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IL-15 controls generation of the restricted TCR repertoire of $\gamma\delta$ intestinal intraepithelial lymphocytes

Hang Zhao, Hai Nguyen, and Joonsoo Kang

Department of Pathology, Graduate Program in Immunology and Virology, University of Massachusetts Medical School, Worcester, MA. 01655

Abstract

$\gamma\delta$ T cells are prevalent in the mucosal epithelia and are postulated to act as sentries to maintain tissue integrity. What these $\gamma\delta$ T cells recognize is poorly defined, but based on the restricted T cell receptor (TCR) repertoire, the notion that they are selected by self-antigens of low complexity has been widely disseminated. We present data demonstrating that generation of the restricted TCR $V\gamma$ gene repertoire of intestinal intraepithelial lymphocytes is regulated by IL-15, which induces $V\gamma$ gene segment-specific local chromatin modifications and enhanced accessibility conducive for subsequent targeted gene rearrangement. This cytokine-directed tissue-specific TCR repertoire formation likely reflects distinct TCR repertoire selection criteria for $\gamma\delta$ and $\alpha\beta$ T cell lineages adopted for different antigen recognition strategies.

Intestinal intraepithelial lymphocytes (i-IELs) found within the intestinal epithelial layer are an essential component of mucosal immunity. Similar to T cell subsets in other lymphoid tissues, i-IELs are composed of two T cell types, $\alpha\beta$ and $\gamma\delta$ T cells. In contrast to other tissues, however, $\gamma\delta$ T cells are prevalent in the mucosal epithelia of most vertebrates and are thought to be a major source of secreted factors necessary for the maintenance of tissue integrity subsequent to infection, transformation, or cell injury¹⁻³.

Despite the long history of i-IELs, their origin and ligand specificity remain uncertain. Earlier work has indicated that $\alpha\beta$ i-IELs originate exclusively from the thymus in normal mice⁴, but whether $\gamma\delta$ i-IELs are subject to similar developmental constraints is unclear. The majority of human and mouse $\gamma\delta$ i-IELs express the homodimeric $\alpha\alpha$ form of the CD8 coreceptor and exhibit restricted Variable (V) γ and $V\delta$ gene usage. In C57BL/6 (B6) mice, for example, $V\gamma 5$ expressing cells constitute the predominant i-IEL population in the small intestine⁵⁻⁷. Mouse strain-specific preferential usage of *Tcrg* and *Tcrd* genes among i-IELs arises from complex molecular and cellular controls with evidence for genetic linkage to the *Tcrg*, *Tcrd* and *H2* loci^{6,8,9}, but the exact mechanism is unknown. Two non-mutually exclusive mechanistic processes can be considered to account for the restricted TCR gene usage: cellular selection and programmed TCR gene rearrangement. A hallmark of adaptive immunity is the cellular selection process geared toward forming a population of cells with antigen receptor repertoires that can recognize the entire external universe of antigens. This selection pressure acts on immature cell subsets expressing randomly generated clonal antigen receptors. Although the TCR-driven selection shaping the $\alpha\beta$ TCR repertoire of conventional $\alpha\beta$ T cells constitutes a fundamental theme in immunology, whether a similar process is needed for $\gamma\delta$ T cell development, and specifically for $\gamma\delta$ i-IELs, remains a matter of debate¹⁰⁻¹².

Alternatively, evidence exists for developmentally programmed antigen receptor gene rearrangements¹³, suggesting that the generation of the restricted TCR repertoire among i-IELs may also arise by a directed TCR gene rearrangement process. In this scenario, polymorphic *cis*-acting elements controlling *Tcrg* and *Tcrd* gene accessibility and transcription may be major contributors to the observed differences in V_γ and V_δ gene usage in different mouse strains. In strong support for this notion, it has been demonstrated that the relatively high prevalence of $V_\gamma 5^+$ cells in thymocytes of the 129 strain (~15% of $\gamma\delta\text{TCR}^+$ thymocytes versus <5% in B6 mice) can be attributed largely to specific genetic traits of the *Tcrg* locus itself¹⁴.

Generation of $\gamma\delta\text{T}$ cells is dependent on interleukin 7 receptor (IL-7R)-mediated signals, which are thought to temporally modulate chromatin accessibility at the *Tcrg* locus^{15,16}. IL-7 belongs to the common γ chain cytokine superfamily (γc) that also includes IL-2, IL-4, IL-9, IL-15 and IL-21. One central biochemical pathway triggered by the γc cytokines to regulate gene transcription is the Janus kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) cascade, and except for IL-4, activation of JAK3-STAT5 is primarily targeted by these cytokines. IL-15 exerts a strong influence on i-IELs, especially in the generation and maintenance of the dominant $\text{CD8}\alpha\alpha\text{Thy1}^-\text{V}\gamma 5^+$ $\gamma\delta$ i-IEL population^{7,17}. The ligand for the $\text{CD8}\alpha\alpha$ homodimer is the non-classical MHC class I (class Ib) molecule TL, which is expressed highly on epithelial cells of the small intestine¹⁸. The ligand(s) for the mouse $\gamma\delta\text{TCRs}$ on i-IELs is unknown, although in humans, the restricted i-IEL $\gamma\delta\text{TCR}$ repertoire has been suggested to focus on surveying for the stress induced ligands, MICA and MICB¹⁹. These molecules can also be recognized with high affinity by the natural killer (NK) cell receptor NKG2D²⁰, which is expressed on human, but not mouse, $\gamma\delta$ i-IELs, and whose expression is regulated by the cytokine IL-15 (ref. ²¹). A small fraction of mouse $\gamma\delta\text{T}$ cells (0.1-2%), including $\gamma\delta$ i-IELs, show reactivity to the MHC class Ib molecule T22 (ref. ²²). These limited documentations of $\gamma\delta\text{T}$ cell ligand specificity have been used to buttress the notion that $\gamma\delta\text{T}$ cells in general recognize a limited set of self-antigens using a restricted TCR repertoire, especially in the mucosal epithelia.

In this study we show that the preferential $V_\gamma 5$ gene usage among $\gamma\delta$ i-IELs is controlled by IL-15, which specifically modulates $V_\gamma 5$ gene segment-associated histone acetylation. The chromatin domain(s) controlled by IL-15 is distinct from those under the control of IL-7, which eventually form the $\gamma\delta\text{TCR}$ repertoire found in the spleen and blood. Moreover, $\text{CD8}\alpha\alpha^+$ $V_\gamma 5^+$ cells can be generated intrathymically by IL-15 and are especially dependent on IL-15-activated STAT5. This study constitutes the first demonstration of a cytokine-directed generation of tissue-specific TCR repertoire by selective control of local V gene chromatin accessibility.

