Review

The Late Stage of T Cell Development within Mouse Thymus

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After positive selection and lineage commitment, the TCRαβ$^+$CD4/CD8 SP medullary thymocytes migrate into and reside in thymic medulla, where they undergo an ordered program of late stage of T cell functional maturation and negative selection to delete self-reactive clones by apoptosis. Accomplishment of this final differentiation pathway, a physiological T cell repertoire is formed: T cells acquire immunocompetence to respond to foreign antigens and tolerance to self-antigens, ready for the emigration to homing to the T cell regions of peripheral lymphoid organs and tissues. In this review, emphases are put on introducing the approaches applied in this area and our own observations. Basically, we have analyzed the late stage of medullary thymocyte phenotypic differentiation pathways of both CD4 SP and CD8 SP medullary thymocytes and the concomitant functional maturation pathway, in particular, of CD4 SP thymocytes. It is to provide a standard to compare the functional capacity of the cells at the developmental stages induced by different conditions. The cellular and molecular basis of this differentiation process has been partially described. *Cellular & Molecular Immunology*. 2004;1(1):3-11.

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Thymus is the essential organ dedicated to the development of T lymphocytes from early thymocyte progenitors (ETPs) (1, 2) to differentiating into functional competent T cells ready for emigration to the periphery. Within thymus, the ETPs undergo a program of multiple stages with the major events occurred at the distinct regions of thymic microenvironment and tightly regulated by cell-cell interactions with local thymic stromal cells of various types and soluble factors. Developing thymocytes at each major stage is differentiated at the specialized thymic region. Thymocytes at disparate stages express distinct phenotypes. As a consequence, the thymus contains heterogeneous populations of thymocytes. According to the cell phenotypes defined by expression of T cell receptor(TCR) complex, CD4 and/or CD8 coreceptors, the thymocytes can be simply divided into four major subsets, namely, CD4$^-$CD8$^-$(DN), CD4$^+$CD8$^+$(DP), CD4$^+$CD8$^-$(CD4 SP), and CD4$^-$CD8$^+$(CD8 SP) thymocytes, which constitute 5%, 80-85%, 10% and 5% of the total thymocytes, respectively. Based on the reconstitution capability of thymocyte subsets and their kinetic appearance in fetal thymus, the developmental sequence of thymocytes can be delineated as: DN→DP→TCRαβ$^+$CD4/CD8 SP pathway. During the stage of CD4$^-$CD8$^-$ (DN) thymocytes, the TCR gene rearrangement and the TCRβ selection are processed in the outercortex, which conducts DN thymocytes to develop into CD4$^+$CD8$^+$ (DP) thymocytes and migrate to the cortex, where TCRα gene is rearranged and functional TCRαβ molecules are expressed on cell surface to initiate the positive and the negative selection. After the primarily positive selection, the survival CD69$^+$DP thymocytes develop into lineage committed TCRαβ$^+$CD4$^+$CD8$^-$ or TCRαβ$^+$CD4$^-$CD8$^+$ (SP) medullary thymocytes and reside in thymic medulla for the late stage of differentiation (2, 3).

A concept of late stage of thymocyte development

After positive selection, the lineages committed TCRαβ$^+$ CD4 SP and TCRαβ$^+$CD8 SP medullary thymocytes reside in thymic medulla for 10-14 days before exiting the thymus (4). It was a long time argument if these cells acquired functional competence once they were positively selected and then what they did during their stay in thymic medulla. The physiological possibility is that they undergo further differentiation in order to accomplish functional maturation and generate immunocompetent T cell repertoire. One claim is that positive selection is sufficient for cortical type thymocytes in RelB-deficient mice to develop into functional CD4/CD8 SP thymocytes after positive selection in the cortex and then directly emigrate to periphery (5). It seems that there is no need for thymic medulla. The evidence was based on the experiments in RelB$^{-/-}$mice, in which the thymic medulla was disorganized as lack of dendritic cells and a type of thymus medullary epithelial cells, but the functional TCRαβ$^+$CD4 SP and TCRαβ$^+$CD8 SP thymocytes were generated. However, the residual thymic medullary stromal cells were present in RelB$^{-/-}$ mice which might contribute to the generation of SP thymocytes (6, 7). In addition, significant thymus atrophy, markedly reduced thymic cellularity, the undeveloped paracortex in lymph nodes, the weak delayed hypersensitivity responsive to antigen stimulation, and the generated T cells are functionally defect in response to TCR signaling (7, 8). All

