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MicroRNAs and other mechanisms regulate interleukin-17 cytokines and receptors

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1. ABSTRACT

Interleukin-17 cytokines are a family of pro-inflammatory cytokines. Our current studies found: *i)* IL-17 cytokines are not ubiquitously expressed, but several receptors and TRAF3IP2 are ubiquitously expressed in tissues with a few exceptions; *ii)* heart and vascular tissue are in the second tier of readiness to respond to IL-17 cytokine stimulation; *iii)* alternative transcription starting sites and alternative spliced isoforms are found in IL-17 cytokine and receptor transcripts; *iv)* higher hypomethylation status is associated with higher expressions of IL-17 receptors; *v)* the binding sites of several RNA binding proteins are found in the 3'UTRs of the mRNAs of IL-17 cytokines and receptors; and *vi)* numerous microRNA binding sites are statistically equivalent to that of experimentally verified microRNAs-mRNA interactions in the 3'UTRs of IL-17 cytokine and receptor mRNAs. These results suggest that mechanisms including alternative promoters, alternative splicing, RNA binding proteins, and microRNAs regulate the structures and expressions of IL-17 cytokines and receptors. These results provide an insight into the roles of IL-17 in mediating inflammation and immunity.

2. INTRODUCTION

Cardiovascular disease (CVD) remains a leading cause of fatality in well-developed countries. Despite a long held understanding and strong characterization of the traditional and non-traditional risk factors for CVD, some mechanisms of CVD onset have only recently been elucidated. As a chronic inflammatory disease, atherosclerosis and its progression involve both the adaptive and innate immune systems(1). For example, we and others reported that $CD4^+CD25^{high}$ regulatory T cells(2-4) (a type of adaptive immune cells) and $Ly6C^{mid/high}$ monocytes (a type of innate immune cells) play suppressive and promoting roles respectively, in the pathogenesis of atherosclerosis and vascular inflammation(5). Since 2003, a new lineage of $CD4^+$RORgammat$^+$ (retinoid-related orphan receptor gamma) T cells has been defined by its production of pro-inflammatory cytokine interleukin-17 (IL-17) and hence named T-helper 17 (Th17) cells. These cells have been found to play an essential role in promoting autoimmune diseases, inflammation(6, 7), and potentially atherosclerosis(8-11) (see our invited review(12)). Increased Th17, rather than Th1 response is associated with

some vasculopathy(13), suggesting an important role for IL-17 cytokines in cardiovascular diseases. However, detailed tissue expression and regulation mechanisms of IL-17 cytokines and receptors remain poorly defined in cardiovascular and other tissues.

IL-17 cytokine family consists of six members designated IL-17A-F. IL-17 cytokine family members are highly conserved between human and mouse with homology of 62% to 88%(14) among the six members across species. The first cytokine of this family studied was IL-17A (also known as IL-17)(15, 16). Sequentially, the other five members were cloned and categorized into the IL-17 cytokine family(17-20). IL-17 cytokines are a family of pro-inflammatory cytokines implicated in numerous autoimmune and inflammatory diseases. IL-17 cytokines have found to induce expression of pro-inflammatory cytokines and chemokines including CXCL1, CXCL2, IL-6, and G-CSF(21, 22) via the activation of nuclear factor - kappaB (NF-κB) and mitogen-activated protein (MAP) kinase pathways(23, 24). It has been shown that IL-17 and IL-17F have the ability to form dimers, and the main functions of IL-17, IL-17F, and IL-17A/F are in autoimmune pathology, extracellular pathogen immunity, and neutrophil recruitment(7, 25). However, the issue of which tissues express both dimer-forming cytokines remains poorly defined.

The IL-17 receptor family is made up of five unique cytokine receptor members: IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE(26). Similar to the cytokines, IL-17 receptors between human and mouse also show a great degree of homology (68% to 90%)(14). IL-17RA and IL-17RC are the receptors for IL-17 and IL-17F, and they are the best characterized receptors among this family of cytokine receptors(27-29). IL-17RB has affinity for IL-17B and IL-17E, IL-17RE binds IL-17C, and the ligands for IL-17RD remain to be elucidated(18, 30). IL-17RA has been shown to be ubiquitously expressed but at higher levels in hematopoietic tissues(27). IL-17 has been shown to activate some of its pro-inflammatory effects via NF-κB and MAPK pathways. A component of the IL-17 receptors that is crucial for IL-17 signaling is an adaptor termed ACT1 (also known as TRAF3IP2; TRAF3 interacting protein 2), which is needed to mediate various downstream event of the IL-17 receptors(31-34). However, the issue of which tissues express receptor complexes remains poorly defined.

Despite significant progress, several important knowledge gaps exist which prevent investigators from defining the detailed roles of these molecules in inflammation and immune responses. First, how IL-17 cytokines and receptors are expressed in cardiovascular and other tissues; second, whether alternative splicing and alternative promoters regulate the structures of IL-17 cytokines and receptors; and third, whether mRNA decay proteins (AU-rich element binding proteins)(35, 36) and microRNAs(37) regulate the mRNA stability and translation of IL-17 cytokines and receptors. Using database mining techniques and statistical analysis similar to that we reported previously(38, 39), we examined the expressions of IL-17 cytokines and receptors in cardiovascular and other tissues from a panoramic viewpoint. In addition, we also examined the potential molecular mechanisms regulating the expression of these IL-17 cytokines and receptors. In depth analysis of the expression patterns of these important cytokines and receptors could prove vital in further understanding the underlying mechanism for immune responses and inflammation. This insight may provide novel avenues for innovative therapeutic treatments for pro-atherogenic inflammation and other cardiovascular diseases.

3. METHODS

3.1. Tissue expression profiles of genes encoding IL-17 cytokines, IL-17 receptors, RORC, and TRAF3IP2

Experimental data mining strategy, as we previously described(38-40) (Figure 1), was used to analyze the expression profiles of mRNA transcripts of IL-17 cytokines, IL-17 receptors, RORC, and TRAF3IP2 in cardiovascular and other tissues in humans and mice by mining experimentally verified human and mouse mRNA transcript expressions in the sequence tag (EST) databases of the National Institutes of Health (NIH)/National Center of Biotechnology Information (NCBI) Unigene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene)(41). Transcripts per million of genes of interest were normalized with that of house-keeping beta-actin in any given tissue to calculate the arbitrary units of the gene expression. The confidence interval of the expression variation of house-keeping genes was generated by calculating the mean and 2 times the standard deviation of the arbitrary units of three randomly selected house-keeping genes (RPS27A, GADPH, and ARHGDIA in human; Ldha, Nono, and Rpl32 in mouse) normalized by beta-actin in given tissues. If the expression variation of a given gene in the tissues was larger than the upper limit of the confidence interval (the mean plus 2 times the standard deviation) of the house-keeping genes, the high expression levels of genes in the tissues were statistically significant. Any given gene transcript, if lower than one per million, was technically presented as no expression.

3.2. Alternative spliced isoforms of IL-17 cytokines and IL-17 receptors

The presence and features of alternative promoters and alternatively spliced isoforms of each gene were examined with the AceView database of the National Institutes of Health (NIH)/National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html).

3.3. Presence of AU-rich elements and functional motifs in 3' untranslated regions of IL-17 cytokines and IL-17 receptors

The gene of interest was searched in the UTRdb (http://utrdb.ba.itb.cnr.it/search) at the Institute for Biomedical Technologies, University of Bari for the existence of functional motifs and signals in the 3' untranslated regions (3'UTR) of each mRNA. The presence of AU-rich elements in the 3'UTRs of IL-17

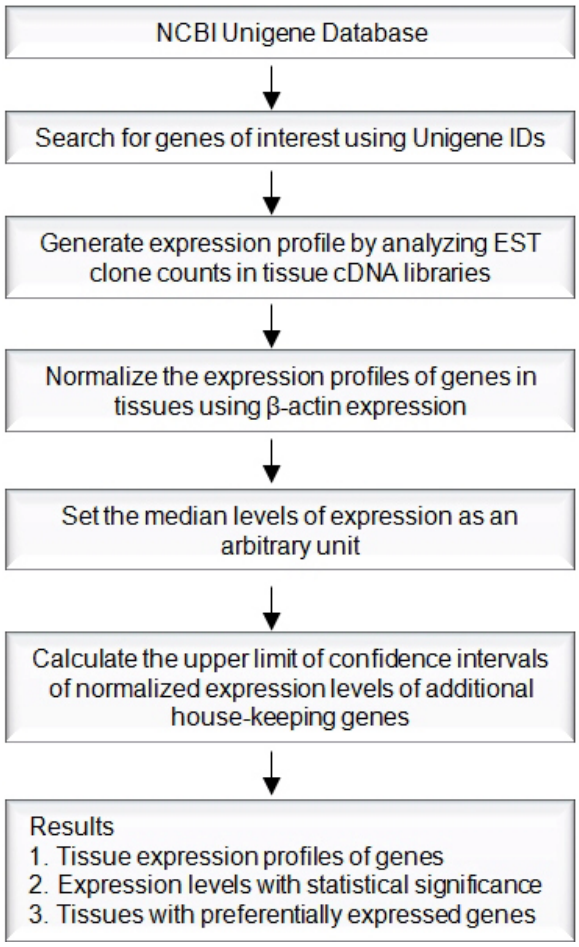

Figure 1. Flow Chart of Database Mining Analysis of Gene Expression Profiles Using NCBI/UniGene Database.

cytokines and receptors was searched using the AU-rich Element Containing mRNA Database (ARED 3.0) (http://brp.kfshrc.edu.sa/ARED/). AU-rich elements or AREs have been classified into three classes based on the presence and distribution of AUUUA sequence (42).