Results

IL-15 regulates $V_\gamma 5^+$ i-IEL development

In preliminary experiments, we performed fetal thymic organ cultures (FTOCs) to determine whether other cytokines could substitute for IL-7 in regulating the *Tcrg* locus. When *Il7r^{-/-}* fetal thymi were cultured with different combinations of cytokines, only IL-15 was able to rescue $\gamma\delta\text{T}$ cell development in the absence of IL-7R signaling (Fig. 1a and data not shown). IL-2, the cytokine most closely related to IL-15 in function that signals via shared β and γ chains, was not effective in promoting $\gamma\delta\text{T}$ cell development in FTOCs (data not shown). The extent of $\gamma\delta\text{TCR}^+$ thymocyte generation in *Il7r^{-/-}* FTOCs by the addition of IL-15 was variable (2-25% of the total thymocytes). Unexpectedly, in cultures where $\gamma\delta\text{TCR}^+$ thymocytes developed, all uniformly expressed the $V_\gamma 5$ TCR chain, the predominant γTCR found among $\gamma\delta$ i-IELs (Fig. 1a).

To verify whether IL-15 can promote the development of $V_{\gamma}5$ expressing cells independent of IL-7 *in vivo*, we generated *Il15* transgenic (TgN) *Il7r^{-/-}* mice. Expression of IL-15 under the control of the Major Histocompatibility Complex (MHC) class I gene promoter²³ led to the exclusive development of $V_{\gamma}5^{+}$ $\gamma\delta$ cells in the thymus, spleen and intestine in the absence of IL-7R-mediated signals (Fig. 1b), confirming and extending the FTOC results. This effect was not simply the result of IL-15 acting as a redundant factor for IL-7, since the low thymic cellularity and the deficit in $V_{\gamma}5^{-}$ $\gamma\delta$ T cell subsets, characteristic of *Il7r^{-/-}* mice, were not corrected in *Il15TgIl7r^{-/-}* mice (data not shown). Remarkably, all $V_{\gamma}5^{+}$ $\gamma\delta$ T cells found in *Il15TgIl7r^{-/-}* mice were $CD8\alpha^{+}$ (Fig. 1c), whereas the majority of normal intrathymic $\gamma\delta$ TCR⁺ thymocytes from B6 mice and the few CD8-lineage $\alpha\beta$ TCR⁺ T cells found in *Il7r^{-/-}* mice expressed the conventional CD8 $\alpha\beta$ heterodimer (Fig. 1c and data not shown). In addition, $\gamma\delta$ TCR⁺ thymocytes of *Il15TgIl7r^{-/-}* mice expressed relatively low amounts of the chemokine receptor CCR9 (data not shown), much like $\gamma\delta$ i-IELs²⁴. As $CD8\alpha^{+}$ $V_{\gamma}5^{+}$ $\gamma\delta$ T cells are only found in the gut in normal mice and are specifically lost when IL-15R signaling is defective^{7,17,25}, these results suggest that IL-15 is a major, if not the central, cytokine responsible for $V_{\gamma}5^{+}$ $\gamma\delta$ i-IEL development and/or expansion. Consistent with this interpretation, the loss of $\gamma\delta$ i-IELs in IL-15-deficient mice predominantly involves the $V_{\gamma}5^{+}$ subset⁷. Conversely, the frequency of $V_{\gamma}5^{+}$ cells was increased among i-IELs of *Il15TgN* (IL-7R-sufficient) mice (Supplementary Fig. 1a). In addition, the effects of IL-15 were also evident in the thymus, as the frequency of $V_{\gamma}5^{+}$ thymocytes was selectively and significantly ($P<0.01$) reduced in newborn *Il15^{-/-}* mice as well as in mice lacking full-length (FL) STAT5 (Fig. 1d), a key intracellular signal transducer of IL-15 activated by JAK3 (ref. ²⁵).

In support of the flow cytometry analysis, thymocytes from *Il15TgIl7r^{-/-}* mice did not appreciably express rearranged genes of the *TcrC γ 1* or *TcrC γ 4* loci other than $V_{\gamma}5$ (Fig. 1e, a schematic drawing of the ~60kb *TcrC γ 1* cluster depicted in Fig. 1f). Interestingly, while *Il7r^{-/-}* thymocytes did not show expression of any rearranged *Tcr γ* genes, as expected, transcripts corresponding to the rearranged $V_{\gamma}5$ gene, but not other V_{γ} genes, were detectable at low levels in intestinal intraepithelial cells of *Il7r^{-/-}*, but not *Jak3^{-/-}* mice (Fig. 1e). Since IL-7 and IL-15 are the only known JAK3-dependent cytokines to affect the frequency of $V_{\gamma}5^{+}$ cells, these results suggest that the production of cells with a rearranged $V_{\gamma}5$ TCR gene can be regulated by IL-15, even when IL-7 function is absent.

IL-15 modulates chromatin accessibility of $V_{\gamma}5$ gene

How might IL-15 allow selective development of the $V_{\gamma}5^{+}$ $\gamma\delta$ i-IEL subset? One possibility was that IL-15 is a specific growth factor for $V_{\gamma}5^{+}$ cells. In the absence of IL-7R function, high concentrations of IL-15 might be capable of expanding a few residual cells in the thymus that had rearranged the $V_{\gamma}5$ gene segment. However, in FTOC and suspension cultures of total i-IELs, IL-15 expanded all $\gamma\delta$ TCR⁺ cells, irrespective of TCR V gene usage (data not shown), ruling out the selective expansion of $V_{\gamma}5^{+}$ cells by IL-15 as a likely explanation^{26,27}. Alternatively, it was possible that IL-15, like IL-7, can regulate *Tcr γ* locus chromatin accessibility, but that IL-15 specifically targets $V_{\gamma}5$ gene rearrangement. Data presented here demonstrate that IL-15 stimulates selective $V_{\gamma}5$ gene segment-associated histone acetylation *in vivo*, which correlates with enhanced chromatin accessibility conducive for V(D)J recombination²⁸.

To determine whether IL-15 has a role in specifically directing $V_{\gamma}5$ gene rearrangement we first re-examined the *Tcr γ* locus activity in the absence of IL-7 function. In *Il7r^{-/-}* mice IL-15 signaling should operate in the thymus, and if IL-15 can affect *Tcr γ* gene rearrangement, it was possible that $V_{\gamma}5$ gene rearrangements are present in *Il7r^{-/-}* thymocytes, but perhaps not transcribed optimally. $V_{\gamma}5$ -J γ 1 gene rearrangements were rare, but detectable, in thymocytes of adult *Il7r^{-/-}* mice, whereas the normally dominant $V_{\gamma}2$ gene rearrangement was not (Fig.

2a). It has been previously shown that the $V_{\gamma}1.1$ - $J_{\gamma}4$ gene rearrangements are not strictly dependent on IL-7 signals transmitted via the γc chain²⁹, and this finding was corroborated in $Il7r^{-/-}$ and $Jak3^{-/-}$ thymocytes, where reduced, but detectable, $V_{\gamma}1.1$ gene rearrangements were observed. Notably, only the $V_{\gamma}5$ gene rearrangements were decreased (by approximately one-fifth) in the absence of IL-15 or FL-STAT5, the predominant signal transducer of the IL-15R, and $V_{\gamma}5$ rearrangements were absent in $Jak3^{-/-}$ cells (Fig. 2a). These results, in conjunction with the fact that IL-7 and IL-15 are the only known JAK3-dependent cytokines to affect the development of $V_{\gamma}5^{+}$ cells, suggest that the residual $V_{\gamma}5$ gene rearrangements in $Il7r^{-/-}$ thymocytes are dependent on IL-15-JAK3 signals. The absence of rearranged *Tcr γ* gene transcripts (other than $V_{\gamma}5^{+}$ transcripts in the intestine, Fig. 1e) in $Il7r^{-/-}$ mice despite the presence of cells with rearranged *Tcr γ* genes, and the corresponding lack of $\gamma\delta$ T cells, suggest that IL-7 is necessary for optimal transcription of rearranged *Tcr γ* genes³⁰, and/or the maintenance of these cells²⁹.

