BMB
*Reports*

**BMB Rep. 2016; 49(1): 11-17**
www.bmbreports.org

**Invited Mini Review**

# Lineage re-commitment of CD4CD8αα intraepithelial lymphocytes in the gut

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**The gastrointestinal tract forms the largest surface in our body with constantly being exposed to various antigens, which provides unique microenvironment for the immune system in the intestine. Accordingly, the gut epithelium harbors the most T lymphocytes in the body as *i*ntraepithelial *l*ymphocytes (IELs), which are phenotypically and functionally heterogeneous populations, distinct from the conventional mature T cells in the periphery. IELs arise either from pre-committed thymic precursors (natural IELs) or from conventional CD4 or CD8αβ T cells in response to peripheral antigens (induced IELs), both of which commonly express CD8α homodimers (CD8αα). Although lineage commitment to either conventional CD4 T helper (Th) or cytotoxic CD8αβ T cells as well as their respective co-receptor expression are mutually exclusive and irreversible process, CD4 T cells can be redirected to the CD8 IELs with high cytolytic activity upon migration to the gut epithelium. Recent reports show that master transcription factors for CD4 and CD8 T cells, ThPOK (Th-inducing BTB/POZ-Kruppel-like factor) and Runx3 (Runt related transcription factor 3), respectively, are the key regulators for re-programming of CD4 T cells to CD8 lineage in the intestinal epithelium. This review will focus on the unique differentiation process of IELs, particularly lineage re-commitment of CD4 IELs. [BMB Reports 2016; 49(1): 11-17]**

## INTRODUCTION

The intestine is the biggest immunological organ in our body with its huge surface that is constantly exposed to foreign matters, such as food and pathogens (1). There is a delicate balance between protective immunity against detrimental pathogens and tolerance to food and beneficial bacteria. To keep the homeostatic balance, various immune cells are located in the gut epithelial lining. Among the immune cells, lymphocytes located within the gut epithelium are intestinal *i*ntraepithelial *l*ymphocytes (IELs), which represent the largest heterogeneous T cell population in the organisms. Due to the constant exposure of antigens at the mucosal barrier, IELs have unique and antigen-experienced activated phenotypes, constitutively expressing CD8α homodimer, CD8αα, and CD103 (αE integrin), distinct from the conventional T cells in the periphery. Further, they are suggested to play a pivotal role in providing protective immunity as the first line of immune defense (reviewed in Ref 2-4).

In this review, we discuss the development, subtypes, and functions of IELs, especially focusing on the unusual CD4 IELs expressing CD8α homodimer, CD4CD8αα double positive (DP) IELs. There are decent reports recently revealed that conventional CD4 T cells post-thymically reprogram to cytotoxic T lymphocyte (CTL)-like DP IELs expressing CTL-lineage associated genes in the gut microenvironment (5, 6). We focus on how conventional CD4 T cells in the periphery reprogram their cellular fate from the T helper lineage to the CTL-like DP IELs in the gut environment.

## SUBSETS OF IELS

IELs can be classified into "natural" and "induced" categories based on their developmental origin and co-receptor expressions (2-4). Natural IELs (nIELs), previously classified as "type b" IELs, differentiate unconventionally from their own precursors and are activated during selection process in response to self-antigens in the thymus. They are barely detectable in peripheral mainstream lymphoid tissues and express either αβ T cell receptor (TCR) or γδ TCR with a unique co-receptor, CD8αα, without conventional CD4 or CD8αβ co-receptors (CD8αα TCRαβ IELs or CD8αα TCRγδ IELs). Induced IELs (iIELs), previously "type a IELs", are derived from the conventional CD4 or CD8αβ T cells in the periphery and post-thymically differentiated in the intestine. They are αβ TCR-bearing CD4 or CD8αβ T cells with a predominance of CD8 T cells, which have much in common with conventional CD4 and CD8αβ T cells found in the spleen and other lymphoid

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http://dx.doi.org/10.5483/BMBRep.2016.49.1.242

**Received 25 October 2015**

**Keywords:** IEL, Lineage commitment, ThPOK, Runx3, T-bet

ISSN: 1976-670X (electronic edition)

tissues. These conventional iIELs also co-express CD8αα together with CD4 or CD8αβ, thus the expression of CD8αα is a hallmark of IEL populations in the gut epithelium (2-4).