3.4. Correlation of the ratios of tissue SAH versus SAM concentrations with the expression levels of IL-17 cytokine and receptor mRNA transcripts

The concentrations of *S*-adenosylmethionine (SAM) levels over *S*-adenosylhomocysteine (SAH) levels were determined previously by Ueland's group (43-45) in tissues from adult male mice under physiological condition. The SAM and SAH levels were measured in perchloric acid extracts by high performance liquid chromatography. The SAM/SAH ratios were calculated for the current analysis based on Ueland's results (43-45). Tissue SAH and SAM concentrations and SAM/SAH ratios were used for further comparison and regression analyses. Simple linear regression analyses were performed using Sigma Plot 9.0 (Systat Software, Inc, San Jose, CA, USA) by plotting mRNA levels of individual gene against SAM/SAH ratios in seven mouse tissues including the brain, kidney, liver, spleen, heart, lung, and thymus. Multivariable regression analyses were performed to evaluate the effect of SAM/SAH ratios on the expressions of IL-17 cytokines and receptors.

3.5. MicroRNA interaction with the mRNAs of IL-17 cytokines and IL-17 receptors

The interactions of the mRNAs of genes of interest with microRNAs were examined using the Bioinformatics and Research Computing software TargetScan Release 5.1. (http://www.targetscan.org/) from the Whitehead Institute for Biomedical Research of Massachusetts Institute of Technology (MIT). The significance of microRNAs binding to the genes of interest was determined using the confidence intervals generated from the microRNAs within the Tarbase, an experimentally verified microRNA online database (http://diana.cslab.ece.ntua.gr/tarbase/)(46, 47). Briefly, human microRNAs, which were single site effective and confirmed with luciferase reporter assays, were used for establishing the intervals. Using single site effective and luciferase assay confirmed microRNAs ensures that the interactions between the microRNAs and their respective mRNA targets are specific. 27 microRNAs that met the criteria were selected and evaluated in TargetScan to construct the intervals and set the lower limit for the context values and score percentile. MicroRNAs with the context score of 70% or higher and context value of -0.22 or lower were determined to be significant.

4. RESULTS

4.1. Most of IL-17 cytokines are not constitutively expressed in the tissues examined, but several IL-17 receptors and TRAF3IP2 are ubiquitously expressed

We hypothesized that in order to keep inflammation in check, IL-17 cytokines and receptors are differentially expressed in cardiovascular and other tissues. To examine this hypothesis, a database mining method as we reported(40) was used to examined experimentally verified expression profiles of mRNA transcripts of IL-17 cytokines and receptors (Table 1). The copy number per million transcripts was calculated based on the experimental data of expression sequence tag (EST) cDNA cloning and sequencing in the NCBI UniGene database. The gene expression data were normalized by beta-actin expression data in the same tissue, thus the arbitrary units of gene expression were comparable among genes (Figure 2). Statistical significance is defined when gene expression is larger than the upper limit of the confidence interval (the mean plus 2 times the standard deviation) of the housekeeping genes. As shown in Table 2A and 2B, the expressions of six IL-17 cytokines, IL-17A-F, five IL-17 receptors, IL-17RA-E, RORC, and TRAF3IP2 (ACT1) gene transcripts were examined in 16 tissues: adrenal gland, blood, bone marrow, brain, eye, heart, intestine, kidney, lung, lymph node, pancreas, placenta, spleen, thymus, trachea, and vascular. The EST profiles for human IL-17A, human IL-17E, mouse IL-17c, and mouse IL-17e were not found in the EST NCBI UniGene database, suggesting lower abundance of these molecules than other IL-17 cytokines expressed in tissues. Of the 16 tissues examined, only a few tissues expressed IL-17 cytokines,

Table 1. The Unigene id of human and mouse genes examined

Genes	Unigene ID	Genes	Unigene ID
L-17B	Hs. 156979	IL-17a	Mm.5419
IL-17C	Hs.278911	IL-17b	Mm.59313
IL-17D	Hs.655142	IL-17d	Mm.390726
IL-17F	Hs.272295	IL-17e	Mm.90154
RORC	Hs.256022	IL-17f	Mm.222807
IL-17RA	Hs.129751	rorc	Mm. 4372
IL-17RB	Hs.654970	IL-17ra	Mm.4481
IL-17RC	Hs.129959	IL-17rb	Mm.269363
IL-17RD	Hs.150725	IL-17rc	Mm.213397
IL-17RE	Hs.390823	IL-17rd	Mm.206726
TRAF3IP2	Hs.561514	IL-17re	Mm.131781
		traf3ip2	Mm.436686

Table 2. IL-17 cytokines and receptors are differentially expressed in human and mouse tissues

Tissues	Cytokine and RORC	Tissues	Receptor and TRAF3IP2
A. IL-17 cytokines and receptors are differentially expressed in human tissues			
1. Adrenal Gland	RORC	1. Adrenal Gland	IL-17RA, IL-17RB, IL-17RC, TRAF3IP2
2. Blood	IL-17F	2. Blood	IL-17RA, IL-17RC, IL-17RD, TRAF3IP2
3. Bone Marrow		3. Bone Marrow	IL-17RA, IL-17RC, TRAF3IP2
4. Brain	IL-17D, RORC	4. Brain	IL-17RA, IL-17RB, IL-17RC, IL-17RD, IL-17RE, TRAF3IP2
5. Eye	IL-17B, IL-17D	5. Eye	IL-17RA, IL-17RB, IL-17RC, IL-17RD, IL-17RE, TRAF3IP2
6. Heart	IL-17B, IL-17D	6. Heart	IL-17RB, IL-17RC, IL-17RD, TRAF3IP2
7. Intestine	RORC	7. Intestine	IL-17RA, IL-17RB, IL-17RC, IL-17RD, IL-17RE, TRAF3IP2
8. Kidney	RORC	8. Kidney	IL-17RA, IL-17RB, IL-17RC, IL-17RD, IL-17RE, TRAF3IP2
9. Lung	IL-17B, IL-17C, IL-17D, RORC	9. Lung	IL-17RA, IL-17RB, IL-17RC, IL-17RE, TRAF3IP2
10. Lymph Node	RORC	10. Lymph Node	IL-17RA, IL-17RB, IL-17RE, TRAF3IP2
11. Pancreas	IL-17D, RORC	11. Pancreas	IL-17RA, IL-17RB, IL-17RC, IL-17RE, TRAF3IP2
12. Placenta	IL-17B, IL-17D	12. Placenta	IL-17RA, IL-17RB, IL-17RC, IL-17RE, TRAF3IP2
13. Spleen		13. Spleen	IL-17RA, IL-17RC, IL-17RD, TRAF3IP2
14. Thymus	RORC	14. Thymus	IL-17RC, IL-17RE, TRAF3IP2
15. Trachea	RORC	15. Trachea	IL-17RC, IL-17RD, TRAF3IP2
16. Vascular	IL-17D	16. Vascular	IL-17RA, IL-17RC, TRAF3IP2
B. IL-17 cytokines and receptors are differentially expressed in mouse tissues			
1. Adrenal Gland		1. Adrenal Gland	
2. Blood		2. Blood	IL-17ra, IL-17rb, traf3ip2
3. Bone Marrow	rorc	3. Bone Marrow	IL-17ra, IL-17re, traf3ip2
4. Brain	IL-17d	4. Brain	IL-17ra, IL-17rb, IL-17rc, IL-17rd, traf3ip2
5. Eye	IL-17b, IL-17d	5. Eye	IL-17ra, IL-17rb, IL-17rc, IL-17rd, IL-17re, traf3ip2
6. Heart	IL-17d, rorc	6. Heart	IL-17rc, traf3ip2
7. Intestine	IL-17b, rorc	7. Intestine	IL-17ra, IL-17rc, IL-17re, traf3ip2
8. Kidney	rorc	8. Kidney	IL-17ra, IL-17rb, IL-17rc, IL-17rd, IL-17re, traf3ip2
9. Lung	IL-17b, IL-17d, rorc	9. Lung	IL-17ra, IL-17rc, IL-17rd, IL-17re, traf3ip2
10. Lymph Node		10. Lymph Node	IL-17ra, traf3ip2
11. Pancreas		11. Pancreas	IL-17ra, IL-17rc, IL-17rd, traf3ip2
12. Placenta		12. Placenta	
13. Spleen	IL-17f	13. Spleen	IL-17ra, IL-17rc, IL-17re
14. Thymus	IL-17a, rorc	14. Thymus	IL-17ra, IL-17rb, IL-17re, traf3ip2
15. Trachea		15. Trachea	
16. Vascular		16. Vascular	