To define potential IL-15-regulated molecular events contributing to the modulation of $V_{\gamma}5$ gene rearrangements, we examined the relative efficiency of recombination activating gene (RAG)-mediated double-strand signal sequence breaks at the $V_{\gamma}5$ and $V_{\gamma}2$ genes in adult thymocytes using ligation mediated-PCR (LM-PCR)³¹. This assay serves as an indirect measure of the frequency of cells undergoing active rearrangement at their *Tcr* loci. DNA breaks at recombination signal sequences (RSSs) were not observed in *Rag1*^{-/-} thymocytes, as expected (Fig. 2b). In normal adult B6 thymocytes, $V_{\gamma}2$ and $V_{\gamma}5$ breaks were detectable, indicative of ongoing *Tcr γ* locus gene rearrangements. Strikingly, $V_{\gamma}5$ breaks were substantially reduced, being barely detectable in $Il15^{-/-}$ thymocytes compared to control $Il15^{+/+}$ thymocytes, whereas the amounts of $V_{\gamma}2$ breaks were unaltered. Hence, the noted amount of reduction in $V_{\gamma}5$ - $J_{\gamma}1$ gene rearrangements (Fig. 2a) was an underestimate of the inefficiency of $V_{\gamma}5$ gene recombination in the absence of IL-15. In $Il7r^{-/-}$ thymocytes no $V_{\gamma}2$ breaks were seen, but rare $V_{\gamma}5$ breaks were detectable, supporting the earlier conclusion that some $V_{\gamma}5$ gene rearrangements are occurring in the absence of IL-7-mediated signals *in vivo*. In $Il15TgNIl7r^{-/-}$ thymocytes, $V_{\gamma}5$ breaks were enhanced when compared to $Il7r^{-/-}$ thymocytes, indicating that IL-15 was increasing the frequency of $V_{\gamma}5$ gene rearrangements. These results demonstrate that IL-15 concentrations correlate with the incidence of ongoing $V_{\gamma}5$ gene-specific DNA cleavage, strongly suggesting that IL-15 controls $V_{\gamma}5^{+}$ thymocyte production by directly affecting the rearrangement process.

We next determined the histone3 (H3) acetylation status of $Il15TgNIl7r^{-/-}$ thymocytes. Rearrangement- and transcription-permissive chromatin is usually associated with acetylated histones 3 or 4 (AcH3 or AcH4)³². It has been shown previously by semi-quantitative chromatin immunoprecipitation (ChIP) assays that the *Tcr γ* locus in $Il7r^{-/-}$ thymocytes is relatively inaccessible^{16,33}. This conclusion was based on the fact that the J_{γ} gene segment, the 3' enhancer ($E_{C\gamma}1$), as well as the locus control region-like element (DNaseI hypersensitive site A, HsA^{14,34}), of the *Tcr γ* locus are hypoacetylated. The latter two *cis*-acting elements are redundantly required for transcription of rearranged $V_{\gamma}2$, $V_{\gamma}4$, and $V_{\gamma}3$ genes, but substantially less so for the $V_{\gamma}5$ gene¹⁴. The histone acetylation pattern at the V_{γ} genes has not been analyzed previously. In $Il7r^{-/-}$ thymocytes, AcH3 was nearly undetectable in all regions of the *Tcr γ* gene cluster, consistent with an inactive, inaccessible locus (Fig. 2c, middle). However, low amounts of AcH3 were detected exclusively at the $V_{\gamma}5$ and $J_{\gamma}1$ gene segments (Fig. 2c). In comparison, quantitative ChIP assay (Fig. 2d and illustrated by semi-quantitative gel assays, Fig. 2c) for AcH3 in $Il15TgNIl7r^{-/-}$ thymocytes showed that the $V_{\gamma}5$ (and $J_{\gamma}1$) gene segment chromatin was selectively hyperacetylated, while other regions of the $C_{\gamma}1$ locus remained hypoacetylated. In the negative control, the fetal-specific $V_{\gamma}3$ gene was not associated with detectable amounts of AcH3 in adult thymocytes, regardless of their genotype. These data suggest that IL-15 specifically regulates the local chromatin domain(s) modification or transcription in the vicinity of the $V_{\gamma}5$ gene. The lack of detectable acetylated histones at

$E_{C\gamma 1}$ and HsA in *Il15TgNl7r^{-/-}* thymocytes, correlative to an inaccessible chromatin state, further solidifies previous findings¹⁴ that the rearrangement of the $V_{\gamma 5}$ gene is largely independent of these regulatory sequences (see below).

As alluded to above, $\gamma\delta$ thymocytes of the 129 strain contain a ~ 3 fold higher frequency of $V_{\gamma 5}^{+}$ cells in comparison to B6 mice (¹⁴ and data not shown). We examined whether this difference in V_{γ} gene usage was independent of a TCR driven cellular selection process by performing a ChIP assay for acetylated histones in thymocytes of *129Rag1^{-/-}* and *B6Rag1^{-/-}* mice. Owing to the absence of TCRs on the cells of *Rag1^{-/-}* mice, no thymic cellular selection can take place, and T cell development is blocked at the precursor stages, providing a relatively uniform population of cells with their TCR loci poised for the actions of the V(D)J recombinase. While the amounts of AcH3 associated with the $V_{\gamma 2}$ gene segment were similar between these two precursor thymocyte preparations, a higher amount (~ 3 fold) of AcH3 was found in the $V_{\gamma 5}$ locus of *129Rag1^{-/-}* precursor thymocytes (Fig. 2e). This result strongly indicates that the strain-dependent enhancement of $V_{\gamma 5}$ usage in thymocytes is correlated at the level of chromatin accessibility, prior to *Tcr* gene rearrangement.

To more strictly demonstrate a link between IL-15 and the TCR V gene-specific epigenetic modifications, we next performed the sterile transcription and ChIP assays using immature lymphoid lineage cells from *Rag1^{-/-}* mice expressing varying levels of IL-15. The amount of germline sterile transcripts emanating from the unrearranged *TcrV_γ* gene segments is an indicator of relative gene activity, and is correlative and predicative of the developmentally-regulated *Tcr* gene rearrangement pattern³⁵. In adult *Rag1^{-/-}* thymocytes, unrearranged $V_{\gamma 2}$ and $V_{\gamma 5}$ gene segment-specific transcripts were detectable, whereas $V_{\gamma 3}$ gene-specific sterile transcripts were absent, reflecting the developmental stage-specific rearrangement pattern of these V genes (Fig. 3a). In addition, the $V_{\gamma 2}$ sterile transcripts were relatively more abundant than those of $V_{\gamma 5}$ in adult *Rag1^{-/-}* thymocytes, correlating with the TCR repertoire of $\gamma\delta$ thymocytes in normal B6 mice (among adult $\gamma\delta$ thymocytes $\sim 50\%$ are $V_{\gamma 2}^{+}$, while $\sim 5\%$ express $V_{\gamma 5}$). Significantly, while the $V_{\gamma 2}$ (and $V_{\gamma 1.1}$ or $V_{\gamma 4}$, data not shown) gene segment-specific transcript abundance was not altered by varying concentrations of IL-15, those of $V_{\gamma 5}$ were decreased by $\sim$ one-fifth when IL-15 was absent, and increased dramatically (>10 fold) when IL-15 was over-expressed.

