome-slicing of embedded tissue), overlaying thymocytes, and dissociating slices for flow cytometric analysis.

2 Materials

2.1 Harvesting a Mouse Thymus for the Generation of Thymic Slices

1. Mouse (*see* **Note 1**).
2. 70 % ethanol spray.
3. Tissue pins.
4. Paper towels.
5. Styrofoam board.
6. Small scissors (e.g. Roboz,. RS-5912) or other similar.
7. Blunt forceps (e.g. Roboz, RS-5136) or other similar.
8. Sharp forceps (e.g. Roboz, RS-5047) or other similar.
9. Micro-dissection scissors (e.g. Roboz, RS-5602) or other similar.
10. 6-cm tissue culture dishes.
11. Phosphate-buffered saline (PBS).

2.2 Embedding and Vibratome Sectioning a Thymic Lobe in Agarose

1. A thymus to be embedded [from 2.1 or human thymus fragment (*see* **Note 2**)].
2. 4 % Low-melting temperature agarose (e.g. NuSieve GTG Agarose; Lonza, 50080, or other similar) in Hank's Balanced

Salt Solution (HBSS): 2 g low-melting-point agarose per 50 mL HBSS (*see* **Note 3**).

3. 6-Well tissue culture plate.
4. Complete DMEM: DMEM supplemented with 10 % FBS, 2 mM Glutamine, 2 mM Penicillin-Streptomycin, and 50 μM 2-mercaptoethanol.
5. 0.4-μm pore-size organotypic cell culture inserts (BD Biosciences, cat. no. 353090) (*see* **Note 4**).
6. 500 mL beaker.
7. Crushed ice.
8. Tissue molds (e.g. Polysciences, 18986-1) or other similar.
9. Paper towels.
10. Blunt forceps.
11. Vibratome (e.g. 1000 Plus Sectioning System) or other similar.
12. Vibratome blades, 2 (feather blades; e.g. Leica Biosystems, 39053234, or other similar).
13. Tissue glue (e.g. 3 M Vetbond tissue adhesive, 1469SB) or other similar.
14. 6-cm tissue culture dishes.
15. PBS.
16. Spatula, bent (spatula, rounded ends, 6.4 mm; e.g. VWR, 57950-000, or other similar) (*see* **Note 5**).

2.3 Overlay of Thymocytes onto Thymic Slices

1. Purified thymocyte population of interest at 1×10^6 cells/10 μL in complete DMEM (*see* **Note 6**).
2. Thymic slices on cell culture inserts (from Subheading 3.2).
3. 37 °C incubator.
4. PBS.

2.4 Dissociation of Thymic Slices for Flow Cytometric Analysis

1. PBS.
2. Microfuge tubes (2/slice).
3. Thymic slices (as generated in Subheadings 3.2 or 3.3) (*see* **Note 7**).
4. Spatula, bent.
5. Microcentrifuge tube sample pestles (e.g. Fisher, 05-559-26, or other similar).
6. Nylon mesh filter.

3 Methods

3.1 Harvesting a Mouse Thymus for the Generation of Thymic Slices

1. Euthanize the mouse by your institution's approved method.
2. Soak the mouse with 70 % ethanol.
3. Using tissue pins, fix the mouse to a Styrofoam board covered with a paper towel.
4. Expose the thymus. To do this, grasp the skin below the sternum, making a small longitudinal cut at the bottom of the ribcage using the small scissors, without cutting through the peritoneum. Then, make one long vertical cut through the skin from the sternum up to the throat followed by two smaller cuts up to the front legs. Peel back the skin to visualize the ribcage under the peritoneum. Grasp the sternum with the blunt forceps and cut along the bottom of the diaphragm using the small scissors. Cut up through the ribs toward the head on both sides to avoid large blood vessels under the ribcage, flip the ribcage toward the head of the mouse, and secure it with a pin.
5. Hold the heart with the blunt forceps and pull caudally. Using the sharp forceps, carefully dissect the connective tissue to separate the thymus from the anterior wall of the ribcage. Grasp the aorta below the thymus and above the heart with the blunt forceps and, using the micro-dissection scissors, cut through the attachments below and behind the thymus, cutting the aorta at a level above the heart last. It is important not to grasp the thymic tissue itself as tears compromise the tissue and the thymic slice quality.
6. Submerge the thymus in PBS in a 6-cm dish on ice until ready to proceed.
7. Place the thymus on a paper towel soaked with PBS. Use the blunt and sharp forceps to carefully separate the lobes and remove any remaining connective tissue. Care must be taken not to grasp the thymus itself to prevent tearing the tissue.
8. Place the individual lobes back into the 6-cm dish while you prepare to embed them.

3.2 Embedding and Vibratome Slicing a Thymic Lobe in Agarose

1. Prepare the agarose: Add 2 g low-melting-point agarose per 50 mL HBSS in a 200 mL Erlenmeyer flask. Microwave on a low setting until the agarose is completely dissolved, about 2 min. Cover with foil and move to a 55 °C water bath until needed.
2. Set up the tissue culture plates by adding 1.5 mL of complete DMEM per well of a 6-well plate, and placing a cell culture insert in each well.
3. Make an ice water bath: fill a 500 mL beaker with ice and add water until the ice is a slushy consistency.

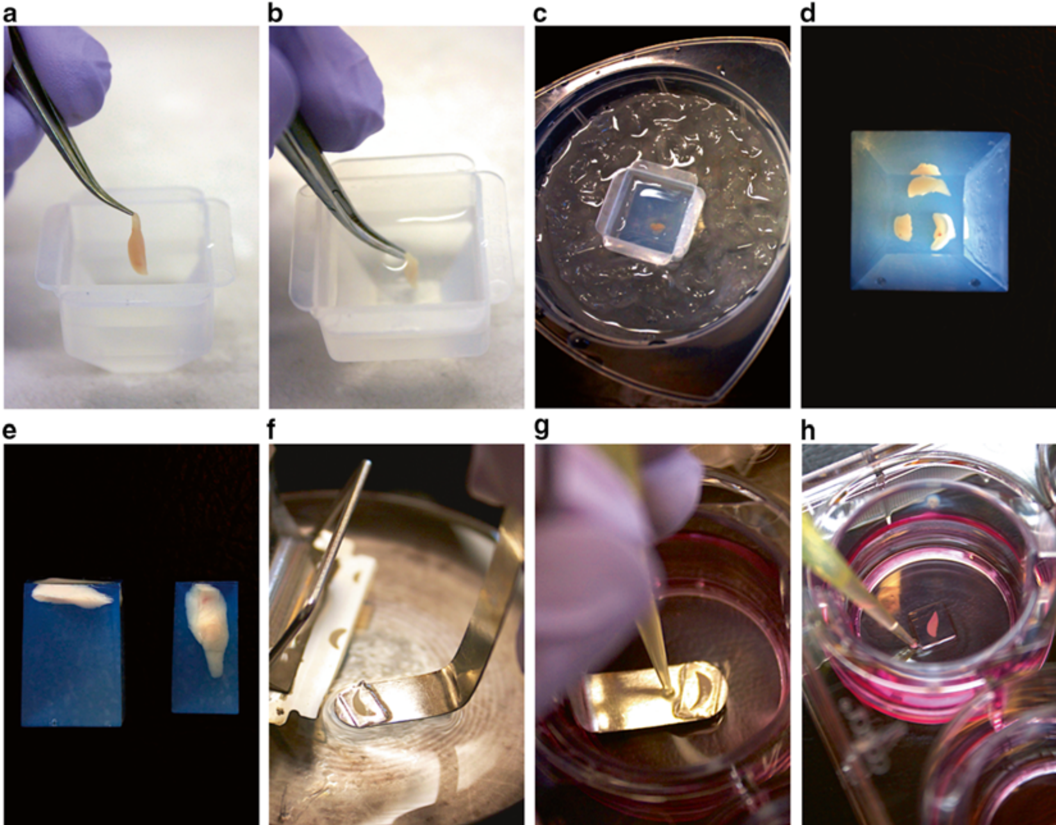

Fig. 1 Embedding and preparing thymic slices. (**a**) Using blunt forceps, grasp connective tissue and transfer individual thymic lobes into a tissue mold filled half way with 4 % agarose solution. (**b**) Gently push the lobe into position within the agarose before the agarose solution sets. (**c**) Once the lobe is positioned, place the mold into an ice water bath to set. (**d**) Example of three lobes embedded in one tissue mold. (**e**) Trim the excess agarose around the embedded lobes. *Left*, thymic lobe embedded horizontally. *Right*, example of a thymic lobe embedded vertically. (**f**) Up to three blocks of embedded tissue can be glued to the vibratome stage and sliced simultaneously. Use a bent spatula to transfer the thymic slices after each cut as they float free of the vibratome blade. (**g**) Use a pipet tip to gently push the thymic slice onto a cell culture insert within a 6-well plate. (**h**) Use a pipet to remove any liquid in the transwell and around the edges of the thymic slice

4. Fill a tissue mold halfway with 4 % agarose solution and wait about 30 s to allow the agarose to cool slightly.
5. Grasp the thymic lobe (as isolated in Subheading 3.1) by any remaining connective tissue and gently blot it on a dry paper towel to remove excess liquid (*see* **Note 8**).
6. Carefully insert the lobe into the agarose using the blunt forceps either vertically to maximize slice number or horizontally to maximize slice area (*see* **Notes 9** and **10**) (Fig. 1a–c).
7. Place the mold in the ice-water bath until the agarose is set (~5 min, agarose will be opaque and firm to the touch) (*see* **Note 11**) (Fig. 1c).

8. While the agarose solidifies, prepare the vibratome by inserting a vibratome blade. The following settings should be used for sectioning thymic tissue: cutting angle 5°, maximum amplitude, minimum speed.
9. Invert the tissue mold and gently press on its center to release the agarose-embedded tissue.
10. Using a sharp blade (*see* **Note 12**), trim the sides of the embedded thymic tissue, leaving ~2 mm of agarose around the individual thymic lobes. It is not necessary to trim the bottom, but make certain to leave a minimum of 0.5 cm of agarose below the thymic lobe as the vibratome can only cut to 0.5 cm above the stage (Fig. 1e).
11. Place the trimmed agarose-embedded tissue into a 6-cm dish filled with PBS for transport to the vibratome.
12. Dry the agarose-embedded tissue block on a paper towel.
13. Using a pipet tip, apply a small amount of tissue glue to the vibratome stage directly in front of the blade and place the agarose embedded lobe on top (*see* **Note 13**). Make sure the stage is clean as excess glue and other debris can float off and obstruct the surface of the slice.
14. Align the vibratome blade with the top of the tissue.
15. Add PBS to fill the vibratome stage and submerge the agarose blocks and blade.
16. Section thymus at the desired thickness (*see* **Note 14**).
17. Retract the blade 200 μm after each slice to prevent dragging along the surface of the tissue.
18. Transfer slices as they are cut to the 6-well tissue culture plates by using a pipet tip to gently slide the slice off the spatula and onto the cell culture insert (Fig. 1f, g) (*see* **Note 15**).

3.3 Overlay of Thymocytes onto Thymic Slices

1. Remove all excess liquid from the insert (any accumulated PBS and/or medium) surrounding the thymic slices by pipetting carefully to prevent tearing or damaging the agarose surrounding the tissue (*see* **Note 16**) (Fig. 1h).
2. Pipet 10 μL of labeled (either congenic or dye, etc.) thymocytes of interest on top of each slice (*see* **Note 17**) carefully without touching the tissue or the surrounding agarose (Fig. 2d–f).
3. Incubate slices at 37 °C for 2 h to allow overlaid cells to migrate into the thymic slices.
4. Rinse the slices with 1 mL/well PBS to wash off any cells that have not yet migrated into the slice.
5. Continue to incubate slices at 37 °C for the desired length of time (*see* **Notes 18 and 19**).

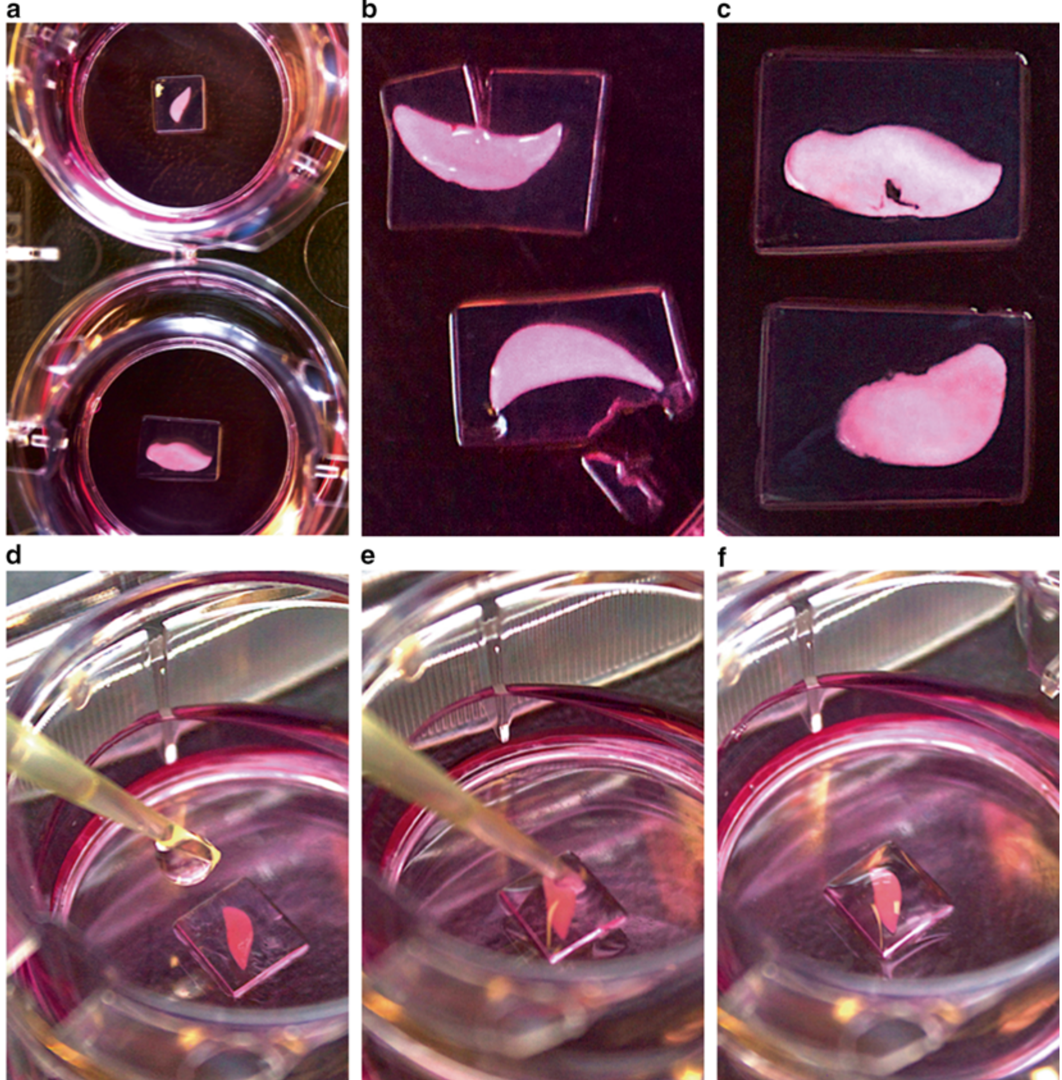

Fig. 2 Quality control and overlay of thymic slices. (**a**) Example slices from a vertically (*top*) versus horizontally (*bottom*) embedded thymic lobe. (**b**) Examples of poor quality slices that will cause overlaid cells to run off. The top slice has a tear in the agarose and the bottom slice has a chunk of agarose torn free. (**c**) Example of a tear in the thymic tissue (*top* slice) versus a good, intact slice (*bottom*). After pipetting off excess liquid, inspection of the thymic tissue itself, as well as the borders of the agarose surrounding the tissue will reveal any slices with defects. These should be discarded and replaced with good quality slices with clean, clear edges of agarose for optimal overlay. (**d**) Add 10–20 μL of cell suspension per slice by pipetting the volume so that it forms a suspended drop. (**e**) Gently touch the drop of resuspended cells to the center of the thymic slice, without touching the pipet tip itself to the slice. (**f**) The overlaid cells will remain on top of the slice

3.4 Dissociation of Thymic Slices for Flow Cytometric Analysis

1. Add 150 μL of PBS to a microfuge tube (1 tube/sample).
2. Add 1 mL of PBS to tissue culture insert containing the thymic slice to release the slices from contact with the culture insert, allowing for ease of transfer.
3. Transfer thymic slice to microfuge tube using the bent spatula.

4. Dissociate slice using tissue sample pestles.
5. Add another 150 μL PBS.
6. Filter the 300 μL volume of cells through a mesh filter into a new microfuge tube. Cells are ready for analysis and can be stained for flow cytometry or prepared for other analyses.

4 Notes

1. Vertical embedding of the thymus of a 1–2 month old wild-type mouse will yield approximately 10–12 400 μm thick slices that contain both cortex and medulla per lobe. The size of the thymus and number of slices depends on age, gender, and genotype.
2. A segment of human thymus (cut to be roughly the same size as a mouse thymus, ~0.5 cm wide × 1–1.5 cm high) can be embedded in the same manner as a mouse thymus. However, connective tissue near the capsule tends to cause the slice to catch on the blade during slicing. Removal of the connective tissue does not increase the chances of successful slicing and may instead lead to tearing of the tissue and generation of slices of poor quality. Plan on reserving several pieces of tissue for sectioning, in the event that the first segment does not cut well.
3. The 4 % agarose solution can be stored in a 55 °C water bath for several days.
4. The tissue culture inserts from BD Biosciences work well for thymocyte overlay. Several other brands did not maintain the overlaid liquid on the slice.
5. A spatula with a 90° bend 1 cm from the end works well as a tool to transfer slices (Fig. 1f).
6. Use labeled thymocytes (for example; fluorescently labeled through transgenic ubiquitin driven GFP [9], a congenic marker such as Ly5.1, or vital dye such as CFSE or SNARF) to distinguish the overlaid population from endogenous thymocytes and/or visualize thymocytes during imaging.
7. Thymic slices can be incubated with or without overlaid cells. If there are no cells being overlaid, the integrity of the agarose surrounding the thymic slice does not have to be intact, as shown (Fig. 2b, c).
8. If the thymic lobe is wet, it may pull out of the agarose block during slicing.
9. Multiple lobes can be embedded simultaneously side-by-side (up to 3) (Fig. 1d) if they are inserted vertically.

10. Ensure that the thymic lobe touches the bottom of the mold while it is being embedded in agarose to provide a 0.5 cm buffer of agarose below the tissue. This will allow for the entire tissue to be vibratome-sliced.
11. There is approximately a 30 s window after the molds are placed into the ice water bath during which the placement of the lobes can be adjusted within the agarose. Use the blunt forceps to move the thymus into the position desired (Fig. 1b).
12. Vibratome blades can be reused for a couple of experiments. Discard after they have been used for trimming the blocks.
13. Up to three tissue blocks aligned side-by-side parallel to the vibratome blade can be sliced simultaneously. Ensure that they are roughly equivalent in height for best results (Fig. 1f).
14. 400–500 μm thick slices work well for tissue culture and 500–1000 μm thick slices work well for imaging.
15. Place as many slices as will fit per insert without touching each other or the insert wall. Ensure that there are no air bubbles below the insert.
16. If the slices are not dried properly, the overlaid cells may spill off. Any tears or holes in the agarose may also lead to loss of overlaid cells/fluid volume (Fig. 2a–c). Finally, properly drying the slices allows for visual inspection of the slice quality. It is a good idea to cut extra slices and replace any as needed.
17. Overlaying 1×10^6 cells/slice for 2 h prior to washing typically results in the cells of interest making up 0.5–2 % of the total thymocytes within the slice. Longer incubations result in a greater proportion of cells migrating into the tissue slice.
18. If a heterogeneous population of thymocytes is overlaid, harvesting an early time-point will establish a baseline of the cells that migrated into the slices. The quality of the slices deteriorates significantly after just a day or two, but development seems to proceed for several days.
19. Over time, thymocytes leak out of the slices due to the absence of a capsule.

Acknowledgements

This work was supported by an NIH grant AI064227-07 (to E.A. Robey), California Institute of Regenerative Medicine clinical fellowship TG2-01164 (to J. Halkias), and postdoctoral training grant TG2-01164 (to H.J. Melichar).

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Chapter 12

FTOC-Based Analysis of Negative Selection

Cody A. Cunningham, Emma Teixeiro, and Mark A. Daniels

Abstract

Potentially harmful T cell precursors are removed from the conventional T cell pool by negative selection. This process can involve the induction of apoptosis, anergy, receptor editing or deviation into a regulatory T cell lineage. As such this process is essential for the health of an organism through its contribution to central and peripheral tolerance. While a great deal is known about the process, the precise mechanisms that regulate negative selection are not clear. Furthermore, the signals that distinguish the different forms of negative selection are not fully understood. Numerous models exist with the potential to address these questions in vitro and in vivo. This chapter describes methods of fetal thymic organ culture designed to analyze the signals that determine these unique cell fates.

Key words FTOC, Negative selection, T cell development, Thymocyte differentiation

1 Introduction

A healthy individual can mount an immune response to exogenous pathogens while avoiding an autoimmune attack on normal tissues. The ability to distinguish between self and non-self is called immunological tolerance. T cell central and peripheral tolerance begins in the thymus with selection [1–3]. T cell selection and development depends on the ability to distinguish between thymocytes that bear useful TCR from those that express useless or potentially harmful ones. Thus, the generation of a healthy immune system depends on the removal or reprogramming of these useless and harmful cells. Useless thymocytes, those that are unable to respond to self-peptide MHC, die by neglect. Those that have a strong response to endogenous self undergo negative selection. The removal of these potentially auto-reactive T cells is achieved by the induction of apoptosis, anergy, receptor editing or reprogramming into a regulatory T cell lineage [4]. Despite the vast amount of data that have been generated on this subject, several questions remain unanswered.

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_12, © Springer Science+Business Media New York 2016

Studies addressing T cell negative selection are inherently difficult in that a thymocyte's favorite activity is dying. The thymus has long been recognized for containing a large number of dying T cells. Thus, it is difficult to distinguish death by neglect (90 % of thymocytes) and death by agonist-induced apoptosis (5 % of thymocytes). The need for intact thymic architecture also means there are no in vitro models that allow one to follow negative selection from beginning to end. Selection is a multistep process with many checkpoints. Thus, research in this area is also complicated by maturation-based changes in a thymocytes response to stimulation as it passes through selection [5–7]. Furthermore, as a T cell matures it moves through the various thymic compartments each with a unique environment and population of phenotypically different thymic epithelium and antigen-presenting cells [8]. Therefore, work performed on whole thymi from a selecting background may miss subtle yet fundamentally important information. Furthermore, the differences between the thymic epithelium of the cortex and the medulla suggest that there may be multiple ways to induce apoptosis. Unfortunately, traditional knock out and transgenic strategies are insufficient as many of the signals that determine selection fates are also essential for earlier stages of T cell development [1–3]. Several in vivo models of selection have provided invaluable information as to the conditions that lead to negative selection. However, considering the complications discussed above, along with the asynchronous nature of in vivo selection, some of the subtler yet critically important details of the process of negative selection remain unknown.

Fetal thymic organ culture (FTOC) remains one of the most informative and manipulatable methods to examine the signals that distinguish negative selection from positive selection. In addition, it has the potential to identify the signals that lead to the different outcomes of agonist selection. As such this is critical for our ability to understand thymic selection's contribution to peripheral tolerance.

The following are examples of manipulations that are easily used to address the signals of negative selection in FTOC:

- Easily manipulate the selecting ligand strength and concentration.
- Assess the contribution of "third" signals through the addition of excess cytokines or antibodies that block or stimulate these pathways.
- Take advantage of the proliferative burst in DN3/4 thymocytes to retrovirally transduce T cells with various constructs and test their affect on selection.

– Use transgenic, tissue-specific and inducible models of antigen expression to test the unique contribution of the different subsets of thymic epithelium.
– Use transgenic, knockout, and conditional expression systems.

Several considerations need to be kept in mind when analyzing negative selection. It is difficult to analyze signaling in dead cells. Dead cells disappear rather quickly, even in FTOC [9, 10]. Thus, while the loss of thymocytes provides information that is essential to understand the conditions that lead to negative selection, we need to catch them before they die to be able to say anything about where they go and how they got there. Along these lines, it is important to distinguish death by neglect and death by negative selection. Thus, we always include both positive selection and non-selection controls in our analysis of negative selection (We have had numerous discussions in our lab which one should be called the positive and the negative controls and finally settled on the term *selection controls* as a result).

We use several standard techniques to assess signals and analyze selection in our system that are further described in Chapter 15.

– For early time points (usually <48 h) immunoblot techniques can be easily used to determine the expression levels and activation states of proteins. For these assays we stimulate, pool and disrupt thymi, lyse, subject to SDS-PAGE, transfer and immunoblot (shown elsewhere in this manual).
– Flow cytometry can be used for nearly all conditions as long as there is an antibody that works by intracellular staining. It is especially useful for late time points (decreasing number of cells) and assays on small/rare populations of cells that can be distinguished by a specific phenotypic marker. For these assays we stimulate, stain surface markers and perform intracellular staining to distinguish the population of interest. In general, we use standard kits (BD Phosflow or Cytofix/Cytoperm kits) and follow their suggested protocols.
– Confocal microscopy provides activation and location information that has added to our understanding of the diversity in the regulation of selection signals [11, 12].
– We assess interactions between different signaling molecules with IP followed by flow cytometry (IP-FCM). This method offers the benefit of being able to precipitate low abundance proteins to generate data with a high signal to noise ratio [13–15]. Additionally, this protocol offers the benefit of being able to use significantly fewer cells for analysis ($\sim 1 \times 10^5$ for IP-FCM vs. 100×10^6 for IP-Western). For these assays we select our cells of interest and perform assays whose methods are described elsewhere in this methods issue.

2 Materials

1. *Culture Dishes:* We generally use 6- or 12-well plates with 2 ml of media in each, but small petri dishes also work.
2. *Sterile filters and gelfoam:*
   (a) Mixed cellulose membrane with a 0.45 μm.
   (b) Surgical Gel-foam.
   (c) ALTERNATIVE: 13 mm Supor-450 membrane filter (e.g. #60170 from PALL, Life Sciences) (*see* **Note 1**).
3. *Media:*
   (a) RPMI 1640 supplemented with 10 % v/v heat-inactivated FCS, 2 mM glutamine, 10 mM HEPES, 0.5 mg/ml folic acid, and 0.2 mg/ml glucose, 100 U/ml Penicillin, 100 μg/ml Streptomycin (*see* **Note 2**).
   (b) *Alternatively,* FTOC can be done in serum-free media. For this use RPMI 1640, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 0.292 mg/ml Glutamine, 1 mM Sodium pyruvate, 1 % Nutridoma SP (Roche) (*see* **Note 3**).
4. Two petri dishes of ice-cold PBS.
5. Two pairs of scissors, one fine.
6. Three pairs of forceps, one regular, one fine curved, one ultrafine.
7. Sterile 4 × 4 gauze.
8. Several 25 g needles, both 1/2″ and 1–1/2″.
9. FTOC chamber (*see* **Note 4**).
10. Dissecting (Stereo) Microscope with a good light source (*see* **Note 5**).

3 Methods

These techniques are adapted from work published by the Hogquist and Anderson labs [16–19] and from the vast experience and guidance of Barbara Hausmann while I was in the lab of Dr. Ed Palmer. In general, while there are several selecting and non-selecting mouse models that can be used for FTOC, we use TCR transgenic, $Rag^{-/-}$, $\beta2m^{-/-}$ mice to perform our assays. They provide a non-selecting environment and a synchronous population of thymocytes. To induce selection of CD8 T cells we simply add our peptide of interest and β2m in the FTOC media, check the selection outcome controls (*see* **Note 6**) and analyze our desired experimental variables [11, 17].

3.1 Timed Matings

The days of the week represent an example of the scheduling procedure used in our lab.

1. Tuesday: Prepare time mating cages by placing a cage divider with feeders and water for both sides in each cage.
2. Wednesday, morning: Place two to three females on one side of the divider and one male on the opposite side of the divider (*see* **Note 7**).
3. Friday, late afternoon/evening: Remove cage divider.
4. Saturday, morning (before 10 a.m.): Remove male. OPTIONAL: check for and record mucous plugs (*see* **Note 8**).
5. Saturday (or the day of the plug) is considered day 0.5 (although technically it could be anywhere from 0 to 0.5 days) (*see* **Note 9**).
6. Two weeks from the following Monday (Day 15.5), harvest fetuses (see below).

3.2 Preparing the Culture Dishes

1. Boil cellulose filters three times in dH_2O. The final time cover with foil and, when cool, transfer into the hood. Place the filters in medium just before use.
2. Prepare medium.
3. Remove a strip of gel-foam and cut it into three squares (KEEP STERILE), placing each square in one well. Using one straight and one curved forceps soak the gel-foam and tease the air bubbles out.
4. Place one filter (from **step 1**) on each gel-foam square.
5. Place the plate in a FTOC chamber (*see* **Note 4**). Pre-warm in the incubator.

3.3 Removing Fetal Thymic Lobes

1. Pregnant mothers are sacrificed according to institutional guidelines.
2. Peel the skin (hide) back from abdomen of pregnant mouse.
3. Make an incision through the abdominal muscle across the mouse just above the bladder. Continue up each side of the abdomen to expose the entire abdominal cavity.
4. Excise both horns of the uterine sac and place in a petri dish with 10 ml of sterile cold PBS.

   From this point on everything should be performed in a horizontal laminar flow hood (or other easily accessibly sterile hood). *Never* work with infected (viral or bacterial) tissues or cells in this hood.
5. Use a sterile scissors to remove the individual embryos from the amniotic sacs.
6. Remove the placenta and amniotic sac tissue from each embryo and place fetuses in fresh cold PBS.

7. Wipe down dissecting scope and light source with 70 % ethanol.

   The actual removal of thymic lobes is relatively easily from a day 15.5 embryo. While, there are several methods by which they can be removed, two of the more common methods are described below.

8. "Pin" method.
   (a) Place petri dish with sterile gauze on the stage of microscope.
   (b) Place the fetus on sterile gauze in petri dish.
   (c) Decapitate fetus with forceps or pith the fetus with a 25 g needle.
   (d) Pin the front limbs of the embryo and cut open the chest to expose the thymic lobes sitting on/above the heart on either side of the trachea.
9. Thoracic stem method: There is an excellent video of this method by Jenkinson et al *J. Vis. Exp.* (2008) at http://www.jove.com/video/906/preparation-of-2-dguo-treated-thymus-organ-cultures.
   (a) Decapitate the embryo with forceps.
   (b) Open the anterior chest cavity by inserting forceps (closed then open) into the thoracic cavity through the neck.
   (c) Use your forceps to reach into the torso and pull out the entire thoracic tree; heart, lungs and trachea. The thymus usually comes out with this and can be found on either side of the trachea, just above the heart (*see* **Note 10**).
10. Pluck the lobes out using the ultrafine forceps and place on filters, taking care not to disrupt the thymic capsule.
11. You can place up to ten lobes per filter disc, place in FTOC chamber (*see* **Note 4**) and culture in 5 % CO_2 at 37 °C.

3.4 Feeding

We use two methods of feeding. Both are described here.

1. Replace the media in the cultures every day.
   (a) To do this, carefully suction off the media with a sterile Pasteur pipette.
   (b) Add 2 ml fresh warm media, carefully basting thymic lobes.
2. Replace the media in the cultures every 2–5 days.
   (a) Carefully baste thymic lobes daily.
   (b) To replace media, carefully suction off the media with a sterile Pasteur pipette.
   (c) Add 2 ml fresh warm media, carefully basting thymic lobes.
3. Seven days of culture is sufficient for good thymic development from an E15.5 lobe. However, they remain viable up to 14 days.

3.5 Analysis

To disrupt the lobes:

1. Place on a small nylon mesh.
2. Add media and crush the lobe(s) against the mesh with the plunger of a 3 cc syringe.
3. On average we recover $2–4 \times 10^5$ T cells per individually processed lobe. Yields go up to $5–7 \times 10^5$ cells/lobe when multiple lobes are pooled during harvesting.
4. Analyze as described in Chapter 15.

4 Notes

1. This brand of filters float nicely in the media (shiny side up) such that expensive (and sometimes difficult to obtain) surgical gel-foam is not needed.
2. We often test our FCS in FTOC in advance to minimize serum-based artifacts of selection.
3. If you wish to add, antibodies, peptides, etc., add them to media prior to plating out thymi.
4. It is critically important to avoid drying of the thymic lobe. To do this we use a covered Pyrex baking dish (available from the local grocery store). We have small holes drilled in the cover (>5 mm). We place cover of a 6- or 12-well plate in the bottom of the baking dish as a pedestal, and fill the bottom of the dish with autoclaved dH_2O. The culture dish containing the FTOC are placed on top of the pedestal and placed in the incubator. See also Jenkinson et al *J. Vis. Exp*. (2008) for an alternative apparatus.
5. With practice day 15.5 embryos are easily removed without the aid of a microscope.
6. It is critically important to have a selection outcome control for each test condition. For CD8 T cells, we always save a lobe or partial lobe (minimum of 1×10^4 cells) and stain for TCR, CD4, CD8α, and CD8β. This is then analyzed by flow cytometry for the following:
   (a) The generation of CD8β SP thymocytes is the test of positive selection.
   (b) The lack of the generation of CD8α and CD8β SP thymocytes and the maintenance of a DP population indicates no selection.
   (c) The lack of the generation of CD8β SP thymocytes with the concomitant loss of DP indicates negative selection.

(d) Finally, the loss of DP with a concomitant lack of CD8β SP and CD8α SP thymocytes indicates negative selection by apoptosis and the generation of only CD8α-positive cells indicates lineage deviation (reprogramming) [11, 20].

7. This procedure helps to induce estrus and increases the efficiency/probability of pregnancies.
8. We generally just wait for the females to show signs of pregnancy.
9. Some consider this day 1.
10. Thymic lobes sometimes remain nestled in the thoracic cavity and are easily removed.

Acknowledgements

The authors thank the members of the Daniels and Teixeiro labs for assistance in compiling these protocols. Funding provided by grants from the Missouri Mission Enhancement Fund and University of Missouri Research Board is greatly acknowledged.

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Chapter 13

Reconstituted Thymus Organ Culture

Zimu Deng, Haifeng Liu, Jinxiu Rui, and Xiaolong Liu

Abstract

Reconstituted thymus organ culture is based on fetal thymus organ culture (FTOC). Purified thymocyte populations, from genetically modified mice or even from other species, are cultured in vitro with thymic lobes depleted of their endogenous thymocytes (by 2′-deoxyguanosine treatment) to form a new thymus. This potent and timesaving method is distinct from FTOC, which assesses development of unmodified thymic lobes, and reaggregate thymic organ culture, in which epithelial cells are separately purified before being aggregated with thymocytes.

Key words Thymus, Thymocyte, Fetal thymus organ culture, 2′-Deoxyguanosine

1 Introduction

After hematopoietic progenitor cells migrate into thymus, they interact with stromal cells and undergo a series of differentiation steps, including V (D) J rearrangement and thymic selection. Fetal thymus organ culture (FTOC) established by Owen [1] and Mandel [2] offers a powerful technique to study both T-precursors and thymic microenvironment during early T lymphocyte development. More recently, reaggregate thymus organ culture (RTOC) [3–6], which allows reconstruction of thymic "aggregates" from thymocytes and stromal cells of distinct origin (and typically of distinct genotype), contributed to elucidate molecular and cellular mechanisms of T cell development [7, 8].

In this chapter, we describe a related method, reconstituted thymus organ culture [4, 9]. In this procedure, fetal thymus lobes are treated with 2′-deoxyguanosine in vitro for 5–7 days to deplete their endogenous T cell progenitors. The depleted lobes are then offered for colonization by and serve as thymic environment for purified thymocytes (Fig. 1). This reconstitution method is useful to study thymocyte development, and especially the development of retrovirally transduced thymocytes; consequently, a succinct

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_13, © Springer Science+Business Media New York 2016

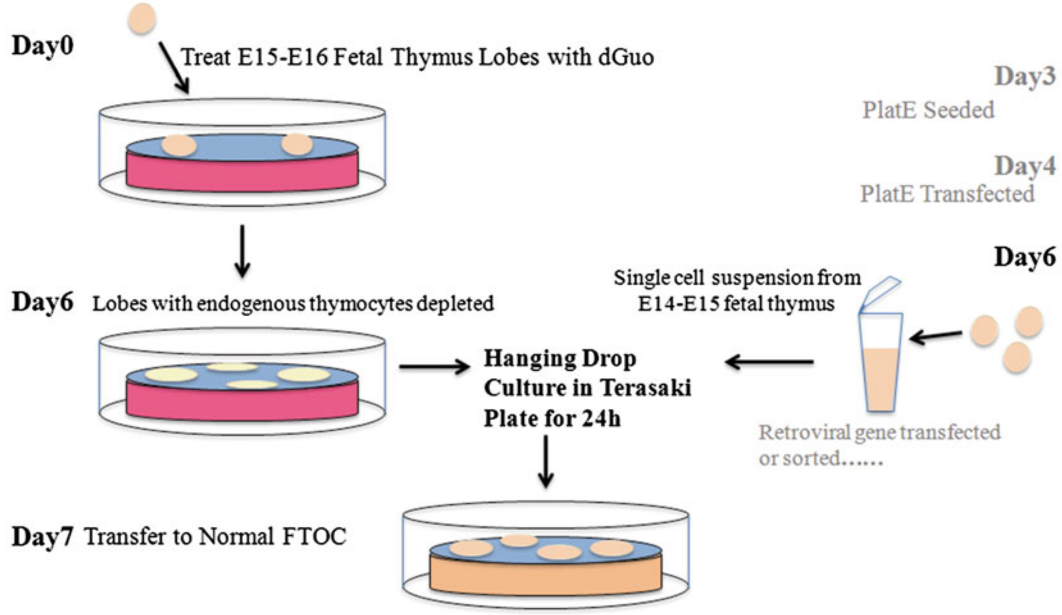

Fig. 1 Schematic overview of reconstituted thymus organ culture system

protocol for retroviral transduction is provided in Subheading 3.6. To study stromal cells, the more sophisticated reaggregated thymus organ culture is preferable.

2 Materials

2.1 Reagents

All reagents must be purchased sterile, prepared with sterile reagents or be sterile-filtered.

1. 1× PBS (Ca^{2+}/Mg^{2+}-free).
2. RPMI-1640 medium: supplemented with 2 mM L-Glutamine, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin, 1× Non-Essential Amino Acids and 10 mM Hepes-pH 7.4. Add 2-Mercaptoethanol in the medium to a concentration of 0.05 mM before use. Referred to as RPMI medium throughout. The concentration of FBS varies from 10 to 20 % as indicated in each step.
3. 2′-Deoxyguanosine (dGuo) (e.g. Sigma-Aldrich, D7145) 10× stock solution: 13.5 mM in 1× PBS (dissolve at 37 °C). Keep the stock solution at −20 °C.
4. IL7 (e.g. Peprotech, 96-217-17-2): 10^5 U/ml 1000× stock in 1× PBS, store at −20 °C.

5. Polybrene (e.g. Sigma-Aldrich, 107689): 10 mg/ml 500× stock in 1× PBS, store at −20 °C.
6. 75 % Ethanol.
7. DMEM, supplemented with 2 mM L-Glutamine, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin.

2.2 Equipment

1. Ophthalmic forceps, 10 cm length.
2. Tying forceps, angled platform, 10.5 cm length.
3. Thin forceps, 0.025 × 0.05 mm tip, 11 cm length (e.g. World Precision Instruments, 50985).
4. Scissors, ophthalmic.
5. Stereo-dissecting Microscope (Olympus, SXZ7).
6. Gelatin sponges, 1 cm^2.
7. 1-ml Syringes.
8. Filters, 0.45-μm pore size (e.g. Millipore, HAWG01300).
9. Nylon mesh with 40-μm pores.
10. Petri dishes, 90-, 60- and 30-mm diameter.
11. 72-Well Conical Terasaki Plates (e.g. Electron Microscopy Sciences, 70439-10).
12. Humidified Box. Fill in a plastic rectangular box (e.g. Lock&Lock, HPL817) with 20 ml sterilized H_2O and place a Petri dish lid as a support. Holes with 1 cm diameter were punched on the plastic lid for air diffusion.
13. Carbon dioxide tissue culture incubator, 37 °C, 5 % CO_2.
14. Centrifuge.

3 Methods

3.1 Preparation of Timed Pregnancies

We usually put two female mice and one male in one cage at day end (5:00 p.m.). Every female is examined for the presence of a vaginal plug at 8 am every subsequent morning. The day a plug detected is counted as Day 1 of gestation. The pregnant mice will be kept in a new cage till use. *See* **Notes 1–4**.

3.2 Isolation of Fetuses from Pregnant Mice

1. Anesthetize a timed pregnant female (gestational age E15-E16) mice with CO_2, then euthanize by cervical dislocation. *See* **Note 1**.
2. Wipe the body with 75 % ethanol and cut open the abdominal wall to reveal the uterus. Use sterilized scissors and ophthalmic forceps to remove the uterus and place it into a 90-mm Petri dish containing 10 ml 1× PBS. *See* **Note 5**.