which correlate with previous reports(15). But our analysis examined more cardiovascular inflammation-related human and mouse tissues for expression of IL-17 cytokines than previous reported. These expression patterns suggest that the non-detected IL-17 cytokines are not required for physiological functions of these tissues; and that the expressions of these non-detected IL-17 cytokines are not beneficial for physiological functions of these tissues.

Among the IL-17 cytokines that had expression (Table 2A), human IL-17D was most widely expressed, and it was found in vascular, placenta, heart, brain, pancreas, eye, and lung. IL-17D has been shown to have an important role in endothelial cell pathology; in HUVECs (Human Umbilical Vein Endothelial Cells) IL-17D upregulates pro-inflammatory cytokines IL-6 and IL-8 production and induces GM-CSF(48). Human IL-17B was expressed in placenta, heart, eye, and lung. IL-17B and IL-17D were expressed significantly in the heart. Human IL-17F and IL-17C were expressed in blood and lung, respectively. In addition, we found that Th17 cell-specific transcription factor RORC (RORgamma) was expressed in trachea, adrenal gland, brain, pancreas, thymus, kidney, lung, and intestine. The tissue expression pattern of RORC was broader than that of Th17 cytokines IL-17A and IL-17F, which was conserved evolutionally in mouse and

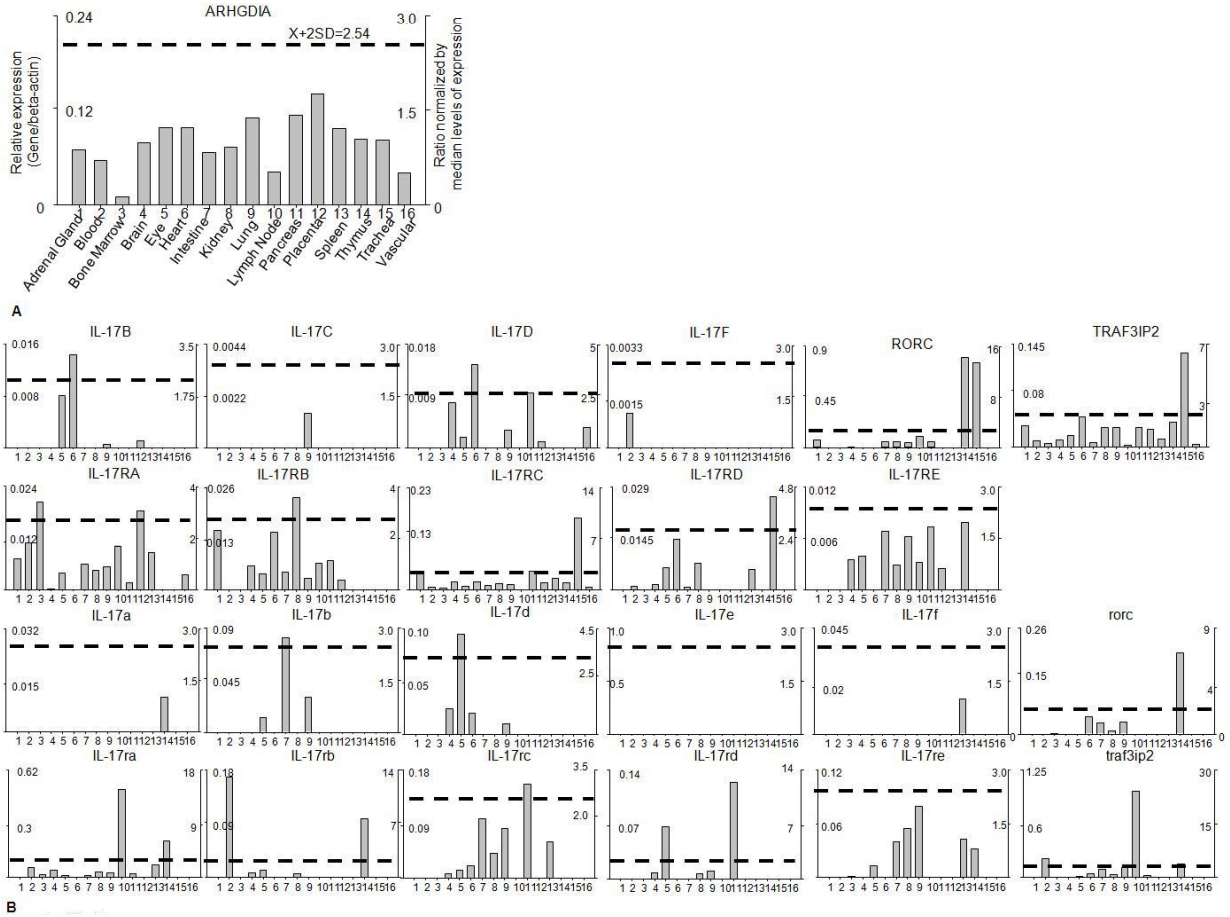

Figure 2. A. Data Presentation Format (The data presented in X-, Y-axis and tissue order are applied to all the genes examined). B. Tissue Expression Profiles of IL-17 cytokines and receptors, RORC, and TRAF3IP2. The gene expression profiles of IL-17 cytokines and receptors, TRAF3IP2, and RORC in mouse and human tissues. A) As an example, the gene expression profiles of human housekeeping gene Rho GDP dissociation inhibitor (GDI) alpha (ARHGDIA) in the sixteen tissues including adrenal gland, blood, bone marrow, brain, eye, heart, intestine, kidney, lung, lymph node, pancreas, placenta, spleen, thymus, trachea, and vascular are presented, with the tissue names and position numbers are shown on the X-axis. The gene expression data were normalized by the β-actin *(*Hs.520640*)* expression data from the same tissue, which are presented on the left Y-axis. The expression ratios among tissues were generated by normalizing the arbitrary units of the gene in the tissues with the median level of the arbitrary units of the gene in all the tissues which are presented on the right Y-axis. In order to define confidence intervals for statistically higher expression levels of given genes, we calculated the confidence intervals of tissue expression [the mean X + 2 x standard deviations (SD) = 2.54] for three housekeeping genes including Rho GDP dissociation inhibitor alpha (ARHGDIA, Hs.159161), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs.544577), and ribosomal protein S27a (RPS27A, Hs.311640). The expression variations of given genes in tissues, when they were larger than 2.54-fold, were defined as the high expression levels with statistical significance (the right Y-axis). To define confidence intervals for statistically higher expression levels of given genes in mouse tissue, we calculated the confidence intervals of tissue expression [the mean X + 2 x standard deviations (SD) = 2.67] for three mouse housekeeping genes including Lactate dehydrogenase A (Ldha, Mm.29324), non-POU-domain-containing octamer binding protein (Nono, Mm.280069), and ribosomal protein L32 (Rpl32, Mm.104368). The expression variations of given genes in tissues, when they were larger than 2.67-fold, were defined as the high expression levels with statistical significance (the right Y-axis). B) The expression profiles of IL-17 cytokines and receptors, TRAF3IP2, and RORC in human tissues (top two rows, with cytokine and receptor family members designated with capital letters) and mouse tissues (bottom two rows, with cytokine and receptor family members designated with lowercase letters). The X-axis indicates the sixteen tissues examined in the same order as that shown in Figure 2A with position numbers shown.

humans. These results suggest that other factors may also be involved in the development of IL-17A- and IL-17F-secreting Th17 cells. Mouse IL-17 cytokines expressed in tissues differently from that in human tissues but the mouse tissue expression of IL-17d was similar to that in human tissues (Table 2B). In mouse, expressions of IL-17b in intestine and IL-17d in eye were statistically significant.