The histone acetylation pattern correlated strongly with the sterile transcription assay. Unlike the situation in *Il7r^{-/-}* mice, thymocytes of *Rag1^{-/-}* mice displayed a histone acetylation pattern consistent with the adult V_{γ} gene usage: $E_{C\gamma 1}$ and HsA were active, as shown by the relatively high amounts of co-localized AcH3 (Fig. 3b and quantitative real-time PCR in Fig. 3c). In addition, among the V_{γ} gene segments, the $V_{\gamma 2}$ gene was associated with the highest amounts of acetylated histones, whereas the fetal-specific $V_{\gamma 3}$ gene was hypoacetylated, consistent with published results³⁶. The $V_{\gamma 5}$ gene had low, but significant, levels of acetylated histones bound to it (Fig. 3b), again correlating with the low frequency of $V_{\gamma 5}^{+}$ $\gamma\delta$ thymocytes in normal adult B6 mice. Similar results have been interpreted to support the chromatin accessibility model of developmental regulation of V(D)J recombination at the *Tcr* locus³⁶. Importantly, in the absence of IL-15, consistently less acetylated histones were found on $V_{\gamma 5}$ gene segments (Fig. 3b, c). This pattern was observed in all experiments using thymocytes and intestinal intraepithelial cells (much more pronounced in the latter, see below), and was consistent with results from the sterile transcription assay (Fig. 3a). However, the difference was difficult to quantify due to the low basal amount of acetylated histones detectable at the $V_{\gamma 5}$ gene of normal thymic precursors using this assay. On the other hand, in thymocytes of *Il15TgNl7r^{-/-}* mice, greatly increased amounts of AcH3 were detected at the $V_{\gamma 5}$ gene, again corroborating the sterile transcription pattern. The H3 acetylation profile in other regions of the *C_γ1* locus was not altered by varying levels of IL-15. In conjunction with the observation that mice lacking IL-15 function have a pronounced deficit in $V_{\gamma 5}^{+}$ i-IELs⁷ (Fig. 1d), these results clearly

demonstrate that IL-15 controls the generation of $V_{\gamma}5^{+}$ cells by modulating histone acetylation, and thereby chromatin accessibility, at the $V_{\gamma}5$ locus prior to V(D)J recombination.

To verify that the histone acetylation patterns observed in thymocytes from *Il7r^{-/-}* and *Rag1^{-/-}* mice are also found in normal thymocytes, we sorted the triple negative thymic precursor cell subset ($CD3^{-}CD4^{-}CD8^{-}$, TN) from B6 mice and repeated the ChIP assay. Increased expression of IL-15 led to an enhanced association of ACh3 specifically with the $V_{\gamma}5$ gene segment in normal precursor cells (Fig. 3d). Collectively, these results demonstrate that the basis for IL-15-mediated generation of $V_{\gamma}5^{+}$ $\gamma\delta$ T cells is the selective enhancement of $V_{\gamma}5$ gene segment activity prior to the RAG-mediated *Tcr* gene rearrangement.

IL-15 control of $V_{\gamma}5$ gene in intestinal lymphoid cell

Since the origin of i-IELs remains an unresolved issue, it was important to address the effects of IL-15 on immature lymphoid cells of the gut. As physiological IL-15 concentration is thought to be high in the intestine^{21,37,38}, we predicted that the selective $V_{\gamma}5$ gene accessibility should be much more evident in lymphoid precursors of the intestine if these cells were subjected to a similar regulation of *Tcr* gene usage as in thymocytes. In *Rag1^{-/-}* intestinal intraepithelial cells, sterile transcription of the unrearranged $V_{\gamma}5$ gene segment was relatively prominent compared to the $V_{\gamma}2$ gene, whereas the reverse was the case in the thymus (Fig. 4a). Sterile transcription of the $V_{\gamma}1.1$ gene, utilized by both the splenic and intestinal $\gamma\delta$ T cells, was not substantially different in thymocytes and intestinal cells of *Rag1^{-/-}* mice. It should be noted that the amounts of $V_{\gamma}5$ sterile transcript in the intestinal intraepithelial cells were very high in comparison to *Rag1^{-/-}* thymocytes. In all experiments, the putative lymphoid lineage cells (identified by the expression of IL-7R and/or CD4, and only sample sets that contained similar proportions of lymphoid lineage cells were compared) among the *Rag1^{-/-}* intestinal intraepithelial cell preparations constituted less than 15% of the total, making it highly probable that the difference in $V_{\gamma}5$ sterile transcript abundance between the intestine and thymus is an underestimate. Hence, the relative V_{γ} gene segment activity before V(D)J gene rearrangement correlates well with the observed tissue-specific V_{γ} repertoire, suggesting a spatial control of *Tcr* gene rearrangement and/or expression.

We next examined the histone acetylation pattern of the V_{γ} gene segments of the *TcrC γ 1* locus in intestinal intraepithelial cells. In *Rag1^{-/-}* intestinal intraepithelial cell preparations, ACh3 was found selectively at the $V_{\gamma}5$ gene segment (Fig. 4b). This result is consistent with the sterile transcription assay showing a biased $V_{\gamma}5$ gene activity (Fig. 4a), and it strongly supports the existence of a distinct pre-rearrangement V_{γ} segment accessibility in favor of the $V_{\gamma}5$ gene in immature intestinal intraepithelial cells compared to thymocyte precursors (Fig. 3a). Critically, this basal $V_{\gamma}5$ gene specific H3 acetylation was dependent on IL-15, as evidenced by the diminution of ACh3 detected in *Il15^{-/-}Rag1^{-/-}* intraepithelial cell preparations (Fig. 4b, c). Conversely, an increased level of IL-15 in the intestine of *Rag1^{-/-}* (Fig. 4b, c) and *Il7r^{-/-}* mice (Fig. 4d, e) resulted in greatly enhanced amounts of ACh3 specifically at the $V_{\gamma}5$ gene segment. These results demonstrate that IL-15 specifically induces local histone modifications consistent with enhanced chromatin accessibility at the $V_{\gamma}5$ gene segment in immature intestinal intraepithelial cells. Taken together, these data indicate that IL-15 establishes permissive $V_{\gamma}5$ gene segment accessibility for directed gene rearrangement in both the thymus and intestine. Whether sufficient functional recombinase activity exists in the putative intestinal lymphoid precursors of normal mice is controversial^{39,40}, but irrespective of anatomical sites, our results show that IL-15 programs targeted enhancement in $V_{\gamma}5$ gene accessibility conducive for selective gene rearrangement.