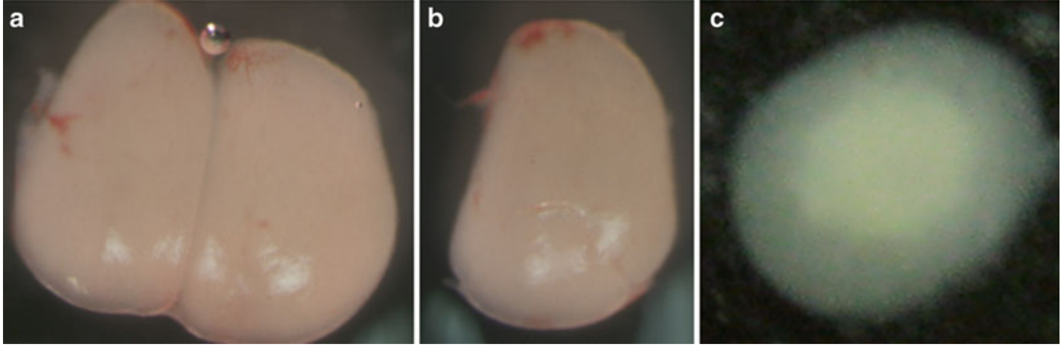

Fig. 2 Fetal thymus lobes before and after treatment with 2′-deoxyguanosine. (**a**) Thymus lobes from E15.5 feta mice. (**b**) An isolated thymus lobe. (**c**) A lobe cultured with dGuo for 6 days

3. Cut open the uterus and collect the embryos. Swirl the Petri dish to wash off the blood from the embryos and transfer them into a 60-mm Petri dish containing 4 ml PBS under the stereo-dissecting microscope.
4. Hold one embryo in a face-up position with thin forceps on the head. Using the other thin forceps, pull each foreleg in a head-to-toe direction, until the chest skin comes off. Two thymus lobes (below the first pair of ribs and above the heart) should be seen. If not, use thin forceps to snip off the first pair of ribs under the armpit and open the chest to reveal the lobes completely.
5. Carefully remove the lobes together using thin forceps and transfer to a new 60-mm Petri dish containing 4 ml RPMI-1640 medium with 10 % FBS. *See* **Note 6**.
6. Gently swirl the dish to wash off any blood. Use thin forceps to tear off any connective tissue between the two lobes and separate them (Fig. 2).

3.3 Elimination of Endogenous Thymocytes

1. Add 0.5 ml of 1 RPMI-1640 medium supplemented with 10 % FBS and 1.35 mM dGuo to each well of a 24-well plate [10]. Immerse one piece of sponge (1 cm^2) in each well and wait until the medium soaks the sponge. Place one filter above each sponge. Make sure no air bubble remains between the interfaces.
2. Scoop up one lobe each time with tying forceps and transfer it onto the filter. In a typical experiment, put no more than four lobes into each well.
3. Put the plate into a humidified box and keep in tissue culture incubator. *See* **Notes 7** and **8**.
4. After 5–7 days, detach the filters from sponges and transfer the filters into a 10-cm dish containing 10 ml cold 10 % FBS-supplemented RPMI-1640. *See* **Note 9**.

5. Keep the plate on ice and carefully detach the lobes from filters under a dissecting microscope (Fig. 2). Scoop up the lobes and transfer them into a new 10-cm dish containing 10 ml fresh 10 % FBS-supplemented RPMI-1640. Swirl the plate and put it in the incubator for 20 min to diffuse away dGuo. Change the medium every 20 min, three times. The lobes are ready for the next step Hanging-Drop reconstitution with T cell precursors.

3.4 Preparation of T Cell Progenitors from Fetal Thymus

1. Isolate E14-E15 fetal thymus as described in Subheading 3.2.
2. Drop 100 μl RPMI-1640 medium supplemented with 20 % FBS on the reverse side of the lid of a 30-mm dish [11].
3. Transfer required number of fetal lobes into the drop (usually put 10 lobes per drop), cover the drop with a piece of nylon mesh (1 cm^2).
4. Bend the needle of a 1-ml syringe to 90° angle. Use ophthalmic forceps to hold one side of the mesh to keep it from slipping, and gently press down the mesh with the bent needle. Gently scrape the mesh back and forth to squeeze the thymocytes out of the lobes.
5. Pipette the thymocyte suspension into a 1.5-ml microfuge tube containing 1 ml RPMI-1640 medium supplemented with 20 % FBS and count cells.
6. Fetal thymocytes are ready for use in Subheading 3.5, or can be purified by cell sorting or retrovirally transduced (see optional Subheading 3.6).

3.5 Hanging-Drop Culture

1. Centrifuge thymocyte suspension at $170 \times g$ for 7 min at 4 °C.
2. Discard the supernatant and resuspend the cells with RPMI-1640 supplemented with 20 % FBS and 100 U/ml IL-7 to a concentration of $(5–100) \times 10^3$ cells/ml.
3. Add 20 μl of the thymocyte suspension into each well of Terasaki plate, and use tying forceps to place one dGuo-treated lobe (prepared as described in Subheading 3.3) to each well.
4. Cap the plate with its lid and invert. Gently flap the plate to make sure every lobe is at the bottom of the drop. Avoid any bubbles in the drops. Place the plate in a humidified box and incubate in tissue culture incubator.
5. 24 h after reconstitution, set up filter cultures by adding 0.5 ml of RPMI-1640 supplemented with 20 % FBS and 100 U/ml IL-7 to wells of a 24-well plate. Immerse one piece of sponge (1 cm^2) in each well and wait until the medium soaks the sponge. Place one filter above each sponge. Make sure no air bubble remains between the interfaces.
6. Transfer the lobes to a 10-cm dish containing 10 ml fresh RPMI-1640 supplemented with 20 % FBS and gently swirl the plate to wash off cells attaching to the surface of the lobes.

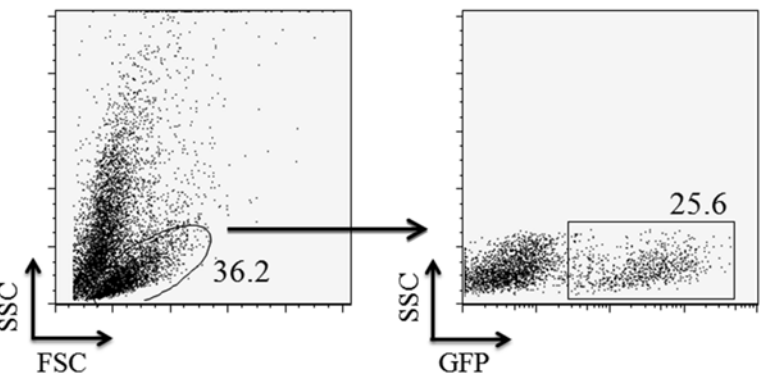

Fig. 3 Viability and GFP expression of progenitor T cells 24 h after pMCS-IRES-GFP retroviral infection

7. Transfer the lobes onto the filters of the culture well set up in **step 5** above.
8. Incubate the plate in the humidified box in tissue culture incubator for 7–10 days. The introduced thymocytes then can be assessed by FACS analysis (Fig. 3).

3.6 Optional Step: Retroviral Transfection of Thymocytes

1. Seed 1×10^6 Plat E cells [12] in a 60-mm Petri dish containing 3.5 ml DMEM supplemented with 10 % FBS. Place for 18–24 h in tissue culture incubator. *See* **Notes 10** and **11**.
2. Transfect 20 μg of retroviral plasmid DNA (e.g. pMCS-IRES-GFP) using adhesion-assisted lipofection or calcium phosphate precipitation method.
3. Seven hours after transfection, remove the supernatants and add 2 ml fresh DMEM supplemented with 10 % FBS. Collect culture (retroviral) supernatants 48–72 h after the transfection and use for infection [13]. Replace with 2 ml fresh DMEM supplemented with 10 % FBS. *See* **Note 12**.
4. Pre-warm up the centrifuge to 32 °C.
5. Filter 500 μl retroviral supernatants with a 0.2-μm filter and add it in a well of a 24-well plate. Dilute polybrene stock 1:500 into the retroviral supernatants to a final concentration of 20 μg/ml. Add 100–10,000 thymocytes (prepared in Subheading 3.4) into each well and seal the plate with parafilm. Spin the plate at $1000 \times g$ in pre-warmed centrifuge for 90 min.
6. After transduction, take an aliquot of thymocytes, culture overnight and assess cell viability and transduction efficiency by flow cytometry (Fig. 4).
7. Use the rest of the cells in hanging-drop culture as described in Subheading 3.5.

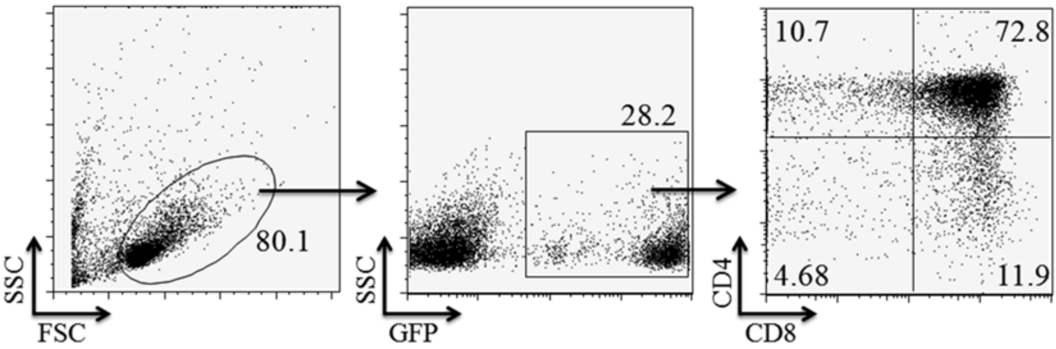

Fig. 4 A typical result of RTOC. The pMCS-IRES-GFP retrovirus-infected thymocytes were reconstituted with dGuo-treated lobes and cultured for 8 days. Cells were isolated and then stained with anti-CD4 and anti-CD8 antibodies

4 Notes

1. Ensure that animal procedures are performed according to appropriate guidelines and approved by relevant animal care authorities.
2. To improve frequency of pregnancies, use 8–12 weeks old females and proven breeder males.
3. Not all females in which a plug is detected are pregnant. Fetuses can be palpated after gestation day 13.
4. We usually get 8–12 E15 fetuses from one pregnant mouse (C57BL/6 background). About 10 E15 thymuses provide 1×10^6 T cell progenitors.
5. All procedures must be carried out sterile, if needed in a tissue culture cabinet. Wipe all instruments with 70 % alcohol every time before and after use. Make sure to remove any remaining alcohol before use.
6. Most laboratories use a mouth-controlled glass pipette transferring the lobes, we use tying forceps handling them. Both approaches require practice. To keep the thymus lobes integrated, avoid damaging or pinching them with forceps tips of any forceps. Use the syphonic effect between the tips of forceps to lift the lobes out of liquid.
7. Quality gelatin sponges are required to prevent sponges from sinking into the medium after 5 or 6 days.
8. During the incubation, check the plate every 2 days to make sure the filter/sponges are intact and firm. After 5–7 days of culture, the endogenous thymocytes are eliminated by dGuo.
9. After treatment with dGuo, the lobes collapse (Fig. 2) and become thinner. Keeping the medium cold makes the detaching process much easier.

10. Ensure that work with recombinant DNA material is performed according to applicable institutional guidelines and regulations.
11. Plat E cells [12] are a high-efficiency packaging line for retroviral transduction. The retroviral gene transfection efficiency depends on the status of Plat E. The higher GFP expressed in Plat E, the higher the viral titer of the supernatant.
12. 24 h after the transfection, the high-FSC/low-SSC population is a good estimate of viable cells (Fig. 4).

Acknowledgements

We thank Graham Anderson (University of Birmingham) for advice on RTOC.

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Chapter 14

Induction of T Cell Development In Vitro by Delta-Like (Dll)-Expressing Stromal Cells

Mahmood Mohtashami, Payam Zarin, and Juan Carlos Zúñiga-Pflücker

Abstract

Recreating the thymic microenvironment in vitro poses a great challenge to immunologists. Until recently, the only approach was to utilize the thymic tissue in its three-dimensional form and to transfer the hematopoietic progenitors into this tissue to generate de novo T cells. With the advent of OP9-DL cells (bone marrow-derived cells that are transduced to express Notch ligand, Delta-like), hematopoietic stem cells (HSC) could be induced to differentiate into T cells in culture for the first time outside of the thymic tissue on a monolayer. We, as well as others, asked whether the ability to support T cell development in vitro in a monolayer is unique to BM-derived OP9 cells, and showed that provision of Delta-like expression to thymic epithelial cells and fibroblasts also allowed for T cell development. This provides the opportunity to design an autologous coculture system where the supportive stromal and the hematopoietic components are both derived from the same individual, which has obvious clinical implications. In this chapter, we describe methods for establishing a primary murine dermal fibroblast cell population that is transduced to express Delta-like 4, and describe the conditions for its coculture with HSCs to support T cell lineage initiation and expansion, while comparing it to the now classic OP9-DL coculture.

Key words T cell development, Lymphopoiesis, Notch, Delta-like, Fibroblasts, Hematopoietic stem cells, Stromal cells, IL-7, Stem cell factor

1 Introduction

Ex vivo fetal thymic organ cultures provided the authentic thymic environment in order to generate T cells through the transfer of hematopoietic stem cells (HSC) [1, 2]. However, the expensive and cumbersome nature of this technique propelled immunologists to seek new methods of generating T cells in culture. It has been well established that the generation of T cells from hematopoietic stem cells (HSC) is dependent on Notch signaling [3]. Based on this insight, our lab established an in vitro coculture system in which a bone marrow-derived stromal cell line, OP9-DL1, ectopically expressing Delta-like 1 (Dll1) could effectively and efficiently support the initiation and expansion of T lineage cells

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_14, © Springer Science+Business Media New York 2016

from HSCs [4]. The OP9-DL1 cells could mimic the thymus in its ability to support early T cell development as well as the generation of mature CD8 single positive (SP) cells [5, 6]. The detailed methods for generating T cells from mouse and human HSCs using OP9-DL cells have been published earlier [7]. We also demonstrated that rather than Dll1, Dll4, which is the Notch ligand physiologically expressed in the thymus, is more efficient at steering HSCs toward T cell lineage when ectopically expressed at physiological levels on OP9 cells (OP9-DL4) [8].

There are several advantages to working with OP9-DL (OP9 cells expressing Dll1 or Dll4) cells, namely the freeing of the reliance on availability of thymic tissue, the ability to perform clonal assays and the ease of expansion of T lineage cell populations [9]. However, one limitation is the ability to test genetic differences within stromal cells supporting T cell development, or to match the genetic background of stem/progenitor cells to that of the supporting stromal cells. To circumvent these issues, we and others recently showed that T cell development can also be supported by thymic epithelial cells [10, 11] and primary murine dermal fibroblasts expressing Dll4 (mFibro-DL4) [12, 13]. Dermal fibroblasts are easily accessible, and though not as efficient as OP9 cells, these cells are nevertheless effective in supporting T cell development, and importantly can be used to establish an autologous T cell coculture system where both the supporting stromal and the T cells are derived from the same individual or genetic background.

Here, we present the protocol for (a) establishing a mouse primary fibroblast, (b) its transduction to express Dll1 or Dll4, (c) purification of mouse HSCs, and (d) maintenance/expansion of cocultures and comparison to OP9-DL coculture, also demonstrating that mFibro-DL1 can also support T cell development (Fig. 1).

2 Materials

2.1 Cell Lines and Media

1. Primary dermal fibroblasts derived from mouse ear biopsies.
2. Fetal bovine serum (FBS) (*see* **Note 1**). Heat-inactivate at 56 °C for 30 min. Store at 4 °C.
3. Phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}.
4. Trypsin 2.5 % (Life Technologies). Dilute with PBS to 0.25 % solution.
5. Fibroblast media: DMEM supplemented with 10 % (v/v) FBS, 2 mM GlutaMAX, 2 mM 2-mercaptoethanol, Penicillin 100 U/mL–Streptomycin 100 μg/mL.
6. Sterilized 22 mm × 22 mm glass coverslips.
7. Sterilized scissors and forceps.
8. Tissue culture ware (10-cm dishes, 6-well plates), tissue culture-treated, serological pipettes.

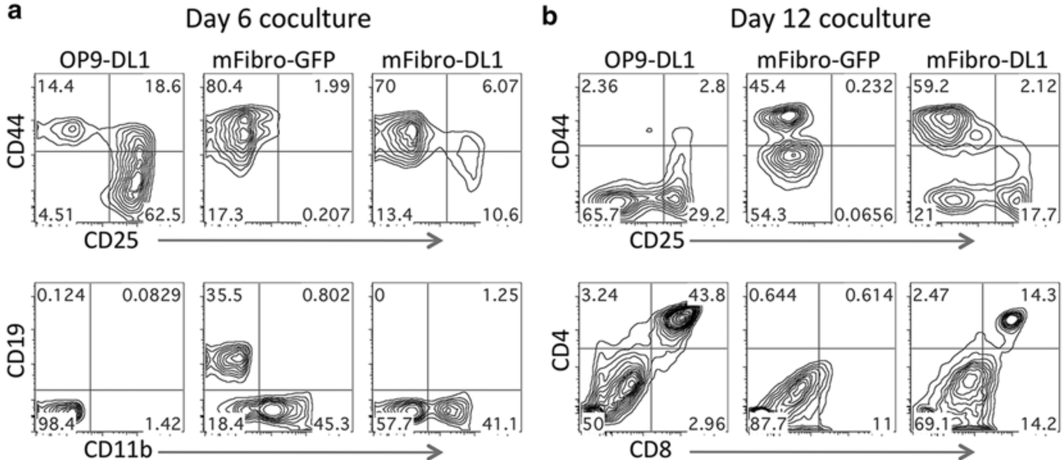

Fig. 1 Cocultures of OP9-DL1, mFibro-GFP, and mFibro-DL1 cell with hematopoietic stem cells. Different hematopoietic cell lineage differentiation outcomes are obtained from E15 fetal liver-derived HSCs cultured with OP9-DL1, mFibro-GFP, and mFibro-DL1 cell lines. Cocultures were set up with 10^3 mouse fetal liver-derived Lineage– CD117⁺ Sca1⁺ HSCs were cultured with the different stromal cell lines as indicated. (**a**) At day 6 of coculture, flow cytometric analysis was performed for the expression of CD25, CD44 for T lineage-specified cells (*upper panel*) and CD19, CD11b for B and myeloid cells, respectively (*lower panels*). (**b**) A fraction of the cocultures were transferred to fresh plates with stromal cells, and at day 12 of coculture, in addition to CD25 and CD44 (*upper panel*), the expression of CD4 and CD8 (*lower panel*) was also examined. Cells were electronically gated on live (DAPI–) and CD45+ hematopoietic cells. Data are representative of at least three independent experiments

2.2 Transfection and Transduction

9. HEK-293 T cells.
10. Viral packaging cell line GP+E-86 cells.
11. pMIG or similar retroviral plasmids with IRES-reporter (e.g., GFP) construct containing the coding region of Dll1 or Dll4 cDNA. Plasmids encoding the VSV-Env and GAG/Pol derived from Moloney murine leukemia virus.
12. $CaCl_2$ 2.5 M (in −20 °C freezer).
13. 2× BES solution (50 mM BES (pH 6.95), 280 mM NaCl, 1.5 mM Na_2HPO_4).
14. 0.2 mm filter units.

2.3 HSC Isolation and Coculture

15. OP9-DL cells: OP9 cells (Riken repository, Tsukuba, Japan, http://www.rtc.riken.go.jp) retrovirally transduced to express the coding region for Dll1 or Dll4 cDNA, as previously reported [8, 14].
16. 40 μm cell strainers.
17. Anti-CD24 mAb: supernatant from J11D.2 hybridoma clone or purified anti-CD24 mAb.
18. Rabbit complement.
19. Lympholyte®-M Cell Separation Media.

20. 70 μm nylon mesh filters.
21. α-MEM—Minimum Essential Medium Eagle Alpha Modification.
22. Hank's Buffered Saline Solution.
23. Coculture medium: α-MEM supplemented with 15 % (v/v) FBS and Penicillin 100 U/mL /Streptomycin 100 μg/mL.
24. Mouse IL-7. Reconstitute at 1 μg/mL in coculture media. Aliquot and store at −80 °C.
25. Human Flt-3L. Reconstitute at 5 μg/mL in coculture media. Aliquot and store at −80 °C.
26. Mouse stem cell factor (SCF). Reconstitute at 25 μg/mL in coculture media. Aliquot and store at −80 °C.
27. CD117-APC, Sca1-PE.
28. Freezing medium: 90 % FBS, 10 % dimethyl sulfoxide, sterile filtered.

3 Methods

3.1 Primary Mouse Fibroblast Culture

Care must be taken to practice sterile culture techniques, especially for the isolation of primary mouse dermal fibroblasts, as the skin is a principal source of contamination. The cultures can be prepared as below [15]:

1. Ear tips are removed from mouse (*see* **Note 4**) that is euthanized, in accordance to the institution's Animal User Protocol approval, using sterile scissors and forceps. The tissue samples are then washed with 10 mL 70 % ethanol for 5 min and transferred to a laminar flow clean bench and rinsed in 10 mL PBS. The samples are minced (~5 mm^2) using sterilized surgical scissors under PBS and treated with trypsin (0.25 %) for at least 1 h at 37 ° C.
2. Using fine sterile forceps, the apposed layers of ear epidermis are pulled away from each other, exposing the dermis. The peeled away tissues are placed under sterile coverslips in wells of a 6-well per plate with 2.5 mL of Fibroblast media. The coverslips are pressed down against the tissue with the hair side against the coverslip using a sterile pipette tip. This has the double effect of forcing out air bubbles and of increasing surface interaction between the dermal tissue and the tissue culture plate. Several wells may be prepared this way.
3. Each well is monitored, and cells are given time to grow out into the well for 7–10 days. Fibroblast cells migrate from the tissue to the plate.
4. To establish a cell culture of primary fibroblasts, the media is removed and 2 mL PBS is used to wash off any remaining

media. Using sterile forceps the coverslips are lifted. PBS is removed and replaced with 1 mL 0.25 % trypsin followed by its transfer to 37 °C incubator for 5–7 min. The skin tissue may remain in the plate to be trypsinized.

5. Following trypsinization, 2 mL of fibroblast media is added to the wells and the cells are removed from the surface of the plate by vigorous pipetting. The trypsinized cells and tissue pieces are passed through a 40 μm cell strainer that is placed on a 50 mL conical tube containing 10 mL fibroblast media. 3 mL PBS is used to rinse the plate and added to the filter on the conical tube.
6. The strainer is discarded and the primary fibroblast cells are centrifuged at 480 × *g* for 5 min at 4 °C. The supernatant is discarded and the pelleted cells are subsequently resuspended in 10 mL of fibroblast media, and transferred to a 10 cm plate.
7. To expand the primary fibroblast, cells are to be cultured up to no more than 90 % confluency. Remove the media then add 5 mL PBS to wash off residual media. Remove PBS and incubate with 2.5 mL 0.25 % trypsin for 5–7 min at 37 °C. Following trypsinization, add 2.5 mL fibroblast media and vigorously pipette the cells to remove them from the surface of the plate. Place in a conical tube and rinse the plate with PBS and add to the contents of the first wash. Pellet the cells at 480 × *g* for 5 min at 4 °C, resuspend in media, and divide among 10 cm plates at a 1:4 passage ratio.

3.2 Mouse Fibroblasts Transduced to Express Dll1/ Dll4

1. Retroviral vector pMIG containing the Dll1 or Dll4 cDNA coding region tagged with hemagglutinin (HA) peptide [8] and an IRES-eGFP (pMIG-DL) are transfected into HEK-293T cells, using $CaPO_4$ transfection, along with plasmids encoding the VSV-Env and GAG/Pol. Briefly, pMIG-DL (10 ug), VSV-env (0.3 ug) and GAG/Pol (1 ug) are mixed in 450 uL of TE and 50 uL of $CaCl_2$ before their addition to 500 uL of 2× BES solution, while vigorously mixing by making bubbles. The mixture is then incubated at room temperature for 20 min followed by their addition to a 10 cm plate of HEK293Ts.
2. After overnight incubation, the medium containing the transfection material is discarded. Cells are washed very gently with 5 mL PBS, and replaced with 7–10 mL fibroblast medium.
3. Within 8 h after replacement with fresh fibroblast medium, supernatant containing the retroviral particles produced and released into the medium by HEK293T cells is collected. To exclude possible HEK293Ts, the supernatant is filtered using 0.2 μm filters. The filtered supernatant is then placed on 50 % confluent GP+E-86 viral packaging cells. The infection

procedure may be repeated once every 4–8 h if more than one round is required.

4. Seven days after the last infection, GFP⁺ GP+E-86 cells are isolated by flow cytometry and expanded in fibroblast media. These cells are named GP+E-DL1 or GP+E-DL4 (GP+E-DL).
5. GP+E-DL cells are allowed to grow to confluency in 10 cm plates. Viral particles produced by GP+E-DL cells are collected within 4–8 h of replacing the medium with fresh fibroblast medium and filtered using 0.2 μm filters. The supernatant is then immediately used to transduce primary fibroblast cells at 60 % confluency in 10 cm plates. Depending on the efficiency of transduction, more than one round of transduction may be done.
6. Two days after transduction, transduced fibroblasts are tested to determine GFP expression levels using flow cytometry.
7. If positive for GFP, the cells may be passaged into several 10 cm dishes. Cells should be passaged at no more than 90 % confluency.
8. Seven days after the last transduction, fibroblast cells are sorted by flow cytometry, based on levels of expression of GFP. Sorted GFP^+ fibroblast cells are named mFibro-DL1 or mFibro-DL4 (mFibro-DL) and its population expanded using fibroblast media as described above in Subheading 3.1, **step** 7.
9. To prepare mFibro-DL for coculture, and compare to OP9-DL cells as the standard, 1.5×10^5 cells per well, seeded overnight, provide an almost confluent well in a 6-well plate for both OP9-DL and mFibro-DL. For OP9-DL cells, coculture medium is used and for mFibro-DL fibroblast medium. Once the right number of cells is placed in 6-well plates, gently rock the plate back and forth for even cell distribution.

3.3 Isolation of HSCs from Mouse d15 Fetal Liver

All experimental procedures related to the use of mice require approval and are performed according to the guidelines specified by local institution's animal user protocol guidelines.

1. CD1 (or desired mouse strain) timed pregnant mice are commercially available from Charles River Laboratories. Alternatively, timed paired matings may be set up in-house in local animal facility.
2. At day 14–15 p.c., fetal mice are obtained and the livers dissected using sterilized scissors and forceps.
3. Using the sterile rubber end of a 3 or 5 mL syringe plunger, day 14–15 fetal livers are then crushed against a 40 μm cell strainer that fits into a 50 mL conical tube. The strainer is washed several times using coculture medium.
4. The cells are centrifuged at $480 \times g$ for 5 min at 4 °C. The supernatant is discarded and the cells in the pellet are resuspended in 4 mL of coculture medium per up to ten fetal livers.

5. To enrich for HSCs, mature cells are lysed using anti-CD24 complement lysis by adding the following to the cell suspension: (a) 1 mL of reconstituted rabbit complement and (b) either 5 mL of filtered culture supernatant from J11D.2 hybridoma clone containing anti-CD24 mAb or 5 mL of coculture media containing 1 ng of purified anti-CD24 mAb per 10^6 cells. Incubate for 30 min at 37 °C.
6. 7 mL of Lympholyte-M density cell separation medium is then carefully laid under the cell suspension as to prevent mixing. The 2-phase solution is centrifuged at $1000 \times g$ for 10 min at room temperature.
7. At the end of centrifugation, the lysed cells will be in the pellet along with the red blood cells and the mononuclear cells (mostly stem/progenitors) will form an opaque band at the interphase between Lympholyte M and the coculture media. The interphase layer is carefully removed using a serological pipette and transferred to a 50-mL tube. Note that there will be some mixing of the two phases while the cells are being taken up. The volume of this mixture is measured and at least twice at much coculture medium is added. The mixture is then centrifuged at $700 \times g$ for 5 min at 4 °C to obtain the HSC-enriched cells in the pellet.
8. At this point, the cell population will be about 85 % $CD117^+$ (c-kit^+). The cells may be cryopreserved by resuspending them in freezing medium at about 4 embryos/fetal livers per mL of freezing medium per vial. At a later date, the cells may be thawed by placing the cryovial containing the fetal liver cells at 37 °C for approximately 1 min. As soon as the cells are thawed, they are gently place in a 15 mL conical tube containing 9 mL of Hank's Buffered Saline Solution and mixed before centrifugation and staining for HSCs.
9. HSCs ($CD117^+$ $Sca1^+$ Lin/$CD24^-$), which should account for about 5 % of the cells, may be obtained using flow cytometric fluorescent-activated cell sorting. The cells are stained with antibodies against CD117 and Sca1 conjugated to fluorochromes of choice—CD117-APC and Sca1-PE are recommended, and sorted for $CD117^+$ $Sca1^+$ cells. After washing, the sorted cells are ready to be placed on OP9-DL4 or mFibro-DL4 to initiate the coculture.

3.4 Initiation and Maintenance of Coculture

Cells may be seeded in 6-well plates or 10 cm plates or other vessels. Here we describe coculture in 6-well plates.

1. Seed 1×10^3 HSCs in 3 mL of coculture medium per 1 well of ~90 % confluent OP9-DL or mFibro-DL cells, and add 5 ng/mL Flt-3L and 1 ng/mL IL-7 and 25 ng/mL SCF (Note, the addition of SCF is not necessary when setting up OP9-DL1 cocultures). Place the culture plates in the incubator.

2. Day 5: Prepare fresh plates of OP9-DL and mFibro-DL (as described above) for transfer on Day 6.
3. Day 6: Small Clusters of round, shiny cells will be present, with some cells detached in suspension. Cells are disaggregated by vigorously pipetting the medium up and down using a serological pipette with pipette-filler. The stromal monolayer may be disrupted during this process and detached from the plate. Once detached, the stromal cells can form clumps that are filtered out by passing the resuspended cells in the medium through 70 μm nylon mesh filter into 15 mL conical tube. The wells may be washed with 3 mL of PBS and passed through the same filter.
4. At this point, the cells are split into two tubes with one-sixth to be transferred to a fresh plate of stromal cells. 1 mL of the mixture is transferred to a 5 mL polypropylene round bottom tube and centrifuged at 480 × *g* for 5 min at 4 °C before discarding the supernatant and resuspension in 3 mL of coculture media containing cytokines. The cells are then transferred to a fresh plate of stromal cells. The remaining 5 mL of cells may be used for cell counts and flow cytometry analysis comparing OP9-DL and mFibro-DL cocultures (*see* Fig. 1a for comparison of OP9-DL1, mFibro-GFP (control), and mFibro-DL1).
5. Day 9 and onward. Follow the instructions outlined above in Subheading 3.4, **step 2** approximately once every 3–5 days. The ratio of the cells transferred may be less or more depending on the number and the frequency of the cells that one is interested in. In addition, the culture can be expanded into larger plates.

4 Notes

1. We routinely test new lots of FBS serum against a standard lot of FBS previously shown to support T lymphopoiesis in culture, such as fetal thymic organ cultures.
2. To preserve early passage stocks of mFibro-DL4 stromal cells, once the plate's confluency reaches 80–90 % of the 10 cm dish, cells are split into four 10-cm dishes. Passaging procedures are continued until the desired number of plates are 80 % confluent. Freeze one plate per cryovial in freezing media and place in liquid nitrogen.
3. To ensure Dll1-HA or Dll4-HA is expressed, a sample of GP+E-DL and mFibro-DL cells may be lysed to perform a Western immunoblot assay using an Anti-HA antibody.
4. We illustrate here that both the fibroblasts and the hematopoietic component are derived from the same strain, but not the same individual mouse; nevertheless, the MHC of the mice from which the stroma and the hematopoietic cells are derived are identical, as shown in Fig. 1.

5. For QRT-PCR of the hematopoietic cells in coculture, it is not enough to filter out the clumps of stromal cells through the nylon mesh filter. There will be some single stromal cells that will go through the filter and may change the values obtained. We recommend placing the filtered resuspended cells onto a tissue culture plate and place in the CO_2 incubator. Both OP9 and fibroblast cells are quite adherent and within 15 min will attach to the surface. The non-adherent hematopoietic component will stay in suspension and can be harvested.

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Chapter 15

In Vitro Analysis of Thymocyte Signaling

Cody A. Cunningham, Emma Teixeiro, and Mark A. Daniels

Abstract

From the moment a developing thymocyte expresses a TCR, it is subjected to numerous interactions with self-peptide/MHC complexes that determine its ultimate fate. These include death by neglect, negative selection (apoptosis and lineage deviation), positive selection, and lineage commitment. The identification of signals that govern these unique cell fates requires the ability to assess the activity, level of expression, subcellular location, and the molecular associations of numerous proteins within the developing T cell. Thus, this chapter describes methods designed to analyze thymocyte signaling under various types of peptide-based stimulation in vitro.

Key words T cell development, Positive selection, Negative selection, TCR signaling, Death by neglect

1 Introduction

The selection of functional T cells is mediated by interactions between the T cell antigen receptor and self-peptide/major histocompatibility complex expressed on thymic epithelium. These interactions either lead to survival and development, or death. The affinity model proposes that the selection outcome is determined by the affinity of the TCR for a peptide–MHC complex. Weak TCR/peptide–MHC interactions do not support thymocyte survival (death by neglect); strong interactions lead to thymocyte apoptosis, lineage deviation or receptor editing (collectively called negative selection); and interactions between these extremes lead to the development of mature T cells (positive selection) [1]. More recently, a number of “third” signals have been given a role in the selection process [2]. A longstanding issue remains as to how the TCR reads the parameters of ligand engagement, along with these third signals, to direct these distinct cell fates.

We use several standard biochemical techniques to assess signals in our system. Immunoblot techniques are used to determine the expression levels and activation states of proteins in large synchronous populations of thymocytes: for these assays we stimulate, lyse,

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_15,

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SDS-PAGE, transfer and immunoblot (see below for stimulation and lysis protocols). Flow cytometry is used to perform similar assays on small/rare populations of cells that can be distinguished by a specific phenotypic marker (also works for large populations): for these assays we stimulate, stain surface markers and perform intracellular staining. In general we use standard kits (e.g. BD Phosflow or Cytofix/Cytoperm kits) and the methods are not described here. However, we have used the fixation and permeabilization protocol reagents below in single cell suspension to assess expression levels of a number of different proteins with some success. Confocal microscopy provides activation and location information that has added to our understanding of the diversity in the regulation of selection signals [3, 4]. These assays are described below.

Historically, immunoprecipitation followed by western blotting (IP-Western) has been invaluable for elucidating the protein–protein interactions that occur during thymocyte positive and negative selection in the thymus. We have used these assays extensively to map out differences in signaling in large synchronous populations of T cells available from thymi of mice on a non-selecting background [3]. However, IP-Western can have significant drawbacks when targeting small/rare populations of T cells, proteins that are expressed at low levels, or proteins whose available reagents are inefficient at IP. Thus, IP followed by flow cytometry (IP-FCM) offers the benefit of being able to precipitate low abundance proteins to generate data with a high signal to noise ratio [5–7]. Additionally, this protocol offers the benefit of being able to use significantly fewer cells for analysis (~1×10^5 for IP-FCM vs. 100×10^6 for IP-Western). Others have even successfully performed these types of analyses on <1×10^3 cells [7]. These assays are described below.

2 Materials

Prepare all solutions using autoclave sterilized MilliQ (or equivalent) water and molecular biology grade reagents. All reagents are stored at room temperature unless otherwise noted.

2.1 Tissue Preparation

1. Complete media: RPMI1640 supplemented with 1 mM non-essential amino acids (NEAA), 1 mM sodium pyruvate, 50 μM β-Mercaptoethanol, 100 U/mL Penicillin 100 μg/mL Streptomycin 0.292 mg/mL Glutamine, and 10 % FBS.
2. TCR transgenic mouse on a non-selecting background. For CD8 T cell selection TCR transgenic $Rag^{-/-}$ $\beta_2m^{-/-}$ mouse preferably <6 weeks of age are ideal (*see* **Note 1**).
3. Alternatively, to assess cohorts of T cells at multiple stages of development, thymi from TCR transgenic and polyclonal mice on a selecting background can also be used (*see* **Note 2**).

2.2 Stimulation with Peptide-Pulsed APC (*See* Note 3)

1. Antigen-presenting cells: examples: bulk splenocytes, T cell-depleted spleens, bone marrow-derived dendritic cells or other cell lines that express the appropriate MHC.
2. Peptide solution, concentration previously optimized.

2.3 Stimulation with Peptide/MHC I Tetramers

1. Peptide/MHC Tetramers (2 mg/mL).
2. Complete media (*see* Subheading 2.1).

2.4 Confocal Microscopy

1. 13 mm round glass coverslips (no. 1: 0.13 mm thick).
2. Microscope slides (pre-cleaned) 25 × 75 × 1.0 mm.
3. Humidity chamber: 6-well plates with a coverslip on diameter filter paper, parafilm and ~1 mL water (Fig. 1) (*see* **Note 4**).
4. Complete media (*see* Subheading 2.1).
5. 0.2 % Triton solution.
6. Fresh 1.5 and 3.0 % (w/v) BSA/PBS solution.
7. Staining conditions *for surface and/or cytosolic proteins:* prepare a stock solution of 4 % formaldehyde fix (1 part 16 % formaldehyde plus 3 parts PBS). Make and keep at RT in chemical hood.
8. Staining conditions *for cytosolic and/or nuclear proteins:* prepare a stock solution of 8 % formaldehyde fix (1 part 16 % formaldehyde plus 1 part PBS). Make and keep at RT in chemical hood.
9. Blocking buffer: 25 mL 3 % (w/v) BSA/PBS. Keep on ice until use.
10. OPTIONAL: DNA/nuclear staining reagent such as DAPI or DRAQ-5AT if nuclear localization is desirable.

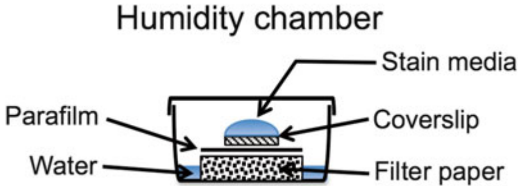

Fig. 1 Schematic of a humidity chamber for confocal microscopy. In general we prepare a 6-well plate with a filter paper, covered by piece of parafilm with approximately 1 mL of water. The filter paper and parafilm are *circles* that we mark and cut using the diameter of a 50 mL conical tube as a template. The cover of the plate is labeled with the conditions (stimulation, stain, time, etc.). These are easily reused from experiment to experiment

2.5 IP-FCM

1. Lysis Buffer: 10 mM Tris pH 7.4, 1 % Triton X-100, 0.5 % NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA. Add 20 mM NaF, 0.2 $NaVO_4$, and 0.2 mM PMSF fresh every time before use.
2. Carboxylate-modified polystyrene latex (CML) beads ~6 μM diameter (Store at 4 °C).
3. Monoclonal antibodies for IP in PBS (≥0.2 mg/mL, must be devoid of BSA).
4. Antibodies for detection (Must be directly conjugated to a fluorochrome or a species other than IP mAb to avoid cross-reactivity).
5. MES Coupling Buffer: 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.0, 1 mM EDTA.
6. EDAC-MES: 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC).
7. Quenching/Blocking/Storage (QBS) Buffer: 1 % BSA + 0.02 % Sodium azide in 1× PBS (Store at 4 °C).
8. FCM Staining Buffer: 1 % FBS + 0.2 % Sodium azide in 1× PBS (Store at 4 °C).

3 Methods

3.1 Harvesting Thymi (*See* Note 5)

1. Prepare sterile tools and small Petri dishes with 1 mL of complete media and 1 piece of sterile nylon mesh.
2. Place Petri dish on ice.
3. Harvest thymi from mice that have been euthanized according to your institutional guidelines. Place in the Petri dish on ice (one thymus per Petri dish).
4. Place another piece of mesh over the thymus and press with a plunger from a 3 mL syringe to create a single cell suspension.
5. Pass cells to a clean 15 mL conical tube that contains 3 mL of complete media.
6. Rinse the mesh with 1 mL of fresh complete media and transfer to the conical tube.
7. Spin cells at 483 × *g* (450–500 × *g*) for 5 min in centrifuge (4 °C).
8. Resuspend cells in fresh complete media (1–2 mL/thymus).
9. Transfer the cells to a new conical (50 mL) through cell strainer (or nylon mesh) to remove large clumps of cells and thymic debris.
10. Count with a hemocytometer (or electronic counter) and dilute cells to a concentration 10×10^6/mL. Proceed to stimulation of your choice.

3.2 Stimulation with APC

3.2.1 Peptide-Loaded APC

1. Prepare media that contains the peptide at the desired concentration (*see* **Note 6**).
2. Resuspend APC at 20×10^6/mL in the peptide solution (from **step 1**).
3. Incubate at 37 °C in the culture incubator from a minimum of 30 min.
4. Wash cells with complete media, spin at $483 \times g$ ($450–500 \times g$) for 5 min and discard supernatant.
5. Resuspend cells at 10×10^6 cells/mL of complete media.

3.2.2 Stimulation with Peptide-Loaded APC

1. Set the thermomixer or water bath at 37 °C.
2. Mix T cells and antigen-loaded APCs at a ratio 1:1. Ideally, for confocal microscopy or ICS-flow cytometry, mix 50 μL APCs (10×10^6/mL) with 50 μL T cells (10×10^6/mL) in a 1.5 mL microfuge tube.
3. Centrifuge in bench top mini-centrifuge. Use pulse/quick spin mode for 7–10 s.
4. Tap the pellet.
5. Place in thermomixer at 37 °C for the planned time course.
6. Place the tubes on ice to stop stimulation.