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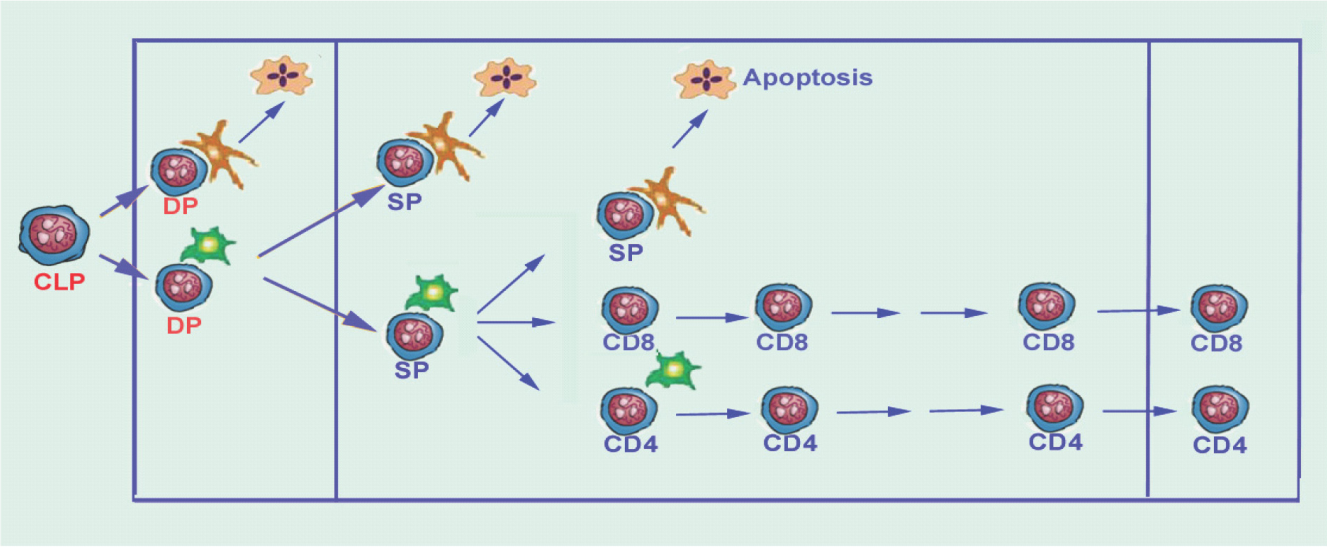

Figure 1. The hypothesis of second thymic selection processed in thymic medulla. In mouse thymus, the DP thymocytes are imposed for the positive and the negative selection mediated by TCR-self peptide/MHC complex. The negatively selected cells are died by apoptosis, whereas the positively selected cells modulate their phenotypes into TCRαβ$^+$CD4/CD8 SP thymocytes and migrated to thymic medulla. The virgin SP medullary type thymocytes are not functional. They undergo further selection imposed in thymic medulla, through which to further delete self-reactive T cell clones and acquire immunocompetence, thereby to form a functional T cell repertoire ready to exit to the periphery. We have termed this process occurred in thymic medulla as the second thymic selection.

these consequent results suggest that cell functional development is incomplete and T cells are functionally aberrant in the absence of thymic medulla in RelB-deficient mice. Moreover, in normal mice, all the SP thymocytes are localized in the thymic medulla and only half of the medullary CD4/CD8 SP thymocytes are functional in response to ConA by proliferation and cytotoxicity (9), whereas the thymocyte emigrants exhibit mature phenotype of medullary thymocytes and fully functions (10). Therefore, the functional maturation is perspectively accomplished in thymic medulla in normal mice. Recently, increasing evidence demonstrates that medullary thymocytes are processed for functional maturation within thymic medulla. The newly emerged TCRαβ$^+$CD4/CD8 SP thymocytes are non-functional or low-functional, whereas the thymic emigrants are highly functional and there are many medullary thymocytes possess heterogeneous phenotypes and distinct functional levels between two extremes (11-13). Thus, the majority of post-selected CD4 SP (also for CD8 SP) thymocytes require the thymus to produce long-lived, functional T cells (14). Apart from functional maturation, thymic medulla has been firmly demonstrated that it is the region for the negative selection of SP thymocytes (15-16). In addition, the medullary thymocytes are expanded at certain degree through cell proliferation in thymic medulla (3, 17) in order to have an appropriate number of competent T cells to fulfill the periphery T cell pool. It is now clear that the TCRαβ$^+$ CD4/CD8 SP medullary thymocytes have undergone a final stage of differentiation in thymic medulla; it is the process consisting both the functional maturation to acquire lineage-specific immunocompetence and the further deletion of self-reactive cells as well as the dysfunctional cells. As this process is also mediated by the interaction with developing medullary thymocytes and medullary thymic stromal cells, it is in the sense of a continuation of both the thymic positive selection and the negative selection. We previously called it as the second thymic selection in the thymic medulla (18) (Figure 1), or termed as post-selection maturation events by others (19, 20).

Characteristics of phenotypic differentiation pathway of medullary thymocytes

Accumulating evidence indicates that both CD4 SP and CD8 SP thymocytes are phenotypically and functionally heterogeneous (12, 21-24). In mice, the functional CD4 SP medullary thymocytes were found to be present in a small proportion of Qa-2$^+$ cells, whereas the majority of Qa-2$^-$ CD4 SP medullary thymocytes were not functional (11). By adoption of other cell surface markers, the newly emerged virgin mouse SP thymocytes expressing a characteristic phenotype of CD69$^+$HSA$^+$6C10$^+$MTS32$^-$Qa-2$^-$, are less functional or functionally inert. However, most of the thymic emigrants are functionally competent SP thymocytes accompanied with a phenotype of CD69$^-$6C10$^-$Qa-2$^+$, of which the majority are HSA$^-$MTS32$^-$ (11, 12, 21-24). In terms of CD8 SP medullary thymocytes in mice, the acquisition of granzyme A proteolytic activity occurs in the relatively more mature HSA$^-$, but not in HSA$^+$, medullary CD8 SP thymocytes (22, 25). In human intrathymic T cell development, the post-selected CD3$^+$CD4SPCD1$^+$ and CD3$^+$CD8SPCD1$^+$ thymocytes were less functional, which require interactions with medullary thymic microenvironment to differentiate into CD3$^+$CD4SPCD1$^-$ and CD3$^+$CD8SPCD1$^-$ thymocytes to acquire functional competence (26, 27). The phenotypic heterogeneity might reflect the multiple steps of SP cell functional maturation process. In the process while newly emerged SP thymocytes in thymic medulla undergoing both functional maturation and negative selection of self-reactive cells, fine phenotypic differentiation is closely