IL-15 control of V γ 5 gene requires STAT5

To determine whether the intracellular components of the IL-15 signaling pathway can directly modulate *Tcr*g V gene segment chromatin accessibility, we tested the contribution of STAT5, a central transcription factor activated by IL-7 and IL-15 signaling that has been proposed to be the key factor controlling general *Tcr*g locus accessibility⁴¹. Although adult mice that cannot express FL-STAT5 (hypomorphic *Stat5*^{-/-} mice^{42,43}) do not exhibit pronounced defects in *Tcr*g gene rearrangement, peripheral $\gamma\delta$ T cells are severely depleted in these mice²⁵. Similar to defects observed in lymphoid lineage cells of *Il15*^{-/-} mice, *Fl-Stat5*^{-/-} mice have greatly reduced numbers of $\gamma\delta$ i-IELs²⁵, while neonatal *Fl-Stat5*^{-/-} mice have a specific reduction in V γ 5⁺ $\gamma\delta$ thymocytes (Fig. 1d), raising the possibility that IL-15-activated STAT5 is responsible for the induction of V γ 5 gene rearrangement. A unique function of STAT5 for IL-15 signaling in *Tcr*g gene regulation is further suggested by the presence of a STAT5 consensus binding site in the V γ 5 gene promoter. Other V γ gene promoters of the *Tcr*C γ 1 locus do not possess this sequence motif (data not shown). Our ChIP analysis of ex vivo *Rag1*^{-/-} thymocytes showed that STAT5 binds to the consensus site in the V γ 5 gene promoter, and that the level of association was substantially increased by *IL15*TgN expression (Supplementary Fig. 1b). In addition, a specific reduction in V γ 5 gene rearrangement was observed in *Fl-Stat5*^{-/-} thymocytes (Fig. 2a), consistent with the role of STAT5 in V γ 5 gene rearrangement. Lastly, among the lymphocyte subsets regulated by IL-15, $\gamma\delta$ i-IELs are the most dependent on FL-STAT5: While the paucity of NK and activated (CD44⁺) CD8⁺ T cells in *Fl-Stat5*^{-/-} (and in *Il15*^{-/-}) mice is corrected in *Il15*TgN*Fl-Stat5*^{-/-} mice, strikingly, $\gamma\delta$ i-IEL defects persist (unpublished results).

To directly test the proposal that STAT5 controls V γ 5 gene segment accessibility, we generated *Il15*TgN*Fl-Stat5*^{-/-}*Rag1*^{-/-} mice and examined histone modification at the *Tcr*g locus. Conspicuously, the IL-15-dependent hyperacetylation at the V γ 5 gene segment in STAT5-sufficient cells was completely abrogated when FL-STAT5 was absent (Fig. 5a, comparing the amounts of AcH3 in the first and last sample sets). The quantitative real-time ChIP data for AcH3 (Fig. 5b) and AcH4 (data not shown) showed the same trend. In comparison, the amounts of AcH3 and AcH4 associated with the V γ 2 gene segment in adult immature thymocytes were not dependent on FL-STAT5. The effects of FL-STAT5 were difficult to discern in precursor thymocytes of *Rag1*^{-/-} mice since the basal amount of AcH3 at the V γ 5 gene was very low and the assay may not be sufficiently sensitive to discriminate potential changes. However, in *Rag1*^{-/-} intestinal intraepithelial cells, the basal AcH3 detectable at the V γ 5 gene is relatively high and the absence of FL-STAT5 led to a reproducible decrease of AcH3 specifically at the V γ 5 gene, showing that the endogenous IL-15-mediated epigenetic modification of the *Tcr*g locus is also dependent on FL-STAT5 (Fig. 5c). Taken together, these results demonstrate a distinct requirement for FL-STAT5 in IL-7 versus IL-15-mediated signals. While IL-7-activated FL-STAT5 is not absolutely essential to the overall *Tcr*g locus regulation, including chromatin modification, gene rearrangement and transcription in adult $\gamma\delta$ T cell development²⁵, FL-STAT5 activated in the context of IL-15 signaling is indispensable for induction of local *Tcr*V γ 5 gene specific chromatin modification.

Discussion

Despite considerable efforts, events leading to the establishment of restricted TCR gene usage among i-IELs in the mucosal epithelia are largely unknown. This is an important issue since it has implications for deducing the nature of $\gamma\delta$ TCR-ligand interactions. Here, we demonstrate that IL-15 programs targeted V γ 5 gene rearrangement in immature precursor cells by modulation of *Tcr*g subdomain chromatin accessibility. This molecular connection between IL-15 and *Tcr*g repertoire formation provides one explanation for the unusual dependence of V γ 5⁺ TCR expressing cells on IL-15, as evidenced by the selective loss of V γ 5⁺ i-IELs in IL-15

signaling-defective mice⁷. It should be stressed that the primary molecular programming of *Tcr* gene rearrangement by IL-15 does not necessarily preclude a subsequent additional checkpoint, perhaps governed by cell surface TCR expression, to generate a stable, functional $\gamma\delta$ IEL repertoire. A precedent for a cellular selection process acting on a uniform population of fetal $V_{\gamma}3^{+}$ $\gamma\delta$ thymocytes generated by targeted TCR gene rearrangement has been recently described⁴⁴. However, the major ligand(s) for $V_{\gamma}5^{+}$ $\gamma\delta$ i-IELs is currently unknown, and the nature of potential secondary cellular selection remains speculative.

This study does not directly address the anatomical origin of $\gamma\delta$ IELs. Recent findings have indicated the exclusive origin $\alpha\beta$ TCR⁺ i-IELs from CD4⁺CD8⁺ thymocytes in euthymic mice⁴, but the situation remains murky for $\gamma\delta$ i-IELs. The developmental requirements for $\gamma\delta$ i-IELs are distinct from those of $\alpha\beta$ i-IELs^{4,10} and suggestive evidence for the extrathymic development of $\gamma\delta$ i-IELs exists^{40,45}. However, in unperturbed normal mice, robust RAG1 and RAG2 expression is difficult to detect in the gut³⁹. Consistent with this finding, we have not been able to detect RAG-mediated DNA breaks at V_{γ} gene segments among i-IELs of normal or *Il15*TgN mice using LM-PCR assays (data not shown), whereas they are easily detectable in thymocytes. Accordingly, IL-15-driven intrathymic generation of CD8 $\alpha\alpha^{+}$ $V_{\gamma}5^{+}$ cells with gut-homing properties, perhaps from a distinct precursor subset than for the peripheral $\gamma\delta$ T cells, may be the primary source of $\gamma\delta$ i-IELs that are then expanded and maintained in the gut by the combination of IL-15 and IL-7. This scenario is reminiscent of the $V_{\gamma}3^{+}$ $\gamma\delta$ dendritic epidermal T cells that arise in the thymus via a programmed gene rearrangement exclusively during the fetal stage, which are positively selected in the thymus to acquire the skin homing ability⁴⁶. Hence, despite the fact that immature lymphoid cells in both the thymus and intestine are governed similarly by IL-15 to become preferentially poised to generate $V_{\gamma}5$ -expressing cells, as assessed by histone acetylation patterns, and $\gamma\delta$ i-IEL development is less compromised than $\alpha\beta$ i-IELs in athymic mice^{39,40}, the apparent paucity of RAG proteins in the gut of euthymic mice may prohibit substantial generation of $\gamma\delta$ i-IELs in the intestine. Caveats to this interpretation are that there might be as yet unidentified intestinal niches where active *Tcr*g and *Tcr*d recombination does occur, but currently employed experimental assays are insensitive to detect such events and/or RAG1 and RAG2 are transiently expressed in the intestine in certain conditions, such as in neonatal stages or during acute infection of the gut to permit extrathymic $\gamma\delta$ T cell development.