3.2.3 Stimulation with Peptide/MHC I Tetramers

1. Add approximately 1×10^6/staining condition to 1.5 mL microfuge tube. Example: for an experiment requiring three staining conditions for each time point, one would add 3×10^6 thymocytes (300 μL of 10×10^6/mL suspension of thymocytes from above).
2. Place cells in thermomixer (37 °C, 200–500 rpm mixing).
3. Add desired concentration of peptide/MHC tetramers and incubate for planned time course (*see* **Note 7**).
4. Wash 2× with ice-cold media (to remove excess tetramer) and place the tubes on ice to stop stimulation.

3.3 Confocal Microscopy

3.3.1 Mounting Cells

1. Wash cells from stimulation (above) and resuspend pellet (10×10^6 cells/mL) by gently tapping the microfuge tube (*see* **Note 8**).
2. Place 100 μL of stimulated cells on the appropriate coverslip. TAKE NOTE OF PLATE LAYOUT.
3. To allow the cells to settle onto the coverslip, carefully place covered 6-well plates in refrigerator for minimum of 10 min. During this time prepare the staining buffers. *Fixation:* (*see* **Note 9**).

4. Move plates to chemical hood and add:
   (a) *For surface and/or cytosolic stains:* 100 μL of 4 % fix. Incubate at RT for 45 min.
   (b) *For cytosolic and/or nuclear stains:* 100 μL of 8 % fix. Incubate at RT for 30 min.
5. Carefully aspirate liquid off each coverslip and wash 2× with 200 μL PBS. (You may stop the procedure at this step. To do this, add 200 μL PBS and place plates at 4 °C). *For cytosolic and nuclear stains proceed to* **step 10**.

3.3.2 Permeablization/ Blocking

1. Gently, but quickly add 100 μL of ice cold 0.2 % triton dropwise and incubate for 2 min. (For the best results, work with six wells at a time. Start the timer after adding the triton to last coverslip.)
2. Aspirate triton and add ice cold 3 % BSA/PBS.
3. Repeat **steps 5** and **6** until all coverslips have been treated.
4. Place plates at 4 °C overnight. Alternatively, block for a *minimum* of 1 h then proceed to staining procedure.

3.3.3 Staining

1. Prepare staining cocktails.
   (a) *For primary surface and cytosolic stains, add the desired antibodies to 1.5 % BSA/PBS (w/v).*
   (b) *For primary cytosolic and nuclear stains, add desired antibodies to 1.5 % BSA/0.2%Triton solution.*
   (c) Prepare secondary antibodies by adding labeled antibodies to 1.5 % BSA/PBS (w/v).
2. Aspirate coverslip and wash 2× with RT PBS. *For cytosolic and nuclear stains skip ahead to* **step 16**.
3. Add primary antibody staining cocktail. TAKE NOTE OF PLATE LAYOUT!!!
4. Incubate at RT in the dark for 1 h. During the staining incubations make labels for slides.
5. Wash 2× with PBS and add secondary staining cocktail.
6. Incubate at RT in the dark for 1 h. *For surface and cytosolic stains proceed to* **step 17**.
7. Add 50 μL of the primary stain cocktail in PBS/BSA 1.5 %/ Triton 0.2 % *for 30 min* at RT
8. Wash three times in PBS/BSA1.5 %
9. Add secondary staining cocktail and incubate for 1 h.
10. OPTIONAL: If DNA/Nuclear staining is desired, dilute DRAQ-5 at 1/5000 (or other DNA stain) in PBS/formaldehyde 1 %. Add 50 μL to the coverslip and incubate for 5 min at RT.

3.3.4 Mounting the Coverslip

1. Using a forceps to pick up coverslip, dab the edge of the coverslip with a lint-free tissue to remove excess stain. Then dip the coverslip in PBS and dab the edge of the coverslip with tissue three times, then dip in water and dab once. Place the coverslip CELL SIDE DOWN, onto a microscope slide that has one drop of mounting media (*see* **Note 10**).
2. Cover the mounted coverslip/slides and let dry overnight at RT, then paint the edges of each coverslip with clear nail polish (to make a seal) and place in slide box and place at 4 °C. NOTE: The slides are then ready for analysis by confocal microscopy. While in theory the stains remain stable for up to a year, we suggest they be analyzed within 3 months of mounting.

3.4 IP-FCM

3.4.1 Generation of Ab-Coupled CML Beads

1. Determine the concentration of CML beads by diluting beads in PBS and enumerating using a hemacytometer under a microscope.
2. Gently vortex CML beads to suspend them evenly and transfer 20×10^6 beads to a 1.5 mL centrifuge tube.
3. Wash the beads three times in 0.5 mL MES Coupling Buffer, centrifuging at ~16,000 × *g* for 5 min at 25 °C.
4. Following last wash, aspirate buffer leaving beads undisturbed. Resuspend beads in 50 μL MES Coupling Buffer.
5. Add 20 μL EDAC-MES (made fresh) and mix by pipetting up and down for 15 min to prevent beads from settling.
6. Wash the beads three times in 0.5 mL MES Coupling Buffer, centrifuging at ~16,000 × *g* for 5 min at 25 °C.
7. Aspirate buffer leaving beads undisturbed and resuspend beads in 50 μL PBS.
8. Add 50 μL mAb of choice.
9. Mix for 3–4 h in a thermomixer at ~1100 rpm at 25 °C, being sure that no beads are settling at the bottom of the tube.
10. Wash beads three times in 1 mL PBS, centrifuging at ~16,000 × *g* for 5 min at 25 °C being sure to discard as much supernatant as possible without disturbing the CML bead pellet.
11. Following last wash, aspirate all PBS and resuspend beads in 100 μL QBS Buffer and store overnight at 4 °C.
12. Count beads by diluting in PBS and enumerating using a hemacytometer under a microscope.

3.4.2 Sample Lysis

1. Lyse 10×10^6 thymocytes in 100 μL Lysis Buffer (ice-cold) in a 1.5 mL microcentrifuge tube for 20 min on ice.
2. Following lysis spin crude lysate for 50 min at ~16,000 × *g* at 4 °C.
3. Pass supernatant to a fresh 1.5 mL microcentrifuge tube (*see* **Note 11**).

4. Add 2.5×10^5 IP beads to the lysate (*see* **Note 12**).
5. Place tubes in a thermomixer at ~1100 rpm overnight (place mixer itself in a 4 °C fridge), making sure that beads do not settle.

3.4.3 Staining of Beads for FCM

1. Wash CML beads that have been incubated with lysate three times in ice-cold Lysis Buffer being sure to discard as much buffer as possible without disturbing the CML bead pellet.
2. Resuspend CML beads in FCM staining buffer and pass beads to 96-well round-bottom plate.
   (a) Split the CML beads into enough wells to assess the proteins of interest plus isotype control(s).
3. Add fluorochrome-conjugated mAbs (or primary Abs) to the samples and incubate for 30 min on ice in darkness. α-POSH, clone M-290, α-JIP-1 clone M-300 and α-JNK1 clone C-17 used for demonstration (Fig. 2) [5].
4. Wash beads two times with FCM Buffer, spinning at ~500 × *g* for 5 min at 25 °C.
5. If unlabeled primary Abs are used, add fluorochrome-conjugated secondary Ab and incubate for 30 min on ice in darkness. If directly conjugated Abs are used, skip this step.
6. Resuspend IP beads in 200 μL FCM Staining Buffer and proceed to analysis on the flow cytometer.

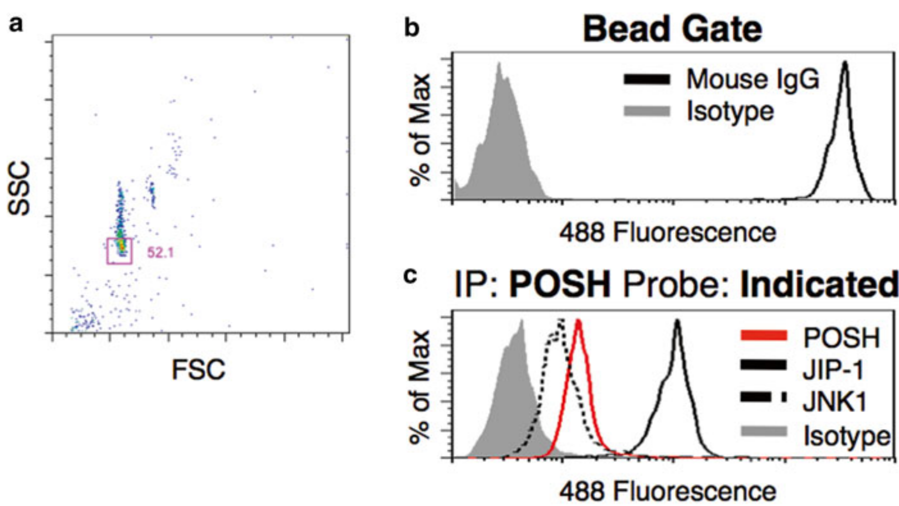

Fig. 2 IP-FCM analysis of protein interactions in pre-selection DP thymocytes. (**a**) Gating on singlet beads based on FSC and SSC properties. (**b**) Proper calibration of fluorescence voltage such that the isotype control falls between 10^0 and 10^1 while a "bright" positive control falls on scale. (**c**) Representative experiment where α-POSH CML beads are used on lysates of pre-selection DP thymocytes. Histograms represent the presence of JIP-1 and JNK1 bound to POSH. *Red* histogram represents the "loading control" to quantify how much POSH was precipitated

3.4.4 Acquisition of Data by FCM

1. Increase the FSC and SSC of the cytometer such that bead singlets are clearly visible (*see* **Note 13**).
2. Draw a gate around the population of bead singlets (Fig. 2a).
3. Apply this gate and visualize the fluorescence data on a log scale for each of the fluorescence channels being used.
4. Pass the isotype control sample and confirm that the generated histogram falls between 10^0 and 10^1 (*see* **Note 14**).
5. Pass the control for a very bright sample (*see* **Note 15**).
6. Pass experimental samples (Fig. 2c).
7. Acquire >1000 bead events. Analyze as described [5, 7].

4 Notes

1. Older mice will work. However, they have significantly fewer pre-selected DP.
2. Due to the heterogeneity of the T cell populations from these thymi, confocal and flow cytometry-based assays are more suitable to assess signaling in these T cells.
3. Due to potential contamination with APC-derived proteins, this method is not appropriate for immunoblotting, IP or IP-FCM.
4. Carefully plan out the stains and time course in advance. Label the cover of the 6-well plate with the stimulation, stain and timepoint for each coverslip. Keep in mind how you want to collect the data at the confocal. One slide holds three coverslips max. An intelligent and logical layout greatly simplifies the collection and analysis of the data. In addition, it greatly reduces the probability of mislabeled coverslips.
5. For short-term assays, those that are performed in less than 24 h, this step can be performed at the bench. Longer time points require FTOC (described in Chapter 12) and should be performed in a sterile hood.
6. When using peptides of different strengths, always start by handling the weakest peptide and move step by step to the strongest peptide.
7. You may substitute anti-CD3 with or without cross-linking. When using cross-linking, pre-incubate thymocytes with anti-CD3 on ice for 15 min, wash, and add pre-warmed media with appropriate concentration of cross-linking antibody, start timing.
8. For analyses involving confocal examination of thymocyte/APC conjugates do not mix cells by pipetting.
9. If your cell surface target is sensitive to fixation, stain on ice after stimulation and prior to fixation).

10. To avoid bubbles, hold coverslip cell side down at 45 % angle and slide the coverslip across slide until it touches the drop of mounting media then gently lay the coverslip down onto slide.
11. Alternatively, one can proceed to standard SDS-PAGE/immunoblot (described elsewhere [3]) procedures at this point.
12. The number of thymocytes used, the volume of lysis buffer, and the number of CML beads used must be determined empirically based on the proteins being analyzed.
13. The CML beads being used are smaller than thymocytes, as such the FSC and SSC gain will have to be increased to make them visible to the cytometer.
14. If fluorescence is too bright lower the channel voltage, if the histogram does not show, increase the channel voltage. α-Rabbit-488 used for demonstration purposes (*see* Fig. 2b).
15. A good control for a very bright sample is anti-mouse Ig (if your Ab coupled to the CML bead is mouse) as it is highly abundant on the CML bead. α-Mouse 488 was used for demonstration purposes (*see* Fig. 2b).

Acknowledgements

The authors thank the members of the Daniels and Teixeiro labs for assistance in compiling these protocols. Funding provided by grants from the Missouri Mission Enhancement Fund and University of Missouri Research Board is greatly acknowledged.

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Chapter 16

Molecular Analysis of Mouse T Cell Receptor α and β Gene Rearrangements

Levi J. Rupp, Liang Chen, Michael S. Krangel, and Craig H. Bassing

Abstract

PCR on genomic DNA isolated from lymphocyte populations is an invaluable technique to analyze T cell receptor (TCR) α and β gene rearrangements. Although this approach is powerful, it also has limitations that must be accounted for in experimental design and data interpretation. Here, we provide background required for understanding these limitations, and then outline standard PCR methods that can be used for analysis of TCRα and β gene rearrangements in mice.

Key words VDJ recombination, T cell receptor (TCR) α and β genes, Variable (V), diversity (D), and joining (J) gene segments, Thymocytes, αβ T cells

1 Introduction

1.1 Biochemistry of TCRα and β Gene Rearrangements

Developing αβ T lymphocytes assemble TCRα and β genes from germline variable (V), diversity (D), and joining (J) gene segments. This recombination process is catalyzed by the lymphocyte-specific RAG1/RAG2 (RAG) endonuclease and ubiquitously expressed non-homologous end-joining (NHEJ) DNA repair proteins. Recombination signal sequences (RSSs) comprised of conserved heptamer and nonamer sequences separated by 12 or 23 nucleotides (12-RSSs or 23-RSSs) flank TCRα and β V, D, and J gene segments. RAG binds a single RSS, captures a second RSS to form a synaptic complex, and then cleaves DNA between each RSS and its flanking gene segment, forming blunt signal ends and hairpin-sealed coding ends [1]. RAG only mediates synapsis and cleavage of gene segments flanked by RSSs of distinct spacer lengths, a level of regulation known as the "12/23" rule. In a subsequent reaction phase, the RAG and NHEJ proteins cooperate to ligate signal ends together to form signal joins and to open, process, and then ligate coding ends together to form V(D)J coding joins [2]. Signal joins are precise, however coding joins are imprecise due to heterogeneity in nucleotide position of hairpin-opening, deletion of coding

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_16,

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end sequences, and addition of non-template nucleotides. Coding joins form the second exons of TCRα and β genes and are transcribed with first exons and downstream constant (Cα or Cβ) region exons. In a population of αβ T cells, the combination of possible joining events and inherent imprecision in V(D)J coding join formation cooperate to generate TCRα and β gene diversity. While combinatorial and junctional diversity are each important for adaptive immunity, they pose substantial obstacles for analysis of TCRα and β gene rearrangements.

1.2 Structure and Recombination of TCRβ Loci

The genomic structure of TCRβ loci is conserved across commonly used lab mouse strains. The TCRβ locus spans 685 kb on chromosome 6. It is comprised of 23 functional Vβ segments and two Dβ-Jβ-Cβ clusters; each Dβ-Jβ-Cβ cluster spans ~6 kb and contains a single Dβ segment (Dβ1 or Dβ2), six functional Jβ segments (Jβ1.1-1.7 or Jβ2.1-2.7 where Jβ1.7 and Jβ2.6 are not functional), and two sets of Cβ region exons (Cβ1 or Cβ2) (Fig. 1). All but two of the 23 functional Vβs reside within a 234 kb Vβ cluster that ends 250 kb upstream of Dβ1. One of the other two Vβs resides 156 kb upstream of this Vβ cluster and the second lies 10 kb downstream of Cβ2. In addition to these 23 functional Vβs, the Vβ cluster contains 12 non-functional Vβs that are pseudogenes or lack competent RSSs. Except for the Vβ downstream of Cβ2, all Vβs reside in the same transcriptional orientation as the Dβ-Jβ-Cβ clusters.

Historically, Vβ segments were named in order numerically as they were identified. Presently, the IMGT (www.imgt.org) and NCBI name Vβ segments based on their genomic location with the Vβ furthest upstream of Dβ1 called TRBV1 and the other functional and non-functional Vβs designated by number in ascending order moving along the locus (with the exception of the TRBV12 and TRBV13 families that are interspersed). Dβ and Jβ segments

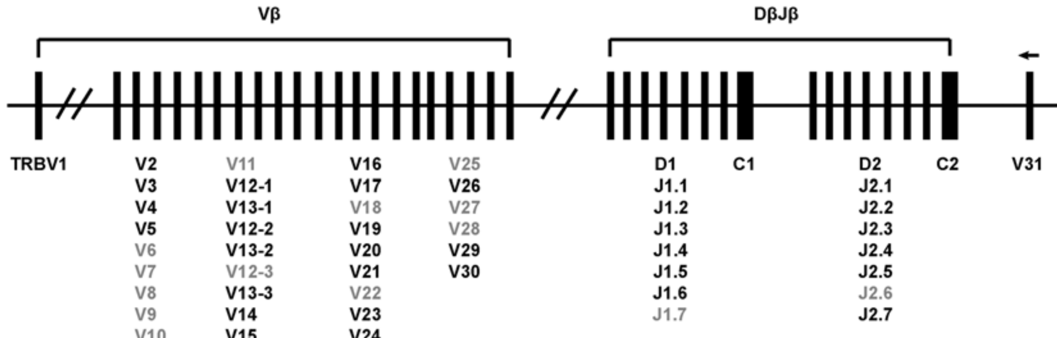

Fig. 1 Schematic of TCRβ locus. Gene segments are listed below each region, from distal to proximal, and are identified by NCBI and IMGT nomenclature. *Gray* lettering indicates that segments are non-functional. Locus is not to scale, and double hash marks indicate large intergenic distances. *Arrow* denotes that TRBV31 is in opposite transcriptional orientation to the rest of the locus

are also numbered in ascending order and labeled TRBD and TRBJ in the current system. Peptides encoded by Vβ segments, however, are still referred to by their historical names. For example, the TRBV1 segment was historically called Vβ2, and TCRβ proteins that contain this Vβ are still referred to as Vβ2^{+} TCRβ chains. In Table 1, we summarize the differences between historical and IMGT/NCBI nomenclature. For this chapter, we use the IMGT/NCBI nomenclature for Vβ gene segments and encoded peptides.

Table 1
Current NCBI and historical nomenclature for TCRβ locus variable (V) region segments

NCBI nomenclature	Historical nomenclature	Join PCR primer [19]
TRBV1	Vβ2	TCCTGGGGACAAAGAGGTCAAATC
TRBV2	Vβ4	AGCTATCAAAAACTTATGGACAATCAG
TRBV3	Vβ16	GATTTTAGGACAGCAGATGGAGTTTC
TRBV4	Vβ10	GCGCTTCTCACCTCAGTCTTCAG
TRBV5	Vβ1	AAATGAGACGGTGCCCAGTCGTT
TRBV6	Vβ26	Non-functional
TRBV7	Vβ27	Non-functional
TRBV8	Vβ28	Non-functional
TRBV9	Vβ24	Non-functional
TRBV10	Vβ25	Non-functional
TRBV11	Vβ29	Non-functional
TRBV12-1	Vβ5.2	CAGCAGATTCTCAGTCCAACAGTTT[a]
TRBV13-1	Vβ8.3	
TRBV12-2	Vβ5.1	CAGCAGATTCTCAGTCCAACAGTTT[a]
TRBV13-2	Vβ8.2	
TRBV12-3	Vβ5.3	Non-functional
TRBV13-3	Vβ8.1	TATATGTACTGGTATCGGCAGGACA
TRBV14	Vβ13	CTGCTGTGAGGCCTAAAGGAACTAA
TRBV15	Vβ12	AGCTGAGATGCTAAATTCATCCTTC
TRBV16	Vβ11	TTCTCAGCTCAGATGCCCAATCAG
TRBV17	Vβ9	TTCCAATCCAGTCGGCCTAACAAT
TRBV18	Vβ23	Non-functional

(continued)

Table 1
(continued)

NCBI nomenclature	Historical nomenclature	Join PCR primer [19]
TRBV19	Vβ6	AAGGCGATCTATCTGAAGGCTATGA
TRBV20	Vβ15	CCCATCAGTCATCCCAACTTATCC
TRBV21	Vβ19	CTACAAGAAACCGGGAGAAGAACTC
TRBV22	Vβ22	Non-functional
TRBV23	Vβ20	CTGGTATCAACAAAAGCAGAGCAAA
TRBV24	Vβ17	TCGAAATGAAGAAATTATGGAACAAAC
TRBV25	Vβ21	Non-functional
TRBV26	Vβ3	GAAAAACGATTCTCTGCTGAGTGTCC
TRBV27	Vβ30	Non-functional
TRBV28	Vβ31	Non-functional
TRBV29	Vβ7	AGCTGATTTATATCTCATACGATGTTG
TRBV30	Vβ18	CCGGCCAAACCTAACATTCTCAAC
TRBV31	Vβ14	AGAGTCGGTGGTGCAACTGAACCT

Segments are listed starting with the most DβJβ distal and moving along the locus toward the DβJβ cluster. Also contains Vβ primer sequences for join PCRs
[a]This primer binds both TRBV12-1 and TRBV12-2

Recombination of TCRβ loci is dictated by RSSs flanking Vβ, Dβ, and Jβ gene segments [3]. Relative to their transcriptional orientation, Vβs are flanked by downstream 23-RSSs, Jβs are flanked by upstream 12-RSSs, and Dβs are flanked by upstream 12-RSSs and downstream 23-RSSs. By the 12/23 rule, recombination between Vβ and Jβ segments should be possible. However, beyond 12/23 compatibility RSS joining restrictions prevent recombination between Vβ and Jβ segments.

Recombination of TCRβ loci is regulated along and between alleles [4]. On each allele, TCRβ recombination is ordered with Dβ-to-Jβ rearrangements preceding rearrangements of Vβ segments to DJβ complexes. The TRBD1 segment can rearrange to any of the six functional TRBJ1 or TRBJ2 segments and possibly to the TRBD2 segment, while the TRBD2 segment can only rearrange to any of the six functional TRBJ2 segments. Each Vβ can rearrange to whatever TRBD1TRBJ1, TRBD1(TRBD2)TRBJ2, or TRBD2TRBJ2 complexes are available. In this regard, Vβ segments can recombine with TRBD2TRBJ2 complexes on alleles that have previously rearranged a Vβ segment to a TRBD1TRBJ1 complex. All TCRβ recombination events occur through deletion

of intervening sequences, except for TRBV31 rearrangements to DβJβ complexes that occur through inversion of intervening sequences. While TCRβ alleles can assemble both a TCRBV31TRBD2TRBJ2 rearrangement and a Vβ–TRBD1TRBJ1 rearrangement involving another Vβ segment, there is no evidence that both of these would be expressed as part of functional TCRβ genes. The Vβ-to-DβJβ recombination step occurs on one allele at a time because of asynchronous initiation of Vβ recombination between alleles and the ability of TCRβ proteins expressed from one allele to signal feedback inhibition of Vβ recombination on the other allele. Although Vβ segments only recombine to assembled DβJβ complexes, it has not been determined whether Dβ-to-Jβ rearrangements occur on both alleles prior to initiation of Vβ-to-DβJβ recombination on a single allele.

1.3 Structure and Recombination of TCRα Loci

The TCRα locus is larger than the TCRβ locus and contains many more gene segments. Moreover, it is polymorphic and structurally distinct in different strains of inbred mice (Fig. 2). Five different Vα haplotypes were described based on restriction fragment length polymorphisms [5]. Detailed sequence analysis is available for strains 129 (which has the same haplotype as BALB/c, AKR, CBA/J, C3H and A/J) and C57BL/6. The 129 TCRα locus spans about 1.6 Mb on chromosome 14, whereas the C57BL/6 locus spans about 1.8 Mb, with the major structural difference mapping to the array of V gene segments. The 129 locus contains 104 V gene segments, of which 88 are likely functional, whereas the C57BL/6 locus contains 138 V gene segments, of which 117 are likely functional. A complicating feature of the locus is the repetitive nature of the V gene array. In strain 129 mice, there is a 400 kb duplication in the central portion of the array, with nearly identical V gene paralogues in each repeat. C57BL/6 mice have copies of these repeats plus an additional repeat sandwiched between them, creating even greater complexity. Both strains have unique V gene segments in non-duplicated regions that flank the repeat region. The repeat structure makes locus analysis rather challenging.

Downstream of the V gene segments are Dδ, Jδ and Cδ gene segments that are used to assemble TCRδ genes. Downstream of Cδ is a single V gene segment in opposite transcriptional orientation to other gene segments in the locus; this is followed by an array of 60 Jα gene segments (of which 43 are likely functional as defined by their ability to undergo recombination and splicing to create mature TCRα transcripts) and Cα. TCRα rearrangement is Vα-to-Jα, whereas TCRδ rearrangement is Vδ-to-Dδ-to-Jδ.

TCRα locus nomenclature has been particularly challenging because there have been several versions; for example, Vα19 was called AV19 by Arden [6] and is now referred to as TRAV1 by IMGT and NCBI, which name the Vα gene segment families in linear order of their first occurrence, from distal to proximal.

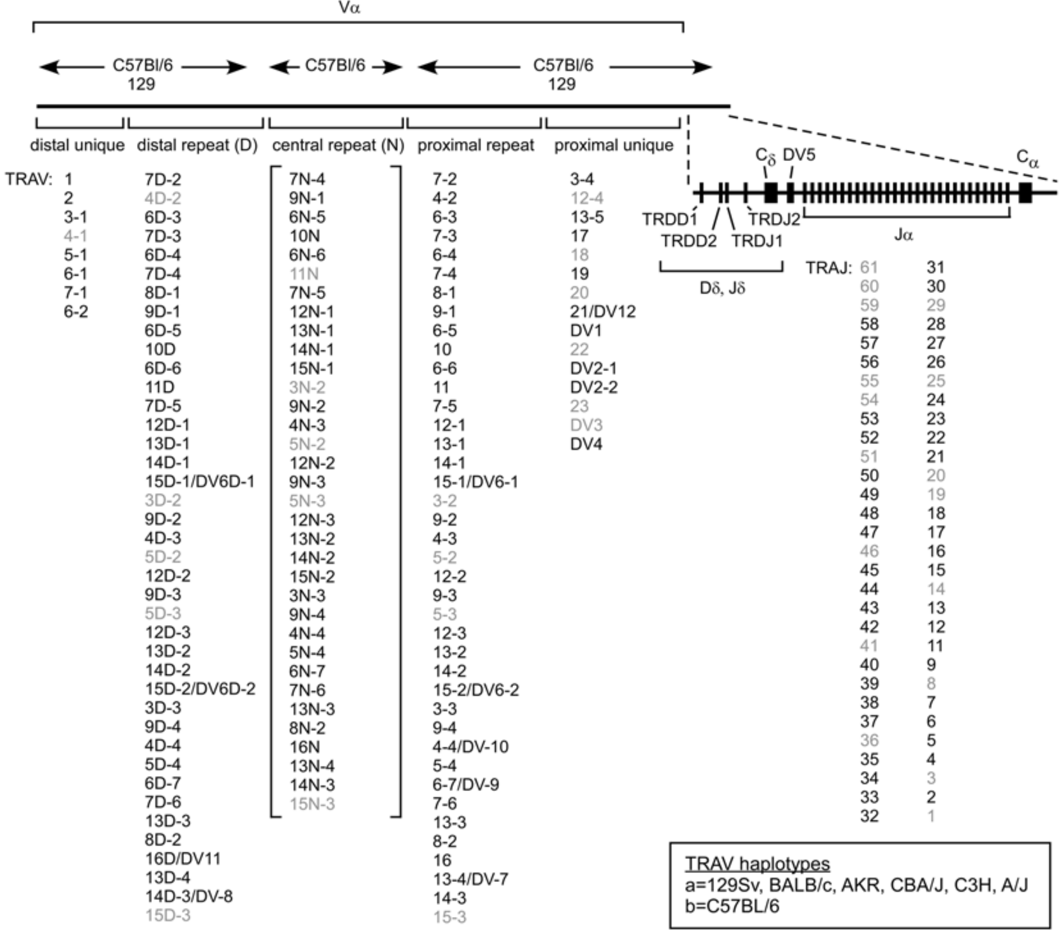

Fig. 2 Schematic of TCRα locus. The structures of the 129 and C57BL/6 TCRα loci are presented. The repeat structure of the V array is noted and the portions shared by 129 and C57BL/6 as well as the portion unique to C57BL/6 are indicated. Gene segments are listed below each region, from distal to proximal, and are identified by NCBI and IMGT nomenclature. *Gray* lettering indicates that genes are non-functional based on the presence of a stop codon or their inability to rearrange or undergo appropriate mRNA splicing. These designations diverge somewhat from those in IMGT based on Jα repertoire studies conducted in our laboratory [15–18] as well as deep sequencing of the peripheral Vα-Jα repertoire [14]. Those V gene segments that are recognized by IMGT to serve as both a Vα and a Vδ have a dual designation (e.g., 15-1/DV6-1, short for TRAV15-1/TRDV6-1); those that are recognized to serve exclusively as a Vδ have a single designation (e.g., DV1, short for TRDV1). Data on TRAV haplotypes were previously reported [5]

The current NCBI nomenclature is compared to the classical Vα nomenclature in Table 3. Similar comparisons of Jα nomenclatures are presented in Table 4. Notably, fifteen of the functional V gene segments in the locus have been classified as Vδ gene segments (TRDV); some of these serve as both Vα and Vδ gene segments (e.g., TRAV15-1/DV6-1) whereas others (e.g., TRDV1, TRDV5) serve exclusively as Vδ gene segments (Fig. 2). All TCRα and TCRδ gene recombination events occur by deletion except for

those involving the inverted TRDV5 gene segment, which undergoes recombination with Dδ gene segments by inversion of the Dδ-Jδ-Cδ region.

1.4 Developmental Control of TCRα and β Recombination

Mature αβ T cells develop through a differentiation program that links expression of TCRα and β proteins from productive V(D)J rearrangements to further developmental progression (Fig. 3). In the thymus, $CD4^{-}CD8^{-}$ (double-negative or DN) thymocytes initiate accessibility of TCRβ loci to promote TCRβ recombination [4]. Assembly and expression of functional VβDβJβ rearrangements leads to TCRβ-mediated intracellular signals that inhibit further Vβ recombination and promote survival, proliferation, and differentiation of DN thymocytes into $CD4^{+}CD8^{+}$ (double-positive or DP) thymocytes. One of the first marks of such TCRβ-selected cells is up-regulated expression of CD28 (from $CD28^{low}$ DN3a cells to $CD28^{high}$ DN3b cells) on DN thymocytes prior to their proliferation and further differentiation [7]. Similarly, up-regulated expression of CD27 is a mark of TCRβ-selected DN thymocytes [8]. Due to inherent imprecision in coding join formation, only ~1/3 of VβDβJβ rearrangements are assembled in-frame and capable of expressing a functional TCRβ protein. In the ~2/3 of DN cells that assemble out-of-frame VβDβJβ rearrangements on the first allele, Vβ recombination can be re-initiated on the other allele in another attempt to assemble an in-frame VβDβJβ rearrangement. In addition, on alleles containing non-functional rearrangements to the Dβ1 cluster, secondary Vβ rearrangements can occur to Dβ2Jβ2 complexes until all possible Vβ-to-DβJβ recombination events are exhausted. As a result, ~60 % of mature αβ T cells contain VβDβJβ rearrangements on one allele and DβJβ rearrangements on the other allele, while ~40 % contain VβDβJβ rearrangements on both alleles where one is out-of-frame in most cells. Although the TRBD1TRBJ1 cluster is rearranged on both alleles in all αβ T

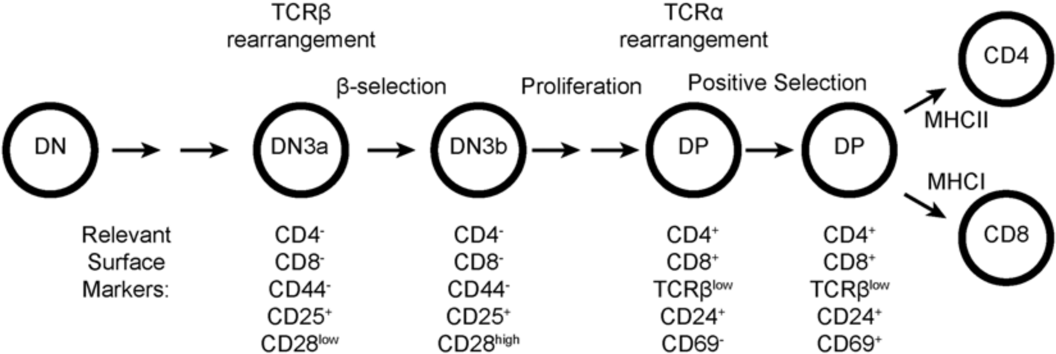

Fig. 3 Diagram of αβ T cell development with markers relevant for cell sorting. Critical checkpoints in αβ T cell development are shown, including surface markers that will differentiate pre- and post-selection DN thymocytes, and pre- and post-selection DP thymocytes

lymphocytes, the TRBD2TRBJ2 cluster remains un-rearranged on one or both alleles in a significant fraction of cells [9].

Thymic DP cells initiate accessibility of TCRα loci to promote TCRα recombination [4]. TCRα recombination generally occurs on both alleles and in multiple rounds on each allele. Initial rearrangements are targeted to the most Cα-distal J gene segments. TRAJ61, although classified as a pseudogene, is the most frequent target of these primary rearrangements, although Jα segments immediately downstream are likely targets as well. These primary rearrangements tend to involve relatively proximal Vα gene segments. However, it should be noted that newly generated DP thymocytes will have already undergone complete or incomplete TCRδ gene rearrangement on both chromosomes. Depending on whether these rearrangements used relatively proximal or distal Vδ gene segments, DP thymocytes initiating TCRα rearrangements will have complete, nearly complete, or only partial Vα arrays to work with. Primary TCRα rearrangements are typically followed by several additional cycles of Vα-to-Jα rearrangement on both alleles, using progressively more proximal Jα and more distal Vα gene segments. The process is terminated either by positive selection based on the specificity of the assembled TCRαβ, or by DP thymocyte apoptosis. The average lifespan of DP thymocytes is thought to be about 3 days. In contrast to the TCRβ locus, TCRα rearrangement is not subject to allelic exclusion. In keeping with this, 25–30 % of TCRαβ T cells have productive Vα-to-Jα recombination on both alleles, although a much smaller fraction actually express two receptors on the cell surface [10–12].

1.5 Analysis of TCRα and β Gene Rearrangements

PCR is the most commonly used technique for analysis of antigen receptor gene rearrangements because it generates large amounts of information in a short amount of time. Both conventional (*see* Subheading 3.1) and real-time (*see* Subheading 3.2) PCR applications may be applied to characterize TCRα and TCRβ rearrangements. Additionally, PCR may be coupled with a Southern blot and probe approach (*see* Subheading 3.3) to increase sensitivity; this application is particularly relevant for analysis of TCRα rearrangements but can be utilized at either locus.

The general PCR strategy is to amplify TCRα and β gene rearrangements from genomic DNA using a forward primer that anneals in a V segment or upstream (5′) of a D segment and a reverse primer that anneals in or downstream (3′) of a J segment. In most cases, these primers anneal to genomic sequences too far apart to amplify products from germline (un-rearranged) alleles, the exceptions being PCR reactions with 5′Dβ and 3′Jβ primers since Dβ segments reside ~700 bp upstream of the 5′ Jβ segment in each cluster. Although PCR reactions on serial dilutions of input DNA are commonly used to determine the linear range of amplification and provide semi-quantitative analyses of V(D)J recombination,

real-time PCR approaches can also be used. For essentially comprehensive analysis of TCRβ gene rearrangements through multiple conventional PCR reactions we employ 43 separate PCR reactions that each use a unique pair of primers. Due to the short distances between the 5′Jβ and 3′Jβ segment in each cluster, one PCR reaction that pairs a Dβ primer with a single primer downstream of one of the Jβ clusters can amplify TRBD1 rearrangements to all six functional TRBJ1 segments, TRBD1 rearrangements to all six functional TRBJ2 segments or TRBD2 rearrangements to all six functional TRBJ2 segments. Similar pairing of a primer specific for any of the 23 individual Vβ segments (or all TRBV12 or TRBV13 family members) with a Jβ1 cluster primer can amplify TRBV-to-TRBD1TRBJ1 rearrangements, whereas pairing of a Vβ primer with a Jβ2 cluster primer can amplify TRBV-to-TRBD1TRBJ2, TRBV-to-TRBD2TRBJ2, or TRBV-to-TRBD1(TRBD2)TRBJ2 rearrangements.

Any attempt at comprehensive analysis of TCRα rearrangements is infinitely more challenging due to the much larger numbers of Vα and Jα gene segments and the large expanse of the Jα array. There are too many Vα-Jα combinations to amplify, and any individual combination would be present quite infrequently and would be that much harder to detect. PCR analyses have generally paired a primer specific for a Vα segment or all members of Vα segment family with a Jα primer. Individual Jα segments are typically spaced by 0.5–2.0 kb, making it difficult to amplify many Jα rearrangements with a single primer. Nevertheless, by using long PCR extension times, a combination of 9 different Jα primers has been used to visualize rearrangements across the entire Jα array using a blot and probe approach [13]. It is more typical to choose short PCR extension times to limit the detected rearrangements to primarily a single Jα gene segment. Due to the low abundance of individual rearrangements, conventional PCR with direct visualization by ethidium bromide staining can be challenging. Rather, it is best to follow conventional PCR by Southern blotting and hybridization to a Vα- or Jα-specific probe, or to analyze by a more quantitative real-time PCR. In designing such experiments it is also important to consider the developmental progression of Vα-to-Jα recombination events that constrains the TCRα repertoire. As borne out by many studies [13, 14], the unique proximal Vα gene segments rearrange most frequently to the most distal Jα gene segments and the unique distal Vα gene segments rearrange most frequently to Jα gene segments in the center and proximal half of the Jα array. Multi-member V gene families that are scattered across the repeat regions tend, as a group, to rearrange broadly across the Jα array. Thus a TRAV19 primer might logically be paired with a TRAJ58 primer, a TRAV1 primer might logically be paired with a TRAJ30 primer, and a TRAV12 family primer could be paired with almost any Jα primer.

Common primers that we have used to amplify TCRβ and α gene rearrangements are listed in Tables 1 and 2 or Tables 3 and 4,

Table 2
Primers for TCRβ join PCRs including oligos 5′ of Dβ and 3′ of Jβ segments

Primer name	Sequence	Location	Southern blot probe
Dβ1	GAGGAGCAGCTTATCTGGTGGTTT	Upstream TRBD1	
Dβ2	GTAGGCACCTGTGGGGAAGAAACT	Upstream TRBD2	
Jβ1.2	CCTGACTTCCACCCGAGGTT	Downstream TRBJ1.2	CATCCTTCCTCTGATTAC [20]
Jβ1.7	AAGGGACGACTCTGTCTT	Downstream TRBJ1.7	GATAAGACAGAATCCTTAGG
Jβ2.2	CTCCAACCCTGACTCAGATCCCCACC	Downstream TRBJ2.2	GGTGGGGATCTGAGTCAGGGTTG
Jβ2.7	TGAGAGCTGTCTCCTACTATCGATT	Downstream TRBJ2.7	TATGAACAGTACTTCGGTCCC [19]

Table 3
Current NCBI and historical nomenclature for representative TCRα locus variable (V) region segments

NCBI nomenclature	Historical nomenclature	Join PCR primers
TRAV1	Vα19	CTATCTCTTCCTGATGGTGG (C)
TRAV2	Vα12	
TRAV3	Vα5	
TRAV4	Vα11	
TRAV5	Vα13	
TRAV6	Vα4	GAAGCAGCAGAGGGTTTGAAG (R)
TRAV7	Vα1	
TRAV8	Vα18	
TRAV9	Vα3	
TRAV10	Vα15	
TRAV11	Vα14	GTCCTCAGTCCCTGGTTGTC (C)
TRAV12	Vα8	GCAGCAGCTCCTTCCATC (R) CAGACAGAAGGCCTGGTCAC (C)
TRAV13	Vα10	AAGAACGTCGCAGCTCTTTG (R)
TRAV14	Vα2	TGGAGACTCAGCCACCTACT (R)
TRAV15	Vα7	TCCATCAGCCTTGTCATTTCA (C,R)
TRAV16	Vα17	TGCAACAGTGGGTCATTATTCT (R)
TRAV17	Vα9	TGGAGCGACTCAGCCAAGTA (R)
TRAV19	Vα20	CCAGCCTGAAGACACAGCAG (R)
TRAV21	Vα6	GTATGGCTTTCCTGGCTATTGC (R)

Also contains Vα primer sequences for join PCRs. (C) and (R) identify primers that have been used in our laboratory for conventional and real-time PCR, respectively. In general, V gene segment primers for conventional PCR are chosen to create amplicons of approximately 300 bp, whereas V gene segment primers for real-time PCR are chosen to create amplicons of approximately 100 bp. Primers for multi-gene families were chosen so that they amplify all members of a given family

respectively. The two major applications of PCR analysis of TCRα and β gene rearrangements are to quantify defects in V(D)J recombination (rates, efficiency, levels) and changes in repertoire. Below we outline certain limitations of PCR analysis of TCRα and β gene rearrangements that must be accounted for in experimental design and data interpretation.