accompanied. To decipher the late stage of SP thymocyte differentiation program, analysis of distinct phenotypes of SP thymocytes and delineating the reasonable phenotypic differentiation pathway is decisive. It will provide a tool to separate these SP cells into distinct subgroups which allow estimating their functional capability. Though the phenotypic and functional heterogenicity of SP thymocytes have been observed in several laboratories, it is unclear the precursor-progeny relationship among these SP subsets because of using different markers in different labs to separate the SP subsets and hardly delineating their relationship. However, a compelling observation from these studies is that some cell surface markers are expressed at early functional immature SP thymocytes, such as CD69, 6C10, CD24 (HSA) and MTS32 molecules; these markers are down-regulated during the process of functional maturation, meanwhile, the Qa-2 molecule is upregulated. We have taken a set of cell surface markers reported to be expressed at different stages of SP thymocytes and analyzed the sequential down-regulation of these markers in combination with the upregulation of Qa-2; we are eligible to separate both CD4 SP and CD8 SP medullary thymocytes into phenotypically distinct subgroups. By such analysis, we have delineated the phenotypic differentiation of CD4 SP and CD8 SP medullary thymocytes. The cell surface markers we have employed include three groups of molecules: 1) TCRβ, CD4 and CD8 to define TCRαβ$^+$CD4 SP and TCRαβ$^+$CD8 SP thymocytes and exclude the immature TCRαβ$^-$CD4 SP and TCRαβ$^-$ CD8 SP thymocytes; 2) 6C10, a glycosylated epitope of Thy-1 (28), 3G11, a ganglioside molecule (29), CD69, CD24 (HSA), these markers are highly expressed at early virgin SP medullary thymocytes and gradually down-regulated, even lost following the progression of the development; 3) Qa-2, β_7-integrin and CD62L (L-selectin), these markers are implicated to be expressed in more mature SP thymocytes; the later two markers are reported to be expressed in thymocyte emigrants, i.e., the molecules mark the latest stages of medullary SP thymocytes (30). The markers of 6C10 and 3G11 were firstly used to identify the maturity of CD4 SP thymocytes in mouse thymus and spleen (31). By flow cytometry four-color analysis, we observed the virgin TCRαβ$^+$CD4 SP and TCRαβ$^+$CD8 SP medullary thymocytes exhibited the phenotype of 6C10$^+$CD69$^+$HSA^{hi} Qa-2$^-$. The sequential down-regulation of cell surface molecules is in the time order of: 6C10, the first; CD69, the second; 3G11, the third; Qa-2 expressed at the end. During this developmental process, the expression of CD24 is modulated from high to intermediate, then to low and eventually lost. Recently the CD8 SP thymocyte emigrants (RTEs) were reported to be CD69$^-$$\beta_7$-integrin^{hi}L-selectin^{hi} (9). We found 88% of Qa-2$^+$ cells expressing high level of β_7-integrin and L-selectin. It implied that a small portion (12%) of Qa-2$^+$ cells might not be able to emigrate to periphery even though they represented the latest group of mature thymocytes. On the other hand, 83% of CD69$^+$ cells, the early stage of medullary CD8 SP thymocytes, expressed high level of L-selectin. It seemed that L-selectin was expressed throughout the major developmental stages of CD8 SP medullary thymocytes. Similarly, β_7-integrin was also expressed during early stage (CD69$^+$) of CD8 SP medullary thymocytes. Along the developmental process of medullary type CD8 SP thymocytes, however, the percentage of β_7-integrin$^+$ cells steadily increased (from 48% to 88%), implying that once β_7-integrin was expressed it was not down-regulated during the remaining differentiation stages. Thus, β_7-integrin and L-selectin can be used as reference markers for RTE, but not as stage-specific markers. They also should not be used as maturation markers for CD4/CD8 SP medullary thymocytes.

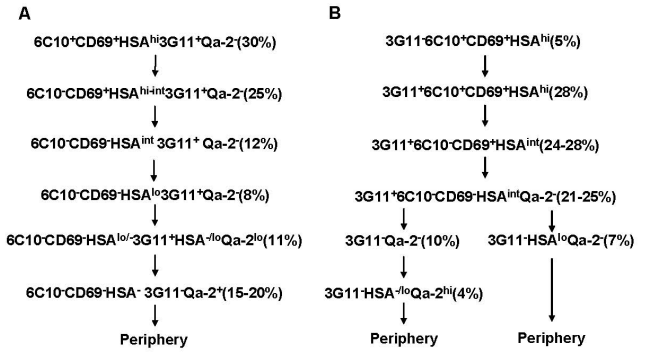

Figure 2. Proposed phenotypic differentiation of mouse medullary thymocytes in thymic medulla. Based on the analysis of phenoltypic differences of TCRαβ$^+$CD4SP and TCRαβ$^+$CD8SP medullary thymocytes, the medullary SP thymocytes are of heterogeneous populations. According to the markers expressed on immature cells, such as 6C10, CD69, HSA (CD24)^{hi} and the molecules expressed on the mature cells, such as Qa-2, and the sequential down-regulation of the molecules of 6C10, CD69 and CD24, the precursor-progeny relationship of the different phenotypes is delineated.