## UNIQUE DEVELOPMENT OF IELS

Originally, nIELs are thought to differentiate thymus-independently. Early experiment described that thymectomy has little effects on the generation of IELs in the intestine (7), and CD8αα IELs are present in neonatally thymectomized mice reconstituted with bone marrow or fetal liver (8-10). Studies show that cryptopatches (CP) in the small intestine contain IEL precursor cells (11, 12), and that CP cells from nude mice are able to reconstitute IELs (12), further supporting the idea that nIELs are derived extra-thymically from CP precursors. However, increasing evidence supports the importance of thymus and thymic progenitors for nIEL development. It has been reportedly observed that the number of CD8αα IELs is extremely reduced in athymic mice (9, 13, 14), indicating that thymus is functional for nIEL development. Moreover, a genetic fate mapping study showed direct evidence that all CD8αα-bearing αβ TCR IELs originate from $CD4^+CD8^+$ DP thymocytes rather than CP precursors (15). The thymic development of nIELs is further clarified by the identification of thymic precursors of CD8αα IELs. Cheroutre and co-workers discovered that $CD4^+CD8\alpha\beta^+CD8\alpha\alpha^+$ triple positive (TP) cells in the thymus, distinct from classical $CD4^+CD8\alpha\beta^+$ DP thymocytes, are unique precursors for CD8αα IELs, which are selected and matured in a "alternative" way by strong interactions with self-antigens, termed "agonist selection" (16) (Fig. 1). As a result, they become mature, memory-like $CD4^-CD8\alpha\beta^-$ αβ or γδ TCR-expressing T cells and acquire gut-homing capacity in the thymus, thereby directly migrating to the intestinal epithelium (16, 17).

Contrary to nIELs, iIELs are the progeny of conventional MHC class I-restricted CD8αβ or MHC class II-restricted CD4 naïve T cells that further undergo post-thymic differentiation process in the intestine (reviewed in ref 2-4). Naïve T cells are activated in response to antigens in the periphery, particularly gut-associated lymphoid tissue (GALT), including Peyer's patches (PPs) and mesenteric lymph nodes (MLN), and then migrate into the intestinal epithelium to become iIELs. It is well known that $CD103^+$ dendritic cells (DCs) in the GALT with their ability to produce the vitamin A metabolite, retinoic acid (RA), confer the gut-homing capacity to naïve T cells they prime (18, 19). RA induces gut-homing receptors, $\alpha_4\beta_7$ and CCR9 on T cells, which bind to mucosal adhesion molecule 1 (MAdCAM-1) and CCL25, respectively (20). These are constitutively expressed on small intestinal endothelium and epithelium, thus directing T cells to traffic into the intestine (21-23). In the intestine, T cells subsequently down-regulate $\alpha_4$ subunit of $\alpha_4\beta_7$ and gradually acquire $\alpha_E$ (CD103) expression to form integrin $\alpha_E\beta_7$ (24, 25), which is induced by transforming growth factor (TGF)-β in the gut environment (26). $\alpha_E\beta_7$ is a major adhesion molecule, which mediates selective localization or retention of IELs to the epithelial compartment of the intestine by interacting with E-cadherin expressed on the basolateral surface of enterocytes (27).

## FUNCTIONS OF IELS

The precise physiological functions of IELs are not still clear. Yet, their strategic location at the front line of defense and concomitant expression of effector and regulatory molecules allows us to predict that these cells are of importance for controlling pathogen infection and preserving the integrity of epithelial barrier in the gut. Although CD8αα TCRαβ IELs are self-reactive and cytotoxic effectors with high granzyme B ex-