Table 4
Current NCBI and historical nomenclature for TCRα locus joining (J) region segments

NCBI nomenclature	Historical nomenclature	Join PCR primers	Southern blot probe
TRAJ61	Jα61	ATGAGTCTTCCAGTCATGGC [R]	
TRAJ58	Jα58	GACTCACTGTGAGCTTTGCC [R,C]	AGCTTAGACCCAGTGCCTTG
TRAJ57	Jα57	AGCTCACTGTCAGCTTTGTCC [R,C]	AAAGATGAGCTTCGCAGACC
TRAJ56	Jα56	ACTCAGAACGGTTCCTTGACC [R,C]	AGCTGACTTTTGGTCAAGGAACCG
TRAJ53	Jα53	GGAGTCACAGTTAAGAGAGTTCC [R,C]	AATTGCTGCCTCCACTGTTC
TRAJ52	Jα52	TTGGATGGACCCTAAGGATG [C]	TGGAGCTAACACTGGAAAGCTC
TRAJ50	Jα50	ACGACTGATAAGGATGTCCC [C]	TTCAGCAAGCTGGTGTTTGG
TRAJ49	Jα49	GGAATGACAGTCAAACTTGTTCC [R,C]	AGAAGTTCTGGTAACCCCGTG
TRAJ48	Jα48		
TRAJ47	Jα47		
TRAJ45	Jα45		
TRAJ44	Jα44		
TRAJ43	Jα43		
TRAJ42	Jα42	AGAGTTTAGTGCCTTTCCCG [C]	TCTGGAGGAAGCAATGCAAAG
TRAJ40	Jα40	TGGTACCTGCTCCAAAGACG [R]	
TRAJ39	Jα39		
TRAJ38	Jα38		
TRAJ37	Jα37	AAATGAGCATAAGCGACAG [R]	
TRAJ35	Jα35		
TRAJ34	Jα34		
TRAJ33	Jα33		

TRAJ32	Jα32		
TRAJ31	Jα31	GCGTCCCATCACCAAAGAAG (R)	
TRAJ30	Jα30	GGGAGAACATGAAGATGTGTCC (R)	
TRAJ28	Jα28		
TRAJ27	Jα27		
TRAJ26	Jα26		
TRAJ24	Jα24		
TRAJ23	Jα23		
TRAJ22	Jα22	TGTCAGTTGGGTTCCAGATCC (C)	TCCAAAGATGAGTTGCCAGC
TRAJ21	Jα21		
TRAJ18	Jα18	TCCCTGGAGTAGAAAGAAACCTACTCAC (C)	CAGGTATGACAATCAGCTGAGTCCC
TRAJ17	Jα17	TGATGGCTAGGCTCCTTTTC (R)	
TRAJ16	Jα16		
TRAJ15	Jα15		
TRAJ13	Jα13		
TRAJ12	Jα12		
TRAJ11	Jα11		
TRAJ9	Jα9		
TRAJ7	Jα7		
TRAJ6	Jα6		
TRAJ5	Jα5		
TRAJ4	Jα4		
TRAJ2	Jα2	TACCGGGTTGCAAATGGTG (R)	

Also contains reverse primers for join PCRs and oligonucleotide sequences for Southern blot probes

The fact that a TCRβ and a TCRα gene rearrangement are both required for αβ T cell development must be accounted for in PCR analyses to assay for and quantify defects in V(D)J recombination. In αβ T cells that develop from normal V(D)J recombination, ~60 % have a VβDβJβ rearrangement on one allele and a DβJβ rearrangement on the other, ~40 % have VβDβJβ rearrangements on both alleles, and nearly 100 % have VαJα rearrangements on both alleles. For TCRβ, this translates to VβDβJβ rearrangements on 70 % of alleles and DβJβ rearrangements on 30 % of alleles in the entire population. While a complete block in V(D)J recombination prevents αβ T cell development, substantially impaired V(D)J recombination could lead to the selection of αβ T cells with reduced frequencies of TCRα and β gene rearrangements. However, there must be at least one fully rearranged allele at each locus. Thus, PCR analysis (even if highly quantitative) would detect only small differences in the amount of TCRβ and TCRα recombination (maximally 30 and 50 %, respectively) in selected αβ T cells. Such defects may be substantially greater when measured in non-selected, immature thymocyte populations that are actually engaged in V(D)J recombination. Defects in TCRα recombination may also be detected as diminished usage of more Cα-proximal as compared to Cα-distal Jα gene segments, since the former require more rounds of Vα-to-Jα recombination than the latter. Additionally, the distribution of Vβ and Jα segments used may also be skewed by selection. Therefore, PCR analysis of TCRβ and TCRα gene rearrangements to assay for and quantify potential defects in V(D)J recombination must be conducted on non-selected DN and DP thymocytes isolated by flow cytometry-based cell sorting with appropriate cell surface markers. Common cell surface markers used to isolate non-selected DN and DP thymocytes are indicated in Table 5 and Fig. 3. Of course, if the goal is to analyze perturbations that affect selection of the TCR repertoire in the thymus, or expansion of particular T cell subsets in the periphery, then PCR analyses should be conducted on either selected single positive (SP) thymocytes or mature peripheral αβ T cells, as appropriate.

Table 5
Cell surface markers for isolation of pre- and post-selection DN or DP thymocytes

Cell population	Gating scheme
DN3a	$CD4^{-}CD8^{-}CD44^{-}CD25^{+}CD28^{low}$
DN3b	$CD4^{-}CD8^{-}CD44^{-}CD25^{+}CD28^{high}$
Pre-selection DP	$CD4^{+}CD8^{+}TCR\beta^{lo}CD24^{+}CD69^{-}$
Post-selection DP	$CD4^{+}CD8^{+}TCR\beta^{lo}CD24^{+}CD69^{+}$

2 Materials

2.1 Conventional PCR

All PCRs should use molecular biology grade H_2O, while standard tap water is acceptable for running buffers.

1. diH_2O—molecular biology grade.
2. Cells of interest (*see* **Note 1**).
3. Rapid Lysis Buffer: 100 mM Tris-base pH 7.4, 0.2 % sodium dodecyl sulfate, 5 mM EDTA, 200 mM NaCl.
4. Proteinase K. Re-suspended at 20 mg/mL in diH_2O.
5. Isopropanol.
6. TE buffer pH 8.0: 10 mM Tris-base pH 8.0, 1 mM EDTA in diH_2O.
7. 10× PCR reaction buffer (as supplied by DNA polymerase manufacturer).
8. DNA polymerase (*see* **Note 2**).
9. 10 mM dNTP mixture (2.5 mM each dATP, dTTP, dCTP, dGTP), available commercially.
10. Primers of interest (*see* Tables 1, 2, 3, and 4). Re-suspend primers at 5 pmol/μL using diH_2O.
11. CD14 control primers. Sense primer: 5′-GCTCAAACTTTCA GAATCTAC-3′; Antisense primer: 5′-AGTCAGTTCGTGGA GGCCGGAAATC-3′; if following with Southern blot (Subheading 3.3), probe: 5′-AGCAGATCTGGGGCAGTT CACTGA-3′.
12. Standard thermocycler.
13. 1× TBE buffer: 89 mM Tris-base, 89 mM boric acid, 2 mM EDTA in H_2O. To make a 5× stock solution: Dissolve 1080 g Tris-base, 550 g boric acid, and 400 mL 0.5 M EDTA in 5 L H_2O. Adjust final volume to 20 L, and dilute to 1× in H_2O prior to use. Adjustment to precise pH is not necessary at any point.
14. Agarose.
15. Ethidium bromide (10 mg/mL stock solution).
16. 6× Gel Loading Dye.
17. 1 kb DNA ladder.

2.2 Quantitative Real-Time PCR

1. diH_2O—molecular biology grade.
2. Primers of interest for join detection (*see* Tables 3 and 4). Prepare 10 mM working stock in diH_2O.
3. 2× SYBR Green PCR Master Mix.
4. Genomic DNA of interest (50 ng/μL).
5. Real-time PCR instrument.

2.3 Southern Blot

Molecular biology grade diH_2O should be utilized for steps involving DNA while standard diH_2O is suitable for running buffer, transfer buffer, hybridization solution, and washing buffers.

1. Join PCR product from Subheading 3.1.
2. 1× TAE buffer: 40 mM Tris-base, 0.11 % glacial acetic acid (v/v), 1 mM EDTA in H_2O. To make a 50× TAE stock solution: Dissolve 968 g Tris-base in 2 L H_2O. Add 228.4 mL glacial acetic acid and 400 mL 0.5 M EDTA solution. Adjust final volume to 4 L in H_2O. Dilute to 1× in H_2O prior to use. Adjustment to precise pH is not necessary at any point.
3. Agarose.
4. Ethidium bromide (10 mg/mL stock solution).
5. DNA ladder (*see* **Note 3**).
6. UV source (gel box or wand).
7. Denaturing solution: 0.5 M NaOH, 0.6 M NaCl in diH_2O.
8. Tupperware (*see* **Note 4**).
9. Orbital shaker such as Stovall Belly Dancer™.
10. Sponges (*see* **Note 5**).
11. Blotting paper (*see* **Note 6**).
12. 10× SSC: 1.5 M NaCl, 150 mM trisodium citrate in diH_2O. To make a 20× SSC stock solution: Dissolve 3506 g NaCl and 1764 g sodium citrate in a final volume of 20 L H_2O. Dilute as necessary in H_2O prior to use. Adjustment to precise pH is not necessary at any point.
13. Nylon membrane (*see* **Note** 7).
14. Paper towels.
15. Parafilm.
16. Disposable pipette.
17. Gel box and electrophoresis power source.
18. Drying oven.
19. 19× SSCPE: 2.85 M NaCl, 285 mM trisodium citrate, 247 mM KH_2PO_4, and 19 mM EDTA in H_2O. Adjustment to precise pH is not necessary.
20. Oligo pre-hybridization solution: 6× SSCPE, 5× Denhardt's solution, 1 % SDS in H_2O. Prepare 100 mL by combining the following: 31.5 mL 19× SSCPE, 10 mL 50× Denhardt's solution, 10 mL 10 % SDS solution, and 48.5 mL diH_2O. Adjustment to precise pH is not necessary.
21. Oligo hybridization solution: 6× SSCPE, 3× Denhardt's solution, 1 % SDS, 100 μg/mL yeast tRNA in H_2O. Prepare 100 mL by combining the following: 31.5 mL 19× SSCPE, 6 mL 50× Denhardt's solution, 10 mL 10 % SDS solution,

0.4 mL yeast tRNA (25 mg/mL stock, Invitrogen Catalog 15401-011), and 52.5 mL diH_2O. Adjustment to precise pH is not necessary.

22. Hybridization oven set to 42 °C.
23. Probe labeling reagents.
    (a) 50–100 pmol oligo DNA (<6 μL total volume). *See* Table 2 or 4 for oligo sequences, including CD14 loading control.
    (b) T4 polynucleotide kinase (10 U/μL).
    (c) γ^{32}P ATP, 6000 Ci/mmol, 150 mCi/mL [e.g. Perkin Elmer Catalog: NEG-035C].
    (d) Heating block set to 37 °C.
    (e) TE buffer, pH 8.0 (*see* Conventional PCR, Subheading 2.1, Item 6).
24. Wash buffer: 2× SSC, 0.1 % SDS in H_2O. To prepare 4 L Wash buffer, combine 400 mL 20× SSC with 40 mL 10 % SDS and adjust volume to 4 L with H_2O. Adjustment to precise pH is not necessary.
25. Heated water bath shaker (*see* **Note 8**).
26. Funnel.
27. Glass roller bottle.
28. Saran wrap.
29. Film (*see* **Note 9**).
30. Autoradiography cassette.
31. Film developer.

3 Methods

3.1 Conventional PCR

1. Isolate genomic DNA by method of choice. We utilize the lysis and isopropanol precipitation protocol described in **steps 2–5**.
2. Pellet 10^4–10^7 cells of interest in 1.7 mL eppendorf tube. Re-suspend cells in 500 μL rapid lysis buffer (*see* **Note 10**) with 5 μL Proteinase K (20 mg/mL stock). Incubate overnight at 56 °C.
3. Add 800 μL isopropanol to Eppendorf tube containing lysed cells. Mix thoroughly by shaking.
4. Centrifuge 10 min at maximum speed in bench-top centrifuge to pellet DNA.
5. Aspirate supernatant. Re-suspend DNA in appropriate volume TE buffer or diH_2O (*see* **Note 11**) and place at 56 °C for 2 h.
6. Determine DNA concentration by method of choice. Dilute an aliquot to 50 ng/μL in diH_2O.

7. Prepare 25 μL PCR reaction as follows, using desired primers from Tables 1, 2, 3, and 4. When performing multiple reactions, preparing a master mix of all components except genomic DNA is recommended:

2.5 μL	10× PCR buffer
0.5 μL	dNTPs (10 mM)
1 μL	Forward primer (5 pmol/μL)
1 μL	Reverse primer (5 pmol/μL)
0.2 μL	DNA polymerase (5 U/μL)
9.8 μL	diH_2O
10 μL	Genomic DNA (50 ng/μL)

8. Include a control reaction for a non-rearranging region, such as CD14.
9. Perform PCR amplification using a standard thermocycler and the appropriate program for the locus being analyzed:

   TCRβ

   (a) 94° for 3 min.

   (b) 94° for 45 s.

   (c) 60° for 1 min 30 s.

   (d) 72° for 2 min 30 s.

   (e) 72° for 10 min.

   TCRα

   (a) 95° for 3 min.

   (b) 95° for 30 s.

   (c) 63° for 30 s.

   (d) 72° for 30 s.

   (e) 72° for 3 min.

   Repeat **steps b–d** for 32–40 cycles total (*see* **Note 12**).

10. Analyze PCR products by standard agarose gel electrophoresis with either 1 % (TCRβ) or 1.2 % (TCRα) agarose gel in TBE.

3.2 Quantitative Real-Time PCR

1. Isolate genomic DNA by the protocol described in Subheading 3.1, **steps 2–5**.
2. Prepare 12 μL PCR reactions in triplicate as follows, using desired primers from Tables 3 and 4. Include control wells for a non-rearranging standard (such as CD14).

6 μL	2× SYBR Green PCR Master Mix
0.3 μL	Forward primer (10 mM)
0.3 μL	Reverse primer (10 mM)
3.4 μL	diH_2O
2 μL	Genomic DNA (50 ng/μL)

3. Perform PCR amplification on real-time PCR instrument.
   (a) 95 °C for 5 min.
   (b) 95 °C for 10 s.
   (c) 65 °C for 30 s.
   Repeat **steps b** and **c** for 45 cycles.
4. Determine relative quantification of Vα-Jα rearrangements in different samples by normalization to CD14 signal or non-rearranging genetic region of your choice (*see* **Note 13**).

3.3 Southern Blot

Unless otherwise described, all procedures may be carried out at room temperature. All solutions may be stored at room temperature, but we store the oligo pre-hybridization solution, oligo hybridization solution, and wash buffer at 42 °C for ease of use. All steps involving radiation should be performed behind a radiation shield and according to relevant institutional guidelines. We utilize a dedicated sink, 37 °C heating block, and heated shaker for radiation purposes.

1. Prepare running gel by dissolving 4 g agarose in 500 mL 1× TAE with ethidium bromide (5 μL of stock 10 mg/mL solution). Combs should be sized to permit 50 μL loading volume (*see* **Note 14**).
2. Load samples on running gel, leaving the outer two lanes on each side empty, as these will be lost in transfer. Load one lane of DNA ladder (*see* **Note 15**).
3. Run gel overnight (18–24 h) at 23 V.
4. Remove gel from gel box and examine by UV to verify samples have amplified (*see* **Note 16**).
5. Activate the gel by treating for 30 s with short-wave UV irradiation (~254 nm) using wand or gel box, with appropriate shielding.
6. Fill a Tupperware container with 1 L of denaturing solution. Place gel in Tupperware. Shake gently on orbital shaker for 1 h at room temperature (*see* **Note 17**).
7. Prepare for transfer. Place a series of sponges (large enough to fully support the gel) side-by-side in large Tupperware container. Lay one sheet of blotting paper over the sponges,

extending blotting paper over the edge of sponges. Add 10× SSC to the Tupperware, filling until buffer level is ½″ below top of sponges.

8. Activate membrane. Cut an appropriately sized piece of nylon membrane and wet in Tupperware containing diH_2O. Remove membrane from water, allowing excess water to drain, and place membrane in a separate Tupperware containing 10× SSC for 1 h at room temperature.
9. Cut two pieces of blotting paper sized to fit the gel.
10. Set-up transfer as follows. Carefully remove gel from Tupperware containing denaturing solution and place on top of sponges/blotting paper prepared in **step 7** (*see* **Notes 18** and **19**). Place activated nylon membrane on top of gel and roll out bubbles using a disposable pipette. Place parafilm around the edge of the membrane in a "framing" pattern. Parafilm should cover the outer ¼″ of the membrane on all sides. Wet one piece blotting paper in 10× SSC and place on top of parafilm layer. Roll out bubbles as before. Place a dry piece of blotting paper on top of the wet first piece. Place paper towels on top of this blotting paper. Place a weight (such as an eppendorf rack) on top of paper towels. The final order, from bottom to top, is: sponge, blotting paper, gel, nylon membrane, parafilm, wet blotting paper, dry blotting paper, paper towels. Transfer overnight (~20 h), even though transfer rate is ~1 kb per hour.
11. Disassemble transfer apparatus. Place membrane between folds of larger piece of blotting paper and dry in vacuum oven, 1–1.5 h at ~110 °C (*see* **Note 20**).
12. Label the membrane and mark the ladder using an industrial style pen to prevent label washing off during subsequent washes. Ladder can be visualized by examining membrane under UV light.
13. Perform pre-hybridization by placing rolled membrane into glass roller bottle. Add 12 mL pre-warmed oligo prehybridization solution to the tube, place in hybridization oven and rotate for minimum of 1 h at 42 °C.
14. Prepare radiolabeled oligo probe per **steps 15–17**.
15. To 1.7 mL eppendorf tube, add 50–100 pmol oligo (total volume ≤6 μL), 1 μL 10× kinase buffer, 1 μL T4 polynucleotide kinase (10 U/μL), and 2 μL γ^{32}P ATP. Bring total volume to 10 μL with diH_2O.
16. Incubate for 30 min at 37 °C.
17. Add 90 μL buffer TE pH 8.0.
18. After minimum 1 h incubation, discard pre-hybridization solution from roller bottle of **step 13**. In a 15 mL conical, add

probe solution from **step 17** to 13 mL hybridization solution pre-warmed to 42 °C. Add immediately to roller bottle containing membrane that has been pre-hybridized. Hybridize overnight at 42 °C.

19. Adjust heated shaker temperature to 42 °C and allow temperature to stabilize.
20. Using a funnel, empty roller bottle contents by pouring radioactive probe into 15 mL conical tube. Cap tube containing radioactive probe, then wash and dry before returning to radiation storage lockbox if planning to re-use probe. Otherwise, discard per appropriate protocol. Dispose of all radioactive materials according to institutional guidelines.
21. Add pre-warmed wash buffer to roller bottle containing membrane and swirl several times. Open roller bottle over sink and remove membrane using forceps. Place in Tupperware containing sufficient wash buffer (pre-warmed to 42 °C) to cover membrane. Seal tupperware and place in heated shaker at 42 °C. Wash with shaking for 15 min. Rinse out roller bottles in sink and decontaminate per standard protocol.
22. After 15 min wash, discard wash buffer in sink. Add fresh wash buffer and repeat, washing three times total (*see* **Note 21**).
23. Remove membrane from last wash and dry by touching the corner to a paper towel. Place on saran wrap and fold saran wrap over blot, completely enclosing. Fix blot in autoradiography cassette using tape. In dark room, place film in autoradiography cassette. Expose per manufacturer's protocol.
24. Develop film per standard protocol.
25. Optional: stripping blots. Often it is desirable to probe a single blot with multiple different probes, for example one probe detecting a rearrangement of interest and the second probe a loading control such as CD14. To strip a blot, boil 1 L of H_2O in a beaker. Place blot in Tupperware container and pour boiling water over blot, swirling for 30 s to 1 min. Discard water and rinse blot until background radiation levels are detected on the Geiger counter.

4 Notes

1. Possible populations include bulk thymocytes, pre- and post-selection thymocytes isolated by flow cytometry, or mature T cells.
2. We have had success with Qiagen HotStarTaq Polymerase but others may be suitable.
3. We use TrackIt λ DNA/HindIII Fragments (Invitrogen).

4. A range of Tupperware/plastic containers are useful for this protocol and should be sized to fit the anticipated gel size.
5. We utilize kitchen sponges ~1 in. thick. These may be cut to size as desired.
6. We recommend VWR Grade 703.
7. We have had success with Zeta Probe GT Genomic Tested Blotting Membranes (Bio-Rad).
8. Analogous to Bellco Catalog 7746-22110.
9. For example, HyBlot CL®, available from Denville Scientific or other suppliers.
10. If using more cells, the lysis buffer volume may be increased accordingly. Isopropanol in the following step should be scaled to maintain a similar ratio of lysis buffer:isopropanol.
11. For low cell numbers (10^4–10^5 cells), re-suspend in 20–50 μL. Larger cell numbers can be re-suspended in 200 μL or sufficient volume to fully re-suspend pellet and obtain accurate DNA quantification. It is better to start with a smaller volume and dilute as necessary.
12. For visualizing products by ethidium bromide staining, high cycle numbers (e.g. 40 cycles) should be used. However, the large amount of input DNA and high cycle number mean that the reaction is not quantitative. To obtain a rough quantitative measure of rearrangement levels one can perform serial two- or fivefold dilutions with low cycle number (e.g. 32 cycles), followed by Southern blotting because low cycle number often makes products difficult to visualize by ethidium bromide staining.
13. The efficiency of primer pairs should be validated for your buffer system/instrument. To do this, perform qPCR on serial dilutions of bulk thymic DNA or plasmid containing the rearrangement of interest. Incorporate this efficiency value into relative quantification ($\Delta\Delta C_t$) calculations.
14. The two outermost lanes on each side will be lost in transfer, so plan gel loading accordingly.
15. Often times it is necessary to examine multiple Jα probes to obtain a complete picture of recombination. Rather than stripping and re-probing a blot multiple times, the same series of samples can be loaded two or more times on a single gel and the membrane cut after transfer. In this case, leave several empty wells between each sample, and be sure to load a ladder with each set.
16. In some cases the control PCR reaction will fail to run. In this case it is not worth proceeding, and the PCR conditions should be optimized before repeating the protocol.

17. Multiple gels may be shaken in a single container, simply place on top of each other.
18. Gels will be very brittle after denaturing. Take care to handle gently. If the gel does crack or split, it is sometimes possible to piece it together and continue with the transfer.
19. Denaturing solution may be re-used three times, after which it should be discarded.
20. After drying the membrane is stable and may be stored at room temperature indefinitely.
21. If the blot is particularly "hot," additional washes can be performed prior to exposure. Typically, if the blot is washed until the Geiger counter gives a reading of 2–3000 cpm, an overnight exposure will be sufficient. If the blot is significantly "hotter," additional washes are typically useful. Note, however, that the appropriate intensity of radiation and duration of exposure must be determined empirically for each probe.

Acknowledgements

This work was supported by NIH grants T32 HG000046 (LJR), R37 GM41052 and R01 AI49934 (LC and MSK), R01 CA125195 and R01 CA136470 (CHB), and a Leukemia and Lymphoma Society Scholar Award (CHB).

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Chapter 17

Intrathymic Injection

Sugata Manna and Avinash Bhandoola

Abstract

Intrathymic injection is used in several T cell-associated immunological studies to deliver cells or other substances directly into the thymus. Here, we describe the intrathymic injection procedure involving surgical incision of the mouse with or without a thoracotomy. Though this procedure can result in poor recovery, postsurgical complications, and distress to the animal, it is actually a simple procedure that can be carried out relatively easily and quickly with experience.

Key words Thymus, Mouse, Intrathymic injection, Thoracotomy, Surgical incision

1 Introduction

Thymus is the essential organ for differentiation and selection of immunocompetent T lymphocytes. It comprises two distinct, symmetrical pyramidal lobes, which are in close contact and connected by an isthmus of connective tissue. This specialized primary lymphoid organ is present in all jawed vertebrates, and is located anterior to the heart and posterior to the sternum in the midline of the thoracic cavity (in the anterior superior mediastinum). Thymus is the first lymphoid organ to develop and in mice, its size is maximal just before puberty followed by a gradual decrease of size and function along with age (age-associated involution) [1].

Intrathymic injection is used to study questions of tolerance induction [2–4], tumor induction [5], cell transplantation [6], T cell-specific gene therapy [7, 8], and T cell development [9–12] in mice. This process can be accomplished by several means including surgical incision of the sternum for direct visualization, accessibility, and injection into the thymus [6–16]; a blind approach following a small skin incision [17, 18] or directly into the thoracic cavity [19]; or an ultrasound-guided approach [20, 21]. As intrathymic injection involving thoracic surgery may cause pain and distress and result in postoperative complications, the latter less

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_17, © Springer Science+Business Media New York 2016

invasive techniques, not requiring a thoracotomy, have been developed. While success rates can be low by the blind injection methods, ultrasound-mediated guidance can alleviate these issues, allowing visualization of both the needle and the injected material as it enters the thymus. Nevertheless, many researchers may not have access to ultrasound equipment for the implementation of this procedure. Here, we describe the techniques involving thoracotomy as well as the blind-injection approach involving a small skin incision.

2 Materials

1. Sterile drape.
2. Sterile latex surgical gloves.
3. Sterile gauge sponge or swab.
4. 1 cc insulin syringe, with 29 1/2 G ultrafine needle.
5. Betadine.
6. Surgical board.
7. Rubber bands.
8. Eye moisture salve.
9. Forceps, scissors appropriate for incision.
10. Wound clip.
11. 10 μL syringe (Hamilton Co; Reno, NV), attached with a 27 G needle (no dead volume).
12. Anesthetics: Ketamine and xylazine (*see* **Notes 1** and **2**). Ketamine and xylazine should be diluted in 1× PBS. Ketamine/xylazine anesthetic should be administered at a dose of 0.1 mg/g mouse weight and 0.02 mg/g mouse weight for ketamine and xylazine, respectively.

3 Procedures

3.1 Intrathymic Injection with Thoracotomy

1. Anesthetize the mice with ketamine and xylazine administration at a dose/volume of 0.01 mL/g mouse weight (~200 μL for a 20 g mouse) via intraperitoneal injection (use 1 cc insulin syringe, with 29 1/2 G ultrafine needle) (*see* **Notes 3** and **4**).
2. Rinse the work area with betadine and lay down a sterile drape.
3. Place the anesthetized mouse on its back on a surgical board and immobilize it by strapping its feet with rubber bands (Fig. 1a) (*see* **Note 5**).

Fig. 1 (**a**) Anesthetized mouse placed and immobilized with rubber bands on surgical board. (**b**) Midline longitudinal incision, with mouse's rib cage cut for visualization of thymus. (**c**) Intrathymic injection, mouse chest held open with forceps and needle with bevel up, is positioned and inserted into the thymus

4. Loosely stretch an additional rubber band across the mouth to hold back the head and gently pull out the tongue with forceps so that the mouse does not asphyxiate.
5. Place the mouse or board so that the head of the animal is toward you.

6. Apply eye moisture salve to each eye to prevent the cornea from drying out.
7. Swab the chest and neck area with betadine.
8. Pinch skin at upper thoracic region with forceps and make a small longitudinal midline cut with a fine delicate scissors. Continue incision through the skin with scissors; make one continuous incision up to the xiphoid process visible as white "V" under the skin (from the maxillary to the middle of the rib cage).
9. Separate skin along fascial plane on either side of incision by gently inserting forceps jaw underneath the skin, and spread the cut skin outward on each side with forceps, creating two "flaps" to expose the sternum.
10. Carefully lift the salivary gland, lying between the larynx and sternal notch, with forceps, make a single cut in the connective tissue attaching it to the rib cage (at the end pointing away from you), and pull the gland superiorly in your direction to visualize the top of the rib cage and trachea, taking care not to tear them (*see* **Note 6**).
11. Using a clean high-quality fine forceps, very carefully pinch thin muscle lying on top of the trachea. Once pinched, do not let it go, and pull muscle inferiorly as far as possible (it should eventually tear) to reveal a small invagination at the top of the rib cage.
12. By introducing a scissors into the invagination make a vertically oriented 3-mm incision (with an upward movement) down through the sternum to bisect the upper sternum at the centerline (slightly to the right, at the level of first two ribs). Gently spread the opened ribcage sideways using the tip of blunt, curved forceps to expose/reveal the thymus, the milky white-translucent-colored organ pulsing through the opening (Fig. 1b) (*see* **Notes** 7 and **8**).
13. Fill 10 μL Hamilton syringe (with a 27 G needle) with 10 μL cell suspension and remove air bubbles.
14. Maintain the split open by pushing it to the side with forceps and with the other hand insert the needle, bevel up, into the parenchyma of the thymus, 2–3 mm under the thymic capsule. Inject 10 μL of cell suspension or solution in the lobe and withdraw the needle carefully to minimize the backflow. If needed repeat the injection for the other lobe (Fig. 1c) (*see* **Notes 9–11**).
15. Put back down the salivary gland in place, to block the rib cage opening. Release the mouse of rubber bands. Hold the two flaps of skin together with forceps and close the wound by stapling the skin gently with two to three wound clips.

16. Return mouse to the cage; check to ensure that tongue is still out. Place subsequent mice next to previous ones to conserve heat until they recover from anesthesia (*may take* up to 2 h for complete recovery) (*see* **Notes 12** and **13**).

3.2 Intrathymic Injection Without Thoracotomy

Intrathymic injection can be performed following intercostal injection, a minimally invasive technique, without thoracic cavity exposure.

1. Anesthetize and immobilize the mice as described above (**steps 1**–**7**).
2. Make a small (4–5 mm) midline incision in the skin above the sternum. Alternatively, you can make a transverse incision in the skin near the second intercostal space, perpendicular to the sternum (Fig. 2a).
3. Spread the cut skin outward with a forceps.
4. Position the needle between the third and fourth ribs, at ~30°–40° angle relative to the sternum. With needle vertex pointing toward the manubrium, inject the thymus lobe through the thoracic wall with 10 μL of cells or solution (if the needle does not pass through the thymus, the injection should be successful) (Fig. 2b).
5. The estimated injection depth is approximately 2–3 mm and both thymic lobes can be accessed through the intercostal space on either side of sternum.
6. Release the mouse of rubber bands. Hold the two flaps of skin together with forceps and close the wound by stapling the skin gently with two to three wound clips.
7. Return mouse to the cage for recovery (*see* **Note 14**).

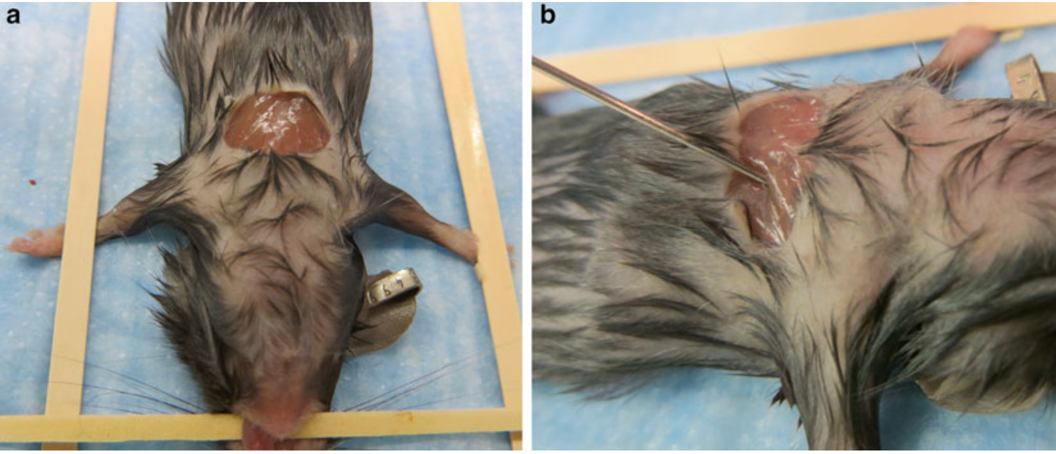

Fig. 2 (**a**) Small incision made perpendicular to the sternum and across the midline of the upper thoracic region. (**b**) Intercostal intrathymic injection, the operator inserts the needle between third and fourth ribs

4 Notes

1. Use sterile vials and aseptic techniques to prepare ketamine/xylazine cocktail for surgical anesthesia.
2. Ketamine and xylazine are controlled substances and therefore should be stored in a locked drawer, at room temperature.
3. It is easier to inject the thymus of young mice (6-week-old recipients). It can be difficult to inject aged mice due to age-related thymic involution.
4. Dosing of the anesthetics is crucial and should be evaluated relative to weight, sex, and strain of the mice. Ketamine/xylazine anesthetic should be administered at 100/20 mg/kg mouse weight, ~200 μL for a 20 gm mouse.
5. Anesthesia takes effect in few minutes and lasts for 20–30 min depending on the mouse strain used. Before starting the procedure, pinch the toe of the mice to assess the anesthetic depth. You can inject a small amount of diluted ketamine solution again if the toe reflex is still active 5 min after ketamine/xylazine anesthetic injection. Do not administer additional ketamine/xylazine anesthetic because the mice will be asleep for a long time and the risk of anesthetic death goes up.
6. If you are working with more than one mouse, inject next mouse with ketamine/xylazine anesthetic at this point.
7. At the time of thoracotomy, keep the tip of the scissors away from the heart and lungs. Bleeding may obscure the opening. If necessary soak up the blood with a clean absorbent pad.
8. Cut only one-third of the sternum. If the tip of the thymus is not visible, you may need to extend the sternum incision.
9. After administration of cell suspension or solution into the thymus, allow the needle to remain in the lobe for few seconds so that the internal pressure decreases. This gives the injected solution time to redistribute, minimizing leakage after withdrawal.
10. The injected volume should not exceed 10 μL per lobe (try to inject close to the middle of the lobe ~2–3 mm deep). Due to enormous size variation of the mouse thymus with age, it is difficult to specify the injection depth.
11. Immediately after injection, flush the syringe three to four times with sterile PBS. Take care not to bend the wire plunger.
12. To prevent hypothermia after surgery, place animals under a heating lamp while still under anesthesia.
13. The efficiency of the technique can be improved by rapid surgical incision and injection.
14. The blind injection technique can be practiced by injecting a dye (e.g., India ink) followed by removal and sectioning of the thymus to observe the site of injection.

Acknowledgments

We thank N. Taylor and V. Zimmerman for reading and critical comments on this chapter. A special thanks to P. Sarkar for photographs.

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Chapter 18

Analysis of Cell Proliferation and Homeostasis Using EdU Labeling

Francis A. Flomerfelt and Ronald E. Gress

Abstract

Determination of cellular proliferation and population turnover is an important tool for research on lymphoid cell function. Historically this has been done using radiolabeled nucleotides or nucleoside analogs, such as BrdU (5-bromo-2-deoxyuridine), that are incorporated into nascent DNA during S-phase. Recently, a new procedure was developed to label nascent DNA using EdU (5-Ethynyl-2-deoxyuridine). This new method overcomes limitations imposed by the procedure used to detect BrdU because EdU detection is based on an easily performed chemical reaction that does not require DNA denaturation, is quick and reproducible, and has a superior signal-to-noise ratio. This technique offers a wide range of opportunities to analyze cellular proliferation, population homeostasis, and cell marking procedures.

Key words EdU (5-ethynyl-2′-deoxyuridine), Click it chemistry, Proliferation, S-phase, DNA replication

1 Introduction

Measurement of the proliferative capacity and life span of defined cell types is fundamental for understanding population dynamics and homeostasis. One of the best approaches to accomplish this goal is to directly measure DNA synthesis. Early studies employed incorporation of radiolabeled thymidine followed by autoradiography and quantification in populations of cells in situ or isolated by a variety of different procedures [1]. However these procedures were laborious and it was difficult to analyze large numbers of cells. Later, methods using BrdU, a halogenated thymidine analog, were introduced to directly measure de novo DNA synthesis based on its incorporation during DNA synthesis and flow-cytometric detection using anti-BrdU antibodies were developed [2]. The great advance of this approach was that it utilized flow cytometry to simultaneously measure phenotypic markers, DNA content, and

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_18, © Springer Science+Business Media New York 2016

BrdU incorporation of a large number of individual cells. With some modifications, this overall approach has been used for the last 30 years to study proliferation dynamics and population homeostasis of defined cell subsets [1–3]. The method and timing of labeling depends on the experimental goals. For example, short term labeling identifies the proportion of cells actively replicating DNA during that period. When this approach is coupled with DNA content analysis, it provides a rich source of information about cellular proliferation. For example, one can calculate the distribution of cells actively dividing according to their distribution in the G1-, S-, and G2/M-phases of the cell cycle. The number of resting cells can also be easily calculated. In addition, information about the duration of DNA synthesis, doubling time, and cell cycle dynamics is available. Due to the fact that EdU is not reutilized, "pulse-chase" studies can be done; dividing cells are labeled for a defined period followed by sampling and analysis at various timepoints. This technique is ideal for tracing step-wise cellular differentiation and population dynamics of post mitotic labeled cells during the chase period, and has been frequently used in studies on neural development and peripheral lymphocyte differentiation, some lasting almost 3 months [4]. Another application, using continuous labeling, allows calculation of proliferation rates of defined cell populations from the rate at which they incorporate labeled nucleotide analogs. The lifetime of any phenotypically defined lymphocyte population can be determined using continuous labeling experiments. The calculation is based on determining how long it takes for 100 % of the cells in a population to become labeled due to complete replacement of that population from precursor cells [4–6]. Specific applications of these procedures have been applied in a vast array of in vivo and in vitro experimental systems in biological models ranging from *C. elegans* and drosophila to humans [7].

In spite of the widespread use of BrdU to measure DNA synthesis, this procedure includes relatively harsh fixation procedures and requires opening of the DNA using heat, acid or DNase to expose the BrdU epitope and allow access for anti-BrdU antibodies. These factors limit phenotypic analysis using antibodies directed to specific proteins due to destruction of epitopes. Additionally, inconsistency in achieving exposure of the incorporated BrdU results in variable signal-to-noise ratios. Recently a new flow-cytometric method to label and detect nascent DNA using EdU has been developed and made commercially available by Life Technologies [8]. EdU, is a nucleoside analog of thymidine that is incorporated into DNA during S-phase just like BrdU and is not reactive in biological systems [9]. The EdU detection procedure technique uses a copper (I) catalyzed click reaction chemistry to covalently couple an azide modified fluorescent dye to incorporated

EdU to form a stable triazole ring [8]. Because of the small size of the click detection reagents, no harsh DNA denaturation steps are required. As a result, EdU detection uses a simple protocol that takes less than 30 min with more reproducible results and greater signal-to-noise ratio. A variety of fluorochromes for EdU detection facilitate analysis of specific populations defined antibody based phenotypic profiling by specific proteins. It is likely that the advent of new and improved techniques using EdU to label nascent DNA will usher in a new wave of creative scientific exploration and exciting findings.

2 Materials

The components for EdU labeling and detection are included in kits purchased from Life Technologies that utilize various fluorochromes: Alexa Fluor 488, Alexa Fluor 647, or Pacific Blue.

2.1 In Vitro EdU Labeling

1. EdU (5-ethynyl-2′-deoxyuridine, Component A) (Life Technologies) (*see* **Note 1**).
2. Media appropriate for type of cell to be cultured.
3. Phosphate buffered saline (PBS).
4. DMSO.

2.2 In Vivo EdU Labeling

1. EdU (5-ethynyl-2′-deoxyuridine, Component A) (Life Technologies) (*see* **Note 1**).
2. Phosphate buffered saline (PBS).
3. Syringes with 25 G needles.
4. Sterile drinking water.

2.3 Stain Cell Surface Markers

1. FACS buffer (1× PBS, 0.5–1.0 % BSA, 0.1 % (W/V) sodium azide) (*see* **Note 2**).
2. Tubes compatible with flow cytometer to be used.
3. Fluorescently labeled antibodies.
4. Anti CD16 (2.4G2) antibody use at 5 μg/ml.

2.4 Fix and Permeabilize Cells

1. Click-iT fixative (Component D).
2. Click-iT saponin-based permeabilization and wash reagent (Component E).
3. De-ionized water.

2.5 EdU Detection

1. $CuSO_4$ (Component F).
2. Click-iT EdU buffer additive (Component G).

2.6 Optional Additional Antibody Staining for Intracellular Antigens or with RPE, PE-Tandem, or Qdot Antibody Conjugates

1. Cells that have been fixed, permeabilized, and stained for EdU with the Click-it reaction.
2. Fluorescently labeled antibodies.
3. 1× Click-iT saponin-based permeabilization and wash buffer.

2.7 Cell Cycle Stain

1. DAPI (4′,6-diamidino-2-phenylindole) solution 5 mg/ml in diH_2O.
2. FACS buffer.

2.8 Flow Cytometry Analysis

1. Flow cytometer and software to analyze data.

3 Methods

3.1 In Vitro EdU Labeling

1. Establish culture conditions where cells are proliferating.
2. Add 4 ml of DMSO or PBS to vial containing 10 mg EdU to make a 10 mM solution (*see* **Note 1**).
3. Add EdU to culture at 2–10 μM. EdU can be dissolved in DMSO or in aqueous solutions. EdU stock solution is 10 mM (*see* **Note 3**). Include cultures treated with vehicle alone for controls.
4. Culture for period to label nascent DNA (*see* **Note 4**). Harvest cells, count, and wash with PBS. Resuspend in FACS buffer at 1×10^7 cells/ml and keep on ice.

3.2 In Vivo EdU Labeling

1. Prepare EdU at 1 mg/ml in sterile phosphate buffered saline (PBS).
2. For short term or pulse labeling of adult mice, perform an intraperitoneal (IP) injection of 100–200 μl dissolved EdU (*see* **Note 5**). Harvest organs/cells at desired timepoints (*see* **Note 6**).
3. For long term labeling, perform IP injection with 100 μl of 1 mg/ml EdU in PBS. Mice are then provided with drinking water ad libitum containing 0.3 mg/ml EdU (*see* **Note 7**). Replace drinking water with freshly prepared EdU every 2–3 days (*see* **Notes 1** and **8**).
4. Harvest lymphoid organs of interest and process into single cell suspensions. Count cells and wash with PBS. Resuspend in FACS buffer at 1×10^7 cells/ml and keep on ice.
5. If cells will not be stained with antibodies, place 100 μl of the cell suspension (1×10^6 cells) into FACS tubes, add 3 ml FACS buffer, pellet cells, remove supernatant and proceed to Subheading 3.4.