It is argued that some of the mature CD8 SP subpopulations might represent recirculating mature cells. It seemed unlikely as the reentry of periphery resting T cells to the thymus was extremely limited (<0.02%) in normal adult mice, in contrast to the S phase T blasts which immigrated to the thymus with substantial number (0.4%) (32). We have also tested the thymic homing of purified CD8$^+$ T cells pooled from mouse spleen and lymph nodes. Ninety-nine percent of these purified CD8$^+$ T cells were small size (forward light scatter *vs* side light scatter analysis) and 98.6% were resting cells (G0-G1 phase). Twenty-four hours after intravenous injection of FITC-labeled purified CD8$^+$ T cells, FACS analysis of cell suspension prepared from different immune organs indicated that localization of these cells was approximately 3% in spleen, 1.5% in lymph nodes, undetectable in thymus. Thus, all the subpopulations of CD8 SP medullary type thymocytes represent the natural residence in thymus. Thus far, the characteristic phenotypes allow us to separate CD8 SP medullary thymocytes into 6 subpopulations (33) and CD4 SP medullary thymocytes into 7 subpopulations (34). Their phenotypic differentiation progress is suggested in Figure 2.

An argument is whether the 3G11$^-$CD4 SP thymocytes are actually the NKT cells. We have assessed the TCR usage in 3G11$^-$CD4 SP thymocytes: 28% of these cells were Vβ6$^+$, 46% Vβ8$^+$, whereas Vβ3 was negative. The other cell surface markers on these cells were CD25 (IL-2Rα) with 8% positive and CD44 with 68% positive. It was reported that NK1.1$^+$ T cells were CD44^{high}, and overused TCRVβ8.

Since the T cells from BALB/c do not express NK1.1 antigen, we have measured the NK1.1 expression in C57BL/6 mice. The $3G11^-6C10^-CD4^+CD8^-$ thymocytes of C57BL/6 mice was NK1.1 negative (35). Thus, the $3G11^-CD4$ SP thymocytes we identified were not NKT cells.

Unlike $TCR\alpha\beta^+CD8$ SP thymocytes, the $TCR\alpha\beta^+CD4$ SP thymocytes are divided into $3G11^+$ and $3G11^-$ cells at the late stage, the $3G11^+$ cells express low level of Qa-2, whereas the $3G11^-$ cells express high level of Qa-2.

Characteristics of functional maturation pathway of medullary thymocytes

With the availability of phenotypic differentiation pathway, we are able to separate the medullary thymocytes into the subgroups and estimate their functional capability individually. Because of no obvious interference of $TCR\alpha\beta^-$ CD4 SP thymocytes, it is relatively easy to separate $TCR\alpha\beta^+$ CD4 SP thymocytes into 6 subgroups and estimate their functional status by the parameters of cell proliferation and cytokine production in response to ConA stimulation without the addition of exogenous cytokines.

The cell proliferation capacity in response to ConA was very low in the first subgroup of $6C10^+3G11^{+/-}CD69^+HSA^{hi}Qa\text{-}2^-$; the $6C10^-3G11^+CD69^+HSA^{int}Qa\text{-}2^-$ cells exhibited sharply increased proliferation; then, the proliferation was steadily and stepwisely increasing along with the developmental pathway of $6C10^-3G11^+CD69^+HSA^{int}Qa\text{-}2^-$, $6C10^-3G11^+CD69^-HSA^{int}Qa\text{-}2^-$, and $6C10^-3G11^+CD69^-HSA^{lo/-}Qa\text{-}2^+$ thymocytes. However, the most phenotypically matured $Qa\text{-}2^+$ cells possess the proliferation capacity still lower than peripheral splenic $CD4^+$ T cells (Table 1). The major functional criterion of CD4 SP medullary thymocytes is the cytokine producing capacity. In response to ConA stimulation, the $6C10^+3G11^{+/-}CD69^+HSA^{hi}Qa\text{-}2^-CD4$ SP cells produced IL-2 only, with marginal activity. The $6C10^-3G11^+CD69^+$ $HSA^{int}Qa\text{-}2^-$ cells extended to produce both IL-2 and IL-4 with low levels, whereas IL-2 activity being slightly higher than that produced by $6C10^+$ $3G11^{+/-}CD69^+HSA^{hi}Qa\text{-}2^-$ cells. In the $6C10^-3G11^+CD69^-$ $HSA^{int}Qa\text{-}2^-$ cells, they secreted intermediate levels of multiple types of cytokines including IL-2, IFN-γ, IL-4,

Table 1. *Proliferation capacity of thymocytes of CD4 SP subgroups to ConA stimulation.*

Subgroups	Proliferation (^{3}H-TdR uptake, cpm±SD)	
	Medium	ConA
$3G11^{-/+}6C10^+CD69^+HSA^{hi}Qa\text{-}2^-$	150±1	512±14
$3G11^+6C10^-CD69^+HSA^{int}Qa\text{-}2^-$	150±1	8521±128
$3G11^+6C10^-CD69^-HSA^{int}Qa\text{-}2^-$	170±9	15875±1400
$3G11^+6C10^-CD69^-HSA^{lo/-}Qa\text{-}2^+$	80±5	26320±1510
Spleen $CD4^+$ T cells	180±40	40260±3590

Cells of different CD4 SP thymocyte subgroups were cultured for 3 days with ConA (2.5μg/ml). Cell proliferation was measured by ^{3}H-TdR uptake pulse added during the last 12h before harvesting. Cells were isolated from spleen $CD4^+$ T cells in adult mice. Cell purity >93%. Data represent means±SD of three experiments.