IL-17 receptor A (IL-17RA), IL-17RC, and TRAF3IP2 were expressed in all human tissues examined

Table 3. The two-tier expression status of IL-17 cytokine receptors are identified in human and mouse tissues

IL-17 Receptor Complex	Human Tissue	Mouse Tissue
First tier ("ready to go" expression status with all components)		
IL-17RA/C Complex +TRAF3IP2	Blood, vascular, placenta, bone marrow, adrenal gland, brain, pancreas, eye, spleen, kidney, lung, intestine	Brain, pancreas, eye, spleen, kidney, lung, intestine
IL-17RA/B Complex +TRAF3IP2	Placenta, adrenal gland, brain, lymph node, pancreas, eye, kidney, lung, intestine	Blood, brain, eye, thymus, kidney
Second tier ("inducible" expression status that requires up-regulation of at least one component)		
IL-17RA/C Complex +TRAF3IP2	Trachea, heart, lymph node, thymus	Blood, vascular, placenta, trachea, bone marrow, adrenal gland, heart, lymph node, thymus
IL-17RA/B Complex +TRAF3IP2	Vascular, trachea, blood, bone marrow, heart, spleen, thymus	Vascular, placenta, trachea, bone marrow, adrenal gland, heart, lymph nodes, pancreas, spleen, lung, intestine

Tissues examined are categorized into first tier or second tier of readiness to respond to IL-17 stimulation based on their expression of IL-17 receptor complexes and TRAF3IP2. Tissues in the first tier of readiness express both IL-17 RA/C or IL-17RA/B and TRAF3IP2 are in a "ready to go" status. Tissues in the second tier of expression are in an "inducible" status which requires up-regulation of at least one component for IL-17 signaling

.except in trachea, heart, and thymus for IL-17RA, and in lymph nodes for IL-17RC, which correlate with others findings that IL-17RA was found in human epithelial cells, fibroblast, B and T lymphocytes, stromal cells(49), and vascular endothelial cells(26). IL-17RA expressions in human bone marrow and placenta were statistically significant. IL-17RB in human kidney was expressed significantly. IL-17RC expressions in human pancreas and trachea were statistically significant. In addition, IL-17RB was expressed in 10 out of 16 tissues; IL-17E was also expressed in 9 out of 16 tissues; IL-17RD was expressed in 8 out of 16 tissues. The tissue expression patterns of IL-17 receptors and TRAF3IP2 were most conserved among mouse and human tissues. Also, in mouse statistically significant expression were found in lymph node and thymus for IL-17ra, in blood and thymus for IL-17rb, in pancreas for IL-17rc, and in pancreas and eye for IL-17rd. Expression of TRAF3IP2 was significant in human trachea while the expression of this gene in mouse was found to be significant in blood, lymph node, thymus, and lung.

4.2. Heart and vascular tissues are in the second tier of readiness to respond to IL-17 cytokine stimulation, which requires the upregulation of IL-17 receptor complex components

IL-17 and IL-17F define a new lineage of IL-17-producing $CD4^+$ T helper (Th17) cells(50, 51). We hypothesized that functional status of IL-17 receptor complex can be different among tissues based on the expression status of IL-17 receptor complex and TRAF3IP2. Since the complex of IL-17RA and IL-17RC is required for IL-17 and IL-17F signaling(29), and TRAF3IP2 is essential for IL-17 signaling(52, 53), we divided the tissues examined into two tiers based on their expression of IL-17 receptor complexes and TRAF3IP2 (Table 3). IL-17RA/B is required for IL-17E signaling(54, 55). Tissues that express IL-17 receptor complexes (A/C or A/B) and TRAF3IP2 are placed in the first tier of "ready to go" status. Tissues that do not express TRAF3IP2 or all the receptors are placed in the second tier of "inducible" status with induction/upregulation of one or more components needed for a complete complex of IL-17 signaling (Figure 3).

Of note, in term of expressions of both IL-17RA/C complex and IL-17RA/B complex, heart was in the second tier. IL-17 signaling may participate in chronic inflammation in heart in response to stimulation by pro-inflammatory risk factors. For the expression of IL-17RA/B complex, vascular tissue was also in the second tier, suggesting that IL-17RA/B complex may be involved in acute and chronic inflammation in vascular tissue, and that IL-17RA/C complex and IL-17RA/B complex may participate in chronic inflammation in heart in response to pro-inflammatory stimuli of risk factors. Moreover, more human tissues were in the first tier than mouse tissues, suggesting that the complexes of IL-17RA/C and IL-17RA/B are more involved in the inflammation of these human tissues than in those tissues of mouse. In the most of the first tier tissues in human including placenta, adrenal gland, brain, pancreas, eye, kidney, lung, and intestine, the expressions of IL-17RA/C and IL-17RA/B signaling pathways were overlapped, which may suggest the assurance of function of IL-17 signaling in these tissues.

4.3. Alternative promoter and alternative splicing regulate the structures and expressions of IL-17 cytokines and receptors

Recent findings justify a renewed interest in alternative splicing, which affects the expression of 60% of human genes. Alternative splicing explains how a vast mammalian proteomic complexity is achieved with a limited number of genes(56). Alternative splicing regulation not only depends on the interaction of splicing factors with splicing enhancers and silencers in the pre-mRNA, but also on the coupling between transcription and splicing. This coupling is possible because splicing is often co-transcriptional, and promoter identity and occupation may affect alternative splicing(57). Due to the lack of expression data for each of the alternatively spliced isoforms of IL-17 cytokines and IL-17 receptors in the database, we focused on the roles of alternative splicing in regulating the structure of IL-17 cytokines and IL-17 receptors. We hypothesized that alternative promoter and alternative splicing regulate the structure of IL-17 cytokines and the receptors. To test this hypothesis, we examined the AceView-NCBI database, the NIH-supported, most comprehensive database of alternative promoters and alternatively spliced isoforms of genes based on experimental data of cDNA cloning and DNA sequencing analysis of tissue mRNA transcriptomes(58). As shown in Table 4A and 4B, human IL-17 and mouse IL-

Table 4. Alternative promoter and alternative splicing regulate the expression and structures of IL-17 cytokines and receptors

A. Alternative promoter and alternative splicing regulate the expression and structures of IL-17 cytokines						
Gene	**Exon(s)**	**Total Isoform(s)**	**ORF Isoform(s)**	**IL-17 domain /secreted**	**Promoter(s)**	
IL-17A	3	1	1	1	1	
IL-17B	6	3	3	2	2	
IL-17C	4	2	2	1	1	
IL-17D	8	5	5	2	3	
IL-17E	3	2	2	2	1	
IL-17F	3	2	2	1	2	
IL-17a	3	1	1	1	1	
IL-17b	8	7	7	4	4	
IL-17c	2	1	1	1	1	
IL-17d	3	3	2	2	1	
IL-17e	3	1	1	1	1	
IL-17f	9	4	4	2	2	
B. Alternative promoter and alternative splicing regulate the expression and structures of IL-17 receptors						
Gene	Exon(s)	Total Isoform(s)	ORF Isoform(s)	Secreted	SEFIR domain	Promoter(s)
IL-17RA	33	23	18		<18	3
IL-17RB	11	2	1		1	1[1]
IL-17RC	39	28	26	5	<26	4
IL-17RD	18	5	4		<4	1
IL-17RE	13	1	1		1	1[1]
IL-17ra	14	4	3	1	<3	1
IL-17rb	11	1	1		1	1[1]
IL-17rc	20	7	7		<7	2
IL-17rd	12	1	1		1	1[1]
IL-17re	23	11	11	3	<11	2

Of note: The data were retrieved from the NIH-NCBI-AceView database except those marked with [1], which were retrieved from the NIH-NCBI-Gene database. AceView-NCBI database was used to examine alternative promoter and alternative spliced isoforms of genes. A majority of the IL-17 cytokine and receptor genes in both human and mouse has numerous isoforms. Many of the genes have alternative promoters and more than one open reading frame (ORF) suggesting that alternative promoters and alternative splicing regulate the structure and expression of IL-17 cytokines and receptors. Human cytokine and receptor family members are designated with capital letters while mouse cytokine and receptor family members are denoted with lowercase letters.