3.3 Stain Cell Surface Markers

1. Prepare a chart for the staining strategy listing fluorochromes, EdU detection and DNA stain. Antibodies using PE, PE-tandem, or Qdot(R) conjugates should not be used before the EdU detection step as their signal is reduced by the Click it reaction conditions. Currently, Life Technologies offers azides with Alexa Fluor 488, Alexa Fluor 647, and Pacific Blue. Also plan for additional samples for controls including unstained cells and single colors of each fluorochrome for compensation. When analyzing rare populations, it is useful to use additional control samples containing "all stains except one" to set the gates for the population identified by the missing fluorochromes.
2. Place 100 μl of the cell suspension (1×10^6 cells) into FACS tubes. Add 0.5 μg anti CD16/CD32 antibody in tubes to be stained with antibodies to block antibody binding by the Fc receptor and incubate on ice 10 min.
3. Set up single stain controls for each antibody. Reserve tubes with cells for EdU detection reagents and DNA stains to be added later. Incubate tubes on ice 15 min, add 3 ml FACS buffer, pellet cells and remove supernatant. Proceed to Subheading 3.4.
4. While control cells are staining, make a mixture with predetermined amounts of all desired antibodies and add an aliquot to samples to be stained. This procedure facilitates dispensing the antibodies and reduces tube-to-tube variability. Incubate tubes on ice 15–30 min, add 3 ml FACS buffer, pellet cells and remove supernatant. Proceed to Subheading 3.4.

3.4 Fix and Permeabilize Cells

1. All samples and controls should be fixed and permeabilized to ensure uniform characteristics when run on the flow cytometer. Prepare needed amount of 1× Click-iT saponin-based permeabilization and wash reagent by diluting the provided 10× stock (Component E) with water (*see* **Note 9**). A total of 3.6 ml per sample is needed for the standard reaction. If intracellular staining will be done, a total of 6.7 ml per sample is needed.
2. Gently drag tubes across a rack or use other method to break up the cell pellets from Subheadings 3.2, **step 5** and 3.3, **steps 3** and **4**.
3. Add 100 μl of Click-iT fixative (Component D) to each tube and mix well. Incubate the cells for 15 min at room temperature in the dark.
4. Add 3 ml of FACS buffer, pellet cells, and remove the supernatant (*see* **Note 10**).
5. Gently break up the cell pellet again and add 100 μl of 1× Click-iT saponin-based permeabilization and wash reagent. Mix well and proceed directly to Subheading 3.5 (*see* **Note 11**).

3.5 EdU Detection

1. Allow kit components to come to room temperature before opening.
2. Prepare a working solution of fluorescent azide for EdU detection by adding 130 μl of DMSO to Component B of the kit (Alexa Fluor 488, Alexa Fluor 647, or Pacific Blue) and mix well. Any remaining working solution will be stable for a year if stored at ≤−20 °C.
3. Add 2 ml of deionized water to the vial containing the Click-iT EdU buffer additive (Component G) to make a 10× stock solution and gently mix until fully dissolved. Any remaining stock solution should be dispensed into single use aliquots and is stable for a year when stored at ≤−20 °C.
4. Prepare a master mix of the components required for the Click-it reaction. For each tube mix together 438 μl PBS or TBS, 10 μl $CuSO_4$ (Component F), 2.5 μl fluorescent azide (prepared in Subheading 3.5, **step 1**), 50 μl 10× reaction buffer additive (prepared in Subheading 3.5, **step 2**). Scale up the mixture for the number of samples to be treated and add 500 μl to each tube. It is important to use the cocktail within 15 min of preparation. It is good practice to include a control sample of cells not exposed to EdU. In addition, these cells are needed for single staining compensation controls for intracellular antigens or antigens stained with RPE, PE-tandem, or Qdot antibody conjugates.
5. Incubate the mixture for 30 min at room temperature in the dark.
6. Add 3 ml of the Click-iT permeabilization and wash buffer (prepared in Subheading 3.4, **step 1**), pellet the cells and remove the supernatant.
7. Gently dislodge the cell pellet. If intracellular antibody staining is desired, add 100 μl Click-iT permeabilization and wash buffer and proceed to Subheading 3.6. Otherwise add 500 μl of the Click-iT permeabilization and wash buffer and go to Subheading 3.7 for DNA staining or Subheading 3.8 for flow cytometry.

3.6 Optional Staining for Intracellular Antigens or with PE, PE-Tandem, or Qdot Antibody Conjugates

1. Add predetermined amounts of antibodies to the cells and mix well. Incubate on ice for 30 min protected from light.
2. Add 3 ml of the Click-iT permeabilization and wash buffer, pellet the cells, and remove the supernatant.
3. Gently dislodge the cell pellet and add 500 μl of the Click-iT permeabilization and wash buffer and go to Subheading 3.7 for DNA staining.

3.7 Cell Cycle Stain

1. Dilute 5 mg/ml DAPI stock 1:100 with diH$_2$O and add 12.5 μl to each tube (3 μM final concentration) and incubate for 15 min at room temperature (*see* **Note 12**). Ensure that a tube of fixed and permeabilized cells is stained with only DAPI for compensation control. After incubation proceed to Subheading 3.8.

3.8 Flow Cytometry Analysis

1. Use unlabeled and single color controls to set up compensation on cytometer and run samples. Use "all but one" controls to set gates if needed (*see* **Note 13**).
2. Gate on the cell population of interest. Collect the fluorescent signal from DAPI or other DNA content dyes using linear amplification; all other fluorescent signals should be collected with logarithmic amplification. When measuring cellular DNA content on most flow cytometers, use a low flow rate (<500 events per second) during data collection. Use the same collection rate for all samples in the experiment (*see* **Note 14**).
3. Cell clumping is often observed when processing samples for flow cytometry. When analyzing DNA content, it is important to distinguish between G1 doublets from a G2-M single event. To discriminate the doublet from the singlet, plot the width (*W*) versus area (*A*) for the channel used in a dot plot graph. A population of single cells will form a diagonal. *W* increases with the diameter of the clump, while the *A* of a G1 doublet and the G2/M single cell is the same. Therefore, discrimination of G1 doublets and clumped cells from G2/M single cells can be made by gating out events that deviate with greater width from the diagonal as shown in Fig. 1a [10].

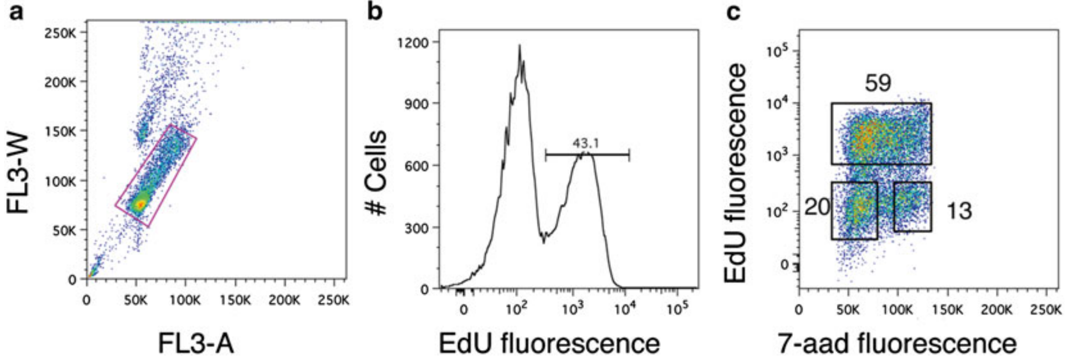

Fig. 1 Human epithelial cells were cultured with EdU for 2 h then stained with Pacific Blue azide and 7-aad. (**a**) 7-aad fluorescence was collected using the FL3 channel and plotted area (FL3-A) versus width (FL3-W) to allow discrimination between clumped doublets and single cells shown within the *rectangular gate*. (**b**) Cells were gated based on size and the percentage of EdU labeled cells is shown as a histogram. (**c**) Bivariate analysis of EdU versus 7-aad staining is shown for cells gated as in (**a**). *Numbers* shown denote percentages. Clockwise from the *top* are gates that show cells in S-phase (59 %), G2/M (13 %) and G0/G1 (20 %)

4. Using the desired phenotypic gate, the percent cells that synthesized DNA and incorporated EdU during the pulse period can be determined with a simple histogram (Fig. 1b). Plotting EdU versus DAPI provides more information about the cell cycle (Fig. 1c). In this example, human epithelial cells were pulsed with EdU for 2 h and stained with 7-aad and Pacific Blue azide. The bivariate analysis shows that 59 % of these cells synthesized DNA during the pulse and represent cells in S-phase (Fig. 1c). Twenty percent of the cells were quiescent and remained in G1 phase while 13 % did not synthesize DNA and were in G2-M phase (Fig. 1c). A population of stable quiescent cells can be identified by lack of EdU incorporation over a longer labeling period, while a population of cycling cells will become uniformly labeled.

4 Notes

1. After being dissolved, remaining EdU stock solution is stable for up to 1 year when stored at −20 °C. EdU solution should be portioned into single use aliquots to avoid freeze thawing. EdU is incorporated into DNA and is a potential mutagen. EdU has been identified as a teratogen in laboratory animals. Proper protective clothing should be used when handling EdU. Also proper procedures should be implemented to minimize contamination and dispose of waste according to institutional guidelines. Waste, including stock solutions, used media; animal cage litter, feces, urine, and water containing EdU should be considered as hazardous.
2. Fetal bovine serum can also be used at 3–10 % instead of BSA. Sodium azide is used as a preservative; its addition to the buffer is optional. Follow proper precautions when disposing of sodium azide to avoid accumulation of potentially explosive deposits in plumbing.
3. Preliminary experiments should be done to determine optimal concentration for labeling and assess potential toxicity of DMSO or EdU on the cells of interest. In general 10 μM EdU has no detectable toxicity for a variety of different cell types. If toxicity is noted, reduction of EdU concentration or shortened labeling times may be indicated.
4. Preliminary experiments should be done to optimize labeling periods. In vitro EdU labeling can be detected in as little as 3 min. In most cases a 1–4 h labeling is sufficient. In general, labeling periods used for BrdU studies can be used as a good guideline for EdU labeling period, however EdU labeling can often be detected with shorter labeling periods than BrdU. Incorporated EdU is very stable and cells can be washed, fixed and stored for months without loss of signal [8].

5. Alternatively mice can be injected with EdU twice 2–4 h apart to initiate the pulse labeling. Start timing the chase after the second injection. Newborn to 20-day-old mice can be injected with 50 μl 1 mg/ml EdU solution IP or SC.
6. Give enough time for EdU to diffuse and label proliferating cells. We have readily detected EdU incorporation in thymocytes after 60–90 min after injection.
7. EdU can also be administered subcutaneously (SC) every 3–4 h for up to 5 days if needed. Successful long term EdU labeling (in brain) has been achieved by injecting EdU every 3–4 h during a 12 h daytime period followed by a 12-h overnight period with no injections for a 5-day period [11]. Sequential injections are indicated for mice under 20 days old since they have not been weaned and do not drink much water. However the extra handling and disturbance may affect the experimental outcome so additional mice injected with vehicle alone on the same injection schedule should be included as controls. Long labeling periods may be toxic to some populations. Signs of distress, ruffled hair, lethargy and decreased thymus size compared to controls [12] suggest possible toxic effects. Labeling periods up to 5 weeks have been done with BrdU to study lymphocyte turnover. We have not observed toxicity with EdU labeling periods up to 7 days.
8. Unlike BrdU, EdU is not light sensitive so water bottles do not need to be covered in foil.
9. The Click-iT permeabilization reagent maintains the morphological light scatter characteristics of leukocytes. It can be used with whole blood or cell suspensions containing red blood cells and will lyse red blood cells. The diluted 1× solution is stable for 6 months when stored at 2–6 °C while the 10× stock is stable for a year stored at −20 °C. In some cases, it may be more convenient to make up 500 ml 1× buffer. Note that this buffer contains sodium azide.
10. If red blood cell debris or hemoglobin is present in the sample, repeat the wash step before proceeding.
11. Cells can be held at this stage for up to 30 min if needed.
12. DAPI is a potential mutagen so use proper precautions when handling and disposing. DAPI stain may not compatible with Pacific Blue depending on whether a violet laser is used. Other DNA dyes include propidium iodide (FL2 channel) or 7-aad (7-Aminoactinomycin D) (FL3 channel). Life Technologies also sells several DNA dyes. RNase treatment is required if the dye binds RNA to ensure accurate DNA content profile and facilitate analysis.

13. Use this chart to determine the correct parameters to detect EdU with the different azides available from Life Technologies.

Azide	Excitation (mn)	Emission filter (or similar)
Alexa Fluor 488	488	Green (530/30 nm)
Alexa Fluor 647	633/635	Red (660/20 nm)
Pacific Blue	405	Violet (450/50 nm)

14. If additional dilution of cells is needed, ensure that wash buffer containing 3 μM DAPI is used to prevent reduction of fluorescent signal.

Acknowledgement

The Intramural Research Program of the National Cancer Institute at the National Institutes of Health supports the authors. The authors thank Kevin Chua for comments on the paper. The authors have no conflicts of interest to disclose.

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Chapter 19

Characterization and Isolation of Human T Cell Progenitors

Inge Van de Walle, Karin Davids, and Tom Taghon

Abstract

During their development, human T cells undergo similar genomic changes and pass through the same developmental checkpoints as developing thymocytes in the mouse. The difference between both species, however, is that some of these developmental stages are characterized by different phenotypic markers and as a result, evidence emerges that the molecular regulation of human T cell development subtly differs from the mouse [1–4]. In this chapter, we describe in detail how the different stages of human T cell development can be characterized and isolated using specific surface markers.

Key words Human, T cell development, Thymocytes, Isolation of T cell progenitors, Fluorescent activated cell sorting, Magnetic activated cell sorting

1 Introduction

In humans, the hematopoietic progenitor cells that seed the thymus are still poorly defined and therefore remain controversial. The group of Canque identified a $CD34^{hi}CD45RA^{hi}CD7^{low}$ pro-thymocyte population in fetal bone marrow, cord blood, and thymus that display preferential T- and NK-cell lineage potential compared to their $CD7^{-}$ counterpart. Given their selective potential to colonize the thymus in a chimeric in vitro setting, they propose these cells to be the direct precursors of uncommitted $CD34^{+}CD1^{-}$ thymocytes (Fig. 1) [2]. In contrast, the group of Crooks identified, besides this $CD34^{+}CD10^{+}CD1^{-}CD7^{low}$ uncommitted pro-thymocyte subset, also a $CD7^{-}$ population with clear myeloid, lymphoid and possibly also erythroid differentiation potential, suggesting that this is a more primitive subset of progenitors that colonizes the human thymus [3, 5]. While these $CD7^{-}$ precursors may not be as efficient to colonize the thymus, it still remains possible that the $CD7^{-}$ and $CD7^{low}$ subsets both contribute to human T cell development since it is anticipated that very few cells effectively seed the thymus. Within the $CD34^{+}CD1^{-}$ uncommitted thymocyte population, a $CD7^{high}$ subset can be

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_19, © Springer Science+Business Media New York 2016

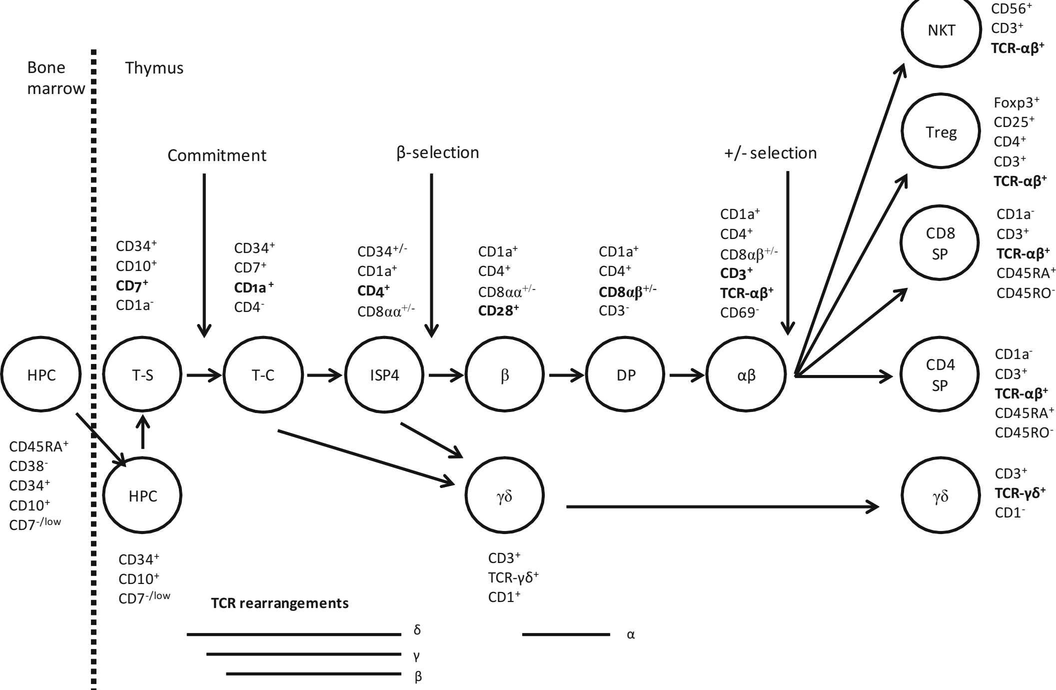

Fig. 1 Schematic overview of the different developmental stages that characterize human T cell development. *HSC* hematopoietic stem cell, *T-S* T-lineage specified progenitor, *T-C* T cell committed progenitor, *ISP4* CD4 immature single positive, *β* rearranged T cell receptor-β chain, *DP* double positive, *αβ* T cell receptor-αβ positive cell, *NKT* natural killer T cell, *Treg* regulatory T cell, *CD8 SP* CD8 single positive, *CD4 SP* CD4 single positive, *γδ* T cell receptor-γδ positive cell, *CD* cluster of differentiation

identified that contains more differentiated T-lineage specified progenitor cells [3]. In each case, it is important to note that the markers CD44 and CD25, used to characterize immature so-called double negative thymocytes in the mouse, are not used to discriminate different development stages of early developing thymocytes in human. While it is anticipated that the most immature human thymocytes express c-kit, analogous to murine ETPs, this marker has not been used thus far to isolate human ETPs, which may be due to the lack of appropriate antibodies.

Irreversible commitment to the T cell pathway is completed when the cells upregulate the human immature T cell marker CD1a [6]. During these specification and commitment processes, T cell receptor (TCR) rearrangements at the TCR-δ, TCR-γ, and TCR-β loci are initiated, in that respective order [7], and peak during the next developmental stage, which is characterized by the gradual loss of the progenitor marker CD34 and the induction of CD4 expression. This is an important difference between mouse and human since these so-called immature single positive thymocytes, characterized as $CD34^{-/+}CD4^{+}CD3^{-}CD28^{-}$, have not

yet passed the β-selection checkpoint, in contrast to mouse ISP thymocytes that are the immediate precursors of DP thymocytes and posses a functional rearranged TCR-β chain. In human, even some $CD4^+CD8\alpha^+$ thymocytes have not passed the β-selection checkpoint, illustrating the importance of using appropriate surface markers to correctly identify the discrete stages of human T cell development [4].

The result of the various recombination processes directly influences the developmental lineage outcome of the T cell precursors. In frame rearrangements of TCR-γ and TCR-δ chains mainly result in the generation of $TCR\gamma\delta^+CD3^+$ T cells that further mature, while a functional TCR-β chain will pair with the surrogate pre-T-cell receptor α chain (pTα) that allows pre-TCR signaling to induce β-selection [4, 8, 9]. Passage through this developmental checkpoint results in the upregulation of CD28 [10], induces strong proliferation as also illustrated by the upregulation of CD71 and finally results in the rapid differentiation into $CD4^+CD8\alpha\beta^+$ double positive thymocytes that will initiate TCR-α chain rearrangements, illustrating the importance of CD8β as a marker of true β-selected DP thymocytes [9].

In frame TCR-α chain rearrangements result in the expression of a full TCRαβ-CD3 complex in these DP progenitors that subsequently mature into naïve $CD1^-CD45RA^+CD4^+$ T helper cells or $CD8^+$ cytotoxic T cells through a process of ligand-dependent positive and negative selection. We refer to Plum et al. [11] for details on these processes. In addition to these subsets, also NKT and regulatory T cells develop intrathymically from DP thymocytes, but these lineages are not discussed further in this chapter. An overview of the human T cell development is presented in Fig. 1.

2 Materials

2.1 Extraction of Human Thymocytes

1. 140 mm petri dish.
2. 10 ml syringe.
3. 50 ml conical tube.
4. 40 μM cell strainer.
5. Iscove's Modified Dulbecco's Medium (IMDM).
6. Penicillin/streptomycin (Pen/Strep).
7. L-glutamine.
8. Fetal bovine serum (FBS, specific serum testing is needed, *see* **Note** 1); heat-inactivated at 56 °C for 1 h.
9. Dimethyl sulfoxide (DMSO).
10. Turk's solution: 10.0 g/l CH_3COOH.
11. Hemocytometer counting chamber.

2.2 Lymphoprep Density Gradient for Isolation of Viable Mononuclear Cells

1. Phosphate buffered saline (PBS).
2. Dulbecco's phosphate buffered saline (DPBS).
3. Lymphoprep.
4. MACS buffer (*see* Subheading 2.3).
5. Turk's solution (*see* Subheading 2.1).
6. Hemocytometer counting chamber.

2.3 MACS Direct CD34 Progenitor Cell Isolation Kit

1. MACS buffer: 2 % FBS (heat-inactivated at 56 °C for 1 h), 2 mM EDTA in PBS. Degas buffer before use (prepared 1 day in advance) as air bubbles could block the column. Store at 2–8 °C up to 1 month.
2. Human wash buffer for analytical staining of human hematopoietic cells (*see* **Note 2**):PBS containing 1 % BSA and 0.1 % NaN_3. Correct pH to 7.2–7.3 and adjust osmolarity to 275–285 mOsm. Filter through 0.22 μM filter. Store buffer at 2–8 °C.
3. 15 ml polypropylene conical tube.
4. Hemocytometer counting chamber.
5. Trypan blue 0.4 %.
6. MACS Direct CD34 Progenitor Cell Isolation Kit, human, Miltenyi, containing FcR blocking reagent and CD34 microbeads (Miltenyi; Bergisch Gladbach, Germany).
7. Magnetic cell separator and LS separation column (Miltenyi; Bergisch Gladbach, Germany).
8. 30 μm pre-separation filter.

2.4 Flow-Cytometric Cell Sorting

1. MACS buffer (*see* Subheading 2.3).
2. 5 ml polypropylene round-bottom tubes.
3. Iscove's Modified Dulbecco's Medium (IMDM).
4. Penicillin/streptomycin (Pen/Strep).
5. L-glutamine.
6. Fetal bovine serum (FBS); heat-inactivated at 56 °C for 1 h.
7. Monoclonal antibodies suitable for flow-cytometric analysis (*see* Table 1 for an overview of monoclonal antibodies used in the described protocols). These can be obtained from different suppliers.
8. Hemocytometer counting chamber.
9. Trypan blue 0.4 %.

2.5 $CD3^+$ and $CD8^+$ Depletion with Dynabeads

1. Mouse anti-human monoclonal antibodies (MAbs): glycophorin-A, CD8 unlabeled, CD3 unlabeled.
2. Sheep anti-mouse immunoglobulin-coated Dynabeads (Invitrogen by Life Technologies; AS, Norway).

Table 1
Overview of monoclonal antibodies to sort different T cell subsets

Start population of interest	Mouse anti-human monoclonal antibodies
Extrathymic progenitors from bone marrow or cord blood	CD34, CD3, CD14, CD56, CD19, CD38
Double positive thymocytes	CD4, CD8, CD3
$CD34^+$ thymocytes	CD34, CD4, CD1
$CD4^+$ ISP thymocytes	CD4, CD34, CD8, CD3, CD28
TCR-γδ T cells from thymus	TCR-γδ, CD3, CD1

3. DynaMag™ Magnet (Invitrogen by Life Technologies; AS, Norway).
4. Dulbecco's Phosphate Buffered Saline (DPBS), 2 % FBS.
5. Fetal Bovine Serum (FBS); heat-inactivated at 56 °C for 1 h.
6. 15 ml polypropylene conical tube.
7. Hemocytometer counting chamber.
8. Trypan blue 0.4 %.

2.6 Anti TCR-γδ Microbead Kit

1. MACS buffer (*see* Subheading 2.3).
2. Anti TCR-γδ microbead kit, human, Miltenyi, containing anti-TCR γ/δ Hapten-Antibody (*see* **Note 3**) and Anti-Hapten MicroBeads-FITC (Miltenyi; Bergisch Gladbach, Germany).
3. Magnetic cell separator and LS separation column (Miltenyi; Bergisch Gladbach, Germany).
4. 30 μm pre-separation filter.
5. Hemocytometer counting chamber.
6. Trypan blue 0.4 %.
7. Alternative method can be performed as well (*see* **Note 4**).

3 Methods

3.1 Human Thymocyte Cell Suspension

1. Transfer thymus tissue, obtained from cardiac surgeries (*see* **Note 5**), to a 140 mm petri dish with 25 ml IMDM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and 10 % heat-inactivated FBS. Mince thymus tissue into 4–5 mm pieces with a sterile scalpel.
2. Gently smash thymus fragments with the end of a plunger from a 10 ml syringe to release the thymocytes. The medium will become turbid.

3. Transfer cell suspension to a 50 ml polypropylene conical tube with a 40 μm cell strainer to remove dispersed cells from tissue fragments.
4. Rinse petri dish with 25 ml IMDM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 10 % heat-inactivated FBS and repeat **steps 2** and **3** until all tissue is processed.
5. Determine cell number with Turk's solution. Thymus suspension can be used immediately for further purification, stored overnight at 4 °C (at a concentration of 30–50 × 10^6 cells/ml) or frozen for later use (*see* **Note 6** and Chapter 20 for details). During overnight incubation at 4 °C and after freezing and thawing of thymus suspension, $CD4^+CD8^+$ double positive thymocytes (>80 % of thymocytes) predominantly die, resulting in an enrichment of more immature thymocytes that makes their purification easier and cheaper since less reagents are required. It is strongly recommended to remove the dead thymocytes using a Lymphoprep density gradient (*see* **Note 7**) prior to further purifications as they will interfere with this process (*see* Subheading 3.2).

3.2 Purification of Viable Cells by Lymphoprep Density Gradient

1. Dilute cord blood 1:3 with PBS. Dilute bone marrow 1:5 with PBS (*see* **Note 8**).
2. Transfer maximum 30 ml of diluted cord blood or thymus suspension in a 50 ml polypropylene conical tube. Gently pipet 15 ml Lymphoprep™ below diluted cord blood or thymus suspension. For bone marrow, 15 ml Lymphoprep™ is transferred in a 50 ml polypropylene conical tube. Gently pipet 30 ml of diluted bone marrow on top of the Lymphoprep™.
3. Centrifuge at 940 × *g* for 20 min at room temperature (acceleration and brake at 30 % of maximum).
4. Aspirate the interface (Fig. 7), containing mononuclear cells from cord blood sample, bone marrow (*see* **Note 9**) or thymus suspension, using a Pasteur pipette (*see* **Note 10**).
5. Pool all interface samples from cord blood, bone marrow or thymus suspension in a 50 ml polypropylene conical tube and wash twice in 50 ml cold PBS for cord blood and bone marrow, or cold DPBS for thymus suspension (centrifuge at 400 × *g* for 10 min at 4 °C).
6. Resuspend cell pellet in 50 ml MACS buffer and determine cell number using Turk's solution.
7. Centrifuge at 400 × *g* for 10 min at 4 °C and remove supernatant.
8. The mononuclear cell fraction is now ready for further purification of specific subsets. Optional: cells can be frozen and stored in liquid nitrogen at this stage for later use (*see* **Note 6**).

3.3 Isolation of Extrathymic CD34 Positive Cells Using Magnetic Activated Cell Sorting (MACS)

Extrathymic CD34 positive progenitor cells can be isolated from the mononuclear fraction obtained after Lymphoprep density gradient of bone marrow or cord blood.

1. Determine cell number (*see* **Note 11**), centrifuge cell suspension at $500\times g$ for 10 min, aspirate supernatant completely.
2. Resuspend cell pellet in 300 μl MACS buffer for up to 10^8 total cells, add 100 μl of FcR blocking Reagent, add 100 μl of CD34 MicroBeads (*see* **Note 12**), mix well and incubate for 30 min in the refrigerator (2–8 °C, *see* **Note 13**).
3. Wash labeled cells twice by adding 15 ml MACS buffer and centrifuge at $500\times g$ for 10 min, aspirate supernatant.
4. Resuspend cell pellet in 3 ml MACS buffer and proceed to magnetic separation.
5. Place the LS separation column in the magnetic field of the MACS Separator, prepare LS column by rinsing three times with 3 ml MACS buffer.
6. Apply cell suspension onto the column (*see* **Note 14**), discard flow-through containing unlabeled cells. Wash the column three times with 3 ml MACS buffer; perform washing steps by adding buffer aliquots only when the column reservoir is empty.
7. Remove the column from the separator and place it in a 15 ml polypropylene conical tube. Pipette 3 ml MACS buffer onto the column and immediately flush out the magnetically labeled cells by firmly pushing the supplied plunger into the column.

   Optional: Cells can be passed on a second LS column if a higher purity is desired without the need for cell sorting as described below. Repeat **steps 4**–**7**.
8. Count the cells and check $CD34^+$ purity on 10,000 cells by adding a fluorescent labeled anti-human CD34 monoclonal antibody.
9. Incubate for 30 min at 2–8 °C.
10. Wash cells by adding 2 ml of human wash buffer, centrifuge at $500\times g$ for 6 min, discard supernatant by decanting 5 ml tube.
11. Run sample on a flow cytometer (Fig. 2).
12. Purity of $CD34^+$ cells obtained from cord blood is between 60 and 80 %.

   Optional: the $CD34^+$ enriched cell fraction can be frozen and stored in liquid nitrogen (*see* **Note 6**).

3.4 Purification of $CD34^+$ Precursors by Fluorescence-Activated Cell Sorting (FACS)

Enriched $CD34^+$ cells, either freshly isolated or frozen (*see* Chapter 20 for thawing cells), are preferentially further purified to >99 % purity by cell sorting to avoid outgrowth of contaminating mature T cells.

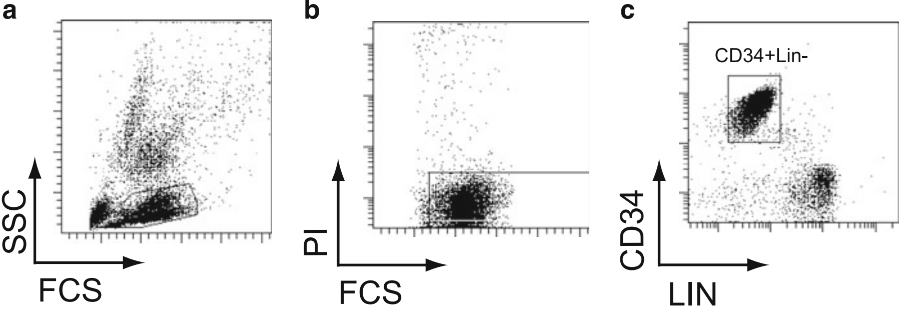

Fig. 2 Gating strategy to sort extrathymic CD34⁺Lin⁻ progenitor cells derived from cord blood. Within the lymphocyte gate (**a**), a viable (**b**) population of CD34⁺ lin⁻ progenitor cells can be gated (**c**). These cells are negative for the monocyte marker CD14, the B cell marker CD19, the NK cell marker CD56 and the T cell marker CD3

1. Resuspend the known number of cells in 100 μl MACS buffer and stain with appropriate antibodies for 30–45 min at 2–8 °C. The antibodies that are required depend on the particular scientific question. For most experiments, it is sufficient to start with $CD34^+$ cells that are negative for lineage markers ($Lin^- = CD3^-CD56^-CD14^-CD19^-$). However, one can also enrich for more primitive precursors by adding CD38 to the panel and sort $CD34^+CD38^{-/low}Lin^-$ cells.
2. Following incubation with monoclonal antibodies, wash the labeled cells once in 10 ml MACS buffer at 500 × *g* for 6 min and resuspend the cells in an appropriate volume MACS buffer (*see* **Note 15**) for cell sorting.
3. Sorted fractions are collected in 5 ml polypropylene round-bottom tubes filled with 1.5 ml IMDM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 50 % heat-inactivated FBS.
4. Transfer the collected cells to a 15 ml polypropylene conical tube, dilute the volume to 10 ml with MACS buffer and centrifuge at 500 × *g* for 10 min.
5. Resuspend the cells in 1 ml IMDM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 10 % heat-inactivated FBS
6. Count the cells (*see* **Note 16**) and take out 10–20 μl (*see* **Note 17**) to check the purity of the sorted population. If the purity is >99 %, cells can be used for downstream T cell development assays (Chapter 20). If the purity is insufficient, cells can be resorted if the remaining cell number is high enough.

Optional: the sorted cell fraction can be frozen and stored in liquid nitrogen (*see* **Note 6**).

In the following sections, we describe the most effective procedures to isolate specific human thymocytes subsets.

3.5 Isolation of Intrathymic CD34 Positive Cells Using MACS

1. Thymus suspension is used as starting material, either fresh, following overnight incubation or after freezing–thawing (*see* Subheading 3.1).
2. For purification of the most immature $CD34^+$ thymocytes, MACS $CD34^+$ microbeads are used, similar as for extrathymic cord blood or bone marrow precursors (*see* Subheading 3.3, **steps 1**–7) (*see* **Note 18**).
3. Purity of $CD34^+$ purified thymocytes should be around 95 % following one column and 99 % if cells are sequentially passed onto two columns.

Optional: the $CD34^+$ enriched cell fraction can be frozen and stored in liquid nitrogen (*see* **Note 6**).

4. Enriched $CD34^+$ cells, either freshly isolated or frozen (*see* Chapter 20 for thawing cells), can be further purified into uncommitted $CD34^+CD4^-CD1a^-$ thymocytes and the earliest committed $CD34^+CD4^-CD1a^+$ thymocytes, depending on the purpose of the experiment.
5. Resuspend the known number of cells in 100 μl MACS buffer and stain with anti-human CD34, CD1 and CD4 antibodies for 30–45 min at 2–8 °C.
6. Follow **steps 2–6** from Subheading 3.4.
7. *See* Fig. 3 for representative pre-sort FACS plots.

3.6 Isolation of Immature Single Positive Cells ($CD4^+CD8^-CD3^-$)

1. Thymus suspension is used as starting material, either fresh, following overnight incubation or after freezing–thawing (*see* Subheading 3.1).
2. Determine the desired cell number (*see* **Note 19**), centrifuge these cells at $500\times g$ for 6 min and aspirate the supernatant. Resuspend 1×10^7 to 4×10^8 cells in 200 μl MACS buffer.

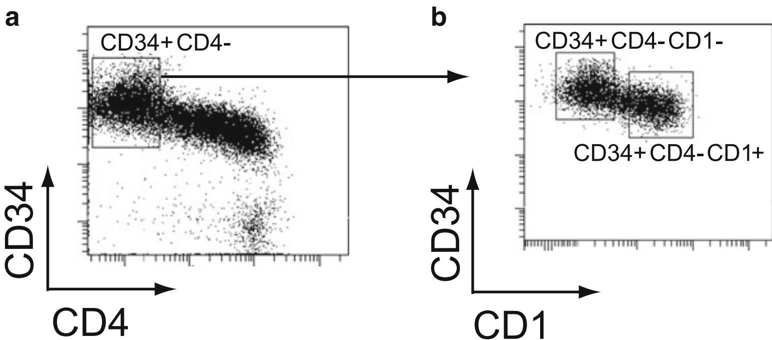

Fig. 3 Gating strategy to sort $CD34^+$ thymocytes. CD4 expression is used to discriminate between the $CD34^+CD4^-$ cells and the more differentiated $CD34^+CD4^+$ that immediately precedes the immature single positive cells (**a**). In the $CD34^+CD4^-$ population two populations can be discriminated based on the expression of CD1 (**b**). Upregulation of CD1 correlates with commitment to the T-cell lineage in postnatal human thymocytes

3. Label the cells first with an unlabeled anti-glycophorin-A monoclonal antibody, and subsequently with unlabeled anti-CD8 and -CD3 monoclonal antibodies for depletion of residual red blood cells and $CD8^+$ and $CD3^+$ cells (*see* **Note 20**).
4. Incubate for 30 min at 2–8 °C.
5. In the meantime, resuspend sheep anti-mouse immunoglobulin coated Dynabeads in the vial by vortexing 30 s, or tilt and rotate for 5 min. Ensure that all magnetic beads are resuspended.
6. Transfer the desired amount of Dynabeads to 5 ml tubes (max 1.25 ml/tube) and dilute the beads to 4 ml in DPBS 2 % FBS and resuspend. Use four beads for each cell that is being labeled, so the starting volume would be 1 ml of a 4×10^8 beads suspension for 100×10^6 cells.
7. Place the tubes in the DynaMag™ Magnet for 2–5 min.
8. Aspirate the supernatant.
9. Take the tubes out of the magnetic field to resuspend the beads in 4 ml DPBS 2 % FBS and place back in the DynaMag™ Magnet for 2–5 min.
10. Aspirate the supernatant.
11. Repeat washing **steps 8** and **9** two more times.
12. Pool all beads and resuspend in the original starting volume (*see* **step 6**) using DPBS 2 % FBS.
13. After 30 min incubation, wash the antibody labeled cells from **step 3** twice in 10 ml DPBS 2 % FBS and centrifuge at $500 \times g$, 4 °C for 6 min.
14. Resuspend the labeled cells in DPBS 2 % FBS at a final concentration of 150×10^6 cells/ml and add 2:3 volume of the prewashed Dynabeads in a round bottom 15 ml conical tube.
15. Incubate on ice for 30 min, while gently shaking every 5 min to keep the beads in suspension.
16. Dilute the cell suspension 4.5 times with DPBS 2 % FBS and distribute the suspension in 5 ml tubes (3 ml/tube).
17. Place the tube(s) in the DynaMag™ Magnet for 5 min.
18. Aspirate the supernatant from the tubes and pool if multiple tubes are used.
19. Centrifuge at $500 \times g$ at 4 °C for 6 min.
20. Resuspend the cells in DPBS 2 % FBS in a volume equal to the volume of the remaining beads and add the remaining Dynabeads (**step 14**) in a round bottom 15 ml conical tube.
21. Repeat **steps 15** and **16**.
22. Use 5 μl of the supernatant in a hemocytometer to verify that all beads are drawn to the magnet. If magnetic beads are still

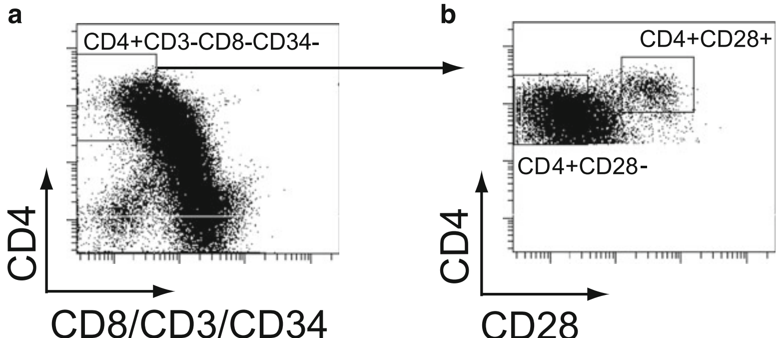

Fig. 4 Gating strategy to sort CD4 immature single positive thymocytes (4ISP) after CD3 and CD8 depletion of total thymocytes. Two populations can be discriminated in the $CD4^{+}CD3^{-}CD8^{-}CD34^{-}$ cell fraction based on the expression of CD28. The differential expression of CD28 discriminates between pre- and post ($CD28^{+}$) β-selected cells

present, elongate the incubation time in the DynaMag™ Magnet for another 5 min. If no beads are present, aspirate the unlabeled cells from the tube, count the cells and centrifuge at 500 × *g* at 4 °C for 6 min.

23. The resulting CD3 and CD8 depleted thymocyte fraction can subsequently be further purified into $CD4^{+}CD3^{-}CD8^{-}CD28^{-}$ pre- and $CD4^{+}CD3^{-}CD8^{-}CD28^{+}$ post-β selection subsets using a cell sorter.
24. Resuspend the known number of cells in 100 μl MACS buffer and stain with anti-human CD4, CD3, CD34, CD8, and CD28 antibodies for 30–45 min at 2–8 °C.
25. Follow **steps 2–6** from Subheading 3.4.
26. *See* Fig. 4 for representative FACS plots.

3.7 Isolation of Double Positive (DP) and Single Positive (SP) Thymocytes

1. Due to their limited life span, $CD4^{+}CD8^{+}$ DP cells need to be isolated directly from freshly prepared thymus suspension. Both $CD3^{-}$ and $CD3^{+}$ thymocytes can be isolated. Using the same labeling, mature SP $CD4^{+}CD3^{+}$ and $CD8^{+}CD3^{+}$ thymocytes can be sorted at the same time, if the available cell sorter has the capacity to sort 4 populations simultaneously.
2. Put the desired cell number (*see* **Note 21**) into a 15 ml polypropylene conical tube, centrifuge at 500 × *g* for 6 min.
3. Resuspend in 100 μl MACS buffer and stain cells for CD4, CD8, and CD3 for 30–45 min at 2–8 °C. Use the desired antibodies in appropriate concentrations as recommended by the manufacturers (*see* **Note 20**).
4. Follow **steps 14–18** from Subheading 3.4 but pay attention to **Note 22**.
5. *See* Fig. 5 for representative FACS plots.