Table 2. *Cytokine secretion property of CD4 SP thymocyte subgroups.*

Subgroups	Cytokines (means±SD)				
	IL-2 (cpm/well)	IFN-γ (U/ml)	IL-4 (pg/ml)	IL-6 (U/ml)	IL-10 (pg/ml)
$3G11^{+/-}6C10^+CD69^+HSA^{hi}Qa\text{-}2^-$	240± 2	<2	<50	<5	<100
$3G11^+6C10^-CD69^+HSA^{int}Qa\text{-}2^-$	720±50	<2	195±10	<5	<100
$3G11^+6C10^-CD69^-HSA^{int}Qa\text{-}2^-$	2760±110	27±9	240±25	26±3	270±9
$3G11^+6C10^-CD69^-HSA^{lo/-}Qa\text{-}2^+$	4110±160	171±19	998± 98	56±46	644±25
Spleen $CD4^+$ T cells	8500±100	853±96	4233±278	53±4	1900±140

Thymocytes of different CD4 SP subgroups were cultured for 2 days with ConA (2.5μg/ml). Supernatants were harvested at 48h and cytokine content measured. IL-2 production was measured by the ^{3}H-TdR uptake of an IL-2 dependent cell line HT-2. The means±SD of HT-2 cells in IL-2-free culture medium (negative control) was 146±13 of three experiments. Data represent means±SD of three experiments.

Table 3. *Th2 like cytokine production by $3G11^-6C10^-CD4$ SP thymocytes.*

Subgroups	Stimulants		Cytokines(means±SD)				
	Medium	ConA	IL-2 (cpm/well)	IFN-γ (U/ml)	IL-4 (pg/ml)	IL-6 (U/ml)	IL-10 (pg/ml)
$3G11^-6C10^-CD69^-HSA^{lo}Qa\text{-}2^-$	+	-	154±4	<2	<50	<10	<100
	+	+	240±10	9±2	1250±160	38±3	495±26
$3G11^-6C10^-CD69^-HSA^{lo/-}Qa\text{-}2^+$	+	-	148±2	<2	<50	<10	<100
	+	+	220±5	9±3	2265±108	80±5	845±96

$3G11^-6C10^-CD69^-HSA^{lo/-}Qa\text{-}2^{+/-}CD4$ SP thymocytes ($1x10^6$/ml) were cultured with or without ConA (2.5μg/ml) for 48h and cytokine content measured. Data represent means±SD of three experiments.

IL-6 and IL-10, typical of Th0 type cytokines. The highest levels of Th0 type cytokines were produced by $6C10^-3G11^+$ $CD69^-HSA^{lo/-}Qa\text{-}2^+$ subgrouped cells (Table 2). By contrast, the $6C10^-3G11^-CD69\text{-}HSA^{lo/-}Qa\text{-}2^+$ thymocytes produced the cytokines of IL-4, IL-6 and IL-10 and little IFN-γ shown as a pattern of Th2 like cytokines (Table 3). These findings have unveiled that there exists a functional maturation process of SP thymocytes with the characteristic of functional acquisition in a stepwise manner and in a hierarchically increasing in the expansion of cytokine profile and in the increase in cytokine levels. In our assays, the end points of CD4 SP medullary thymocytes are branched into $3G11^+Qa\text{-}2^+$ and $3G11^-Qa\text{-}2^+$ cells, which are endowed with the capacity of Th0- and Th2-like cytokine production. In view of cytokine producing capacity, all cytokines are lower, except IL-6, than those produced by the peripheral splenic $CD4^+$ T cells (36). It raises an old dilemma if the post thymic maturation process is required for the T cells to acquire full functions.

The functional capacity of CD8 SP medullary thymocytes is more difficult to analyze. The main reason is the interference of $TCR\alpha\beta^-$ immature CD8 SP thymocytes. We are limited by the advanced flow cytometry with more than four color analysis and sorting. In addition, the purified CD8 SP medullary thymocytes failed to respond to ConA without exogenous addition of IL-2. With the stimulation by PMA plus ionomycin, both $Qa\text{-}2^-CD8$ and $Qa\text{-}2^+CD8$ SP subgrouped thymocytes displayed similar proliferation capacity (Table 4). The functional capacity of $TCR\alpha\beta^+$ $Qa\text{-}2^-CD8$ SP and $TCR\alpha\beta^+Qa\text{-}2^+CD8$ SP subgrouped thymocytes are distinct shown in cytokine producing levels. Under the stimulation of PMA plus ionomycin, the $TCR\alpha\beta^+Qa\text{-}2^+$ CD8 SP thymocytes produced higher levels of IFN-γ, TNF-α and IL-2 than those produced by $TCR\alpha\beta^+Qa\text{-}2^-$ CD8 SP (Table 5). It is clear that more mature of CD8 SP medullary thymocytes, the higher levels of cytokines they produced. However, the cytokine producing capacity of $TCR\alpha\beta^+Qa\text{-}2^+$ CD8 SP thymocytes was significantly lower than that of peripheral $CD8^+$ T cells in spleen (37).