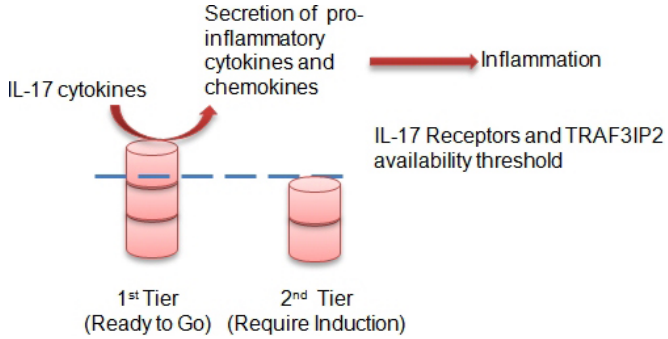

Figure 3. The Two-Tier Model of IL-17 Receptors and TRAF3IP2 Expression. in Human and Mouse Tissues. Tissues in the first tier expression status will secrete pro-inflammatory cytokines and chemokines to induce inflammation in response to IL-17 cytokine stimulation while tissues in the second tier need up-regulation of at least one component in the IL-17 receptor complex to induce secretion of pro-inflammatory cytokines and chemokines to drive the inflammatory process.

17 had no alternatively spliced isoform, suggesting the functional intolerance for structural variations of these molecules. In contrast, other human IL-17 cytokines and mouse IL-17 cytokines had numerous isoforms. Human IL-17B, IL-17D, and IL-17F had alternative promoters, suggesting that the importance of alternative promoters of IL-17 cytokines in response to tissue-specific and/or stimulation-specific transcriptional regulation (Figure 4). Human IL-17RA (18 isoforms), IL-17RC (26 isoforms), and IL-17RD (4 isoforms) had more than three open reading frames (ORFs). These results suggest that alternative splicing plays more important roles in regulating the structures of IL-17 receptors than regulating the structures of IL-17 cytokines, which may serve as a compensatory mechanism since IL-17 receptors were more widely expressed than IL-17 cytokines in the tissues examined. Future work is needed to determine whether pro-atherogenic risk factors regulate alternative splicing and alternative promoters.

4.4. Higher hypomethylation status is positively associated with higher expressions of IL-17 receptors and lower expression of IL-17d in mouse tissues

Previously, it has been shown that epigenetic changes at the IL-17A/F locus are associated with Th17 differentiation(59). To demonstrate the possibility that the expressions of IL-17 cytokines and receptors are regulated by intracellular metabolic stimuli, we hypothesized that the

Table 5. SAH and SAM levels found in mouse tissues

	Metabolite Concentrations (nmol/g wet wt)		
Tissue	**SAM**	**SAH**	**SAM:SAH**
Brain	35.8 ± 4.0	0.8 ± 0.1	47.1 ± 9.6
Heart	58.5 ± 4.2	0.4 ± 0.3	142.7±87.6
Kidney	107.4 ± 5.5	4.2 ± 0.8	25.6±5.0
Liver	112.8 ± 12.4	25.5 ± 3.9	4.4±0.8
Lung	47.7± 3.6	5.5 ± 0.9	8.6±1.5
Spleen	65.2 ± 8.1	1.7 ± 0.4	38.4±10.2
Thymus	41.3 ± 11.5	1.2 ± 0.3	34.7±13.6

Concentrations of SAM and SAH in mouse tissues were previously examined by Ueland *et al.*

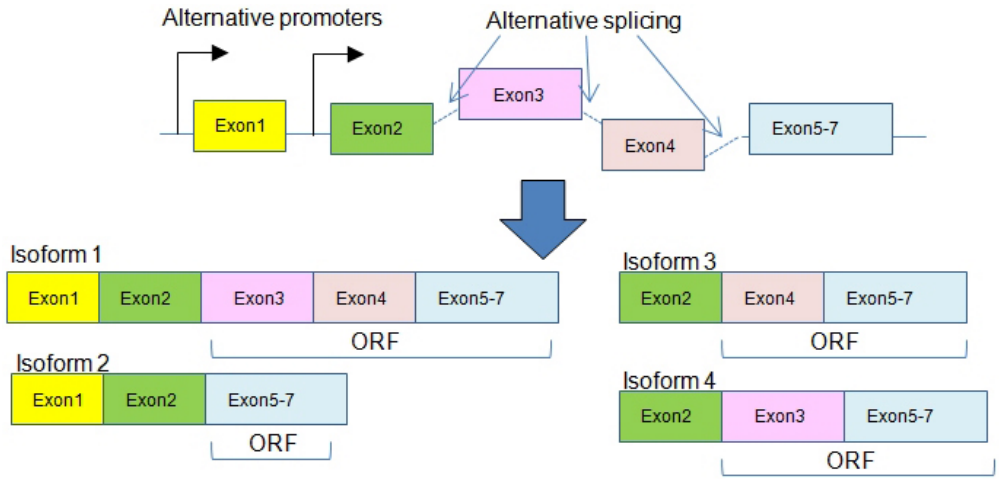

Figure 4. Schematic Representation of Alternative Promoter and Alternative Splicing Mechanism in Generating Different Isoforms of IL-17 Cytokines and Receptors. Alternative promoter and alternative splicing regulate the expression and structure of IL-17 cytokines and receptors. Schematic representation of how alternative promoter and alternative splicing affect the open reading frame of a gene and contribute to transcription of various isoforms of the gene. In the 3'UTR of gene there is also possible modification by alternative splicing.

expressions of IL-17 cytokines and receptors are under the regulation of methylation/demethylation, a major metabolic stress-related epigenetic modification(60). As we discussed in our invited review(61), the ratio of *S*-adenosylmethionine (SAM) levels over *S*-adenosylhomocysteine (SAH) levels is an important metabolic indicator of cellular methylation status (Figure 5A)(62, 63). To test this hypothesis, we summarized tissue concentrations of SAH and SAM and the ratio of SAM over SAH in seven mouse tissues including brain, heart, kidney, liver, lung, and thymus(39), which were reported previously by Ueland's group(43-45) (Table 5). In this study, we used SAH and SAM data generated by this group for the consideration of methodology consistency as we reported(39). We performed multivariable regression analyses to determine the effect of cellular methylation indicated by the SAM/SAH ratio on the expressions of IL-17 cytokines and receptors. As shown in Figure 5B, the SAM/SAH ratios negatively correlated with the expression levels of five IL-17 receptors and traf3ip2, especially with those of IL-17rc and IL-17re ($p<0.05$). The SAM/SAH ratios did not correlate with the expressions of cytokines IL-17a, IL-17e (IL-25), IL-17f, and RORgamma (RORC). However, the SAM/SAH ratios were somehow either positively or negatively correlated with the expressions of cytokines IL-17d ($p<0.7196$) and IL-17b ($p<0.2739$), respectively. These results suggest that higher cellular hypomethylation status as judged by the lower methylation status (SAM/SAH ratios) is positively associated with higher expressions of IL-17 receptors and lower expression of IL-17d.

4.5. RNA binding proteins may regulate the mRNA stability and translation of IL-17 cytokines and receptors

The results from tristetraprolin (TTP) knock-out mice and other studies suggest that TTP targets pro-inflammatory cytokine tumor necrosis factor (TNF)-alpha for decay in the exosome complex(54) via specific binding to AU-rich element(42) in the 3'UTR of TNF-alpha(35, 64). Since both TNF-alpha and IL-17 are pro-inflammatory cytokines, we hypothesized that mRNA stability mechanisms may regulate the mRNA stability of IL-17 cytokines and receptors. Using web-based AU-rich element mRNA database(65), we analyzed all the mRNA 3'UTRs of IL-17 cytokines and IL-17 receptors in the most comprehensive UTR database UTRdb (http://utrdb.ba.itb.cnr.it/search) (Table 6). As shown in Table 6, human IL-17A was the only molecule that contains an AU-rich element in the 3'UTR. In addition, other binding motifs for RNA binding proteins were found in some 3'UTRs of IL-17 cytokines and receptors. For example, Mos-PRE (Musashi Binding Element), which regulates the temporal order of mRNA translation(66), was