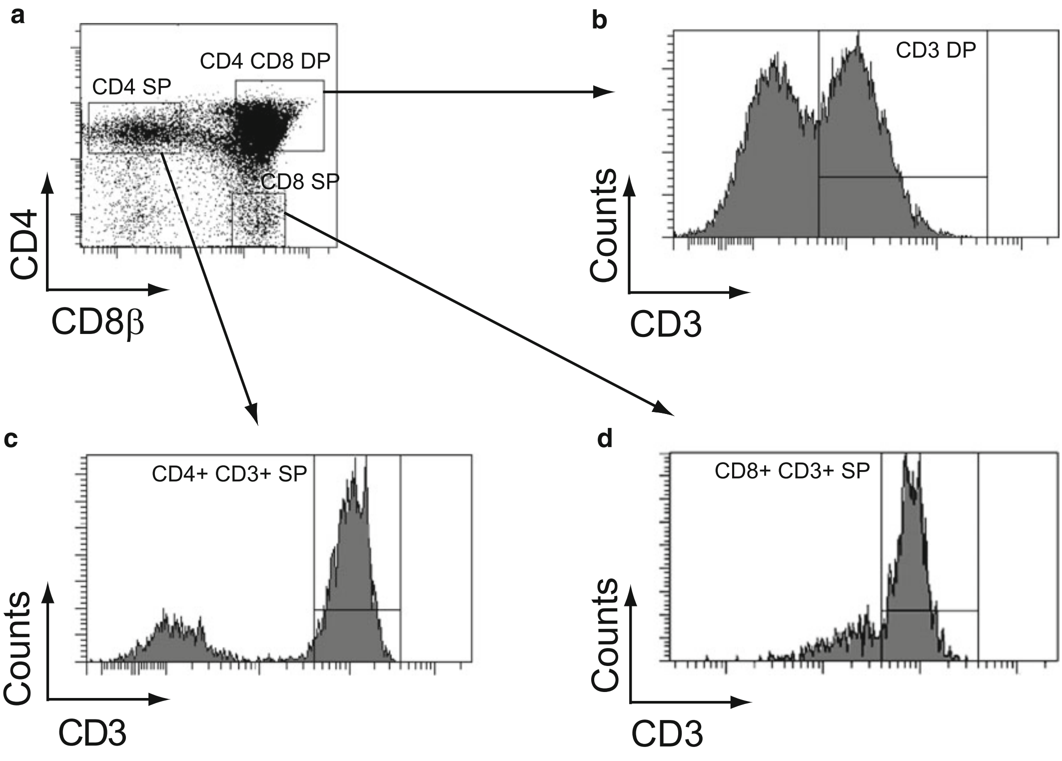

Fig. 5 Gating strategy to sort DP and SP thymocytes. $CD4^+CD8^+$ double positive cells as shown in (**a**) can be separated in $CD3^-$ and $CD3^+$ subsets as shown in panel (**b**). Single positive cells $CD4^+CD8^-$ and $CD8^+CD4^-$ thymocytes (**a**) express high levels of CD3 (**c**, **d**) that can be incorporated as a marker to sort for CD4 and CD8 SP cells

3.8 Isolation of TCR γδ Cells

1. Freshly prepared thymocytes (*see* Subheading 3.1) are used as starting material.
2. Determine the cell number (*see* **Note 23**), centrifuge the cells at 500 × *g* for 6 min and aspirate the supernatant.
3. Resuspend the cell pellet in 20 μl MACS buffer per 10^7 total cells (*see* **Note 24**).
4. Label thymocytes with anti-TCR γδ haptenated (*see* **Note 3**) antibodies (10 μl per 10^7 cells). The amount of antibody added is based on 1:4 of the total cell number (*see* **Note 25**).
5. Mix well and incubate for 10 min at 4–8 °C.
6. Add 15 μl MACS buffer per 10^7 total cells and add MACS Anti-Hapten MicroBeads-FITC (20 μl per 10^7 cells) based on 1:4 of the total cell number.
7. Mix well and incubate for 15 min at 4–8 °C.
8. Proceed with MACS protocol (*see* Subheading 3.3, **steps 3–7**).

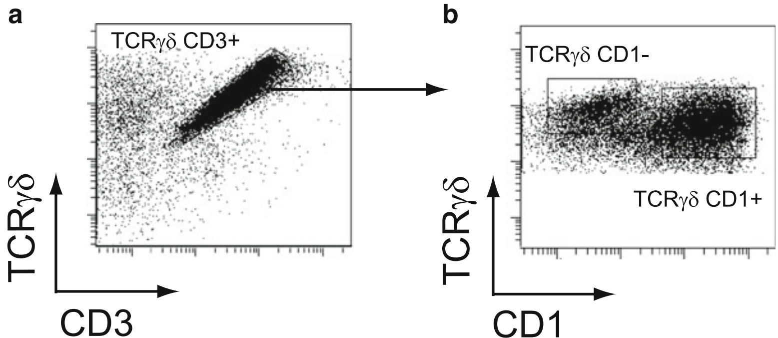

Fig. 6 Gating strategy to sort immature and mature TCR-γδ thymocytes. Two populations can be obtained in the TCR-γδ population based on the expression of CD1. Downregulation of CD1 correlates with a more mature phenotype

9. Count the cells and centrifuge at 500 × *g* at 4 °C for 6 min.
10. To purify and separate immature and mature TCR-γδ positive, stain the MACS enriched cells with CD3 and CD1 monoclonal antibodies for 30–45 min at 2–8 °C (*see* **Note 26**).
11. Follow **steps 2–6** from Subheading 3.4.
12. *See* Fig. 6 for representative FACS plots to sort immature $TCR\gamma\delta^{+}CD3^{+}CD1a^{+}$ and mature $TCR\gamma\delta^{+}CD3^{+}CD1a^{-}$ TCRγδ T cells.

4 Notes

1. Technical disadvantages to using serum include the undefined nature of serum, batch-to-batch variability in composition, and the risk of contamination. Therefore, it is recommended to perform serum testing. To test for T cell development, we culture fetal thymic lobes (fetal day 14) of B6 mice in fetal thymus organ culture for 14 days and check for efficiency to support T cell development by cell counting and flow-cytometric analysis of T cell differentiation.
2. This buffer is not recommended for staining cells that will be cultured due to the presence of NaN_3.
3. The anti-TCRγ/δ MicroBead Kit consists of a hapten-conjugated anti-TCRγ/δ antibody and FITC-conjugated anti-hapten MicroBeads.
4. An example of an alternative method, instead of anti-TCRγ/δ MicroBead kit, is to first stain thymocytes with a TCRγ/δ-PE conjugated antibody, followed by magnetically labeling of the cells with anti-PE Microbeads. Subsequently, cell suspension is

loaded on MACS Column that is placed in the magnetic field of a MACS separator. The magnetically labeled cells are retained in the column.

5. Thymus tissue is obtained from children undergoing cardiac surgery, with ages ranging from a couple of days old to mostly 3–4 years. We do not recommend to use the thymus from children that are only a couple of days old since we experience that fewer thymocytes are obtained compared to older children and the thymocytes are less viable. The reason for this difference is unclear. Please be aware that you must request and obtain permission of the Ethical Commission of your research Institute to obtain and use human cells and tissues.
6. Human hematopoietic progenitor cells can easily be frozen and stored in liquid nitrogen for a prolonged period until needed to start a culture experiment. However, it is highly recommended to freeze–thaw hematopoietic progenitor cells and especially thymocytes only once. Repeated freezing–thawing will significantly affect cell survival. Details on freezing are provided in Chapter 20. Freezing or overnight incubation at 4 °C is not recommended for RNA analysis.
7. As an alternative to remove dead cells, the Dead Cell Removal kit from Miltenyi Biotec can also be used.
8. After dilution, the bone marrow is filtered with a cell strainer to remove aggregates.
9. Following centrifugation of bone marrow, a fat layer is formed on top of the mononuclear cells. Aspirate this fat layer before aspiration of the mononuclear cells.
10. Lymphoprep™ is used for the isolation of mononuclear cells from cord blood and bone morrow, or for the isolation of viable thymocytes after overnight incubation or after thawing of thymus suspension. Lymphoprep™ is a density gradient medium (1.077 g/ml). Granulocytes and erythrocytes from cord blood/bone marrow samples have a higher density than mononuclear cells and therefore sediment through the Lymphoprep™ layer, while mononuclear cells are retained at the interface (Fig. 7). For thymus suspension, dead cells sediment through the Lymphoprep™ layer and viable mononuclear cells are retained at the interface due to their lower density.
11. The cell number is dependent on the amount of cells needed for further analysis or experiments. Bone marrow mononuclear cells contain around 5 % $CD34^+$ cells of which up to half are $CD34^+CD19^+$. Cord blood mononuclear cells contain 1–3 % $CD34^+$ cells.
12. When working with fewer than 10^8 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly.

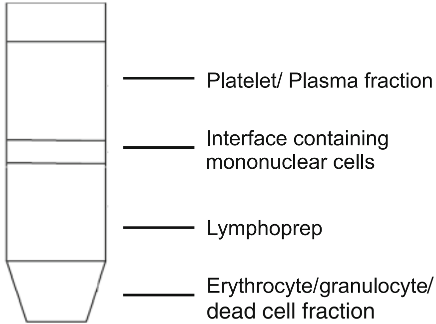

Fig. 7 Lymphoprep density gradient. The different fractions are shown following centrifugation

13. It is not recommended to incubate the cells with microbeads on ice since this results in a lower temperature that interferes with the binding efficiency.
14. For optimal performance, it is important to obtain a single cell suspension before applying the cells onto the column. If needed, pass the cell suspension through a 30 μm nylon mesh to remove cell clumps which may clog the column. Moisten filter with MACS buffer before use.
15. MACS buffer volume depends on the amount of cells present before the sort. As a guideline, use at least 200 μl of MACS buffer as lower volumes will lead to loss of a substantial fraction of the cells during setup of the sort gates.
16. Be sure to count the cells following sorting as the numbers shown by the cell sorter are never accurate and depend on the sorting efficiency which may differ from sort to sort.
17. This volume depends on the amount of cells that were effectively sorted. A minimum of 10^3 cells is desired to have a representative image of the purity of the sort.
18. The cell number is dependent on the amount of cells needed for further analysis or experiments. Only 1 % or less of total thymocytes are $CD34^+$ cells. This frequency increases following overnight incubation at 4 °C or freezing and thawing, followed by Lymphoprep density gradient.
19. The cell number is dependent on the amount of cells needed for further analysis or experiments. $CD4^+$ ISP thymocytes compose around 1–3 % of total thymocytes.
20. Antibodies should be titrated to determine the optimal amount. In general, 1 μg of monoclonal antibody per 10^6 cells can be used as rule of thumb, but for thymocytes depletions, this can be reduced to 1:4.

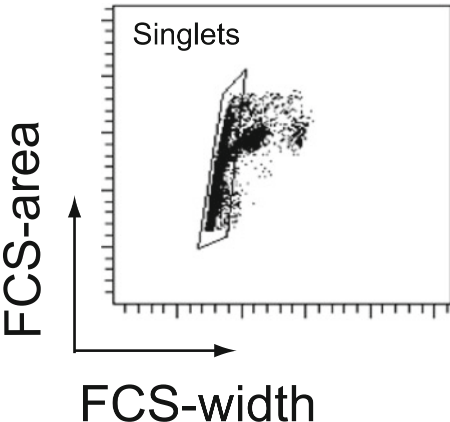

Fig. 8 Gating strategy to exclude doublets. The population of interest (singlets) have an average width signal of 75,000. The formed doublets can be discriminated based on the width signal strength, that is double (150,000) in comparison with the singlets

21. The cell number is dependent on the amount of cells needed for further analysis or experiments. $CD4^+CD8^+$ DP thymocytes constitute 70–85 % of total thymocytes, mature $CD4^+CD3^+$ T cells around 8–14 % and mature $CD8^+CD3^+$ T cells around 4–8 %.
22. Doublets or aggregates can be formed by two particles that stuck together and can be seen by the sorter as a single cell. Problems with purity can be caused by the inclusion of doublets that appear as single particles, especially when sorting DP thymocytes. The doublets can be identified and excluded by appropriate sort gates as depicted in Fig. 8. By using signal area (FSC-A) and width (FSC-W), it is possible to discriminate between real double positive cells and doublets because of the larger FSC-W signal.
23. The cell number is dependent on the amount of cells needed for further analysis or experiments. Roughly 1–3 % of thymocytes are TCR-γδ positive cells.
24. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly.
25. Cells labeled with the anti-TCR γδ Microbead Kit cannot be additionally stained with another fluorochrome-conjugated anti-TCR γδ antibody against the same epitope, since the anti-TCR γδ Hapten antibody occupies most of the epitopes.
26. TCR-γδ antibody is not required because of the FITC-labeled hapten-antibody in the MACS procedure.

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Chapter 20

Approaches to Study Human T Cell Development

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Abstract

Not only is human T cell development characterized by unique changes in surface marker expression, but it also requires specific growth factors and conditions to mimic and study T cell development in vitro. In this chapter, we provide an overview of the specific aspects that need attention when performing T cell differentiation cultures with human progenitors.

Key words Human, T cell development, OP9 coculture, Viral transduction, Gene perturbation, In vitro, Hematopoietic stem cell, Lineage differentiation

1 Introduction

The ability to study human T cell development is of critical importance from a clinical perspective. Not only is there accumulating evidence that there are fundamental differences between human and mouse T cell development, there is also an increasing need to be able to perform functional genetic experiments in a human setting since the accumulating high-throughput sequencing data reveal an endless amount of genetic variation that awaits functional analysis. While some genetic abnormalities reveal a clear functional defect, other variations might result in more subtle phenotypes [1]. As described in Chapter 19, the distinct developmental stages of human T cell development can be identified using specific cell surface markers that differ from mouse. However, there is increasing evidence that also the molecular mechanisms that control this developmental process slightly differ between both species [2]. While the molecules involved seem to be similar, the kinetics and their activity sometimes differ between both species as exemplified by the Notch signaling requirements [2, 3]. This may help to explain the differences in surface marker expression that also display different kinetics with respect to developmental progression as for instance, illustrated for CD4 as described in Chapter 19. In addition, execution of the full T cell developmental program starting from human

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_20, © Springer Science+Business Media New York 2016

hematopoietic progenitor cells demands more time compared to mouse precursors and this is an important aspect to consider when studying human T cell development. In this chapter, we describe how the OP9 coculture system can be employed to achieve efficient human T cell differentiation, specify the growth factors and conditions which are required and highlight specific aspects that require special attention in comparison to when studying mouse T cell development. Fetal thymus organ culture also remains an invaluable tool to study human T cell development in vitro since this offers a more physiological setting for developing thymocytes and even allows the use of specific gene-deleted thymic microenvironments [3]. However, this protocol has been described in great detail previously [4] and is therefore not described in this chapter.

2 Materials

2.1 Freezing and Thawing of Human T Cell Progenitors

1. 15 ml polypropylene conical tube.
2. Sterile cryogenic vials.
3. Trypan blue stain 0.4 %.
4. Hemocytometer counting chamber.
5. Fetal bovine serum (FBS) heat-inactivated at 56 °C for 1 h (*see* **Note 1**).
6. FBS with 20 % dimethyl sulfoxide (DMSO).
7. IMDM complete: Iscove's Modified Dulbecco's Medium (IMDM), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 10 % heat-inactivated FBS.

2.2 Viral Transduction of Human T Cell Progenitors

1. 96-well round-bottom tissue culture plate.
2. 96-well flat-bottom non-tissue culture plate.
3. Humidified cell culture incubator at 37 °C, 7 % CO_2.
4. Fetal bovine serum (FBS) heat-inactivated at 56 °C for 1 h.
5. IMDM complete: Iscove's Modified Dulbecco's Medium (IMDM), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 10 % heat-inactivated FBS.
6. Cytokines: human interleukin-7 (IL-7), human Flt-3 ligand (Flt-3 L), human stem cell factor (SCF), human thrombopoietin (TPO).
7. RetroNectin: 24 μg/ml in sterile PBS, filtered through a 0.22 μm filter. Unused solution can be stored at −20 °C.
8. Phosphate buffered saline (PBS) containing 2 % BSA, passed through a 0.22 μm filter.
9. Recombinant retrovirus or lentivirus (homemade or commercially available).
10. PBS without Ca and Mg.

2.3 Preparation of OP9 Feeder Layers

1. Humidified cell culture incubator at 37 °C, 7 % CO_2.
2. OP9 stromal cells expressing human DLL4 (OP9-hDLL4) at different levels (OP9-hDLL4low and OP9-hDLL4high) or OP9 cells expressing no or other Notch ligands as described [5].
3. Fetal bovine serum (FBS) heat-inactivated at 56 °C for 1 h.
4. Medium to maintain OP9 cell lines (OP9 medium): Liquid Minimum Essential Medium Alpha, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 20 % heat-inactivated FBS.
5. PBS without Ca and Mg.
6. 0.25 % trypsin–EDTA.
7. Trypan blue stain 0.4 %.
8. 15 ml polypropylene conical tube.
9. 24-well flat bottom tissue culture plate.
10. 100 mm × 20 mm cell culture dish.
11. Hemocytometer counting chamber.

2.4 Initiation and Maintenance of OP9 Coculture Experiments

1. Humidified cell culture incubator at 37 °C, 7 % CO_2.
2. 24-well tissue culture plate containing confluent layer of OP9 cells (*see* Subheading 3.3, **step 4**).
3. Fetal bovine serum (FBS) heat-inactivated at 56 °C for 1 h.
4. Homemade Minimum Essential Medium Alpha (MEMα): 950 ml sterile water (*see* **Note 2**), 29.3 ml $NaHCO_3$ (7.5 %), 10 g of alpha-MEM powder, stirred for 5–10 min. pH corrected to 7.1–7.2 and osmolarity corrected to 260–285 mOsm. Filter solution using a 0.22 μM bottle top filter.
5. OP9 coculture medium:

   Homemade MEMα media, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 20 % heat-inactivated FBS.
6. Cytokines: human interleukin-7 (IL-7), human Flt-3 ligand (Flt-3L), human stem cell factor (SCF), human interleukin-15 (IL-15), human thrombopoietin (TPO), human granulocyte colony-stimulating factor (GCSF), and human granulocyte-macrophage colony-stimulating factor (GM-CSF).
7. PBS without Ca and Mg.
8. 15 ml polypropylene conical tube.
9. Trypan blue stain 0.4 %.
10. Hemocytometer counting chamber.

2.5 Analysis of OP9 Coculture Experiments

1. Flow cytometer capable of multi-color analysis (e.g., FACS LSRII).
2. Anti-human FcR Blocking Reagent.

3. Anti-mouse FcRγII/III (clone 2.4.G2).
4. Human Wash Buffer: PBS without Ca and Mg, 1 % BSA, 0.1 % NaN_3, pH corrected to 7.2–7.3, osmolarity corrected to 275–285 mOsm and passed through a 0.22 μm filter. Store buffer at 2–8 °C.
5. Mouse anti-human monoclonal antibodies obtained from different suppliers.
6. 5 ml polystyrene round-bottom tubes.

3 Methods

3.1 Freezing and Thawing of Human T Cell Progenitors

1. Human $CD34^+$ T cell progenitors, isolated as described in Chapter 19, can easily be stored in liquid nitrogen and preserved for initiating differentiation cultures when desired. Transfer cells into a polypropylene conical tube and determine cell number with Trypan Blue. Cord blood progenitors and early thymocytes can be frozen at a density of maximum 150×10^6 cells and 250×10^6 cells per vial respectively.
2. Centrifuge cells at $500 \times g$ for 6 min at 4 °C and remove medium.
3. Resuspend cell pellet in 1:2 volume of pure FBS.
4. Add 1:2 volume of FBS supplemented with 20 % DMSO (*see* **Note 3**). This should be done dropwise, while mixing the cell suspension by swirling the conical tube.
5. Aliquot cells per 1 ml into sterile cryogenic vials and put immediately on ice.
6. Transfer cryogenic vials containing cells to a prechilled freezing box at −80 °C.
7. After 24 h, transfer vials to liquid nitrogen (*see* **Note 4**).
8. To thaw the cells, remove cryogenic vials containing frozen cells from liquid nitrogen and place in 37 °C water bath until 80 % is thawed.
9. Transfer cells to a 15 ml polypropylene conical tube on ice. Gradually increase volume by adding IMDM complete dropwise while continuously shaking the tube (1 ml–2 ml–4 ml) and put on ice for 1 min after addition of each volume.
10. Centrifuge at $500 \times g$ for 6 min at 4 °C and remove supernatant.
11. Resuspend cells in IMDM complete and determine cell number and survival with Trypan Blue. For cord blood $CD34^+$ precursors, recovery can be over 90 % while for $CD34^+$ thymocytes the survival rate is generally less.

3.2 Culture Progenitor Cells and Transduction

Prior to the transduction of progenitor cells, cytokine-stimulated culture is essential for optimal proliferation of the cells, necessary for high transduction efficiencies.

1. Resuspend progenitor cells ($CD34^+Lin^-$ cord blood precursors or $CD34^+CD4^-CD1^-/1^+$ thymocyte precursors, *see* Chapter 19) in IMDM complete medium supplemented with combinations of cytokines and seed cells in 96-well round-bottom tissue culture plate in 200 μl medium per well. Cord blood progenitors are seeded at a density of 500×10^3 cells/ml in medium containing 100 ng/ml SCF, 100 ng/ml Flt3-L, and 20 ng/ml TPO, thymocyte precursors at 1×10^6 cells/ml in medium containing 10 ng/ml SCF and 10 ng/ml IL-7.
2. Culture thymocyte precursors or cord blood progenitor cells at 37 °C, 7 % CO_2 for 24 h or 48 h respectively.
3. Transduce cells using RetroNectin™-coated 96-well flat-bottom plates (*see* **Note 5**).
   (a) Add 80 μl RetroNectin (at 24 μg/ml) per well and incubate for 2 h at RT.
   (b) Aspirate RetroNectin, transfer to a sterile vial, and store at −20 °C (RetroNectin can be reused at least once).
   (c) Add 80 μl PBS containing 2 % BSA and incubate for 30 min at RT.
   (d) Remove PBS containing 2 % BSA and rinse the well by adding 200 μl IMDM complete and remove immediately.
   (e) Add 100 μl viral supernatant to the well, followed by the desired cell number in 100 μl IMDM complete with appropriate cytokines (*see* **Note 6**).
4. 48 h after transduction, collect the cells by vigorous pipetting and ensure that all cells are removed from the well.
5. A fraction of the cells can be used to determine transduction efficiencies using flow cytometry (*see* **Note 7**). Remaining cells can be sorted based on the expression of EGFP (optional, *see* **Note 8**) and cultured on OP9 feeders or used in FTOC.

3.3 Preparation of OP9 Feeder Layers

1. Thaw OP9 cells expressing human DLL4 (OP9-hDLL4) (*see* **Note 9**) 1 week prior to initiating coculture experiments.
   (a) Prepare a 15 ml polypropylene conical tube with 10 ml OP9 medium (RT).
   (b) Thaw a vial with frozen OP9-hDLL4 cells in 37 °C water bath.
   (c) Quickly add cells to 15 ml polypropylene conical tube, spin $500 \times g$ for 6 min, discard supernatant.

(d) Flick pellet to help resuspension of the cells. Gently resuspend pellet in 10 ml OP9 medium (RT), transfer to 100 mm × 20 mm cell culture dish, and place in a humidified incubator at 37 °C and 7 % CO_2.

(e) Change medium the next day.

Optional: Besides their T cell developmental capacity, T cell progenitors, depending on their developmental progression, can also be assayed for differentiation towards other hematopoietic lineages. This may require different OP9 stromal cells. We refer to ref. [5] for more details.

2. Passage the OP9 stromal cells that are not being used for cocultures each time when the confluence reaches about 80–95 %. Do not let them reach confluency! To split OP9 cells:
   (a) Carefully aspirate the medium from the cell culture dish and wash the monolayer with 5 ml PBS to wash out the serum.
   (b) Add 2 ml 0.25 % Trypsin–EDTA, incubate for 5 min at 37 °C.
   (c) Verify that all OP9 stromal cells have detached. If not, incubate for another 5 min at 37 °C.
   (d) Neutralize hydrolysis reaction by adding 4 ml of OP9 medium and transfer the cells from the culture dish into a 15 ml polypropylene conical tube.
   (e) Spin at 500 × *g* for 6 min at 4 °C and aspirate supernatant.
   (f) Resuspend cells in 1 ml OP9 medium and determine cell number with Trypan Blue.
3. Maintain OP9 stromal cells by seeding 200×10^3–250×10^3 cells per 100 mm × 20 mm cell culture dish in a total volume of 10 ml OP9 medium. Split OP9 cells every 2–3 days.
4. For coculture experiments, prepare a single layer of OP9 stromal cells 24 or 48 h before initiating the coculture. Therefore, plate 20×10^3 or 10×10^3 cells, respectively, in a total volume of 500 μl OP9 medium in a 24-well flat bottom tissue culture plate (*see* **Note 10**).

3.4 Initiation and Maintenance of OP9 Coculture Experiments

1. Verify using a microscope that the OP9 cells have generated a confluent layer of cells and remove medium from the wells.
2. Resuspend the purified progenitor cells in the appropriate volume of "OP9 coculture medium" (*see* **Note 11**), supplemented with the required cytokines. The medium volume depends on the number of progenitor cells that will be used to initiate the culture (*see* Table 1). Supplemented cytokines depend on the hematopoietic lineage being studied (*see* Table 2).

Table 1
Maximum number of cells from different progenitor populations for initiation of cocultures onto different well types

Plate format	Medium volume (μl)	Number of cord blood progenitors	Number of pediatric thymus progenitors
96-well	150	1–300	1–500
48-well	300	$300–5 \times 10^3$	$500–7 \times 10^3$
24-well	500	$5 \times 10^3–10 \times 10^3$	$7 \times 10^3–20 \times 10^3$

Table 2
Different cytokine cocktails for the generation of different cell types

T cell mix	NK cell mix	B cell mix	Myeloid cell mix
5 ng/ml SCF	5 ng/ml SCF	20 ng/ml SCF	20 ng/ml SCF
5 ng/ml Flt3-L	5 ng/ml Flt3-L	20 ng/ml Flt3-L	20 ng/ml Flt3-L
5 ng/ml IL-7	5 ng/ml IL-7	20 ng/ml TPO	20 ng/ml TPO
	10 ng/ml IL-15		10 ng/ml GM-CSF
			10 ng/ml GCSF

3. Seed the resuspended progenitor cells onto the confluent OP9 cells.
4. Place the coculture plate in an incubator at 37 °C, 7 % CO_2.
5. Check cocultures every 3 days. If cell density is to high due to expansion of the progenitor cells or if OP9 cells start to detach, split coculture progenitor cells by transferring them to new OP9 feeder cells (*see* **Note 12**).
6. Harvest cocultures at different time points to split cocultures or to perform flow-cytometric analysis.
   (a) Disaggregate the cells by forceful pipetting (*see* **Note 13**) and transfer them to a 15 ml polypropylene conical tube. If necessary, rinse well with sterile PBS to remove any remaining cells and transfer to the same 15 ml polypropylene conical tube.
   (b) Centrifuge at $500 \times g$ for 6 min at 4 °C.
   (c) Carefully remove the supernatant and resuspend the cell pellet in 500 μl pre-warmed OP9 coculture medium with appropriate cytokines.
   (d) Determine cell number with Trypan Blue.

(e) Transfer 1:2 or maximum 100×10^3 cells to new wells of a 24-well tissue culture plate (if sufficient cells, otherwise use 96- or 48-wells) that contain a fresh confluent layer of OP9 cells for further coculture.

(f) The rest of the cells can be used for flow-cytometric analysis (*see* Subheading 3.5).

7. Repeat **steps 5** and **6** until the cells have sufficiently differentiated into the T cell lineage pathway as evident from the flow-cytometric analysis (*see* Subheading 3.5). Given that human T cell development progresses with slower kinetics compared to in mouse, it is not trivial to maintain the cultures for a prolonged time. Timing of analysis depends on the starting population. When cocultures are initiated with $CD34^+CD4^-CD1^{-/+}$ thymocytes, it is recommended to analyze the cells at day 6, 12, 19, and 25 after initiating coculture. At this last time point, cells will no longer expand and most will have successfully differentiated into either TCR-γδ or TCR-αβ T cells. When cocultures are initiated with cord blood or bone marrow derived $CD34^+Lin^-$ precursors, analysis is performed at day 12, 19, 26, 33, 40, 40, 55 of coculture. A representative image of the kinetics of human T cell development form $CD34^+Lin^-$ cord blood precursors is given in Fig. 1.

3.5 Analysis of OP9 Coculture Experiments

1. Before labeling, centrifuge the cells for 6 min at $500 \times g$.
2. Discard the supernatant.
3. Resuspend the cells in 100 μl human wash buffer and preincubate on ice for 5 min with 1 μl anti-human FcR Blocking Reagent and 2 μl anti-mouse FcRγII/III 20 μg/ml (clone 2.4.G2) to avoid nonspecific binding.

Fig. 1 Kinetic analysis of human T cell development from $CD34^+Lin^-$ cord blood progenitors on OP9-DLL4low vs. OP9-DLL4high cells. Dot plots also show effects of low (OP9-DLL4low) versus high (OP9-DLL4high) levels of Notch activation on human T cell development as discussed in more detail elsewhere [2]. Early specification towards the T cell lineage is favored by high Notch activity and can be tracked by the upregulation of CD7 while $CD34^+$ expression is maintained. Further differentiation is characterized by the upregulation of CD5 and downregulation of CD34. Addition of HLA-DR to the panel is important to discriminate T cell progenitors from monocytic/dendritic cells. While $CD34^+$ cells coexpress HLA-DR, also following upregulation of CD7 (*green cells*), $CD7^+CD5^+$ T cell progenitors will downregulate $CD34^+$ as well as HLA-DR (*blue cells*), while $CD5^+$ cells also contain $HLA\text{-}DR^+CD4^+$ monocytic/dendritic cells (*red cells*). Thus, CD5 or CD7 are insufficient as sole markers for early T cell progenitors. Further differentiation into $CD4^+CD8\beta^+$ double positive thymocytes and $TCR\text{-}\alpha\beta^+$ $CD3^+$ T cells is more efficient when Notch activity is reduced (OP9-DLL4low) but requires over 50 days of coculture to develop. TCR-γδ T cells preferentially develop in conditions of high Notch signaling activity (OP9-DLL4high) (color figure online)

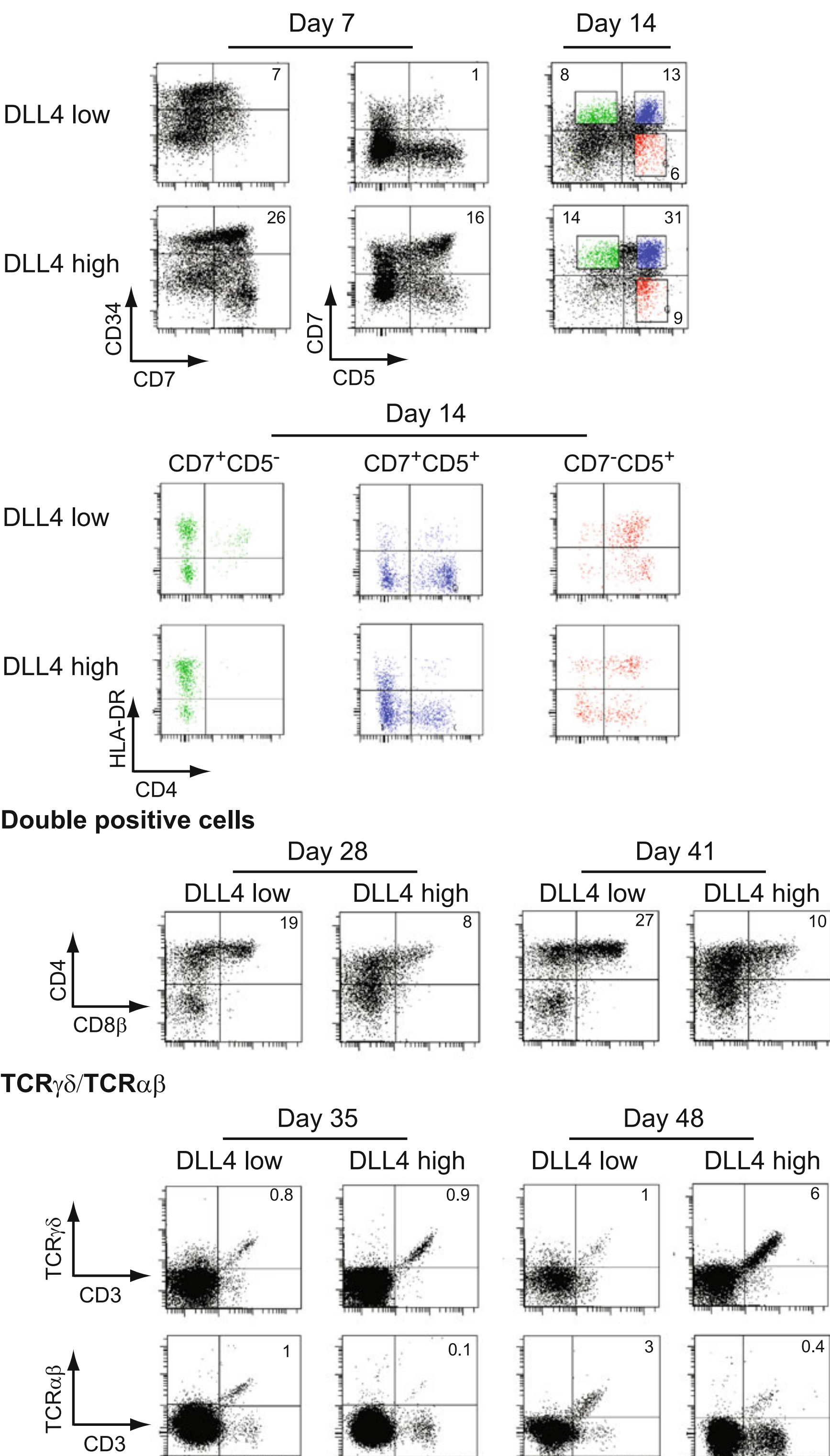
Early T cell precursors
Day 7
Day 14
DLL4 low
DLL4 high
7
1
8
13
6
26
16
14
31
9
CD34
CD7
CD7
CD5
Day 14
CD7+CD5-
CD7+CD5+
CD7-CD5+
DLL4 low
DLL4 high
HLA-DR
CD4
Double positive cells
Day 28
Day 41
DLL4 low
DLL4 high
DLL4 low
DLL4 high
19
8
27
10
CD4
CD8β
TCRγδ/TCRαβ
Day 35
Day 48
DLL4 low
DLL4 high
DLL4 low
DLL4 high
0.8
0.9
1
6
TCRγδ
CD3
1
0.1
3
0.4
TCRαβ
CD3

Table 3
Antibody panels to study different early stage of human T cell development in vitro (*see* Note 14)

Early T cells	Double positive cells	TCRαβ/TCRγδ T cells	Myeloid cells	B cells
PI	PI	PI	PI	PI
CD45 *(HI30)*	CD45 *(HI30)*	CD45 *(HI30)*	CD45 *(HI30)*	CD45 *(HI30)*
CD34 *(AC136)*	CD8b *(2ST8.5H7)*	TCRαβ *(BW242/412)*	CD4 *(RPA-T4)*	CD19 *(LT19)*
CD7 *(M-T701)*	CD8 *(SK1)*	TCRγδ *(11F2)*	HLA-DR *(LN3)*	HLA-DR *(LN3)*
CD5 *(UCHT2)*	CD4 *(RPA-T4)*	CD3 *(UCHT1)*	CD14 *(TUK4)*	
CD1a *(HI149)*	CD3 *(UCHT1)*	CD4 *(RPA-T4)*	CD15 *(VIMC6)*	
CD4 *(RPA-T4)*	HLA-DR *(LN3)*	CD8b *(2ST8.5H7)*	CD123 *(6H6)*	
HLA-DR *(LN3)*		CD1a *(HI149)*	CD303 *(AC144)*	

Antibody clones are indicated in italicized parenthesis

4. Transfer 10–100 × 10^3 cells into 5 ml polystyrene round-bottom FACS tube for each staining.
5. Cells are stained with panels of anti-human monoclonal antibodies according to the guidelines of the manufacturer. Antibody panels to study different early stages of T cell development are represented in Table 3.
6. Incubate for 30–45 min at 4–6 °C, in the dark.
7. Wash the cells by adding 2 ml prechilled human wash buffer per sample. Centrifuge at 500 × *g* for 6 min at 4 °C and remove the supernatant. Keep the cells on ice and covered from daylight until flow-cytometric analysis.
8. A representative image of the kinetics of human T cell development form $CD34^{+}Lin^{-}$ cord blood precursors is given in Fig. 1.

4 Notes

1. Technical disadvantages to using serum include the undefined nature of serum, batch-to-batch variability in composition, and the risk of contamination. Therefore it is recommended to perform serum testing. To test for T cell development, we

culture fetal thymic lobes (fetal day 14) of B6 mice in fetal thymus organ culture for 14 days and check for efficiency to support T cell development by cell counting and flow-cytometric analysis of T cell differentiation.

2. Use a 1-L glass graduated cylinder for the preparation of homemade MEMα. Do not use the cylinder for any other purpose and do not clean with any detergent! After each use, rinse the cylinder with milliQ H_2O and then with 100 % EtOH, autoclave occasionally. Store homemade MEMα at 4 °C and use in cocultures for maximum 4 weeks after preparation!

3. For the preparation of FBS supplemented with 20 % DMSO, DMSO should be added dropwise to FBS while keeping the solution on ice to minimize the exothermic reaction.

4. DMSO is a cryoprotective agent, which slows down the cooling rate, reducing the risk of ice crystal formation. The gradual decrease in temperature, by temporarily storing the cells at −80 °C, improves amorphous freezing. However, DMSO is toxic to cells and should be added dropwise to minimize the exothermic reaction that occurs when DMSO is added to water.

5. Large number of cells can be transduced using 24-well flat-bottom RetroNectin™-coated plates. Therefore, volumes need to be adjusted using the following protocol:

   Add 320 μl RetroNectin 24 μg/ml per well and incubate for 2 h at RT.

   Aspirate RetroNectin, transfer to a sterile vial and store at −20 °C (can be used once again).

   Add 320 μl PBS containing 2 % BSA and incubate for 30 min at RT.

   Remove PBS containing 2 % BSA and rinse well using 600 μl IMDM complete.

   Add 400 μl viral supernatant and 400 μl of precultured cells, correct cytokine concentration as viral supernatant do not contain cytokines.

6. Be sure to add sufficient cytokines for compensating the viral supernatant that does not contain cytokines. Cytokine concentrations from the preculture should remain stable during the complete transduction procedure.

7. It is recommended to use EGFP (or another fluorescent marker gene) expressing retroviruses or lentiviruses (such as LZRS or MSCV) to monitor transduced cells during the subsequent T cell developmental assays. Transduction efficiency peaks around 48 h after adding the virus to the cells.

8. To initiate cocultures with transduced cells, it is recommended to start with a 100 % sorted $EGFP^+$ transduced cell population

as this is the most accurate way to calculate cell numbers. While a mixture of EGFP⁻ and EGFP⁺ cells has the advantage of containing an internal control for evaluating the efficiency of the cultures (EGFP⁻ cells should behave similarly for each virus being tested), it is more difficult to track the fate of the transduced cells since gene perturbations that lead to a negative effect on cell growth and/or differentiation may lead to a transition of EGFP⁺ to EGFP⁻ cells, making it difficult to make accurate calculations.

9. DLL4 is the physiological Notch ligand that induces T cell development in vivo and is therefore recommended although we do not see major differences between DLL4 and DLL1. We also don't see any major differences in effects on human T cell development when using OP9 cells that express a human or mouse Notch ligand. Effects of other Notch ligands have been described [3, 5] and can be used depending on the particular experiment.
10. Coculture experiments can be performed with 96-well, 48-well or 24-well plates. It is important that OP9 stromal cells reach 100 % confluency before adding lymphoid progenitor cells to ensure close interaction between the lymphoid progenitor cells and the Notch ligand expressing OP9 cells.
11. It is essential to use OP9-coculture medium that is prepared with fresh, homemade MEMα medium since MEMα that is purchased as a liquid seems to have lost an unknown component that is important for efficient human T cell development. Therefore, the freshly prepared medium cannot be used for more than 4 weeks to ensure reproducible results.
12. The growth rate of human hematopoietic progenitor cells is highly variable due to the donor variety. As a result, cultures need to be monitored continuously to avoid that cultures become too dense as this will result in cell death. Cord blood or bone marrow derived CD34⁺ progenitor cells usually display limited expansion during the first week of OP9-hDLL4 coculture.
13. Forceful pipetting of OP9 cocultures will result in the detachment of both OP9 and human hematopoietic progenitor cells. There is currently no efficient protocol that allows the selective isolation of the hematopoietic cells without OP9 contamination. This, however, does not affect downstream analysis or further culture of the cells when replated on a fresh monolayer of OP9 cells.
14. An anti-human CD45 monoclonal antibody is added to each tube to distinguish human hematopoietic cells from OP9 stromal cells. Alternatively, anti-mouse CD51 can also be used to stain OP9 stromal cells. Propidium Iodide (PI) is added to exclude dead cells.