The above data were obtained by the *in vitro* analysis of developing thymocytes from adult mice. To avoid any artificial bias which may be caused by *in vitro* assays, the kinetics of naturally developmental process of SP medullary thymocytes in the *in vivo* thymus have been monitored during the mouse ontogeny. The results, in principle, have verified those that we have obtained in the analysis of SP thymocytes in adult mice, but more characteristic developmental properties were disclosed.

The specialized thymic architecture in medullary region is suited for late stages of SP medullary thymocyte development to accomplish functional maturation program and further delete self-reactive T cells by apoptosis. It is no doubt that the signal mediated by thymocyte expressed TCR to recognize and bind to self-peptide-MHC complex molecules triggered positive selection and lineage commitment into $TCR\alpha\beta^+$CD4/CD8 SP medullary thymocytes. For the condition to induce functional maturation, different laboratories have used different methods and reached different conclusions. In the two-stage culture model, Germain's lab reported that TCR-self-peptide-MHC interaction was also required for positively selecting $CD69^+CD4^{lo}CD8^{lo}$ thymocytes to differentiate into functional mature CD4 SP thymocytes (3, 38). Unlike this, the report from Singer's lab was that IL-7 was sufficient to support lineage committed intermediate CD4 SP thymocytes to develop into CD8 SP thymocytes and to be functional (39). In our early experiments, we tried to add the supernatant (SN) of MTEC1 (a mouse medullary

Table 4. *Proliferation capacity between two CD8 SP medullary thymocytes.*

Stimulants	Subgroup (^{3}H-TdR uptake, cpm±SD)		
	$CD69^-Qa\text{-}2^-$	$CD69^-Qa\text{-}2^+$	Spleen $CD8^+$T cells
Medium	560±25	284±31	236±76
Anti-TCRβ	456±89	332±47	771±109
ConA	146±10	183±26	60±11
PMA+Ionomycin	27357±3373	27020±4475	20599±3480

The purified cells ($2x10^5$/ml) were cultured under the stimulation indicated for 5 days at 37°C, 5%CO_2, then, the cell proliferation was measured by ^{3}H-TdR uptake.

Table 5. *Proliferation capacity between two CD8 SP medullary thymocytes.*

Stimulants	Cytokines	Subgroups		
		$CD69^-Qa\text{-}2^-$	$CD69^-Qa\text{-}2^+$	Spleen $CD8^+$T cells
ConA	IFN-γ	2.1%[1]	3.55%[1]	1.91%[1]
	TNF-α	3.1%[1]	1.7%[1]	2.1%[1]
	IL-2	ND[2]	ND[2]	ND[2]
PMA+ Ionomycin	IFN-γ	2.9%[1]	9.85%[1]	29.3%[1]
	TNF-α	3.4%[1]	11%[1]	32%[1]
	IL-2	40[3]	61.5[3]	160.5[3]

[1]The purified subgrouped cells ($1x10^6$/ml) were stimulated for 5h with the respective stimulants. The intracellular cytokine was detected by FACS analysis after inhibition with monensin to block the cytokine secretion.
[2]ND not done.
[3]The purified cells ($1x10^6$/ml) were stimulated and cultured for 48h, then, the supernatants were harvested and the IL-2 was measured by IL-2 dependent cell line HT-2. The IL-2 activity was assessed as U/ml.

Table 6. *Effect of mMTEC1 cells on the cytokine production property of thymocytes of CD4 SP subgroups to ConA stimulation.*

Subgroups	ConA	ConA + mMTEC1	Cytokines (means±SD)				
			IL-2 (cpm/well)	IFN-γ (U/ml)	IL-4 (pg/ml0	IL-6 (U/ml)	IL-10 (pg/ml)
$3G11^{+/-}6C10^{+}CD69^{+}HSA^{hi}Qa\text{-}2^{-}$	+	-	236±2	<2	<50	<5	<100
	+	+	320±10	<2	123	<5	<100
$3G11^{+}6C10^{-}CD69^{+}HSA^{int}Qa\text{-}2^{-}$	+	-	720±53	<2	192	<5	<100
	+	+	1150±45	8	218	18	<100
$3G11^{+}6C10^{-}CD69^{-}HSA^{int}Qa\text{-}2^{-}$	+	-	2757±114	32	235	25	268
	+	+	3890±108	64	796	64	571
$3G11^{+}6C10^{-}CD69^{-}HSA^{lo/-}Qa\text{-}2^{+}$	+	-	4109±158	128	985	60	636
	+	+	6349±253	256	1890	100	1120
Spleen $CD4^{+}$T cells	+	-	8496±103	1024	4400	50	1900

Thymocytes of different CD4 SP subgroups were co-cultured with mMTEC1 cells for 2 days with ConA (2.5μg/ml). Supernatants were harvested at 48h and cytokine content measured. IL-2 production was measured by the ^{3}H-TdR uptake of an IL-2 dependent cell line HT-2. The means±SD of HT-2 cells in IL-2-free culture medium (negative control) was 146±13/well of three experiments. Data represent means±SD of three experiments.