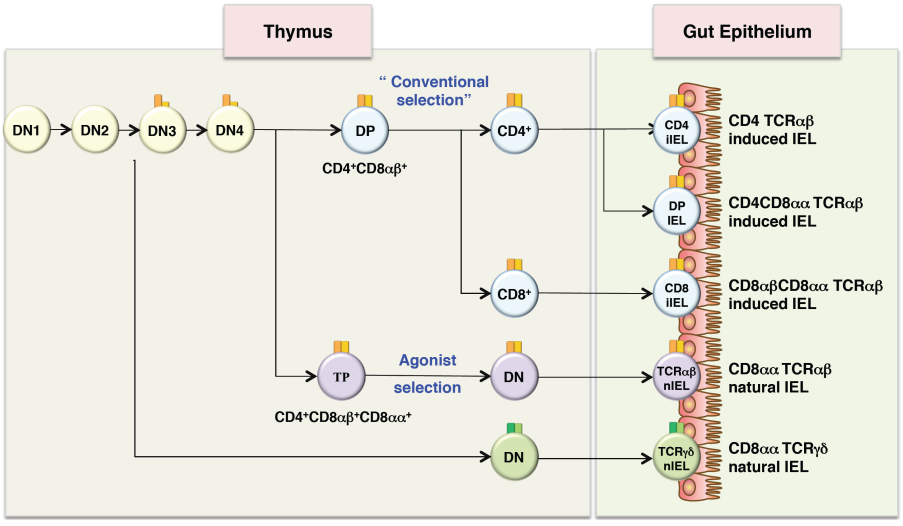


**Fig. 1.** Development and subsets of IELs. Natural CD8αα IELs (nIELs) differentiate from unique thymic precursors, $CD4^+CD8\alpha\beta^+CD8\alpha\alpha^+$ triple positive (TP) cells, which go through a self antigen-based thymic selection process ("agonist selection"). On the other hand, induced IELs (iIELs) are derived from the circulating conventional CD4 or CD8αβ T cells, which further undergo post-thymic differentiation process.

pression (3), they are not self-destructive, and instead they have been shown to mediate immune regulation by expressing several regulatory molecules including interleukin-10 (IL-10), TGF-β and lymphocyte activation gene 3 (LAG 3) (28, 29). In a mouse model of induced colitis, self-specific CD8αα TCRαβ IELs exert a regulatory function by controlling the migration and activity of pathogenic CD4 T cells in the intestine (28). Nevertheless, it is still possible that these self-reactive CD8αα TCRαβ IELs may drive autoimmune pathology under inflammatory or damaged conditions (4). TCRγδ IELs also contribute to protective immunity, secreting TNF and IFN-γ (30) and keep gut integrity through producing TGF-β1, TGF-β3 and keratinocyte growth factor (KGF) to repair epithelial damage (31).

CD8αβ TCRαβ IELs are reported to serve as long-residing effector memory cells within the epithelium to provide protection against various invading pathogens, including simian immunodeficiency virus (SIV) (32, 33), lymphocyte choriomeningtis virus (LCMV) (34), and rotavirus (35). Studies suggest that CD4 IELs, though a minor subset compared to natural or CD8αβ TCRαβ IELs, may contribute to the prevention of immunopathology in the intestine by exerting their protective and regulatory functions (36, 37).

## CD4CD8αα IELS

In general, CD4 T cells are known as helper cells in terms of their ability to modulate the functions of other immune cells, such as cytotoxic T cells, B cells and antigen presenting cells. However, their effector functions as CTLs and the existence of CD8-expressing CD4 T cells have continuously been reported over the decades, particularly during virus infection (38-40). Nevertheless, the cytotoxic activity of CD4 T cells has been considered an *in vitro* artifacts or a functional variant of T helper 1 subset *in vivo*, and there is little convincing evidence to support this subset of CD4 T cells as an independent subtype of CD4 T cells (41).

Two important reports recently identified CD4 CTLs as a distinct subset of effector CD4 T cells, which express co-receptor CD8αα and CTL lineage genes distinguishing them from any other conventional CD4 T helper subsets or regulatory T cells (5, 6). At steady state, these cytotoxic CD4 T cells are mostly found in the small intestine as CD4CD8αα double positive (DP) IELs (42) and can be experimentally generated in the T cell transfer model of colitis, in which naïve T cells are transferred into lymphopenic hosts (5, 6). Remarkably, these reports show that the cytotoxic DP IELs lack ThPOK, the master transcription factor for CD4 Th lineage commitment, of which expression is maintained highly in the peripheral conventional CD4 T cells and critical for their helper functions. DP IELs are initially originated from conventional $CD4^+ThPOK^+$ T cells, which lose ThPOK expression post-thymically upon migration to the gut (5). The loss of ThPOK is induced by Runx3-mediated silencing, the master transcription factor for the CD8 CTL lineage program, thus $CD4^+ThPOK^-$T cells reprogram to MHC-II-restricted cytotoxic CD4 T cells (6).