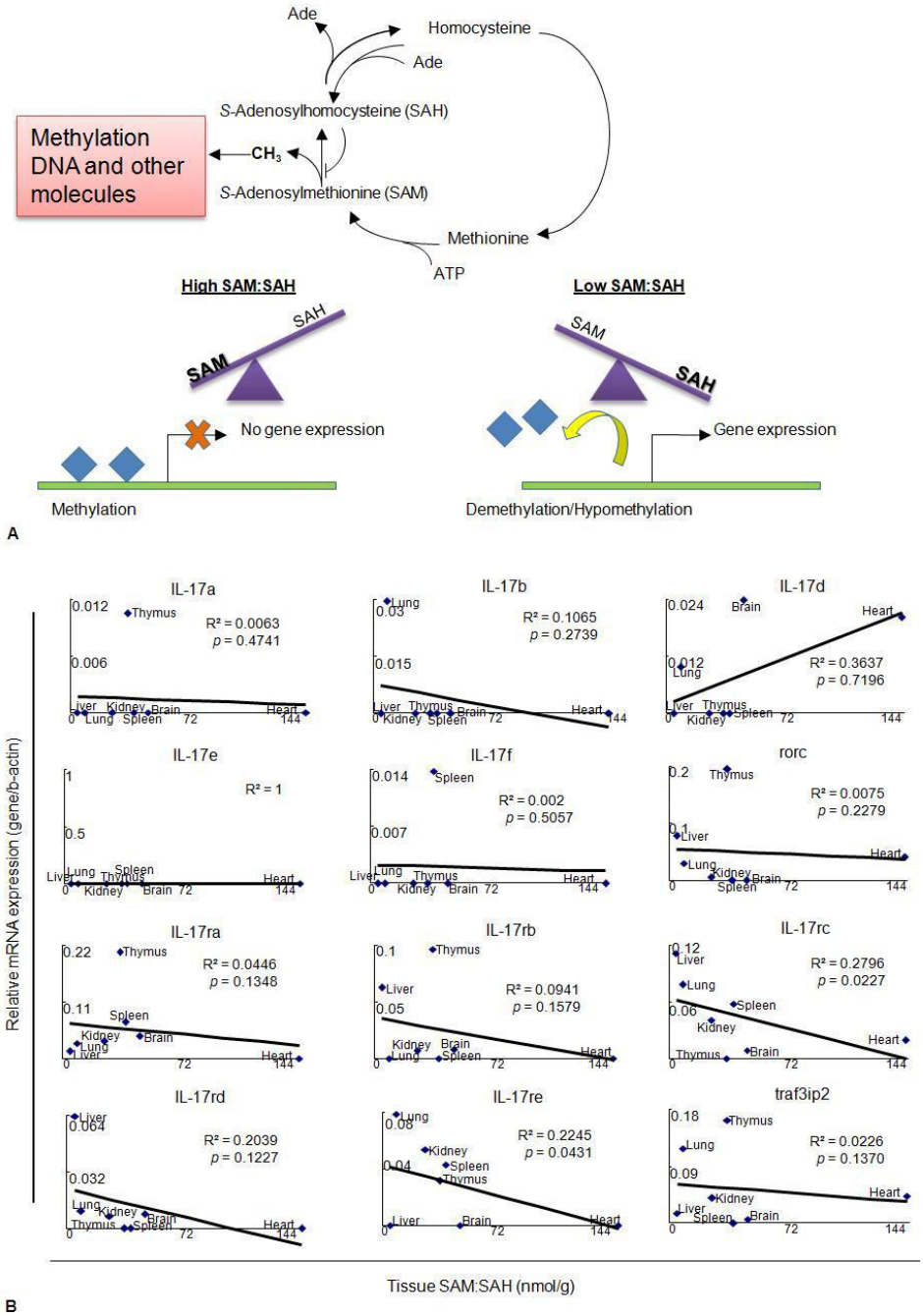

Figure 5. A. *S*-Adenosylhomocysteine (SAH) and *S*-Adenosylmethionine (SAM) Ratio is Associated with the Methylation Status of Tissues. B. Correlation Between IL-17 Cytokine and Receptor and Traf3ip2 and SAM/SAH Ratio in Mouse Tissues. Higher Hypomethylation Status is Positively Associated with Higher Expression of Gene. A) *S*-Adenosylhomocysteine (SAH) and *S*-Adenosylmethionine (SAM) are intermediate metabolites of the Homocysteine-methionine metabolism cycle. SAH is a potent inhibitor of cellular methylation; accumulation of SAH in tissues prevents methylation of DNA and other molecules by SAM. Abnormal DNA methylation has been reported to contribute to the development of cardiovascular and metabolic diseases. High SAM/SAH ratio in tissues is associated with hypermethylation of DNA and gene repression and low SAM/SAH ratio is associated with hypomethylation that leads to gene overexpression. B) Correlation of IL-17 cytokines, IL-17 receptors, and traf3ip2 expression with SAM/SAH ratio in mouse tissues. Relative expression of genes examined was determined as described in Figure 2 and expressed by relative mRNA expression levels. Tissue relative expressions of IL-17 cytokines, IL-17 receptors, and Traf3ip2 mRNA were plotted against tissue SAM/SAH ratios shown in Table 5. Linear regression analyses were performed using data points from 7 mouse tissues. Higher hypomethylation status (lower SAM/SAH ratio) is positively associated with higher expression of IL-17 receptors.

Table 6. 3'-Untranslated Region in mRNAs of IL-17 cytokines and receptors in human and mouse contain signals for RNA protein binding

Human Gene	Length	Signal	Position	Mouse Gene	Length	Signal	Position
IL-17A	1346	Mos-PRE, ARE[1]	703-726, 261-14, 383-446, 624-628	IL-17a	637	BRD-BOX	462-468
IL-17B	102			IL-17b	100		
IL-17C	402			IL-17c	71[2]		
IL-17D	1144			IL-17d	526	BRD-BOX	118-124
IL-17E	523			IL-17e	475		
IL-17F	245			IL-17f	621	UNR-bs	244-257
IL-17RA	695			IL-17ra	548		
IL-17RB	492	GY-BOX	293-299	IL-17rb	456		
IL-17RC	37			IL-17rc	19		
IL-17RD	6411	GY-BOX	1019-1025	IL-17rd	5900	K-BOX, GY-BOX	3131-3138, 4166-4172, 4526-4532
IL-17RE	595	GY-BOX	242-248	IL-17re	608		
TRAF3IP2	465			traf3ip2	778		

Mos-PRE: Musashi Binding Element; ARE: AU-Rich Element; GY-BOX: GTCTTCC; BRD-BOX: AGCTTTA; UNR-bs: Upstream of N-ras Binding Site; K-BOX: cTGTGATa; [1]Class II ARE with 3 clusters. The data were retrieved from UTRdb (http://utrdb.ba.itb.cnr.it/search) at the Institute for Biomedical Technologies, University of Bar except that marked with [2], which was from the NIH-NCBI-AceView database.

Table 7. MicroRNA binding sites are found in the 3'UTR of IL-17 cytokine and receptor mRNAs

Molecule	Total Predicted miR binding Sites	Significant predicted miR binding sites
IL-17A	125	42
IL-17B	11	2
IL-17C	36	8
IL-17D	73	13
IL-17E	10	1
IL-17F	33	9
IL-17RA	58	4
IL-17RB	41	19
IL-17RC	2	0
IL-17RD	476	23
IL-17RE	44	4

found in the 3'UTR of human IL-17A mRNA. The GY-BOX (GTCTTCC)(67) was found in the 3'UTRs of human IL-17RB, IL-17RD, IL-17RE, and mouse IL-17rd mRNAs, respectively. Bearded (BRD)-BOX (AGCTTTA)(68) was found in the 3'UTRs of mouse IL-17a and IL-17d. BRD boxes and GY box confer negative regulatory activity on heterologous transcripts *in vivo*. UNR-bs (Upstream of N-ras Binding Site)(68) was found in the 3'UTR of mouse IL-17f and K-BOX (cTGTGATa)(69) was found in mouse IL-17rd. These results suggest that various RNA binding proteins may participate in regulating mRNA stability of IL-17 cytokines and receptors(36).