Although our data indicate that IL-15 was sufficient to program $V_{\gamma}5$ gene rearrangement intrathymically, the absence of $V_{\gamma}5^{+}$ (or $V_{\gamma}1.1^{+}$) $\gamma\delta$ T cells in *Il7r*^{-/-} mice, despite the evidence for gene rearrangements involving these V genes, indicate that IL-7 is necessary for full maturation of $\gamma\delta$ i-IELs in normal physiological condition. It has been shown that IL-7 is required for normal transcription of rearranged *Tcr*g genes^{30,47}. The 3'E_{CY1} and the HsA, although not essential, are cooperatively required for optimal transcription of the rearranged $V_{\gamma}5$ genes in thymocytes¹⁴. The greatly impeded accessibility of these two regulatory elements in *Il7r*^{-/-} cells³³ may lead to suboptimal expression of the TCR and potentially contributes to the loss of cells that had rearranged the $V_{\gamma}5$ gene. In the presence of high amounts of IL-15 and STAT5, $V_{\gamma}5$ gene expression appears less dependent on the regulatory elements and $V_{\gamma}5^{+}$ cells are more likely to develop even when IL-7 activity is absent. This interpretation is consistent with the detectable expression of the rearranged $V_{\gamma}5$ gene in intestinal intraepithelial cells of *Il7r*^{-/-} mice, which are presumably exposed to high levels of IL-15, but not in *Il7r*^{-/-} thymocytes. Definitively establishing the quantitative aspect of complex IL-15 signaling⁷ in distinct tissues *in vivo* will require the development of reliable reagents to detect IL-15 and the IL-15R α chain. It is also possible that IL-7 signaling is required for the maintenance and survival of i-IELs and that in its absence, some cells with productive $V_{\gamma}5$ gene rearrangements directed by IL-15 may arise, but these cells cannot persist. Although mature i-IELs, unlike resting peripheral T cells, do not express high amounts of IL-7R, the requirement for IL-7 during certain periods of i-IEL maintenance cannot be ruled out.

Both IL-7 and IL-15 predominantly activate JAK3-STAT5, but the unique dependence of IL-15 on FL-STAT5 (ref. ²⁵) further accentuates the differing signaling properties of these two γ c cytokines. Lymphocytes of *Fl-Stat5*^{-/-} mice still express partly functional NH2-terminal truncated proteins (H. N. and J. K., unpublished results) and these STAT5 isoforms, which are also found in normal cells, are sufficient to ensure normal intrathymic T cell development regulated by IL-7, but they are insufficient for IL-15 signaling^{25,43}. The N-terminal region of STAT5 has been implicated in both positive and negative regulatory functions of nuclear STAT5, including nuclear retention, protein dephosphorylation, oligomerization, and co-factor recruitment⁴⁸. Among these properties, STAT5 tetramerization has emerged as a central feature of optimal STAT5 activity, especially critical for stimulating the expression of genes with suboptimal STAT5 consensus binding motifs⁴⁹. The underlying basis for the relatively normal IL-7-regulated adult intrathymic T cell development mediated by N-terminal truncated STAT5 protein versus aberrant development of lymphoid lineages controlled by IL-15 in *Fl-Stat5*^{-/-} mice is likely to entail cell-type specific differences in both the quantitative and qualitative aspects of IL-7 and IL-15-mediated signal transduction.

A highly related issue to the unique signaling properties of IL-7 and IL-15 is why the increased IL-15 signaling via STAT5 is incapable of establishing an accessible chromatin across the entire *Tcr* locus since it has been shown that a constitutively active form of STAT5 (CA-STAT5) can bypass the requirement for IL-7R signaling in TCR γ gene rearrangement in FTOC^{16,41}. One possibility is that the *Tcr* locus of the cells capable of responding to IL-15 is irreversibly maintained in a relatively inaccessible chromatin configuration. An attendant prediction of this model is that all i-IELs with *Tcr* gene rearrangements involving the C γ 1 cluster on both chromosomes exclusively exhibit V γ 5 gene rearrangements. However, analysis of *Tcr* gene rearrangement pattern in V γ 5⁺ $\gamma\delta$ thymocyte clones does not support this model: In four V γ 5⁺ T cell clones with gene rearrangements in the C γ 1 locus in both alleles, the non-productive rearrangement involves V γ 2, indicating that V γ 5⁺ cells are not inherently restricted to rearranging V γ 5 genes⁵⁰. An alternative explanation is that IL-7 activates an unknown cofactor(s) that cooperate with STAT5 in making the locus globally accessible and/or IL-15 activates factors that inhibit accessibility of other V γ genes and/or preferentially target active STAT5 to the V γ 5 gene segment. Implicit in this scheme is that cells triggered by IL-15 do not receive sufficient IL-7 signals and/or the outcome of IL-7 signaling is qualitatively different. Indirect evidence indicating that the history of γ c cytokine exposure can alter a cell's response to other γ c cytokines exists^{51,52}. Our study clearly underscores exquisitely distinct functions of IL-7 and IL-15 on the *Tcr* locus, which cannot be accounted for simply by activation of STAT5 alone. We have reported previously that FL-STAT5's function in T cell development is developmentally regulated, being more critical in fetal stages²⁵. The reported ability of CA-STAT5 to restore $\gamma\delta$ T cell development in the absence of IL-7R⁴¹ may also be restricted to the fetal stage (or FTOC) since in adult *CA-Stat5TgNl17r*^{-/-} mice, $\gamma\delta$ T cell development is not restored to the numbers observed in WT mice (M. Farrar, Univ. of Minnesota, personal communication).

The γ c cytokines IL-7 and IL-4 can globally and regionally permit increased chromatin accessibility for *Tcr* and *Igh* chain gene rearrangement and immunoglobulin class switching, respectively. The results from this study establish another level of regulation of chromatin accessibility by a cytokine *in vivo* that displays an exquisite selectivity: STAT5-dependent IL-15 regulated V gene segment-specific chromatin modification leading to the generation of a tissue-restricted $\gamma\delta$ TCR repertoire. Hence, cellular selection is unlikely to be the initial driving force behind the formation of the gut-restricted $\gamma\delta$ TCR repertoire. To date, no firm evidence for the requirement of MHC molecules in the overall $\gamma\delta$ TCR repertoire formation has been obtained^{10,12} and the cytokine-directed TCR repertoire shaping most likely represents a substitute mechanism for the MHC selection processes. Whether development of other

lymphocyte subsets with a restricted TCR repertoire, such as NKT⁵³ and mucosal-associated invariant T cells⁵⁴, is similarly influenced by cytokines will require further investigation.