## ThPOK-Runx3: MASTER TRANSCRIPTIONAL REGULATORS FOR CD4CD8αα IEL DEVELOPMENT

The co-receptor expression of either CD4 or CD8αβ is precisely correlated with T cell function as helper or killer T cells, respectively. Therefore, this lineage decision process from $CD4^+CD8^+$ DP thymocytes to either lineage is crucial in T cell biology and extensively studied over the decades. With the identification of the ThPOK transcription factor (43, 44) and its counteraction with Runx3 (45), it is now widely accepted that this key developmental decision is made at the transcriptional level.

ThPOK is a key transcription regulator, which drives $CD4^+CD8^+$ thymocytes to become mature CD4 T cells, and Runx3 is required for CD8 T cell development (46, 47). The fate decision of helper or CTL is driven by the antagonistic interaction between these two transcription factors, as ThPOK is highly expressed and maintained in mature CD4 T cells in the periphery, preventing Runx3-mediated CTL program. On the other hand, Runx3 expression is preferentially up-regulated during CD8 lineage differentiation in the thymus and remains high in mature CD8αβ T cells, which represses ThPOK expression by binding to silencer of *ThPOK* gene in immature CD8αβ thymocytes (46, 47). In addition to silencing CD4 by repressing ThPOK, Runx3 also reactivates CD8 by directly binding to *Cd8* enhancer, E8I, of the *CD8* gene locus (48).

It has long been thought that CD4-CD8 lineage commitment is mutually exclusive and irreversible. Although mature CD4 T cells are able to differentiate into separate subtypes of helper T cells in response to peripheral antigens, such Th1, Th2, Th17, and regulatory T cells, they maintain their ThPOK expression and helper lineage gene profiles (5, 49). Therefore, little progress has been made in the study of CD8-expressing cytotoxic CD4 T cells in terms of lineage reprogramming despite their existence in the gut and other tissues during viral infection. Recently, two groundbreaking studies bring a new concept of plasticity of mature CD4 T cell development, revealing that CD4 T cells can reprogram to cytotoxic CD8 lineage-like cells, CD4CD8αα T cells, in the small intestine in response to cognate antigens by losing their ThPOK expression, which is mediated by Runx3 up-regulation in the specific gut environment (5, 6).

Mucida *et al.* (5) found that CD4CD8αα DP IELs, the most observed cytotoxic CD4 T cells, lack ThPOK expression, which is normally expressed high in the conventional CD4 T cells in the periphery. Concomitantly, DP IELs display cytotoxic phenotypes like CD8αβ CTLs, including high expression of granzyme B and the activation-induced degranulation marker CD107a. By using well-designed fate-mapping mouse model, they showed that these cells are derived from $ThPOK^+$ CD4 T cells, which lose their ThPOK expression after activa-

tion in the periphery and migration into the gut. In line with this, the development of cytotoxic CD4CD8αα IELs are significantly hampered in mice with germline deletion of *ThPOK* silencer or mice deficient for the zinc-finger transcription factor *MAZR* that activates the *ThPOK* silencer. This indicates that reactivation of *ThPOK* silencer thereby terminating ThPOK post-thymically is essential for the differentiation of DP IELs and their CTL program.