4.6. MicroRNAs may regulate the mRNA stability and translation of IL-17 cytokines and receptors independently or via interaction with RNA binding protein-mediated mechanism

MicroRNAs (miRNAs or miRs) is a newly characterized class of short (18-24 nucleotide long)(70), endogenous and non-coding RNAs, which contribute to the development of particular disease states through the regulation of diverse biological processes such as cell growth, differentiation, proliferation, and apoptosis(37). This regulation occurs through base-pairing predominately with messenger RNAs (mRNAs) at the 3'UTR (71, 72), and leads to target mRNA cleavage and degradation or inhibition of mRNA translation(73). Sequence analysis identified miR-16 as possessing complementary sequence to the canonical AUUUA and demonstrated a role for this microRNA in interaction with the AU-rich element(74). Since we found an AU-rich element in the 3'UTR of human IL-17A, we hypothesized that mRNAs of IL-17 cytokines and receptors contain the structures in their 3'UTR for microRNA binding and regulation (Table 6). To examine this hypothesis, we used the online microRNA target prediction software, TargetScan (http://www.targetscan.org/) developed in MIT. The rationale for the use of this prediction database is presented in the discussion. To ensure that the predicted microRNAs have the binding quality equivalent to that of the experimentally verified microRNAs, we hypothesized that experimentally verified microRNAs have certain shared binding features between microRNAs and targeted 3'UTRs of mRNAs that are reflected in the context value and context percentage. To test this hypothesis, the confidence intervals for context value (the mean ± 2 x SD = -0.25 ± 0.12) and that for context percentage (76.07 ± 19.07) were generated, respectively, from the 45 interaction between 27 experimentally verified human microRNAs and 36 different genes (not shown) within the Tarbase, an online database of experimentally verified microRNAs (http://diana.cslab.ece.ntua.gr/tarbase/)(46, 47). These human microRNAs were confirmed using luciferase reporter assays and had effectively targeted a single unique mRNA sequence.

Using the microRNA target prediction software TargetScan, 909 microRNA binding sites were found in the 3'UTRs of the mRNAs of IL-17 cytokines and receptors. Using the confidence intervals of context value and context percentage, 125 microRNAs out of total predicted 909 microRNAs (125/909=13.75%) were selected to target the mRNAs of IL-17 cytokines and IL-17 receptors except IL-17RC (Table 7 and 8) with the binding quality equivalent to that of the experimentally verified microRNA-mRNA

Table 8. MicroRNAs may regulate the mRNA translatability and mRNA stability of IL-17 cytokines and receptors via 3'untranslated region-dependent mechanisms

mRNA	MicroRNA	Position on 3'UTR	mRNA	MicroRNA	Position on 3'UTR
IL-17A	hiR-1267	1142-1148	IL-17B	miR-1271	55-61
	miR-1248	198-204		miR-96	55-61
	miR-1266	279-285			
	miR-127-5p	140-146	IL-17C	miR-1184	217-223
	miR-1290	702-708		miR-1275	90-96
	miR-129-3p	247-253		miR-1301	216-222
	miR-1299	1304-1310		miR-369-3p	374-380
	miR-1324	1195-1201		miR-485-5p	164-170
	miR-142-5p	326-332		miR-544	219-225
	miR-146a	747-753, 872-878		miR-650	86-92
	miR-146b-5p	747-753, 872-878		miR-939	38-44
	miR-147	1211-1217			
	miR-30b	234-240	IL-17D	miR-1204	976-982
	miR-30c	234-240		miR-1229	865-871
	miR-324-3p	1236-1242		miR-129-3p	938-944
	miR-383	1198-1204		miR-194	493-499
	miR-423-5p	56-62, 1130-1136		miR-220b	862-868
	miR-485-5p	85-91		miR-324-5p	713-719
	miR-507	512-518		miR-331-5p	423-429
	miR-515-5p	1118-1124		miR-423-3p	99-105
	miR-520f	460-466		miR-548n	428-434
	miR-548a-5p	237-243		miR-549	899-905
	miR-548i	237-243		miR-579	478-484
	miR-548n	238-244		miR-606	1103-1109
	miR-552	397-403		miR-625	820-826
	miR-557	512-518			
	miR-559	237-243	IL-17E	miR-370	116-122
	miR-578	1046-1052			
	miR-618	110-116	IL-17F	miR-106a	167-173
	miR-626	82-88		miR-1257	140-146
	miR-629	688-694		miR-1324	70-76
	miR-635	1271-1277		miR-142-5p	168-174
	miR-643	1044-1050		miR-17	167-173
	miR-655	617-623		miR-20a	167-173
	miR-664	314-320		miR-20b	167-173
	miR-671-5p	1110-1116		miR-340	169-175
	miR-886-5p	48-54		miR-555	45-51
	miR-888	835-841			
	miR-938	246-252	IL-17RD	miR-1236	1975-1981
				miR-1248	4595-4601
IL-17RA	miR-331-3p	197-203		miR-1270	180-186
	miR-377	99-105		miR-134	570-576
	miR-597	542-548		miR-182	242-248
	miR-661	143-149		miR-21	530-536
				miR-330-3p	396-402
IL-17RB	miR-1225-5p	320-326		miR-338-3p	3773-3779
	miR-1274a	50-56		miR-383	2063-2069
	miR-129-5p	431-437		miR-412	3139-3145
	miR-155	160-166		miR-490-5p	460-466
	miR-221	156-162		miR-515-3p	410-416
	miR-222	156-162		miR-519d	412-418
	miR-376c	345-351		miR-519e	410-416
	miR-380	424-430		miR-532-3p	279-285
	miR-382	190-196		miR-583	1003-1009
	miR-522	248-254		miR-587	2342-2348
	miR-548a-3p	227-233		miR-589	483-489
	miR-548d-3p	444-450		miR-590-5p	530-536
	miR-548e	227-233		miR-643	100-106
	miR-548f	227-233		miR-758	1035-1041
	miR-548g	226-232		miR-769-5p	93-99
	miR-590-3p	131-137		miR-943	569-575
	miR-664	358-364			
	miR-671-5p	30-36	IL-17RE	miR-384	468-474
	miR-7	294-300		miR-511	569-575
				miR-516b	258-264
IL-17RC	Not Identified			miR-516b	266-272

binding sites. The results suggest that the statistical confidence intervals are highly selective for the qualified microRNA-mRNA bindings. Among 125 high quality microRNAs, 39 selected microRNAs were found to target human IL-17A. Eight human IL-17A-targeting microRNAs, including miR-129-3p, miR-30b, miR-30c, miR-548a-5p, miR-548i, miR-548n and miR-938, targeted the same/nearby sequence region, bases 234-253 of the

Table 9. Some IL-17 cytokine- and receptor-targeting MicroRNAs also target cardiovascular disease molecules, inflammation molecules, and cancer-related molecules

MicroRNA	Target IL-17 cytokines and receptors	Target CVD molecules	Target inflammation molecules	Target cancer-related molecules
MiR-146	IL-17A		TRAF6/IRAK-1(87), IRF-5(88)	
MiR-17	IL-17F	p21, Jak1(89)	AML-1(90)	
MiR-20a	IL-17F	VEGF(91)	AML-1(92)	TGFBR2(90)
MiR-20b	IL-17F	VEGF(92)		
MiR-155	IL-17RB	AT1R(93)	MMP-3(94), PU.1(95), TAB2(96)	
MiR-221	IL-17RB	c-Kit(97), eNOS(98)		
MiR-222	IL-17RB	c-Kit(97), eNOS(98)		
MiR-21	IL-17RD			RECK, TIMP3(99), TPM1(100), PTEN(101), PDCD4(102)

3'UTR of human IL-17A. Similarly, three out of eight microRNAs targeted to the same sequence region (bases 216-225) in the 3'UTR of human IL-17C, and six out of nine microRNAs targeted to the same sequence region (bases 167-175) in 3'UTR of human IL-17F. These results suggest that the same sequence region in the 3'UTR of the mRNAs of IL-17 cytokines and receptors can be targeted by several different microRNAs, and that these "hot spots" in the 3'UTRs of mRNAs may be important for microRNA-mediated post-transcriptional regulation of IL-17 cytokine and receptor expression. In addition, some microRNAs were found to target more than one mRNA. For example, miR-129 targeted human IL-17A, IL-17D, and IL-17RB; miR-383 targeted human IL-17A and IL-17RD; and miR-1248 targeted human IL-17A and IL-17RD. This finding correlated well with others' report that some microRNAs have numerous mRNA targets(75, 76). Furthermore, our analysis on experimental reports showed that 10 microRNAs, that target IL-17 cytokines and receptors, also targeted the mRNAs involved in cardiovascular disease, inflammatory molecule mRNAs, and cancer-related mRNAs (Table 9). The results suggest that signaling pathways regulating the expression and translation of IL-17 cytokines and receptors may be related to pathogenic processes of cardiovascular disease, inflammation, and cancer.