Table 7. *Th2 like cytokine production by $3G11^{-}6C10^{-}CD4$ SP thymocytes to ConA stimulation with or without co-cultivation with mMTEC1 cells.*

Subgroups	Stimulants			Cytokines (means±SD)				
	Medium	ConA	mMTEC1	IL-2 (cpm/well)	IFN-γ (U/ml)	IL-4 (pg/ml)	IL-6 (U/ml)	IL-10 (pg/ml)
$3G11^{-}6C10^{-}CD69^{-}HSA^{lo}Qa\text{-}2^{-}$	+	-	-	154±4	<2	<50	<10	<100
	+	+	-	240±10	9±2	1250±160	38±3	495±26
	+	+	+	245±7	9±2	1085±110	243±12	384±40
$3G11^{-}6C10^{-}CD69^{-}HSA^{lo/-}Qa\text{-}2^{+}$	+	-	-	148±2	<2	<50	<10	<100
	+	+	-	220±5	9±3	2265±108	80±5	845±96
	+	+	+	228±7	11±4	1828±96	271±10	570±28

$3G11^{-}6C10^{-}CD69^{-}HSA^{lo/-}Qa\text{-}2^{+/-}CD4SP$ thymocytes ($1x10^{6}$/ml) were cultured with or without ConA (2.5μg/ml) in the absence or presence of mMTEC1 for 48h and cytokine content measured. The cytokines produced by mMTEC1 cells cultured in medium were: IL-2, 150±3cpm/well; IFN-γ, <2U/ml; IL-4, <50pg/ml; IL-6, 11± 2U/ml; IL-10, <100pg/ml. In the presence of ConA, mMTEC1 cells did not increase cytokine production. Data represent means±SD of three experiments.

type epithelial cell line) culture, which contained mixed cytokines including IL-7, to the isolated $Qa\text{-}2^{-}CD4$ SP thymocytes. Though the MTEC1- SN could support the proliferation of $Qa\text{-}2^{-}CD4$ SP thymocytes, the mixed cytokines could not induce the cells to express Qa-2, nor increase the cytokine production. Strikingly, after co-cultured with mMTEC1 cells for 3 days at 37°C, 5%CO_2, the $Qa\text{-}2^{-}CD4$ SP thymocytes differentiated into $Qa\text{-}2^{+}$ cells with the positive rate of 87% and expressed high levels of IL-2, IL-4 and IL-6, resembling the levels produced by the natural $Qa\text{-}2^{+}CD4$ SP thymocytes directly isolated from mouse thymus (40). In our recent study, we have isolated 6 subgroups of $TCR\alpha\beta^{+}CD4$ SP medullary thymocytes and estimated their cytokine production capacity with or without co-cultivation with mMTEC1 cells. The results showed that the functional capacity of all four subgroups of $3G11^{+}CD4$ SP thymocytes and two subgroups of $3G11^{-}CD4$ SP thymocytes was increased in the co-cultivation with mMTEC1 cells. A salient feature is that mMTEC1 cells could only induce the cells of each individual subgroup to increase their functional capacity reaching to the level possessed by the naive cells positioned at the next differentiation stage as shown by both cell proliferation and the profiles and levels of produced cytokines (Table 6 and 7). That implies that the cells at each stage have inherent potential to develop into the next stage along with differentiation pathway. Apparently, the functional maturation of CD4 SP thymocytes is a programed process executed in a stepwise maturation manner. It is important to note that co-cultivation with mMTEC1 cells did not change the Th0 pattern of cytokine production by $3G11^{+}Qa\text{-}2^{+}CD4$ SP medullary thymocytes, nor change the Th2-like pattern of cytokine production by $3G11^{-}Qa\text{-}2^{+}CD4$ SP medullary thymocytes. These cytokine patterns are still reserved when the $3G11^{+}Qa\text{-}2^{+}CD4$ SP and $3G11^{-}Qa\text{-}2^{+}CD4$ SP medullary thymocytes co-cultivated with freshly isolated mixed thymic

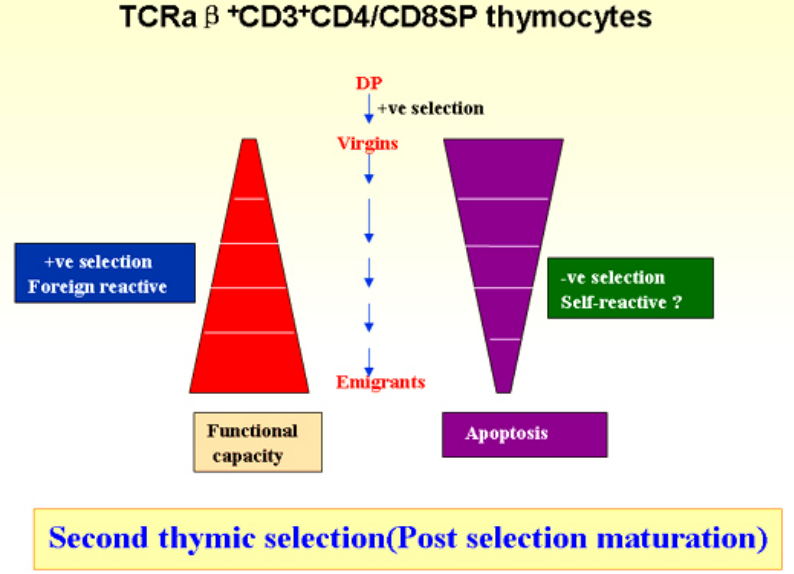

Figure 3. Sketched late stage of SP medullary thymocyte development. According to our experimental data, the characteristics of the late stage of SP medullary thymocyte development are proposed. The functional maturation is a stepwise acquisition and in a hierarchical increased shown as "erected pyramid"; meanwhile, the susceptibility of negative selection by apoptosis of self-reactive T cell clones is conversely relevant to the functional maturation status of the SP medullary thymocytes shown as "inverted pyramid".