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Chapter 21

Humanized Mice to Study Human T Cell Development

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Abstract

While in vitro models exist to study human T cell development, they still lack the precise environmental stimuli, such as the exact combination and levels of cytokines and chemokines, that are present in vivo. Moreover, studying the homing of hematopoietic stem (HSC) and progenitor (HPC) cells to the thymus can only be done using in vivo models. Although species-specific differences exist, "humanized" models are generated to circumvent these issues.

In this chapter, we focus on the humanized mouse models that can be used to study early T cell development. Models that study solely mature T cells, such as the SCID-PBL (Tary-Lehmann et al., Immunol Today 16:529–533) are therefore not discussed here, but have recently been reviewed (Shultz et al., Nat Rev Immunol 12:786–798).

Key words Immunodeficient mice, Humanized mice, In vivo mouse model, T cell development, BLT mouse model, SCID-Hu mouse model

1 Introduction

1.1 Mouse Strains Used to Study In Vivo T Cell Development

Engraftment of human hematopoietic cells and fetal hematopoietic tissues was initially described in severe combined immunodeficient (SCID) mice [1–3]. Due to the *Prkdc*^{scid} (protein kinase, DNA activated, catalytic polypeptide) mutation, these mice lack functional T- and B-lymphocytes, allowing higher and more diverse engraftment than was described earlier in nude and beige-nude-Xid (bnx) mice, which support T cell but not B cell development [4–6]. However, engraftment of human cells in SCID mice was still suboptimal, owing to high levels of host natural killer (NK) cell activity and innate immunity. Another way to delete T and B cells in the murine host, is to target one of the recombination activating gene 1 and 2 (*Rag1* and *Rag2*) genes, crucial for rearrangements of both T and B cell receptor genes during T and B cell development [7, 8]. However, these mice also retain high NK cell

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_21, © Springer Science+Business Media New York 2016

activity that leads to low human cell engraftment. The *scid* mutation, as compared to the *Rag* mutation, also results in defective DNA repair (as the *Prkdc* gene encodes for a polypeptide important in repair of double-stranded DNA breaks) and, consequently, higher radiosensitivity [9].

The development of nonobese diabetic (NOD)-*scid* mice, by crossing the *scid* mutation onto the NOD background, led to the next breakthrough in the development of immunodeficient mouse models. Additional defects in innate immunity in NOD-*scid* mice, lacking hemolytic complement and showing functional defects in both macrophages and NK cells, led to higher engraftment of human hematopoietic cells, compared to *scid* mice [10–12]. However, the residual activity of both NK cells and macrophages in NOD-*scid* mice still limited human hematopoietic engraftment, and especially T cell development was very inefficient. To address this problem, existing models were adapted, e.g., by injecting mice with TM-β1, an IL-2 receptor beta chain (IL2Rβ) blocking antibody [13–15], further decreasing NK cell activity in these mice, allowing the engraftment of T cell precursors in the murine thymus with in vivo T cell development in about 50 % of the cases. The major drawback of this model was that large amounts of antibody and several intraperitoneal injections of the mice were necessary. Later, new mouse strains lacking a functional IL-2 receptor gamma chain (*Il2rg*^{null}) were developed such as NOD/Shi-*scid Il2rg*^{null} (NOG) [16], NOD/LtSz-*scid Il2rg*^{null} (NSG) [17], BALB/c--*Rag2*^{null} *Il2rg*^{null} (BRG) [18], NOD/*Rag1*^{null} *Il2rg*^{null} (NRG) [19]. Since the IL2Rγ chain is an important component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, these mice completely lack NK-cell development, as well as functional T- and B-lymphocytes, features that lead to superior human hematopoietic engraftment and allow for T cell development in the murine thymus in a near to 100 % frequency. An important disadvantage of the NOD-*scid* strain is the relatively short life span of these mice due to thymoma development [20], thereby not allowing evaluation of the injected and/or in vivo generated cells for an extended period of time. *Il2rg*^{null} mice, however, have a longer median life span (more than 90 weeks for NSG mice compared with 37 weeks for NOD-*scid* mice) and thymoma development is very rare to nonexistent [21]. Also in mice lacking beta2-microglobulin (*B2m*$^{-/-}$) (NOD/SCID/*B2m*$^{-/-}$) [22] B, T, and NK cell development and functions are disrupted, as β_2m is necessary for major histocompatibility complex (MHC) class I-mediated immunity. These and other immunodeficient mouse strains are extensively reviewed in ref. 21.

The thymic microenvironment and especially the thymic epithelial cells support positive and negative selection processes through interaction of MHC molecules on the epithelial cells with the TCR

of the developing T cell. Therefore, in the above-mentioned models, human T cells are selected on murine MHC molecules. To overcome this hurdle, humanized mouse models have been developed in which a human thymus is transplanted in an immunodeficient mouse (*see* Subheading 1.2 SCID-hu and BLT models).

Currently, new immunodeficient mouse strains are being developed that allow differentiation of fully functional T-lymphocytes from injected human HSC after selection on human MHC molecules, without the need for transplantation of human organs. Such strains include mice that lack MHC class I or MHC class II molecules and that are engineered to express human MHC class I and/or II molecules: HLA-A2 (NSG-A2, [23]), HLA-DR (NOG/HLA-DR4, [24]), and both HLA-A2 and HLA-DR (NSG-A2/DR1, [25]). In this way, xenogeneic graft-versus-host disease (GVHD) is reduced and the development of human MHC-restricted T-cells, able to recognize antigens presented by human MHC-expressing antigen-presenting cells (APCs) in the periphery, in the absence of mouse MHC-restricted T-cells can be achieved using the Hu-HSC model [26] (*see* Subheading 1.2). The drawback here is that only T cells expressing a HLA-A2 and/or HLA-DR restricted TCR are being selected, and there is a need for mouse strains expressing other human MHC molecules, both class I and class II.

Also, transgenic mice engineered to express human cytokines are able to enhance engraftment and/or proliferation of human hematopoietic cells. Engraftment of especially HSC and T lymphocytes is improved in stem cell factor (SCF)-expressing mice [27], whereas human interleukin 7 (IL-7) (after retro-orbital injection of a hIL-7 lentiviral supernatant into BRG mice) enhances homeostatic proliferation of human T cells [28].

An overview of the available mouse strains to study T cell development in a humanized mouse model is given in Table 1. T cell development and HSC engraftment scores are based on published data but a side by side comparison of the different strains has not yet been performed, and the scores should be treated as such.

1.2 Immunodeficient Mouse Models

Such immunodeficient mice can be reconstituted with human progenitors and organs to generate humanized mice, according to several outlines. The Hu-HSC model, where human HSC are adoptively transferred in immunodeficient mice, was primarily developed to study and characterize human HSC, as it is the only model in which all properties of HSC (multilineage development, self-renewal, high proliferative capacity and ability to reconstitute) can be evaluated [12]. This model revealed the SCID repopulating cell or SRC, as the ultimate definition of the HSC. After transfer of HSC in the Hu-HSC model, these cells undergo multilineage differentiation, including both T and B cell development. Multipotent progenitors will home to the thymus, where they are educated on

Table 1
Currently available mouse strains optimally supporting human T cell development

(a) Basic							
Mouse strain	**Alternative name**	**Abbreviation**	**Background**	**Genetic defect**	**T cell development**	**HSC engraftment**	**Refs**
NODShi.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}	NOD/Shi-*scid Il2rg*^{null}	NOG	NOD/SCID	IL-2Rγ chain lacks IC domain	+++	++	[16]
NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}	NOD/LtSz-*scid Il2rg*^{null}	NSG	NOD/SCID	Null mutation of IL-2Rγ	+++	+++	[17]
NOD.Cg-*Rag1*^{tmMom} *Il2rg*^{tmWjl}	NOD/*Rag1*^{null} *Il2rg*^{null}	NRG	NOD	Null mutation of Rag1 and IL-2Rγ	+++	+++	[19]
C.Cg-*Rag2*^{tmFwa} *Il2rg*^{tmSug}	BALB/c-*Rag2*^{null} *Il2rg*^{null}	BRG	Balb/c	Null mutation of Rag2 and IL-2Rγ chain lacks IC domain	++	++	[18]
C.Cg-*Rag1*^{tm1Mom} *Il2rg* ^{mWjl}	BALB/c-*Rag1*^{null} *Il2rg*^{null}	BRG	Balb/c	Null mutation of Rag1 and IL-2Rγ chain lacks IC domain	++	++	[19]
C;129S4-*Rag2*^{tmFlv} *Il2rg*$^{tm1Flv/J}$	/	BRG or C;129RG	Mixed (Balb/c x 129Rag2^{null})	(Null mutation of Rag2) Null mutation of IL-2Rγ chain	++	++	[29]

(b) Supplementally engineered mice to enhance T cell development

Mouse strain	Abbreviations	Background (*see* ref. 30)	Additional genetic defect	Supplemental effects on T cell development	Refs
NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}Tg(HLA-A/H2-D/B2M)1Dvs	NSG Tg(HLA-A2/B2M) NSG-A2/B2M	NSG	Transgene HLA-A2 and human β2-microglobulin	Supports development of human HLA-A2 restricted T cells	[31] [32]
NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}Tg(HLA-A2.1)1Enge	NSG Tg(HLA-A2) NSG-A2	NSG	Transgene HLA-A2	Supports development of human HLA-A2 restricted T cells	[23]
NOD.Cg-*Rag1*^{tm1Mom}*IL2rg*^{tmWjl}Tg(HLA-DRB1)31Dmz	NSG Tg (HLA-DR4) NSG-DR4	NSG	Transgene HLA-DR4	Supports development of human HLA-DR restricted T cells, enhanced immune function	[33]
NOD.Cg-P*rkdc*^{scid}H2-*Ab1*^{tm1Dol}*Il2rg*^{tm1Sug}Tg(HLA-DRA, HLA-DRB1*0405)1Jic/Jic	NOG Tg(HLA-DR4) NOG-DR4	NOG	Transgene HLA-DR4	Supports development of human HLA-DR restricted T cells, enhanced immune function	[24]
NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/Sz Tg(HLA-A2*0201/HLA-DRA*0101, HLA-DRB1*0101)	NSG Tg(HLA-A2/DR1) NSG-A2/DR1	NSG	Transgene HLA-A2 and HLA-DR1	Supports development of human HLA-A2 and HLA-DR restricted T cells, enhanced immune function	[25]

Abbreviation: *IC* intracytoplasmic; *nc* not compared
Adapted from [26]

murine epithelial cells, and therefore will be tolerant to the mouse host, resulting in a low frequency of GVHD [21]. However, the generated T cells will be mouse MHC-restricted instead of human MHC-restricted [26]. As mentioned above, newer mouse strains with deleted mouse MHC molecules and transgenic expression of human MHC molecules (*see* Table 1b) have answered this issue. For the Hu-HSC model, HSC from several sources have been used, from fetal liver [34], umbilical cord blood [16, 18, 35], bone marrow [34], or granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood [17], all leading to acceptable levels of engraftment. Originally, HSC were injected intravenously (i.v.) into adult hosts, but engraftment was improved when the cells were delivered intrahepatically (i.h.) into newborn mice (probably due to a more tolerant environment) or after intrafemoral (i.f.) injection into adult recipients. Intrafemoral injection circumvents the need for homing of HSC to the bone marrow, but therefore does not allow evaluation of homing characteristics.

To enhance the selection process of human thymocytes, the SCID-Hu mouse model, wherein human fetal liver and thymus are co-implanted under the renal capsule of SCID mice (SCID-Hu thy/liv) [36], or human bone marrow is implanted subcutaneously in SCID mice (SCID-Hu bone) [37], can be employed. Several reports have demonstrated that this is a useful and relevant in vivo system to study the developmental potential of transplantable human hematopoietic progenitor and stem cells [38–40]. The advantage of this assay is the presence of a human microenvironment; the obvious disadvantage is the necessity for fetal material and the lack of quantitative data. Moreover, although it is suitable for studying T cell development, only low levels of hematopoietic cell lineages other than thymocytes can be generated and the overall human immune system function is poor.

To overcome these hurdles, the bone marrow/liver/thymus (BLT) model was developed. This model is in origin the same as the SCID-Hu model, but, in addition, human HSC isolated from the same fetal liver are injected i.v. and engraft the bone marrow, resulting in a complete human immune system arising in the immunodeficient mouse [41, 42].

An overview of the different humanized mouse models supporting T cell development is given in Fig. 1 and Table 2. Depending on their research goal, investigators need to choose the appropriate humanized mouse model for their studies. All above-mentioned strains and models, including advantages and disadvantages, are reviewed in refs. 26 and 21. In terms of establishment of engraftment of HSC and resulting T cell development, NSG mice have been proven to be superior compared to other mouse strains, especially at limiting HSC doses [43]. Moreover, female NSG mice show higher engraftment levels than male NSG mice [44].

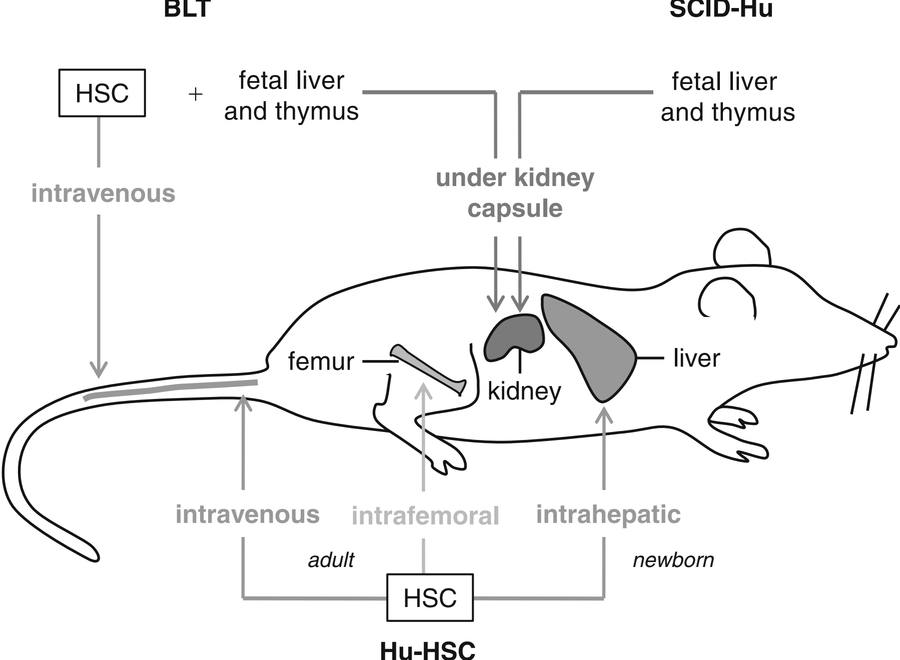

Fig. 1 Schematic overview of humanized mouse models

Human peripheral blood lymphocytes (PBL) can also be injected when the function of mature T cells is being studied. As this is beyond the scope of this chapter, which focuses on T cell development, we refer to other reviews for the Hu-PBL model [21, 30].

1.3 Methods to Enable Engraftment in Mice

Immunodeficient mice are usually preconditioned using total-body sublethal γ-radiation before injection of HSC to enable optimal engraftment in these mice. The purpose of this irradiation is to eliminate residual immune cells, to induce the production of cytokines (improving engraftment and differentiation) and to create space for the introduced HSC. The level of the employed radiation differs, depending on the age of the mice (newborn or adult) and the strain used (*scid* or *Rag* mutation). Newborn mice are more sensitive than adult mice to irradiation, and as the *scid* mutation leads to heightened radiosensitivity, a lower dose of irradiation given to NSG or NOG mice causes the same effect as a higher dose in BRG mice. Typically, mice are irradiated using a ^{137}Cs gamma irradiator, but any radiation source can be used when applying settings specifically developed for mouse irradiation. Newborn mice harboring the *scid* mutation (NSG, NOG) are typically irradiated with 100 cGy [45], while newborn BRG mice can be irradiated with up to 400 cGy [18]. Adult NSG or NOG mice tolerate radiation

Table 2
Humanized mouse models supporting T cell development in vivo

Humanized mouse model (abbreviated)	Brief description	Thymic microenvironment	Organs showing T cell reconstitution	Advantages	Limitations	Refs.
Hu-HSC	Injection of human HSC (from FL, CB, BM or mPB) i.v. or i.f. in adult and i.h. in newborn irradiated immunodeficient mice	– Murine (selected on mouse MHC) – Murine with HLA expression (class I and/or class II) only in HLA-tg mice	Murine thymus, blood, spleen, bone marrow, lymph nodes (seldom)	– Multilineage development (B/T cells, myeloid cells, NK cells) – Higher T cell engraftment in newborn mice	– T cells educated on murine TEC and murine MHC restricted – Low engraftment levels of some hematopoietic lineages in blood	[12] [16] [17] [18] [34] [35]
SCID-Hu	Co-implantation of human FL and FT (of the same donor) under the kidney capsule	Human	Transplanted thymic organ	– T cells educated on human autologous TEC and human MHC restricted – Availability of large amounts of human T cells in thymus	– Specialized transfer method (surgery) – Human fetal material required – Low engraftment levels of hematopoietic lineages other than thymocytes	[36]
BLT	After establishment of SCID-Hu	Human (and murine)	Transplanted thymic organ, murine thymus, blood, spleen, bone marrow, lymph nodes (seldom)	– Complete human immune system – T cells educated on human autologous TEC and human MHC restricted – Availability of large amounts of human T cells in thymus – Higher levels of total human HSC engraftment than in Hu-HSC	– Specialized transfer method (surgery) – Human fetal material required	[41]

Abbreviations: *Hu* human; *SCID* severe combined immunodeficiency; *BLT* bone marrow/liver/thymus; *FL* fetal liver; *CB* cord blood; *BM* bone marrow; *mPB* mobilized peripheral blood; *i.v.* intravenous; *i.f.* intrafemoral; *i.h.* intrahepatic; *MHC* major histocompatibility complex; *HLA* human leukocyte antigen; *TEC* thymic epithelial cells
Adapted from [26]

doses up to 400 cGy [17] but mostly 200–300 cGy is used [46–49]. For adult BRG mice, radiation doses of 500–600 cGy are used [50]. The only mouse strain that seems to overcome the need for irradiation, and therefore might provide a more physiological environment, is the newborn NSG mouse expressing membrane-bound SCF [27] and the adult *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*Kit*$^{Wv/Wv}$ mouse [51], in which the mutant Kit receptor opens up the stem cell niches across species barriers [52]. As human lymphoid development in the *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*Kit*$^{Wv/Wv}$ mouse has not yet been published, this mouse strain is not discussed above.

An alternative method that substitutes irradiation is injection of the mice with the chemotherapeutic agent Busulfan. Choi et al. [53] showed that this leads to a higher survival rate and enhanced T cell development.

Other engraftment-enabling methods that have previously been used are injection of the mice with TM-β1, before injection of human cells [13], a strategy which has been made redundant by the newer strains, lacking IL2Rγ or β_2m, and in vivo injection of various recombinant human cytokines, such as IL-15, IL-7, and SCF, after injection of human cells [54–56].

Also, intrafemoral injection of HSC can be considered as a method to enable engraftment, as injection of HSC directly into the bone marrow circumvents the need for homing of these cells to the bone marrow and therefore allows for immediate engraftment.

2 Materials

2.1 Common Materials

1. Phosphate-buffered saline (PBS), sterile.
2. ^{137}Cs gamma irradiator (*see* **Note 1**).
3. Sterile, autoclaved housing device for mice during irradiation (*see* **Note 2**).
4. Sterile alcohol swab or 70 % ethanol spray.

2.2 CD34$^+$ Isolation

1. Hematopoietic stem cell (HSC) source (umbilical cord blood, mobilized peripheral blood, bone marrow, or fetal liver) (*see* **Note 3**).
2. Lymphoprep or Ficoll, stored at room temperature.
3. Method to isolate CD34$^+$ cells (*see* **Note 4**).

2.3 Intrahepatic Injection

1. Immunodeficient mice breeding pairs (*see* **Note 5**).
2. 1-cc tuberculin syringes with 25-G needle or 1-cc insulin syringe with 29-G needle.
3. Optional: topical nasal decongestant.

4. Ice bucket with ice.
5. Sterile gauze.
6. Sterile 100 mm petri dishes.
7. Heat lamp or heating device.

2.4 Intravenous Injection

1. Immunodeficient mice: male or female, 6–10 weeks old (*see* **Note 5**).
2. 1-cc tuberculin syringes with 25-G needle.
3. Heat lamp or heating device.

2.5 Intrafemoral Injection

1. Immunodeficient mice: male or female, 6–10 weeks old (*see* **Note 5**).
2. Anesthetic and/or anesthesia equipment (according to institutional guidelines).
3. Surgical instruments: curved forceps, scalpel, wound closure staple and clip or suture, needle holder, suture scissors (*see* **Note 6**).
4. Hamilton syringe (Hamilton, Bonaduz, Switzerland).
5. Hair clipper.
6. Bone wax (Braun, Diegem, Belgium).
7. 25-G needle.

2.6 Generation of a Transplantation Needle

1. 18-G hypodermic needle.
2. 18-G spinal needle.
3. Sandpaper, very fine.

2.7 Subcapsular Kidney Transplant

1. SCID mice (preferably male, 6–8 weeks old) or NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ (NOD/LtSz-*scid* $Il2rg^{-/-}$) mice (male or female, 6–10 weeks old) (*see* **Note 5**).
2. Human fetal liver and fetal thymus of 14–23 weeks of gestational age (preferably HLA-typed, *see* **Note 7**) (*see* **Note 8**).
3. Sterile PBS supplemented with 50 μg/ml gentamicin (Gibco, Invitrogen, Merelbeke, Belgium).
4. Sterile RPMI-1640 medium (Gibco, Invitrogen, Merelbeke, Belgium) supplemented with 50 μg/ml gentamicin.
5. Anesthetic and/or anesthesia equipment (according to institutional guidelines).
6. Special injection device/transplantation needle (*see* **Note 9**).
7. Surgical instruments: scalpel, microdissecting scissors, Dumont tweezers, microdissecting forceps, microdissecting scissors, wound closure staple and clip and/or absorbable suture, needle holder and suture scissors, scissors and toothed tweezers (*see* **Note 6**).

8. Sterile 100 mm petri dishes.
9. Hair clipper.
10. 10 ml syringe with 18-G needle.
11. Heat lamp or heat bags.
12. Optional (BLT model): sterile RPMI-1640 supplemented with 1 mg/ml collagenase/dispase (Roche, Mannheim, Germany) and 0.5 U/ml DNase I (Roche).
13. Optional (BLT model): 70 μm cell strainer, sterile.
14. Optional (BLT model): 1-cc tuberculin syringes with 25-G needle.

3 Methods

3.1 CD34⁺ Isolation

1. Isolate mononuclear cells from HSC source using Lymphoprep or Ficoll density gradient centrifugation (*see* Chapter 19 by K. Davids et al. for details).
2. From the obtained mononuclear cells, isolate $CD34^+$ cells (*see* **Note 4** and Chapter 19 by K. Davids et al.).
3. Resuspend the isolated $CD34^+$ cells in sterile PBS at the desired cell density (volumes and cell numbers typically used are discussed in **Note 10**) and keep on ice until transplanted (*see* **Note 11**).

3.2 Mice Irradiation

1. Place mice in sterile autoclaved housing device for irradiation (*see* **Notes 12** and **13** for additional information on handling newborn mice).
2. Irradiate mice with the appropriate amount of total-body irradiation (*see* **Notes 1** and **14**).

3.3 Intrahepatic Injection in Newborn Mice

1. Monitor breeding pairs for the birth of new litters (this can be estimated using timed matings). The procedure should be performed on 1- (not earlier to avoid rejection by the mother) to 4- (not later as engraftment efficiency will decrease) day old newborn pups.
2. Isolate $CD34^+$ as described in Subheading 3.1.
3. Irradiate pups as described in Subheading 3.2. Pups are placed back with their mother afterwards.
4. Wait for at least 2 h and not more than 24 h before starting with injection procedure.
5. Place irradiated pups on a sterile gauze pad in a sterile petri dish on ice for 5–10 min until anesthetized (when gross movement ceases).
6. Load the $CD34^+$ cell suspension into a 1-cc tuberculin syringe with 25-G needle or a 1-cc insulin syringe with 29-G needle.
7. Swab abdomen of mice with sterile alcohol swab.

8. Deliver 50 μl of cell suspension directly into the liver (*see* **Notes 15** and **16**).
9. Place the pups under a heat lamp for 1–2 min (*see* **Note 17**).
10. Optional: before putting the pups back with the mother, apply a small amount of topical nasal decongestant to the snout of the mother (*see* **Note 18**).
11. Pups can be weaned from the mother at 3–4 weeks of age and human cells can be detected in the blood from 4 weeks after HSC injection on. Full evaluation is optimally done at 10–12 weeks of age by sacrificing mice and collecting organs (bone marrow, thymus, liver, spleen, and blood by cardiac puncture).

A representative flow cytometric analysis of T cell reconstitution after intrahepatic injection of human CD34$^+$ cells from cord blood in newborn NSG and *Rag*$^{-/-}$ mice at 12 weeks after injection is shown in Fig. 2.

3.4 Intravenous Injection in Adult Mice

1. Isolate CD34$^+$ as described in Subheading 3.1.
2. Irradiate mice as described in Subheading 3.2.
3. Wait for at least 4 h and not more than 24 h before injecting mice with HSC.
4. Put mice with their tail under a heat lamp or heating device to cause vasodilatation (*see* **Note 17**). Mice can be immobilized using restraining devices.
5. Swab tail vein with sterile alcohol swab.
6. Inject 200 μl of cell suspension into the lateral tail vein using a 1-cc syringe with 25-G needle.
7. Depending on the research question, different analyzing time points can be set. Evaluation of homing of HSC can be done immediately after injection of the cells. T cell development can be analyzed from 3 to 4 weeks after injection on. Full evaluation is optimally done at 10–12 weeks after HSC injection by sacrificing mice and collecting organs (bone marrow, thymus, liver, spleen, and blood by cardiac puncture).

3.5 Intrafemoral Injection in Adult Mice

1. Isolate CD34$^+$ as described in Subheading 3.1.
2. Irradiate mice as described in Subheading 3.2.
3. Wait for at least 4 h and not more than 24 h before injecting mice with HSC.
4. Anesthetize mice according to institutional protocols.
5. Shave the fur broadly over the knee joint area of the mice using a hair clipper.
6. Locally spray 70 % ethanol and let it dry.
7. After flexing the knee to 90°, make an incision of 2–3 mm in the skin just above the patella using the scalpel.

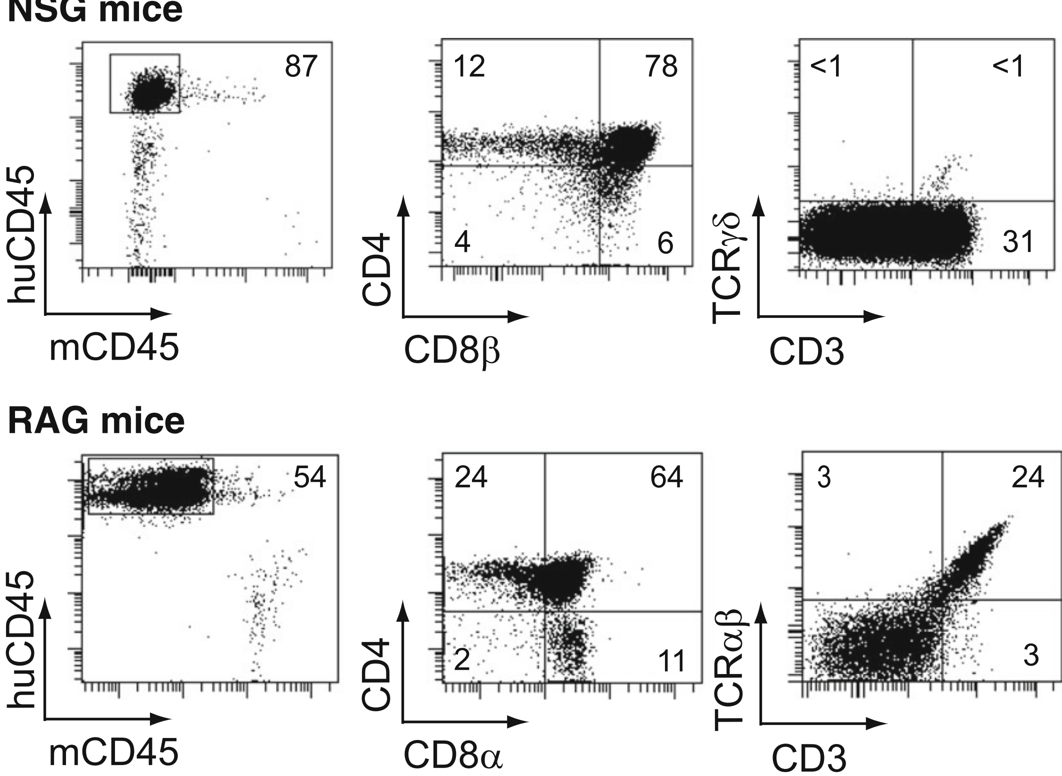

Fig. 2 T cell reconstitution after intrahepatic injection of cord blood CD34⁺ cells in newborn NSG and *Rag*$^{-/-}$ mice at 12 weeks after injection

8. Move the patella to the side.
9. Place a 25-G needle between the condyles at the end of the femur and twist gently until resistance is lost to make an intra-femoral tunnel in the bone marrow cavity. Remove the needle.
10. Fill the Hamilton syringe with the CD34^{+} cell suspension and insert into the hole made in the bone marrow cavity. Inject the cell suspension into the bone marrow cavity.
11. Seal the needle hole with bone wax and reposition the patella.
12. Close the skin incision with wound staples or absorbable suture (*see* **Note 19**).
13. Depending on the research question, different analyzing time points can be set. T cell development can be analyzed from 3 to 4 weeks after injection on. Full evaluation is optimally done at 10–12 weeks after HSC injection by sacrificing mice and collecting organs (bone marrow, thymus, liver, spleen, and blood by cardiac puncture) (*see* **Note 20**).

3.6 Fetal Thymus Preparation for Subcapsular Kidney Transplant

1. Place the fetal thymus in a petri dish and rinse twice with PBS/gentamicin.
2. Transfer the thymus to a petri dish filled with cold RPMI medium.
3. Remove the connective tissue from the thymus.
4. Cut the thymus into small pieces (1 × 1 × 1 mm) using microdissecting scissors (*see* **Note 21**). Keep on ice.

3.7 Fetal Liver Preparation for Subcapsular Kidney Transplant

1. Place the fetal liver in a petri dish and rinse twice with PBS/gentamicin.
2. Transfer the liver to a petri dish filled with cold RPMI medium.
3. Cut the liver into small pieces (1 × 1 × 1 mm) using microdissecting scissors (*see* **Note 21**). Keep on ice.
4. Optional (BLT model): Digest small tissue pieces using RPMI-1640 supplemented with collagenase and DNase I at 37 °C for 1 h. Gently disrupt the tissues by mixing every 15 min. Filter the cell suspension through a sterile 70 μm cell strainer. Isolate $CD34^+$ cells as stated above. If for the experiment cells arising from injected FL $CD34^+$ and transplanted FL pieces need to be discriminated, $CD34^+$ cells can be labeled (*see* **Note 22**).

3.8 Generation of a Transplantation Needle

1. A transplantation needle can be generated if necessary (*see* **Note 9**).
2. Smooth the tip of an 18-G hypodermic needle with sandpaper.
3. Cut the internal piece of an 18-G spinal needle to a length 2 mm longer than the hypodermic needle. Also smooth the surface of the internal needle with sandpaper.
4. Place the internal piece of the spinal needle inside the hypodermic needle.

3.9 Subcapsular Kidney Transplant

1. If necessary (*see* **Note 9**): generate a transplantation needle as described in Subheading 3.8.
2. Prepare fetal thymus and fetal liver as described in Subheadings 3.6 and 3.7, respectively.
3. Optional (BLT model): irradiate mice as described in Subheading 3.2. Wait for at least 4 h and not more than 24 h before starting transplantation procedure.
4. Anesthetize mice according to institutional protocols.
5. Shave the left side of the mouse from hip joint to shoulder joint using a hair clipper.
6. Load transplantation needle with fetal tissues: using Dumont tweezers, place a piece of fetal liver on the tip of the hypodermic needle, and aspirate it inside by drawing back the internal needle. Similarly, load a piece of thymus and a second piece of liver on the same needle, making a liver–thymus–liver sandwich.

7. Swab the left side of the mouse using alcohol swabs.
8. Make a longitudinal skin incision from approximately the last rib to the hip joint (2 cm).
9. Loosen connective tissue under the skin using the blunt side of scissors, until you see the kidney bulge beneath the musculature.
10. Pull the musculature above the kidney upwards with tweezers and make a small incision perpendicular to the axis of the kidney in the abdominal wall.
11. Using thumb and index finger of both hands, squeeze the kidney out of the abdominal cavity. Direct the pressure to keep spleen and gut in place while the kidney pops out. When the incision in the abdominal wall is small, the kidney will now remain in place outside the abdominal wall (as a button in a button hole).
12. Keep the kidney wet by regularly dropping a small amount of sterile PBS onto it using a 10 ml syringe with an 18-G needle.
13. Using Dumont tweezers, gently tear the kidney capsule near the lower pole, making a hole. Be careful not to damage the parenchyma of the kidney as to prevent bleeding.
14. Insert the transplantation needle (loaded with human fetal liver and thymus) through the hole and move the needle upwards towards the upper pole of the kidney, between the renal capsule and the kidney parenchyma. Make sure the kidney remains wet (by dropping sterile PBS onto it).
15. Push the internal needle to drive out the human fetal liver and thymus pieces. Make sure that the three pieces stay together while retracting the needle.
16. Gently pull two edges of the muscular wall around the kidney upwards with toothed tweezers, allowing the kidney to reenter the abdomen.
17. Close the abdominal wall using absorbable sutures.
18. Close the skin incision with wound staples or absorbable suture (*see* **Note 19**).
19. After surgery, make sure the mice maintain their body temperature by placing the cage on heat bags or by using a heat lamp.
20. Optional (BLT model): tissue implanted mice can be intravenously injected with CD34$^+$ cell suspensions from the same fetal liver on the day of tissue transplantation (a few hours after surgery). CD34$^+$ isolation is performed as described in Subheading 3.1.
21. Engraftment can be analyzed 10–12 weeks after transplantation (*see* **Note 20**).

4 Notes

1. Typically, a ^{137}Cs gamma irradiator is used but in theory any radiation device can be used (as described in Subheading 1.3).
2. Housing devices during irradiation may be Hepa filter top cages or special irradiation devices.
3. The use of human HSC requires approval by the ethical committee of your institution. Informed consent should be obtained. When using human organs, be sure to follow the appropriate (inter)national guidelines.
4. $CD34^+$ cells can be isolated using anti-CD34 magnetic activated cell sorting (MACS) beads (Miltenyi, Leiden, The Netherlands) or by fluorescence activating cell sorting (FACS).
5. Immunodeficient mice should be housed in specific pathogen free (SPF) conditions, in microisolator cages. When performing animal studies, be sure to follow the appropriate (inter) national guidelines.
6. Surgical instruments need to be sterilized by autoclaving before use or disposable instruments should be used.
7. HLA typing of fetal liver and fetal thymus can be done to enable matching or discrimination, depending on the experimental setup.
8. Typically, fetal thymus is of 14–23 weeks of gestational age and fetal liver 18–23 weeks [57].
9. A transplantation needle for injecting fetal thymus and liver can be constructed using 18-G hypodermic and spinal needles [58] or an 18-G trocar can be used [57].
10. Total volumes typically used for injection are: 50 μl for intrahepatic injection in newborn mice, 200 μl for intravenous injection in adult mice and 10 μl for intrafemoral injection in adult mice. Cell numbers typically used are: $5–50 \times 10^4$ for intrahepatic injection, $1 \times 10^5–5 \times 10^6$ cells for intravenous injection (when using more than 10^7 cells, the high viscosity will result in difficulties and mice may die soon after injection) and $5–10 \times 10^4$ cells for intrafemoral injection. Depending on the experimental setup, cell numbers may vary.
11. Cells need to be injected as soon as possible when resuspended in PBS, to avoid cell death.
12. All investigators should wear sterile gloves and rub clean bedding between their hands before handling newborn mice, to mask any foreign odors on pups and improve the chance that the mother will accept her pups back into the cage after completion of the procedure.

13. Pups should have milk in their stomach (seen as a white spot under the relatively transparent skin) before irradiation procedures can be started.
14. Depending on the immunodeficient mice strain used, a different amount of irradiation should be given (*see* Subheading 1.3). Newborn NSG or NOG mice are typically irradiated with 100 cGy, newborn BRG mice with 400 cGy.
15. The liver can be seen as a dark spot under the relatively transparent skin of the newborn pup and is situated above the white stomach (filled with milk).
16. Mice are best restrained by one investigator with one hand at their scruff and another holding hind legs and tale, and injected by another investigator. When performing the injection without a second investigator, mice can be held with two fingers (thumb and forefinger) at the scruff and little finger at the tail and injecting can be performed using the other hand.
17. Make sure not to overheat mice, the temperature should not exceed 30–32 °C (85–90 °F).
18. The topical nasal decongestant applied to the snout of the mother masks the foreign odors left by the investigators and improves the chance that the mother will accept her pups back into the cage after completion of the procedure (*see also* **Note 12**).
19. Staples are preferred as they are less easily removed by the mice.
20. Mice should be monitored regularly for wound healing and possible signs of distress. Wound staples should be removed after 7–10 days.
21. The diameter of the fetal thymus at 14–23 weeks of gestational age is typically 1–1.5 cm. The length of the fetal liver at 14–23 weeks of gestational age is typically 15–35 mm.
22. $CD34^+$ cells can be labeled with an intracellular dye (e.g., CFSE, PKH, or cell trace) or by transduction with a fluorescent marker (e.g., EGFP), depending on the experiment.

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Chapter 22

Using the Zebrafish Model to Study T Cell Development

Yong Zhang and David L. Wiest

Abstract

While zebrafish have for some time been regarded as a powerful model organism with which to study early events in hematopoiesis, recent evidence suggests that it also ideal for unraveling the molecular requirements for T cell development in the thymus. Like mammals, zebrafish possess an adaptive immune system, comprising B lymphocytes as well as both the γδ and αβ lineages of T cells, which develop in the thymus. Moreover, the molecular processes underlying T cell development in zebrafish appear to be remarkably conserved. Thus, findings in the zebrafish model will be of high relevance to the equivalent processes in mammals. Finally, molecular processes can be interrogated in zebrafish far more rapidly than is possible in mammals because the zebrafish possesses many unique advantages. These unique attributes, and the methods by which they can be exploited to investigate the role of novel genes in T cell development, are described here.

Key words Zebrafish, T cell development, Morpholino, Functional rescue, In situ hybridization

1 Introduction

Zebrafish are becoming a powerful vertebrate model for in vivo genetic studies of human development [1–3]. Zebrafish and mammals share similar blood cells and use common molecular pathways to regulate the production of blood cells including thymocytes [4–6]. In zebrafish, the first definitive HSCs derive from the ventral wall of the dorsal aorta at about 28–30 h post fertilization (hpf), then migrate to the caudal hematopoietic tissue, and finally home to the thymus and the kidney, where T cell development and adult hematopoiesis occur, respectively [7]. T cell progenitors marked by *ikaros* expression appear in the thymus by 3 days post fertilization (dpf) [7]. As in mammals, zebrafish T cell development gives rise to two T lineages, γδ and αβ, and is accompanied by the rearrangement of four T-cell receptor (TCR) loci (TCRα, TCRβ, TCRγ, and TCRδ) [8]. As in mammals, TCR genes in zebrafish are assembled by a V(D)J recombination process that is dependent upon the recombination activating genes, *rag1* and *rag2* [9].

Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5_22, © Springer Science+Business Media New York 2016

Table 1
Transgenic lines for studying thymocyte development in zebrafish

Reporter line	Gene promoter	Cells marked	References
Tg(itga2b:EGFP)^{la2}	*cd41*	Lymphoid stem cells	[13, 14]
Tg(ikzf1:GFP)^{fr101}	*ikaros*	Lymphoid stem/progenitor cells	[15]
Tg(rag2:EGFP)^{zdf8}	*rag2*	Lymphoid progenitors	[16]
Tg(lck:EGFP)^{cz1}	*lck*	T progenitors/T cells	[17]

The T cell specific nonreceptor tyrosine kinase *lck* is also found in the lymphoid precursors and maturing T lymphocytes in the bilateral thymic lobes of zebrafish [3, 10]. A recent review summarizes additional genes that play an important role in zebrafish and serve as markers of T cell development (*c-myb*, *ikaros*, *rag1*, *rag2*, *lck*, TCRα, TCRβ, TCRγ, TCRδ, *IL7R*, *jak3*, *ccr9a*, *ccr9b*, *zap70*, *gata3*, *runx1*, *foxn1*, etc.) [11].