Reis *et al*. (6) further revealed that loss of ThPOK is initiated by Runx3 expression. By using knock-in reporter mice for Th-POK and Runx3, they observed that expression of CD8αα on CD4 IELs is directly associated with the high expression of Runx3, normally repressed in conventional CD4 T cells in the periphery. Runx3$^{hi}$ CD4 IELs have a very low level of ThPOK expression and other T helper genes, but have increased expression of CTL lineage genes. TGF-β and RA are the environmental cues in the intestine to induce Runx3 and CD8αα while down-regulating ThPOK, as evidenced by *in vitro* and *in vivo* experimental settings using mice deficient for TGF-β and RA signaling. Commensal microorganisms in the intestine are also directly or indirectly involved in cytotoxic DP IEL development, since DP ILEs are absent in the germ-free animals (5). Remarkably, the presence of Runx3$^{hi}$ThPOK$^{hi}$ CD4 T cells, but not Runx3$^{lo}$ThPOK$^{lo}$ cells, indicates that Runx3 induction precedes the down-regulation of ThPOK (6). Indeed, mice with a conditional deletion of Runx3 in T cells fail to down-regulate ThPOK expression in CD4 IELs, thereby exhibiting reduced frequency of CD4 IELs expressing CD8αα, CD103 and CTL genes. Overall, upon migration of CD4 T cells to the intestine, Runx3 is up-regulated within the gut environment, which in turn down-regulates ThPOK, promoting cytotoxic CD4CD8αα DP IEL development.

## T-bet AS A SWITCH FOR CD4CD8αα IEL DEVELOMENT

Subsequently, Reis *et al*. (50) also reported that transcription factor, T-bet (T-box expressed in T cells), is a critical upstream regulator of Runx3 and ThPOK in the development of cytotoxic CD4CD8αα DP IELs. T-bet is a well-known transcription factor for Th1 differentiation and CD8 T cells for their IFN-γ production, whereas inhibiting the development of other T helper subtypes. They found that the expression of *Tbx21* encoding T-bet is up-regulated in ThPOK$^{lo}$ IELs, and DP IEL differentiation is impaired in T-bet-deficient CD4 T cells, comparable to Runx3$^{-/-}$ CD4 T cells. In the presence of gut microenvironmental cues, such as TGF-β and RA, and T-bet inducing cytokines in the intestinal milieu, such as IFN-γ or IL-27, preferentially induce cytotoxic CD4CD8αα DP IEL differentiation by inducing Runx3 and suppressing ThPOK expression. Notably, these combinations suppress TGF-β/RA-mediated Foxp3 induction and T-bet-mediated Th1 differentiation, suggesting a distinct role of T-bet in the CD4CD8αα DP IEL development.

Interestingly, overexpression of T-bet in the absence of Runx3 suppresses Th17 differentiation, and T-bet prevents Foxp3 induction regardless of Runx3 expression, suggesting that T-bet inhibits other CD4 T cell subsets in a Runx3-independent manner. A series of experiments using genetically modified CD4 T cells as well as chromatin immunoprecipitation (ChIP) analysis indicate that T-bet directly binds to the -39 kb and the -17 kb *Runx3* regulatory elements and induces Runx3 expression. T-bet also binds to *ThPOK* regulatory binding sites to down-modulate ThPOK expression in a Runx3-dependent manner. Collectively, this study suggests that T-bet that is induced by gut environmental factors serves as an upstream regulator for Runx3 induction thereby promoting CD4CD8αα DP IEL development. Yet once Runx3 expression is induced, T-bet and Runx3 have complementary roles in ThPOK down-regulation and cytotoxic DP IEL generation (Fig. 2).

## FUNCTIONS OF CD4CD8αα IELS

Although DP IELs are equipped with cytotoxic properties since their development, DP IELs do not exert CTL function immediately and instead stay immunologically quiescent at steady state. Antigen-specific CD4CD8αα DP IELs, as generated by transferring OVA-specific OT-II cells into immunodeficient recipients following OVA-feeding, remain immunologically inactive even in the continuous presence of cognate dietary antigens, though they possess cytotoxic potential (5). This in-