Methods

Mice

B6Il7r^{-/-}, *Fl-Stat5^{-/-42}*, *B6Il15TgN²³* and *B6Il15^{-/-17}* mice have been described. *Fl-Stat5^{-/-}* mice were backcrossed to B6 mice five times. *129Rag1^{-/-}* mice were purchased from Taconic. Routinely, 4-6 weeks old mice were used for experiments. For fetuses, the day of vaginal plug was designated day 0. All mice used in this report were housed in a pathogen free rodent barrier facility. All animal experiments were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (Worcester, MA).

Cell preparation and culture

i-IELs, intestinal intraepithelial cell preparations and nylon wool column-passed splenic cell preparations enriched for T and NK lymphocytes were obtained using published protocols²⁵. For comparison of intestinal intraepithelial cell subsets from *Rag1^{-/-}* genetic background, preparations containing similar proportions (routinely 12-15% of the total) of lymphoid progenitors (IL-7Rα⁺ and/or CD4⁺) were used. For FTOC, *Il7r^{-/-}* and littermate control embryonic day (E) 14 or E15 thymic lobes were cultured for 5-12 days on transwell plates (Corning). Standard culture media (RPMI 1640 with 10% FCS, 50μM 2-mercaptoethanol, 2mM L-glutamine, 20mM HEPES and antibiotics) supplemented with recombinant IL-15 (10-20ng/ml) or IL-7 (20ng/ml) (R&D) was used. In some experiments supernatants from *Il7* or *Il2* transfected cell lines were used at 2% v / v. After culture, single cell preparations were analyzed by FACS.

Antibodies and Flow Cytometry

Anti-TCR V_γ5 mAb GL1⁵ was generously provided by L Puddington (UConn, Farmington, CT) and anti-mouse CCR9 Ab²⁴ was from Dr. P. Love (NIH, Bethesda, MD). The following Abs were purchased from E Biosciences or BD Pharmingen: Abs specific for CD4 (Cy5 conjugated), CD8α (Cy5), CD8β (biotin), CD3ε (Cy5), TCRβ (fluorescein isothiocyanate, FITC), TCRγδ (biotin), V_γ2TCR (biotin and FITC), V_γ3TCR (FITC), CD122 (biotin), CD127 (biotin), and NKRP1.1 (FITC). Streptavidin-phycoerythrin was purchased from BD Pharmingen. Flow cytometric analyses were performed on an EPICS XL cytometer (BD-Coulter). Data were analyzed using FlowJo software (Tree Star). Cells were sorted using MoFlo (Cytomation).

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was carried out according to manufacturer's instructions (Upstate Biotechnology) with minor modifications. Briefly, $1-2 \times 10^6$ cells (each pooled from 3-5 mice) were fixed with 1% formaldehyde. Cells were washed in ice-cold PBS containing 1 μg/ml pepstatin A (Sigma) and a complete protease inhibitor cocktail (Roche). Cell pellets were resuspended in SDS lysis buffer supplemented with the protease inhibitor mixture for 10 min on ice. Samples were sonicated to an average DNA length to 0.1-0.6 kb. Cellular debris was removed by centrifugation for 10 min at 16,000g at 4 °C. The supernatant was diluted 10-fold in buffer supplemented with the protease inhibitor mixture. An aliquot of the diluted supernatant was used as "input" DNA control after reverse cross-linking. The cell supernatant was pre-cleared with salmon sperm DNA/protein A agarose for 30 min at 4 °C with agitation. Immunoprecipitation was carried out (overnight at 4°C on a rotator) with a 1:200 dilution of the anti-AcH3, AcH4 (Upstate) or STAT5 Abs (Santa Cruz) along with no Ab controls. Immune complexes were mixed with salmon sperm DNA/protein A agarose followed by

incubation for 1 h at 4 °C on a rotator. After extensive washing, the complexes were eluted by adding a 250- μ l aliquot of a freshly prepared 1% SDS, 0.1 M NaHCO₃ solution. The sample was briefly vortexed and incubated at room temperature for 15 min on a rotator. After microcentrifugation, the protein-DNA complex was washed with a second aliquot, and the resulting supernatant was pooled with the first aliquot. The protein-DNA cross-link was reversed at 65 °C for 4 h. Samples were digested with 2 μ l of proteinase K (10 mg/ml) at 45 °C for 1 h, and the DNA was recovered by phenol and chloroform extractions. The precipitated DNA was dissolved in 20 μ l of Tris-EDTA pH 7.4 buffer (designated the “bound DNA fraction”) and subjected to PCR or real-time PCR analysis. PCR was carried out for 28-35 cycles, and the products were visualized by ethidium bromide staining. The sequences of the primer pairs used are listed in Supplemental Table 1. SYBR green PCR core reagents (Applied Biosystems) were used for the real time PCR assay using the BioRad iCycler. The amount of the immunoprecipitated “bound” DNA was normalized against input DNA.

Semi-quantitative PCR, RT-PCR and LM-PCR

PCR and RT-PCR assays to detect *Tcrg* gene rearrangements and transcripts were performed as described^{14,34,35} using primers published and listed in Supplementary Table 1. LM-PCR to detect double-strand signal sequence breaks 3' of the V γ 5 and V γ 2 gene segments were carried out as described^{31,44} using primers listed in Supplementary Table 1. Semi-quantitative radioactive PCR (40 or 41 cycles) was used to detect PCR products, using a 5% polyacrylamide gel. Genomic DNA was extracted using Wizard genomic DNA purification kit (Promega). RNAs were isolated using a micro-to-midi total RNA purification Kit (Invitrogen) or Trizol. RNA was treated with DNase I to digest contaminating genomic DNA. RNA samples were reverse transcribed (RT) with Superscript II (RNase H⁻) reverse transcriptase using oligo-dT₍₁₂₋₁₈₎ primers (Invitrogen).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Generation of $V_{\gamma}5^{+}$ $\gamma\delta$ thymocytes by IL-15 in the absence of IL-7 signaling. **(a)** Development of $\gamma\delta TCR^{+}$ thymocytes in FTOC (5-7 days) of E14 *II7r^{-/-}* lobes in the presence of IL-15 (10ng/ml). Cultures with IL-7 (left) were used as negative controls. For the middle profile, percentage of $V_{\gamma}5^{+}$ cells refers to the boxed area. Total thymocyte numbers in each culture ranged from 1.5×10^5 to 3.8×10^6 /lobe when IL-15 was added ($n=15$) and $<1 \times 10^5$ to 1.4×10^6 /lobe ($n=22$) when other cytokines were included. **(b, c)** Flow cytometric analysis of *II15TgNII7r^{-/-}* mice (4-8 weeks old). Analysis of thymocytes (top, $n=6$), nylon-wool passed spleen cells (NWPS) enriched for T cells (middle, $n=9$) and i-IELs (bottom, $n=8$), stained for the indicated markers, is shown. Numbers in the plots indicate percentage of the total ($\pm$ SEM). In **(c)**, profiles show CD8 α or CD8 β expression on gated $\gamma\delta TCR^{+}$ cells. **(d)** Flow cytometric analysis of CD4⁻CD8⁻ $\gamma\delta TCR^{+}$ thymocyte subsets in 1 day old mice with defects in IL-15 signaling. Total thymocyte numbers were comparable in *II15^{-/-}* and B6 newborn mice, but reduced to 1/3 to 1/2 in *Fl-Stat5^{-/-}* animals. Numbers in the plots indicate average percentage of the total ($\pm$ SEM) based on the analyses of minimum of three mice of each genotype. **(e)** RT-PCR analysis for rearranged *Tcrγ* gene transcripts in *II7r^{-/-}* tissues with different concentrations of IL-15. Relative tubulin (*Tubb*) mRNA amounts were used as loading controls. One of two independent experiments with similar results is shown. **(f)** A schematic depiction of the murine TCR $C_{\gamma}1$ cluster (not to scale) showing the relative position of V_{γ} genes and two *cis*-acting regulatory sequences, 3'EC $_{\gamma}1$ and HsA. V_{γ} -specific primers for all ChIP experiments are from the promoter regions (black boxes).