stromal cells from mouse thymus. Therefore, these cells have been committed to produce the characteristic patterned cytokines. We never found that CD4 SP medullary thymocytes developed into polarized Th1 cytokine producing cells along with cell functional maturation process. A question raised is why mMTEC1 cells could only induce one step forward, not the whole process, in the functional maturation of $6C10^{-}3G11^{+}$CD4 SP thymocytes. It might be attributed to the limitation in the two dimensional culture system that cell differentiation was arrested at certain stage, or due to the lack of the multiple types of thymic stromal cells, or due to the short term co-culture, or even combination of these reasons that made the early functionally immature CD4 SP medullary thymocytes unable to develop through the entire stepwise process into the late stage of fully functional cells. However, it is through the short time co-culture that the multi-steps and the specific feature of CD4 SP thymocyte functional maturation program have been revealed. We have investigated if the addition of exogenous IL-7 and IL-2 cytokines could modulate the cytokine producing pattern of $6C10^{-}3G11^{+}$CD4 SP medullary thymocytes. Exogenous IL-7 and IL-2 markedly promoted ConA stimulated cell proliferation; IL-7 or IL-2 or both, however, did not change these cells to produce Th2-like cytokine pattern (IL-4, IL-6 and IL-10), nor was IL-2 produced, and the production of IFN-γ was still a little, without increasing (data not shown). Taken together, our data indicate the cell-cell interaction between SP medullary thymocytes and medullary thymic stromal cells is the critical factor to determine functional maturation rather than the cytokine such as IL-7. Our conclusion is that medullary CD4 SP thymocytes undergo a stepwise functional maturation process and that the process is induced and modulated by thymic stromal cells. Although it is not perfect, the unveiled ordered program of late stage of T cell development within thymus medulla has provided an objective standard to determine the TCR signaling, their strength and duration, the costimulatory molecules, cytokines and the combination in the induction of such development and to what stage of functionally developing SP medullary thymocytes can be induced by these molecules. By the stages of the stepwise maturation pathway, we are able to identify the particular types of thymic stromal cells interacting on the particular stage of SP thymocytes and looking at the particular receptor-ligand to mediate the signaling for the activation of the transduction pathway leading to the target gene activation and the cells develop forward.

Unveiling the late stage of thymocyte developmental pathway is also needed for the understanding of the molecules involved in the negative selection to induce SP thymocyte apoptosis within thymic medulla. It is so far reported that many apoptotic molecules, such as Fas, Bim, Bak and Bax, are identified to induce apoptosis of both DP and SP thymocytes (41-43). However, we do not know if these molecules are sufficient to induce the apoptosis of all the self-reactive T cell clones. One possibility is that each individual molecule may act on the SP thymocytes developing at certain stage, not all the self-reactive cells which are destined to die. Without unveiling each step of late stage thymocyte development and analyzing the apoptotic potential of the cells at each stage, we are not eligible to evaluate if any molecule is sufficient to induce a cell to accomplish the entire late stage of developmental pathway. Neither can we compare at what developmental stage of the cells different molecules could induce their differentiation, as the developing SP thymocytes express distinct capacity of functions at distinct stages. The identification of the late stage of developmental program provides a standard to compare the results from different labs, through which to determine the critical factors required for this process and more factors should be identified. For example, we have also found a molecule called PF18-3, which may be involved in negative selection by apoptosis in thymic medulla (44). It implies that more cellular and molecular mechanisms will be identified in order to better understand the late stage of thymocyte development.

To summarize our researches in late stage of thymocyte developmental pathway within thymic medulla, we propose that the functional acquisition is in a stepwise hierarchical

increase, like erect pyramid, i.e., the cells at ealier stage, the less function they possess; in contrast, the cells at much later stage, the more function they exhibit. Contrarily, the negative selection of self-reactive T cells by apoptosis displays a form of reversed pyramid, i.e., the cells at much earlier stage, the more susceptible to apoptosis to the self-antigen they express; in contrast, the cells at more late stage, the more resistance to apoptosis as they acquire the response to foreign antigen in the context of self-MHC molecules (Figure 3). It is partially shown in our experiments that all the $6C10^{+}CD69^{+}$CD4/CD8 SP thymocytes were eliminated after peritoneal injection of cortisone for 24h in mice. In the peritoneal injection of anti-TCRβ mAb, the number of $6C10^{+}CD69^{+}$CD8 SP thymocytes reduced by 16-fold, the $6C10^{-}CD69^{+}$CD8 SP thymocytes reduced by 8-fold, in contrast, the $6C10^{-}CD69^{-}$ Qa-2^{+} increased by 1.4-fold (45). Intensive studies need to be done to verify our hypothesis.

The latest approaches to studying intrathymic development are the extensive analysis of thymic stromal cells which constituting the specialized thymic niche for particular stage of thymocyte development (46) and to use cDNA micro-array to analyze the gene which may function in T cell development (47). Combination of our primarily identified late stage of thymocyte developmental pathway with cDNA microarray and proteomics, we wish to decipher the cellular and molecular mechanism by which the medullary thymocytes are induced to undergo a final stage of development; to decipher the checkpoints for the branched signals to generate Th subsets with distinct functions such as the regulatory T cells (48) and helper T cells among CD4 SP medullary thymocytes.

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