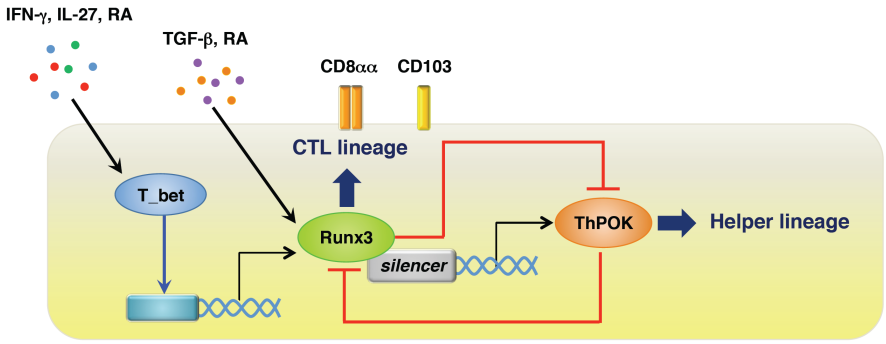


**Fig. 2.** Transcriptional regulation in the development of CD4CD8αα DP IEL. Runx3, known as a key transcription factor for CD8 T cell lineage, which are normally repressed in conventional CD4 T cells, can be reactivated in peripheral CD4 T cells by gut environmental factors including TGF-β and RA and T-bet. Runx3 repressess ThPOK, a transcription factor for CD4 T cell lineage, through direct binding to ThPOK silencer elements, thereby promoting CD8αα and CD103 expression and CTL-associated gene program in the CD4 T cells.

dicates that DP IELs are "activated yet resting" like other IEL subsets, which is confirmed by increased uptake of thymidine analog BrdU and week signals of cell-cycle marker Ki67 (5).

Upon exposure to inflammatory IL-15 with cognate antigens, however, diet-induced DP IELs become immunologically active, producing great amounts of IFN-γ and TNF and up-regulation of CD107a expression, implying the pathogenic potential of cytotoxic DP IELs (5). On the other hand, Reis *et al*. showed the plasticity of CD4CD8αα T cells in their functional aspects (6). In a T cell-transfer model of colitis, ThPOK deletion in CD4 T cells, which enhance the generation of CD4CD8αα T cells, coincides with reduced intestinal inflammation, suggesting non-pathogenic function of DP IELs. In line with this, mice receiving Runx3-deficient CD4 T cells accelerate colitis, which is associated with reduced CD4CD8αα T cells, but increased Th17 differentiation. Mice deficient for CD4-specific Runx3 also develop more pathogenic Th17 cells with increased inflammation and tissue damage compared to control mice after *Citrobacter rodentium* infection; however, they are more efficient at clearing *C. rodentium* than the wild-type control (6). These results suggest that two facet of inflammation, protection and tissue damage, can be modulated at the transcriptional level by Runx3 and ThPOK. Further studies are needed to define the roles of CD4CD8αα T cells during infection and disease progress.

## CONCLUDING REMARKS

Cytotoxic CD4 T cells have not been recognized for a long time since there is a lack of distinct functions, developmental mechanisms, and physiological relevance. Nevertheless, cytotoxic phenotypes of CD4 T cells have been repeatedly reported in human and rodents, mostly CD4CD8αα DP IELs in the intestine. Furthermore, there is accumulating evidence showing the presence of cytotoxic CD4 T cell subsets during anti-viral (38-40) and anti-tumor immune responses (51, 52). Finally, recent elegant studies provide us unequivocal answers for the cytotoxic CD4 T cells as a distinct, independent subtype of effector CD4 T cells, which are derived by the unique modulation of transcription factors that drive their development and functional differentiation (5-7). Although the precise role of CD4CD8αα DP IELs and the relevance of their cytolytic phenotypes *in vivo* are not understood yet, the identification of a new CD4 subset and novel developmental program will improve our understanding on the functional differentiation of effector T cells. New insights gained from these studies will contribute to the identification of new targets for the design of new therapies to prevent and cure infection and diseases.

## ACKNOWLEDGEMENTS

This work was supported by a grant of the Korean Health Technology R&D project (HI14C2171) and Korean Health Technology R&D project through the Korean Health Industry Development Institute (KHIDI) (HI14C2640), funded by the Ministry of Health & Welfare, Korea, and a BK21 Plus grant (grant number: 10Z20130012243) funded by the Ministry of Education, Korea. S-J Moon was supported by National Research Foundation of Korea (NRF) funded by the Korean Government (NRF-2015-Fostering Core Leaders of the Future Basic Science Program/Global Ph.D. Fellowship Program).

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