Figure 2.

Histone acetylation patterns correlate with V γ gene usage. **(a)** Semi-quantitative PCR assay (5-fold serial dilution series, one of two experiments) for *Tcrg* gene rearrangement in thymocytes of various γ c cytokine signaling-defective mice compared to B6 controls. **(b)** LM-PCR assay to determine relative abundance of RAG-mediated DNA cleavage at V γ 5 and V γ 2 RSSs in thymocytes of various genotype. For PCR, genomic DNA samples were used at the highest dilution for B6 and *Il15^{-/-}* thymocytes (left two lanes, $\approx$ 100ng of ligated DNA), while 5-fold serial dilution series were used ($\approx$ 100ng and 20ng) for the rest of the samples. Amounts of *Tubb* PCR products were used to normalize for input DNA quantity. One of three experiments with similar results is shown. **(c)** ChIP assay for the relative presence of AcH3 in HsA, 3'EC γ 1, the promoter regions of V γ 5, V γ 2, V γ 3 gene segments, the immediate 5' upstream region of J γ 1 gene segment and positive control *Gapdh* promoter in thymocytes of *Il15TgNIl7r^{-/-}*, *Il7r^{-/-}* and control *Il7r^{+/+}* mice. Control (Ctrl) lanes represent no Ab added during immunoprecipitation for all ChIP experiments. Results shown are representative of three independent experiments. **(d)** Quantitative real-time ChIP data of the experiment described in c. The ratios of co-immunoprecipitated DNA (AcH3 bound) over arbitrary DNA input quantitative PCR assay control (input) are the mean $\pm$ SEM of triplicate data points from a representative experiment (one of three) using pooled thymocytes from 4 mice of each genotype. J γ 1 and *Gapdh* gene regions were not examined quantitatively. **(e)** Quantitative real-time ChIP assay for relative AcH3 associated with the V γ 5 versus V γ 2 promoter regions of *Rag1^{-/-}* mice of 129 or B6 strain.

Figure 3.

IL-15 regulates transcriptional activity and alters histone chromatin modification specifically at the V_γ5 gene segment independent of RAG-mediated *Tcrg* gene rearrangement in thymocytes. **(a)** A representative semi-quantitative RT-PCR assay (one of four experiments) for relative amounts of un-rearranged sterile V_γ gene-specific transcripts in thymocytes of *Rag1^{-/-}* mice exposed to different amounts of IL-15. A 5-fold serial cDNA sample dilution series is shown. RT⁻, no reverse transcriptase control. **(b, c)** ChIP assays for relative AcH3 patterns across the *TcrC_γ1* cluster in thymocytes of *Rag1^{-/-}* mice with differing amounts of IL-15. Shown are a representative agarose gel **(b)** and quantitative real-time PCR assay **(c)**. In **b**, input DNA control was serially diluted 4-fold. In **c**, bound/input DNA ratios are the mean ± SEM of triplicate data points from a representative experiment (one of three) using pooled thymocytes from 4 mice of each genotype. **(d)** Quantitative real-time ChIP assay for relative AcH3 patterns across the *TcrC_γ1* cluster in sorted (>95% pure) precursor TN thymocytes of *Il15TgN* or control B6 mice. Bound/input DNA ratios are the mean ± SEM of triplicate data points. One of two independent experiments with similar results is shown.

Figure 4.

Comparison of V_γ5 gene segment-specific transcriptional activity and chromatin accessibility in the intestine versus thymus. **(a)** A representative semi-quantitative RT-PCR assay (one of 5 experiments) for relative abundance of un-rearranged sterile V_γ gene-specific transcripts in thymocytes versus intestinal intraepithelial cell preparations of *Rag1*^{-/-} mice. A 5-fold serial cDNA sample dilution series is shown. RT⁻, no reverse transcriptase control. **(b)** A representative ChIP assay (one of three) for the relative abundance of ACh3 in the promoter regions of V_γ5, V_γ2, V_γ3 gene segments in intestinal intraepithelial cell preparations of *Rag1*^{-/-} mice with differing amounts of IL-15 (4 to 5 mice of each genotype). **(c)** Quantitative real-time ChIP data of the experiment described in **b**. Ratios are the mean ± SEM of triplicate data points from a representative experiment (one of three) using pooled cells from 4 mice. * denotes statistically significant difference ($P < 0.05$, Student's *t* test). **(d)** A representative ChIP assay (one of three) for the relative presence of ACh3 in the promoter regions of V_γ5, V_γ2, V_γ3 gene segments and the 5' upstream region of J_γ1 gene segment in intestinal intraepithelial cell preparations of *Il17r*^{-/-} mice with or without *Il15TgN* expression. Relative ACh3 amounts at the *Gapdh* promoter were used as positive control for the assay. **(e)** Quantitative real-time ChIP data of the experiment described in **d**. Ratios are the mean ± SEM of triplicate data points from a representative experiment (one of two) using pooled cells from 4 mice. J_γ1 and *Gapdh* gene regions were not examined quantitatively.

Figure 5.

Increased AcH3 modification at the V_γ5 gene segment that results from *Il15TgN* expression is dependent on FL-STAT5. **(a)** A representative ChIP assay (one of three experiments) for the relative abundance of AcH3 in the promoter regions of V_γ5, V_γ2, V_γ3 gene segments in thymocytes of *Il15TgN Rag1^{-/-}* mice with or without FL-STAT5. **(b)** Quantitative real-time ChIP data of the experiment described in **a**, assessing relative amounts of AcH3 associated with specific V_γ gene promoters. Ratios are the mean ± SEM of triplicate data points from a representative experiment (one of two) using pooled thymocytes from 3 mice. **(c)** A representative semi-quantitative ChIP assay (one of two) for the relative presence of AcH3 in the promoter regions of indicated V_γ gene segments in intestinal intraepithelial cell preparations of *Rag1^{-/-}* mice with or without FL-STAT5.