Although the mouse remains the gold standard for immunological research, the zebrafish model provides a number of distinct advantages that complement the use of mice. Specifically, zebrafish females lay a large number of externally fertilized eggs that develop ex utero and this allows easy kinetic analysis of the effect of mutations that are lethal at early embryonic stages and would thus be very difficult to analyze during mouse development. Most zebrafish genes have mammalian orthologs, and the roles they play in hematopoiesis in general, and T cell development in particular, are highly conserved. Accordingly, insights gained into processes underlying T cell development in zebrafish will be applicable to mammalian biology [12]. Another significant advantage of using the zebrafish model is that embryos are transparent for the first 3 weeks of life. The optical clarity of zebrafish embryos allows visualization of development using fluorescent lineage tracers. These tracers enable real time imaging to study migration, colonization, and cell-fate determination of T progenitors without the need for sophisticated intravital two-photon microscopy [13, 14]. Transgenic fluorescent lineage tracers available for studying T cell development are shown in Table 1. For gene expression analysis, both the expression level and cell type distribution of transcripts can be easily assessed using whole mount in situ hybridization with antisense probes. Finally, gene function can be rapidly assessed using gain- and loss-of-function approaches. Indeed, the requirement of a gene can be readily examined by loss-of-function using antisense oligonucleotides called "morpholinos," which interfere with either splicing or translation of mRNA [18]. Morpholinos can be obtained by providing Gene Tools with the target sequence, following

which they will design sequence-specific oligos to block either mRNA translation or splicing (https://oligodesign.gene-tools.com/request/). Conversely, gain-of-function, overexpression experiments can be done either by simply injecting the egg with mRNA encoding the protein of interest or by using heat-inducible plasmids that allow manipulation of both the timing or level of induction. These advantages, which make zebrafish such a powerful model for the dissection of T cell development, are described here. Expected experimental outcomes are illustrated using our recent analysis of the role of the ribosomal protein Rpl22 in T cell development.

2 Materials

2.1 Zebrafish Facility, Husbandry, and Culture of Embryos

1. AHAB zebrafish housing systems (Aquatic Habitats, Aquatic Eco-Systems, Inc.): The fish room is maintained on a 14-h day/10-h night cycle. We maintain and breed Zebrafish at 28.5 °C under the standard aquaculture conditions published in The Zebrafish Book (http://zfin.org/zf_info/zfbook/zfbk.html). Embryos were staged as described previously [19].
2. Egg water stock (60×): pH 7.0, 300 mM NaCl, 10.2 mM KCl, 19.8 mM $CaCl_2$, 19.8 mM $MgSO_4$. Prepare 60× Stock by adding 17.5 g of NaCl, 0.76 g of KCl, 2.8 g of $CaCl_2$, and 4.9 g of $MgSO_4$ to 1 l Millipore Q water. Adjust pH to 7.0, and sterilize by autoclaving. Make a 1× working solution by diluting 60× stock into distilled water. Add methylene blue to a final concentration of 0.01 %. The solution can be stored for several weeks at room temperature (RT) (*see* **Note 1**).
3. Phenylthiourea (PTU): Prepare the 60× stock solution by dissolving 1.8 g powder in 1 L Millipore Q water and heating to 60 °C for several hours under agitation. Store at 4 °C. Make up the working solution by dilution to 1× with egg water. The use of PTU can prevent the embryos from developing pigment, thereby prolonging the developmental window during which they embryos are transparent.
4. Pronase: Dissolve 1 g powder in 100 ml egg water to make 10 mg/ml stock solution. Aliquot into 15 ml tubes and store at −20 °C.
5. Petri dishes (100 × 20 mm).
6. Transfer pipettes
7. Incubator for zebrafish embryos

2.2 Zebrafish Lines

Wild-type and transgenic zebrafish lines are available from Zebrafish International Resource Center (ZIRC, Eugene, OR). For examples described in this chapter, we employed the following fish lines: (1) AB wild-type fish; and (2) *Tg(lck:EGFP)*^{cz1} [17].

2.3 Morpholino Design and Preparation

1. Morpholinos (MO) (Gene Tools, Philomath, OR): We use morpholinos to knock down gene expression. The 25-base morpholinos are complementary with their target RNAs and can either inhibit mRNA translation or mRNA splicing. Translation-blocking morpholinos often decrease protein expression to levels undetectable by Western blot and begin to act immediately in fertilized embryos. Splice-blocking morpholinos act after the switch from maternal to zygotic transcription at 3.5dpf and allow selective deletion of particular protein domains.
2. Danieau buffer: pH 7.6, 5.0 mM HEPES, 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, dissolved in nuclease-free water.
3. The morpholino stock is dissolved in either Danieau buffer or nuclease-free water at a concentration of 1–3 mM in 5 μl aliquots. Stocks should be stored frozen at −80 °C or in airtight microtubes at RT to prevent evaporation (*see* **Note 2**). These are sample sequences for MOs targeting the zebrafish *rpl22* start codon (5′-CCGACAGTTTTGGCAGAAAGCCAGT-3′, designated "Rpl22 MO") and as well as a 5-base mismatch control (5′-CCCACACTTTTCGCACAAACCCAGT-3′, designated "Rpl22 MM").

2.4 Microinjection

1. Needle puller (Flaming/brown micropipette puller, Model P-97, Sutter Instrument Co.).
2. Borosilicate filamented glass capillaries (World Precision instruments, Inc.).
3. Microinjector (PM1000 cell microinjector, Microdata Instrument, Inc.). This microinjector requires a separate compressed air pump (Senco, Model: PC1010).
4. Micromanipulator holder (GJ-8 magnetic stand, Narishige Scientific Instruments).
5. Micromanipulator arm: M-152 (Narishige Scientific Instruments).
6. Dissecting stereomicroscope.
7. Plastic Mold for making injection plates: *See* Fig. 1a for a description of the plastic mold to make injection chambers. This can also be found in the Zebrafish Book (http://zfin.org/zf_info/zfbook/chapt5/5.1.html).
8. Stage micrometers.
9. Straight probes for micromanipulating zebrafish embryos (Fine Science Tools, Cat#: 10140-01).
10. Petri dishes (100 × 20 mm).

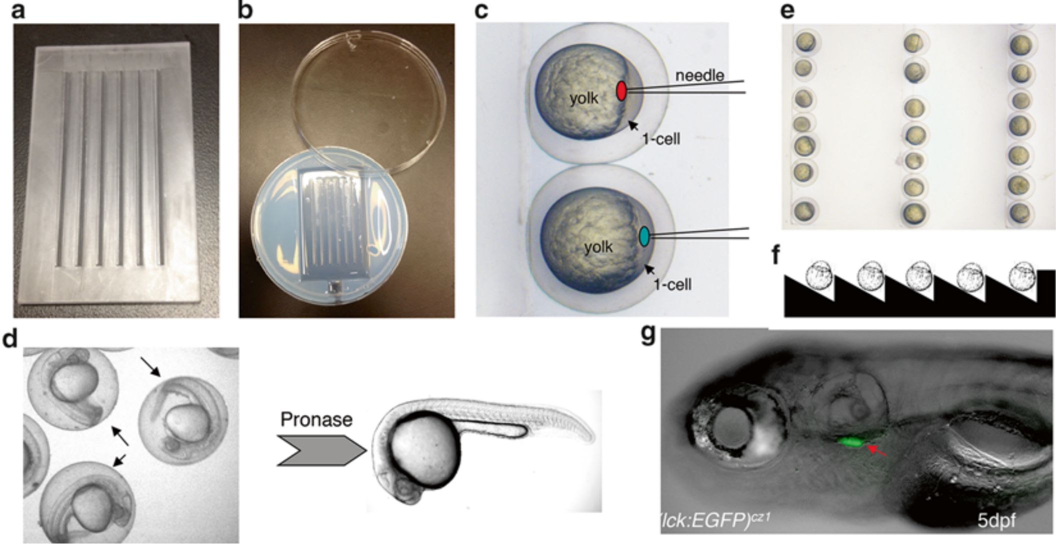

Fig. 1 Microinjection and dechorionation for zebrafish embryos. (**a**) Injection mold. (**b**) Injection chamber/plate. (**c**) Schematic of morpholino or plasmid injection. For morpholino knockdown, we inject the liquid (*red spot*) to the interface between cell and yolk. For plasmid overexpression, we directly inject the solution to the cytoplasm (*green spot*) at 1-cell stage. (**d**) Dechorionation process. The *arrows* indicate the chorions around the 24hpf embryos. (**e**) Alignment of the one-cell stage embryos in the trenches of the injection plate. (**f**) Schematic of the vertical section of the injection plate. (**g**) Lateral view of a 5dpf *Tg(lck:EGFP)*^{cz1} embryo. *Arrow* indicates the EGFP+ T cells in the thymus

2.5 Transient Rescue Experiments

1. Plasmid DNA preparation kit for highly purified DNA.
2. Water bath.
3. Construct: For heat shock mediated inducible expression, clone cDNA encoding the gene of interest into the heat-inducible pSGH2 vector [6, 20]. The pSGH2 vector contains two I-SceI restriction sites.
4. I-SceI meganuclease: (New England Biolabs) Aliquot 2 μl each upon arrival and store at −80 °C) along with I-SceI buffer (New England Biolabs), supplied with the NEBuffer I-SceI pack (*see* **Note 3**).

2.6 Western Blot [21]

1. Deyolking buffer: 55 mM NaCl, 1.8 mM KCl, 1.25 mM $NaHCO_3$.
2. Wash buffer: 10 mM Tris–HCl pH 8.5, 110 mM NaCl, 3.5 mM KCl, 2.7 mM $CaCl_2$.
3. 200 μl Precision tips.
4. Protease inhibitor cocktail (Roche).
5. PMSF.

6. SDS lysis buffer: 100 mM Tris pH 6.8, 4 % SDS, 200 mM DTT, and 20 % glycerol, supplemented with complete mini protein inhibitor cocktail (Roche).
7. MOPS running buffer.
8. Transfer buffer: 20 mM Tris, 150 mM glycine, 20 % methanol, and 0.038 % SDS.
9. 20× TBST solution: 500 mM Tris pH 7.4, 60 mM KCl, 2.8 M NaCl, and 1.0 % Tween 20 in high purity H_2O. Make 1× TBST by diluting 20× TBST solution with distilled H_2O.
10. Blocking buffer: 5 % nonfat milk powder in TBST.
11. Antibodies as needed. The following antibodies were used in the experiments described in this chapter: Anti-human Rpl22 serum (detects N-terminus of human Rpl22, Transduction Labs, San Jose, CA [22]), dilution: 1:1000; Anti-Actin (Sigma, AC-40), dilution: 1:2000. Secondary anti-rabbit or mouse IgG HRP-conjugated Antibodies (Cell Signaling), dilution: 1:1000.
12. 0.45 μm PVDF transfer membrane.
13. ECL substrate and chemiluminescent reagents.
14. Protein gel running and transfer equipment (e.g., Novex XCell *SureLock*® Mini-Cell and XCell II™ Blot Module, Life technologies or other similar).
15. Autoradiography films.

2.7 Whole-Mount TUNEL Assays

1. In Situ Cell Death Detection Kit, TMR red (Roche).
2. Acetone.
3. Permeabilization solution (0.1 % v/v Triton X-100 and 0.1 % w/v sodium citrate in PBS): 100 μl Triton X-100 and 1 ml 10 % sodium citrate in 98 ml of pH 7.4 PBS, freshly prepared.

2.8 Whole-Mount In Situ Hybridization

1. Fixative: 4 % paraformaldehyde in PBS. Dissolve 40 g of powder in 1 L of PBS by heating to 60 °C, then adjust the pH to 7.4. Fixative can be aliquotted into 50-ml tubes and stored at −20 °C (up to ~6 months).
2. Nuclease-free water.
3. PBS (10× Stock solution, pH 7.4).
4. Tween 20.
5. PBST: 1× PBS plus 0.1 % Tween 20.
6. Methanol.
7. Proteinase K stock: 10 mg/ml of proteinase K (Roche Diagnostics) in PBST.

8. RNA polymerase (T7, Sp6, or T3) and transcription buffer (Ambion).
9. DIG RNA labeling mix (Roche Diagnostics).
10. RNAse inhibitor (Ambion).
11. NucAway™ spin columns (Ambion,).
12. UltraPure formamide
13. 20× SSC stock solution
14. Prehybridization buffer (Hyb−): 50 % formamide, 5× SSC, 0.1 % Tween 20. Store at −20 °C.
15. Hybridization buffer (Hyb+): Hyb (−) plus 5 mg/ml RNA from Bakers Yeast (Sigma) and 50 μg/mL heparin (Sigma,). Store at −20 °C.
16. 2× SSCT: 2× SSC, 0.1 % Tween 20.
17. 2× SSCT/50 % formamide: 2× SSCT, 50 % formamide.
18. 0.2× SSCT: 20× SSC diluted to 0.2× with nuclease-free water, 0.1 % Tween 20.
19. Blocking reagent for nucleic acid hybridization.
20. Heat-inactivated FBS: Incubate FBS at 60 °C for 1 h, filter-sterilize, then store at −20 °C.
21. MAB buffer: 10 mM Tris pH 9.5, 100 mM maleic acid, 150 mM NaCl.
22. MABT: MAB plus 0.1 % Tween 20.
23. 10 % BMB Block: 10 % blocking reagent in MAB solution.
24. Complete Block: MABT plus 10 % heat-inactivated FBS, 2 % BMB Block.
25. Anti-digoxigenin–alkaline phosphatase antibody (Roche Diagnostics).
26. Staining buffer: 100 mM Tris–HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1 % Tween 20, 1 mM levamisole (add fresh from a 0.5 M stock).
27. Vector BCIP/NBT Staining solution: We use BCIP/NBT AP substrate kit IV (Vector Laboratories). Add two drops of kit solution per 5 mL of staining solution, mix; two drops solution 2, mix; and two drops solution 3, mix well.
28. 15 mm Netwell™ Insert with 74 μm Mesh Size Polyester Membrane (Corning,).
29. Orbital shaker.
30. Tecan NanoQuant machine for RNA/DNA quantitation (Tecan, Infinite M200).
31. Hybridization oven.

2.9 Mounting Embryos for Observation

1. Fluorescence stereomicroscope equipped with green fluorescent protein (GFP) and DsRed filters, DS-Fi1 digital camera, and imaging software.
2. Microscope depression slides (Science Enthusiast).
3. Tricaine: Prepare a 25× solution (4 mg/ml) by dissolving 1.2 g in 300 ml of water. Store at 4 °C.
4. Methylcellulose: Prepare a 3 % solution in egg water. The living or stained embryos are mounted in 3 % methylcellulose and photographed.

2.10 Fluorescence-Activated Cell Sorting (FACS)

1. 1× trypsin–EDTA.
2. TRIzol reagent.
3. 35 mm culture dish.
4. 40 µm nylon mesh filter.
5. FACSAria II (BD) or comparable cell sorter.

3 Methods

3.1 Identifying Zebrafish Ortholog

Many mammalian genes have zebrafish orthologs. We use both homology and synteny-based approaches to identify the zebrafish orthologs of mammalian genes. Conserved synteny (i.e., contiguous genes or ESTs with conserved map order on the chromosomes of different species) is a prominent feature of vertebrate genomes. Analysis of zebrafish genomic mapping data has revealed conserved rearrangements and homology segments between zebrafish and human genomes [5]. Thus, the homology based syntenic analysis can be used to effectively predict and find orthologous genes between zebrafish and human.

1. Go to http://www.ensembl.org/Homo_sapiens/Info/index.html and enter the name of the human gene (e.g., *RPL22*) in the SEARCH section.
2. Click on ContigView, then the gene name, to get the amino acid sequence as well as genomic position.
3. Go to http://www.ncbi.nlm.nih.gov/, select tBLASTn and paste the amino acid sequence in the dialog box. Select "Nucleotide selection (nr/nt)" in the "Choose search set-Database" window, and "zebrafish" in the "Organism" window, and click on BLAST, followed by clicking on "Formatting" in a new window to get the search summary.
4. Record the zebrafish gene name (e.g., *zgc:123327*, *rpl22*), then go to http://www.genome.ucsc.edu/ to identify the genomic position of this gene.

5. Repeat these steps for other genes located near the gene of interest until it is clear that there is a good syntenic relationship.
6. Next align the amino acid sequences of the human, mouse and zebrafish orthologs of your gene of interest. The Clustal W2 algorithm is an effective tool for this task (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3.2 Preparation of Microinjection Plates/Needles

1. Prepare a 1.5 % agarose solution with 1× egg water.
2. Pour enough molten agarose into a 100 cc petri dish to cover the bottom with a thin layer, then allow to solidify at room temperature. When the agarose is solid, pour another layer of molten agarose on top and place the plastic injection mold (Fig. 1a) into the agarose solution. Make sure no bubbles are trapped under the mold. Cover and allow it to cool.
3. Overlay the injection plate with ddH_2O and store at 4 °C (*see* Fig. 1b).
4. Pull microinjection needles using the P-97 micropipette puller. Load the glass capillary tubes into the heating chamber with the middle part of the capillary surrounded by a platinum heating film. The platinum heating film electrically heats up the glass capillary, enabling a horizontal linear force to pull the heated glass apart to produce two separate needles. Store needles into a petri dish on a row of plasticine to prevent the needles from breaking.
5. Under a stereomicroscope, use a blade to cut the tip off of the needle. A good microinjection needle should be long and sharp but not flexible. This will minimize damage to the embryo and help to pierce the chorions.

3.3 Preparation of Zebrafish Embryos

1. The procedures to follow to set up matings are described in detail in the zebrafish book (http://zfin.org/zf_info/zfbook/zfbk.html). Briefly, set up well-fed fish (6–18 months old) the night before in pairs (two males and two females) in the mating chamber with the divider in place. The next morning, remove the divider to allow the fish to spawn.
2. Collect embryos approximately 20 min after female and male zebrafish begin to mate. Visually inspect the embryos to ensure that the clutch is uniformly at the early single cell stage. For gene knockdown analysis, as well as for most other manipulations of zebrafish embryos, it is best to inject into the cytoplasm of one-cell stage embryos, which produces the most consistent results.

3.4 Dechorionation

The chorion is the membrane around the developing zebrafish embryo (*see* Fig. 1d, arrows). Embryos should be removed from their protective chorions to facilitate further observation, fixation, or other manipulations. Removal of the chorion, or dechorionation, is accomplished by pronase digestion of embryos older than 18hpf. We generally dechorionate at 24hpf.

1. Dilute 10 mg/ml pronase stock with egg water to 2 mg/ml working solution.
2. Transfer the embryos to a petri dish, removing as much egg water as possible. Add 2 ml of the diluted pronase solution to the dish and swirl the embryos. Incubate at room temperature for 10 min.
3. Use a Pasteur pipette to gently transfer the embryos to a fresh petri dish full of egg water. Most of chorions will have been removed at this time (*see* Fig. 1d).
4. Rinse the embryos thoroughly (at least three times) with egg water to remove the pronase.

3.5 FACS Sorting of Lineage Marked T Cell Progenitors

1. Transgenic zebrafish embryos produced as above from appropriate lines [Table 1; e.g., *Tg(lck:EGFP)*^{cz1}] are dechorionated by pronase treatment. For FACS sorting of fluorescently labeled progenitors, we use about 100 transgenic embryos (5dpf, *see* Fig. 1e) that have been grown in the 28.5 °C incubator.
2. To anesthetize the embryos, transfer them to a petri dish containing Tricaine in egg water at 0.2 mg/ml and incubate until they are no longer swimming.
3. Transfer anesthetized embryos to deyolking buffer (keep on ice) and pass them through a 200 μl pipette tip several times to remove their yolk.
4. Dissect and macerate deyolked whole embryos in ice-cold 0.9 × PBS plus 5 % FBS using a scalpel blade to facilitate digestion.
5. Transfer embryos to a 35 mm culture dish containing 1× trypsin–EDTA solution and incubate for 30–60 min at 32 °C. During incubation, gently agitate by pipetting every 10 min.
6. Terminate the digestion by adding $CaCl_2$ to a final concentration of 1 mM and fetal calf serum to 10 %.
7. Pellet the cells for 5 min at 400 × *g*, then wash twice with 0.9 × PBS at 400 × *g*.
8. Filter the suspension through 40 μm nylon mesh to eliminate debris (Keep cold).
9. Pellet and resuspend the cells in 0.9 × PBS/5 % FBS. Stain with 1 μg/ml propidium iodide (PI) 30 min on ice to identify dead cells and debris.

10. Perform FACS on the resulting single cell suspensions at room temperature. Use non-transgenic AB strain zebrafish cells to set the gate control, then sort the progenitors of interest (e.g., lck-GFP$^+$ T cell progenitors) and GFP$^-$ cells directly in TRIzol, if you are preparing RNA.
11. Isolate total RNA from the purified cells by TRIzol extraction according to manufacturer's instructions. Quantitative or conventional PCR can then be used to assess mRNA expression of the gene of interest.

3.6 Morpholino Microinjection

1. Before injection, heat the morpholino (MO; antisense oligonucleotide) in a thermocycler for 10 min at 65 °C to dissolve any precipitates that can clog the microinjection needle.
2. Assemble the needle to the microinjection arm.
3. Turn on air the compressor pump and PM1000 microinjector.
4. Press the "Timer" and "BALN" buttons to activate balance pressure. Press the "VENT" button.
5. Use the "Fill" button to load the needle with the MO targeting your gene of interest.
6. Use the "Inject" knob on the injector panel to adjust the injection air pressure and set the injection time. For the PM1000 cell microinjector, we use 60 ms injection time and 10-psi injection pressure as the starting point. Use the "Balance" knob to adjust the balance pressure between 0 and 0.4 psi to prevent both liquid from leaking out of the needle and medium from flowing back into the needle.
7. Place a drop of mineral oil on the microscope stage micrometer. Submerge the tip of the injection needle into the mineral oil and press the foot pedal to finish the injection. The released droplet will form a perfect sphere in the oil. Measure the diameter of the droplet and calculate the volume according to $4/3\pi R^3$. Adjust the injection pressure and injection time to produce the desired injection volume and dose of morpholino (*see* Table 2).
8. Transfer embryos into the trenches of the injection plate using a Pasteur pipette (Fig. 1e).
9. Use the fine probe to align the embryos, with the animal pole facing you (*see* Fig. 1e, f).
10. Manipulate the injection arm to advance the needle, gently piercing the chorion of the embryos. Inject the morpholino into the interface between cytoplasm and yolk of one-cell stage embryos (*see* Fig. 1c, lower panel and **Note 4**).
11. Move the injection plate and repeat until all of the embryos on the plate have been injected, then transfer the embryos to a clean dish containing egg water, and place in 28.5 °C incubator.

Table 2
Morpholino (MO) dose per microinjection

Diameter of drop (μm)	Radius of drop (μm)	Volume (pL) $4/3\pi R^3$	MO dose (ng) of 0.5 mM = 4.15 ng/nl	MO dose (ng) of 2 mM = 16.6 ng/nl
1	0.5	0.52	0.00	0.01
2	1	4.19	0.02	0.07
3	1.5	14.13	0.06	0.23
4	2	33.49	0.14	0.56
5	2.5	65.42	0.27	1.09
6	3	113.04	0.47	1.88
7	3.5	179.50	0.74	2.98
8	4	267.95	1.11	4.45
9	4.5	381.51	1.58	6.33
10	5	523.33	2.17	8.69
11	5.5	696.56	2.89	11.56
12	6	904.32	3.75	15.01
13	6.5	1149.76	4.77	19.09
14	7	1436.03	5.96	23.84
15	7.5	1766.25	7.33	29.32

3.7 Western Blot Analysis

1. To examine protein expression, dechorionate embryos using pronase as above, then wash three times in egg water to eliminate residual pronase.
2. Transfer both control and morpholino injected embryos to 1.5 ml microcentrifuge tubes. We found that approximately five embryo equivalents were required to detect Rpl22, but this will vary with protein abundance and antibody quality.
3. Centrifuge at 300 × *g* for 5 min and remove all residual egg water. Add 200 μl deyolking buffer and pipet up and down with a 200 μl tip until the yolk is disrupted. 200 μl deyolking buffer is enough for up to 100 embryos and the volume should be adjusted in proportion as the number of embryos is varied.
4. Agitate the tubes on the orbital shaker for 5 min at 12.85 × *g* to dissolve the yolk.
5. Centrifuge at 300 × *g* for 30 s and discard the supernatant. Wash two more times, then add 0.5 ml wash buffer to the pellets, agitate for 2 min at 12.85 × *g*, and pellet as above.
6. Solubilize the pelleted embryos in 2 μl 2× SDS-sample buffer per embryo and heat for 5 min at 95 °C. No homogenization is necessary, as the cells should dissolve rapidly in the buffer.

7. Remove insoluble material by centrifugation at 4 °C for 10 min at 12,000 × *g*. After collection, the supernatant can be immediately run on an SDS-PAGE gel or stored at −80 °C in aliquots.
8. Approximately five embryo equivalents are loaded per lane, resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted. The sample immunoblot employed mouse anti-actin monoclonal antibody and rabbit anti-Rpl22 serum, followed by incubation with appropriate secondary HRP-conjugated-Abs. Bound antibody was visualized by enhanced chemiluminescence (Fig. 2b) (*see* **Note 5**).

3.8 Fluorescent Microscopy

1. T cell development in *Tg(lck:EGFP)*^{cz1} embryos is clearly evident by 5dpf. To examine the effect of gene knockdown on T cell development in this model, the embryos must be immobilized. To accomplish this, place 5dpf morphant embryos (i.e., morpholino-injected) in a petri dish containing Tricaine in egg water at 0.2 mg/ml and incubate until the embryos are no longer swimming. This usually requires about 5 min.
2. 3 % methylcellulose works well for mounting 5dpf embryos for regular microscopic observation and photography. To mount embryos, add a drop of 3 % methylcellulose to the microscope depression slide.
3. Transfer the embryo using a Pasteur pipette and position it in the methylcellulose.
4. Use the steel probe to precisely orient the embryo for lateral or dorsal view.
5. Photograph using a fluorescence stereomicroscope (*see* Fig. 2c).

3.9 Whole-Mount TUNEL Assays

To determine if thymocytes are undergoing apoptosis, we employ TUNEL staining.

1. Fix Embryos in 4 % PFA overnight at 4 °C.
2. Dehydrate in graded methanol/PBS (50, 70, 95, 100 %), incubating for 5 min at room temperature at each step and then storing at −20 °C overnight.
3. Incubate for 10 min in acetone at −20 °C.
4. Wash embryos twice in PBS for at least 5 min per wash.
5. Permeabilize the embryos for 15 min at RT in 0.1 % Triton X-100 plus 0.1 % sodium citrate/PBS (freshly prepared). Longer incubation times may be necessary when working on older embryos.
6. Wash twice in PBS, 10 min per wash.

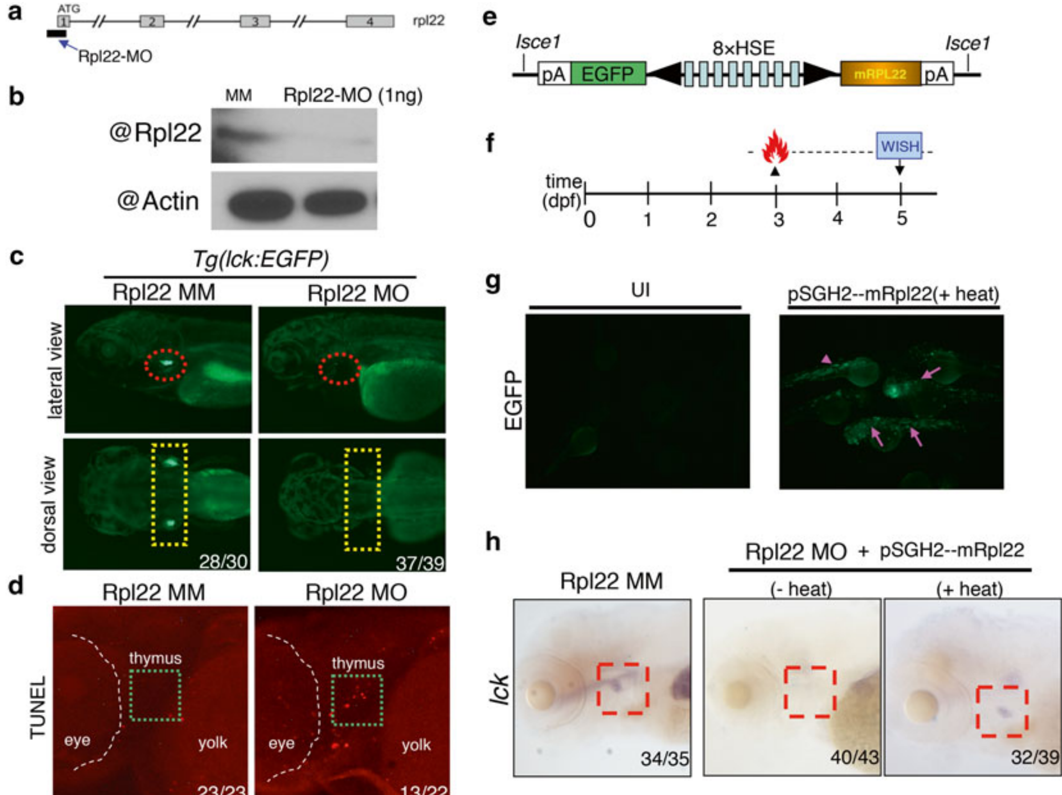

Fig. 2 Using zebrafish model to investigate the role of Rpl22 in T cell development. (**a** and **b**) Targeted knockdown of Rpl22 in zebrafish embryos. (**a**) For Rpl22, the MO is designed to specifically block the initiation of translation at the start codon in exon 1. The *short black line* and *blue arrow* indicate the positions of sequences targeted. (**b**) The effect of 1 ng Rpl22-MO knockdown on expression of endogenous Rpl22 is assessed by immunoblotting on 5dpf embryos. (**c**) Rpl22-MO injected (1 ng), but not MM (5-base pair mismatch) control injected, *Tg(lck:EGFP)* fish exhibit a loss of EGFP-marked T cells at 5 dpf (lateral view, *red circles*; dorsal view, *yellow rectangles*). *Numbers* refer to fraction of morphants with the depicted phenotypes. (**d**) Injection of 1 ng Rpl22-MO causes apoptosis in the thymus at 3.5 dpf, while injection of the same quantity of 5-base pair mismatch control morpholino (Rpl22-MM) caused no change in the thymus (*green dashed rectangles*). The *white dashed line* delimits the eye. *Numbers* refer to fraction of morphants with the depicted phenotypes. (**e**–**h**) The use of a heat-shock inducible, bidirectional expression construct to restore T cell development in *rpl22* morphants. (**e**) *Upper panels* depict schematics of the heat-inducible expression plasmid pSGH2-mRpl22. (**f**) The heat shock-inducible expression plasmid associated with I-sceI mediated transient overexpression is injected into one-cell stage embryos. At 3dpf, the embryos are heat shocked at 37 °C for 1 h and then effects on T cell development are analyzed at 5 dpf using WISH for *lck*, a marker of thymic progenitors. (**g**) After heat-shock, GFP+ embryos are selected for subsequent WISH analysis (*pink arrowheads*). (**h**) Heat shock induction of mouse Rpl22 expression rescues T cell development in *rpl22* morphants (5dpf *lck* WISH staining, *red rectangles*). *Numbers* refer to fraction of morphants with the depicted phenotypes

7. Add 50 μl enzyme solution (containing terminal deoxynucleotidyl transferase) to 450 μl label solution (containing nucleotide mixture in reaction buffer) to obtain a total of 500 μl TUNEL reaction mixture, then mix well (Roche TMR Kit instructions).
8. Incubate embryos for 1 h at 37 °C in the dark in TUNEL reaction mixture in a 1.5 ml, round-bottom microcentrifuge tube. 100–150 μl should be enough for up to 50 embryos.
9. In the dark, wash for 2 h (4 × 30′ in PBST), then photograph (Fig. 2d).

3.10 Whole-Mount In Situ Hybridization

Whole mount in situ hybridization (WISH) employs an antisense ribonucleotide probe and can be used for a number of purposes including determining the tissue distribution and gross expression level of an mRNA species and/or the presence of a particular cell population marked by a lineage-restricted mRNA.

1. To prepare DNA for making riboprobes, linearize the plasmid encoding your probe and purify by two rounds of phenol–chloroform extraction. Check 1 μl of digested DNA on a 1 % agarose gel to ensure the DNA template is linear. Quantify the DNA concentration of the template (e.g., Tecan NanoQuant).
2. To transcribe the riboprobes, add the following to the transcription reaction: 1 μg of linearized DNA template, 2 μl of 10× transcription buffer, 2 μl of DIG-labeling RNA mix, 1 μl of RNase inhibitor (40 U/μL), 2 μl of RNA polymerase (T7, T3, or Sp6), and enough nuclease-free water to bring the reaction to 20 μl. Incubate the reaction at 37 °C for 2 h, then purify the riboprobe using a Ambion NucAway spin column. Assess the quality and yield of the probe by running 1 μl of the probe on an agarose gel and quantifying the concentration. Probes can be stored at −80 °C for up to 2 years.
3. Dechorionate and fix 24 h embryos using pronase. After dechorionation, grow the embryos in egg water containing 0.003 % PTU to prevent pigmentation, which starts to develop at around 24 hpf. Fix embryos at 5dpf (or another developmental stage appropriate for your experiment) with 4 % fix solution and rock overnight at 4 °C (*see* **Note 6**). Thymocyte seeding can be observed by 3.5–4dpf, but the optimal time to assess whether knockdown of a gene blocks development is 5dpf. Wash embryos in PBST three times, 5 min each. Wash with 100 % methanol, incubate for 5 min, then transfer the embryos to fresh methanol and store at −20 °C.
4. Rehydrate embryos for 5–10 min each in 75 % Methanol/PBST, 50 % Methanol/PBST, and 25 % Methanol/PBST. Wash embryos in PBST once quickly, then twice more for 5 min each.

5. Digest embryos with 10 μg/ml Proteinase K in PBST for 30 min at room temperature with gentle rocking. These conditions work well for zebrafish embryos from 3 to 5 dpf. Proteinase K is made from a 10 mg/ml stock stored at −20 °C.
6. Remove the proteinase K with three quick PBST washes.
7. Re-fix embryos for 1 h at room temperature. This step is necessary to eliminate residual proteinase K.
8. Remove the fixative with three quick PBST washes.
9. Hybridization: Preheat in situ hybridization buffer Hyb(−) and Hyb(+) solutions to 65 °C. The temperature should be consistent throughout the hybridization procedure. Add Hyb(−) to the 1.5 ml tubes containing embryos and shake gently for 15 min at 65 °C. Replace the Hyb(−) with Hyb(+) and shake gently for 1 h at 65 °C. Add probes (typical range is 0.3–1.0 ng/μl) and rock gently overnight at 65 °C.
10. Wash embryos in 2× SSCT/50 % formamide twice for 30 min at 65 °C.
11. Wash embryos in 2× SSCT twice for 15 min at 65 °C.
12. Wash embryos in 0.2× SSCT twice for 30 min at 65 °C.
13. Wash embryos in MABT three times at room temperature, 10 min each.
14. Transfer the embryos from MABT to complete blocking solution in 12-well plates containing 15 mm Netwell™ inserts with polyester membrane and incubate with gentle shaking for at least 2 h at room temperature (*see* **Note 7**).
15. Remove complete blocking solution and replace it with anti-digoxygenin-AP antibody at 1:5000 dilution in complete blocking solution and rock overnight at 4 °C.
16. Wash embryos in complete blocking solution, 1 h at room temperature.
17. Wash embryos in MABT solution, 30 min at room temperature.
18. Wash embryos in staining buffer at room temperature, 3 × 5 min, with gentle rocking.
19. Wash in 0.1 M Tris pH 9.5 for 5 min at RT with gentle rocking.
20. Add Vector BCIP/NBP staining solution. Wrap the plate in aluminum foil and incubate in the dark at room temperature for 20–60 min monitoring the development of staining every 20 min. Alternatively, the embryos can be stained overnight at 4 °C.
21. Stop the staining reaction by washing in PBST with gentle rocking 3× for 5 min at room temperature.

22. Replace PBST with 50 % glycerol/PBS and wash for 15 min at room temperature. Glycerol is the mild clearing reagent that makes the stained embryos more transparent for photography. Transfer embryos to a six-well plate containing 100 % glycerol, then store at 4 °C.
23. Photograph. Effects of gene knockdown on development of the T lineages can be assessed using the *lck* probe (*see* Fig. 2h).

3.11 Heat Inducible Gene Expression to Rescue Developmental Arrest Caused by Gene Knockdown

In zebrafish, it is critical to perform rescue experiments to ensure that phenotypes resulting from MO-mediated gene knockdown are truly due to the absence of the intended target, rather than due to off-target effects. This can be conveniently accomplished by injecting MO treated embryos with MO-resistant forms of the mRNA encoding the intended target; however, because injected mRNA lasts only a few days, mRNA overexpression is only effective for phenotypes at early embryonic stages. For later developmental stages more than 3dpf, heat-shock inducible DNA constructs can be used to temporally overexpress exogenous genes to study T cell development [6, 20]. The I-SceI meganuclease was originally utilized in *Medaka* to induce stronger promoter activity in the F0 founder. Coinjection of I-SceI significantly improves the specificity of transient expression and decreases the mosaicism in zebrafish [23]. We have employed this approach to ensure that ectopic expression of Rpl22 can restore the block in T cell development observed in *rpl22* morphants at 5dpf. In doing so, we heat induced expression of mouse Rpl22 at 3dpf and we identified phenotypic rescue of thymocyte development by performing WISH with an *lck* probe at 5dpf (Fig. 2e–h).

1. To enable heat-inducible expression of a gene of interest, clone the cDNA of the gene in question into pSGH2 [20]. pSGH2 enable simultaneous expression of the gene of interest as well as GFP to mark embryos that have productively incorporated the vector (*see* Fig. 2e).
2. Upon cloning, prepare purified pSGH2 expressing the gene of interest, using a high purity plasmid preparation kit.
3. Collect the one-cell stage embryos and align them on the microinjection plate (*see* **Note 8**).
4. Mix pSGH2 plasmid with 0.5× I-SceI buffer and 0.5 U/μl I-SceI meganuclease (New England Biolabs) and microinject (100 pg) directly into the cytoplasm of one-cell stage embryos.
5. At 24hpf, check the quality of the injected embryos and remove the dead embryos. Dechorionate the embryos by pronase treatment and then culture at 28.5 °C to 3dpf.

6. At 3dpf, transfer the injected embryos into the 1.5 ml tubes filled with 1 ml egg water, heat shock at 37 °C water bath for 1 h, then resume culturing the embryos at 28.5 °C (*see* **Note 9**).
7. pSGH2 vector has the heat shock-inducible bidirectional promoter consisting of multimerized heat shock elements (HSE). Thus, this heat shock-inducible expression construct allows temporal control of ectopic expression of the gene of interest, coupled with EGFP marking of expressing embryos (*see* Fig. 2e–g). After heat-shock, GFP+ embryos are selected for analysis (Fig. 2g). In Fig. 2h, WISH with an *lck* probe was performed to identify developing T cells. This revealed that the developmental arrest caused by Rpl22 knockdown can be rescued by a morpholino resistant form of Rpl22 (pSGH2-Rpl22) in a heat-inducible manner.

4 Notes

1. Methylene blue is used to prevent fungal growth in egg water; however, it can also induce autofluorescence, especially for embryos after 3dpf. Therefore, for observation under fluorescent microscopy, we recommend that embryos should be cultured in egg water without methylene blue.
2. For morpholino storage, GeneTools recommends to store aliquots at RT. We use airtight microtubes to prevent evaporation. If morpholino aliquots are stored for a long time, GeneTools recommends freeze-drying the oligo. MOs should be heated up at 65 °C for 10 min before microinjection to dissolve possible precipitates.
3. Due to the low stability of I-sceI meganuclease, aliquots of enzyme should be prepared (e.g., 2 μl) upon arrival and stored at −80 °C. The microinjection mix should be prepared immediately before injection and kept on ice.
4. We inject the morpholino at one-cell stage to get more consistent results for T cell phenotypes at 5dpf.
5. We have an excellent antibody for testing the efficiency of Rpl22 translation-blocking MO. Figure 2b shows a sample immunoblot of Rpl22 expression in controls and embryos treated with 1 ng of a morpholino that blocks translation (Fig. 2a, b). However, sometimes antibodies are not available for detection of zebrafish proteins. Thus, an alternative way of evaluating the efficacy of start-site morpholinos that do not alter mRNA size, is to produce a chimeric mRNA comprising the target sequence surrounding the target genes' ATG fused in frame with GFP-coding sequence. Co-injection of this fusion mRNA with MO will reveal whether the morpholino

blocks GFP synthesis, which serves as a surrogate marker for expression of the endogenous target protein. Splice-blocking morpholinos can also be used to knockdown target genes, and the efficiency checked by assessing the predicted size shift by RT-PCR.

6. For embryos older than 18 hpf, the chorion should be removed before fixation to avoid a curved tail after fixation.
7. We use inserts with a polyester membrane to conveniently transfer the embryos between wells during WISH washing and staining steps.
8. The heat-inducible plasmid should be directly injected into the cytoplasm of the cell. We strictly use only one-cell stage embryos to ensure that the transient overexpression has consistent effects on phenotypic rescue.
9. Temperature or incubation time of heat-shock treatment may be varied for different ectopically expressed genes. Increasing the temp/incubation time may increase the extent of overexpression; however, excessive heating may also kill embryos. Different conditions should be tested to identify the best compromise between overexpression and survival.

Acknowledgments

We are grateful to the support and help from Dr. Jennifer Rhodes, Allison Ulrich and Alison N. Bilbee for the zebrafish work. We gratefully acknowledge the assistance of the following core facilities of the Fox Chase Cancer Center: Flow Cytometry, DNA Sequencing, Imaging and Laboratory Animal/Zebrafish. This work was supported by NIH grants AI081814, AI073920, NIH core grant P01CA06927, Center grant P30-DK-50306. Y.Z. is a W.J. Avery Postdoctoral Fellow of Fox Chase Cancer Center.

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Rémy Bosselut and Melanie S. Vacchio (eds.), *T-Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1323, DOI 10.1007/978-1-4939-2809-5, © Springer Science+Business Media New York 2016

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