5. DISCUSSION

IL-17 cytokines are a family of pro-inflammatory autoimmune cytokines(also see our invited review(12)). Despite significant progress, several important knowledge gaps exist which prevent investigators from defining the detailed roles of these molecules in inflammation and immune responses. Our current studies have made the following findings: *i)* most IL-17 cytokines are not constitutively expressed in the 16 tissues examined, but several IL-17 receptors and TRAF3IP2 are ubiquitously expressed with a few exceptions, suggesting the upregulation of IL-17 cytokines in response to pro-inflammatory stimuli is one of the major mechanisms for IL-17 signaling; *ii)* heart and vascular tissue are in the second tier of readiness to respond to IL-17 cytokine stimulation, which require the upregulation of IL-17 receptor components; *iii)* alternative promoters and alternative spliced isoforms are found in the transcripts of IL-17 cytokines and receptors, suggesting that tissue-specific and stimulation-specific alternative promoters and alternative splicing regulate the structures and expressions of IL-17 cytokines and receptors; *iv)* Higher hypomethylation status as judged by the lower SAM/SAH ratios is positively associated with higher expressions of several IL-17 receptors and lower expression of IL-17d in mouse tissues, suggesting that the expression of these molecules is also regulated by epigenetic methylation mechanism; *v)* An AU-rich element is found in the 3'UTR of human IL-17A and the binding sites of several RNA binding proteins are found in the 3'UTR of IL-17 cytokines and receptors, suggesting that RNA binding proteins may regulate the mRNA stability and translation of IL-17 cytokines and receptors; and *vi)* using the statistical confidence intervals generated with experimentally verified microRNA-mRNA binding sites, 125 binding sites in the mRNAs of IL-17 cytokines and receptors for qualified microRNAs to target were selected out of the 909 total predicted binding sites. The finding of microRNA binding sites in the 3'UTR of IL-17 cytokines and receptors statistically equivalent to that of experimentally verified microRNAs-mRNA interaction suggests that microRNAs may regulate the mRNA stability and translation of IL-17 cytokines and receptors. Of note, the microRNA-mediated regulations of mRNA stability of IL-17 cytokines and receptors are realized independently or via interaction with RNA binding protein-mediated mechanism.

It is worth to point out that the expression data retrieved from the expression sequence tag (EST) database analyzed in this study are more precise than that detected with traditional approaches including Northern blot analysis and PCR analysis due to the un-biased cDNA cloning and DNA sequencing procedures of EST database deposits(77). Thus, the expression patterns of IL-17 cytokines and receptors are experimentally based and precise.

Alternative promoters play an important role for gene transcription in response to tissue/cell-specific, and/or stimulation-specific transcription signaling(78). One of the best examples about multiple promoter usage is fibroblast growth factor-1 (FGF1) transcription, which is controlled by at least four distinct promoters in a tissue-specific manner. The 1.A and 1.B promoters of FGF1 are constitutively active in their respective cell types. In contrast, different biological response modifiers, including serum and transforming growth factor-beta, can induce the 1.C and 1.D promoters of FGF1(78). Identification of alternative promoters in human IL-17B, IL-17D, and IL-17F genes suggests that the transcription of these three human IL-17 cytokines may be under regulation of tissue-specific and/or stimulation-specific transcription signaling.

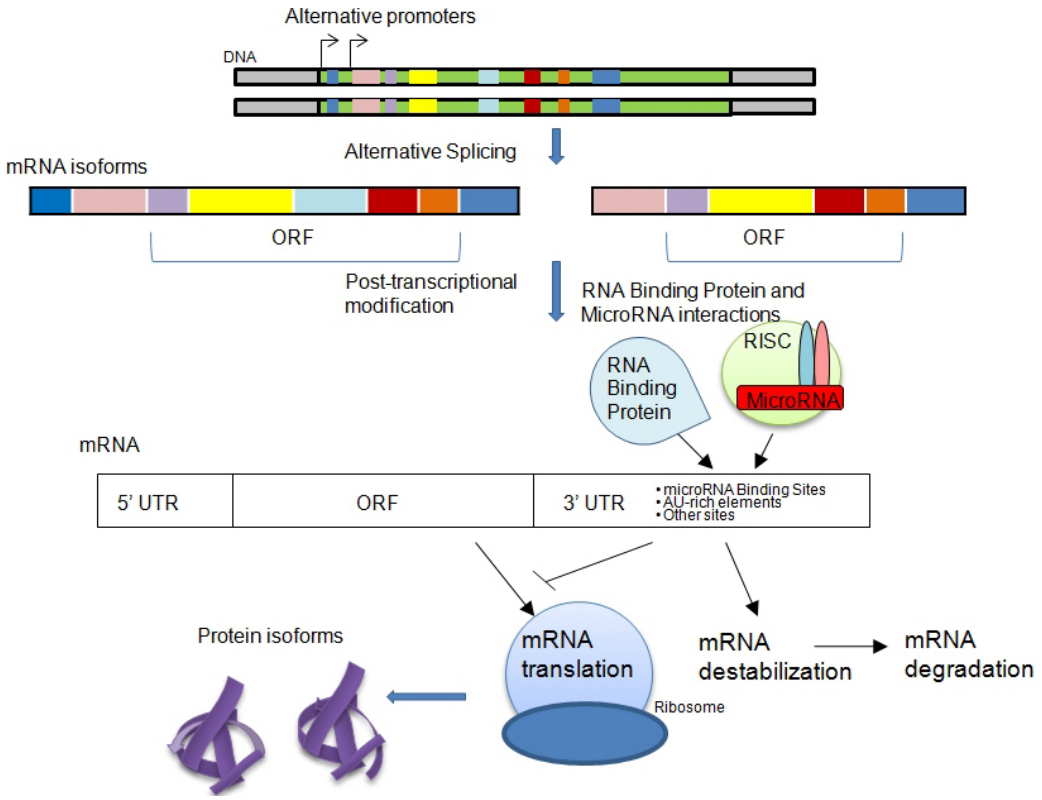

Figure 6. Working Model of IL-17 Cytokine and Receptor Expression and Structure Regulations. Alternative promoters, alternative splicing, and post-transcriptional modifications all contribute to IL-17 cytokine and receptor expression and structure regulations. Approximately 50% of IL-17 cytokine and receptor genes contain multiple promoters which may have important role in tissue-specific and/or stimulation specific transcriptional regulation. Alternative splicing affects the open reading frames(ORF) of RNA transcript which are translated into different protein isoforms. RNA transcripts are regulated by post-transcripitonal modifications including RNA binding protein and microRNA interactions. Interactions with RNA binding protein and microRNA can enhance RNA stability or induce RNA destabilization leading to protein translation or RNA degradation, respectively.

Post-transcriptional regulation controls the abundance, turnover, and translation of mRNA and offers the capacity to integrate signal transduction events with very rapid changes in gene expression during cellular differentiation, which can be realized via different mechanisms including RNA binding proteins such as Tristetraprolin family proteins and microRNAs(79). Previous report showed that mitogen-activated protein kinase (MAPK) stabilizes mRNAs through the inhibition of stabilizing proteins such as tristetraprolin. Tristetraprolin binds to AU-rich elements in mRNA transcripts and delivers them to the exosome complex, where they are degraded(54). Identification of AU-rich element in the 3'UTR of human IL-17A mRNA suggest that the mRNA stability of human IL-17A may be under regulation of tristetraprolin-activated MAPK pathway.

RNA binding proteins may also interact with microRNAs through mechanisms that are not fully understood, and there is evidence that both mechanisms can target the same mRNA(80). MicroRNAs are a newly characterized class of short (18-24 nucleotide long)(70), endogenous, and non-coding RNAs, which are processed by nuclear RNase Drosha and cytosolic RNase Dicer. MicroRNAs are capable of controlling complex biological functions through post-transcriptional gene silencing(73). A recent report showed that miRNA-326 promotes Th-17 cell differentiation by targeting Ets-1, a negative regulator of Th-17 cell differentiation(81), suggesting a possibility that IL-17 cytokines and receptors are under regulation of microRNAs. However, the issue of whether microRNAs regulate the expression of IL-17 cytokines and receptors remains unknown. Using the most widely used target prediction program TargetScan (http://www.targetscan.org/) (82-84), we predicted a list of microRNAs that could target the 3'UTRs of IL-17 cytokines and receptors mRNAs. We hypothesized that experimentally verified microRNAs have certain shared binding features between the microRNAs and the targeted 3'UTRs of mRNAs that are reflected in the context value and context percentage, which can further be analyzed with statistical methods. By examining this hypothesis, the confidence intervals were generated, which allowed us to identify the microRNAs with the binding features statistically equivalent to that of experimentally verified microRNA-mRNA interaction. In our study, introduction of statistical method to generate the

confidence intervals of experimentally verified microRNA-mRNA binding features sort out a small portion of high quality microRNAs among total predicted ones and significantly improves our prediction. Our result suggest that microRNAs as a new mechanism may regulate the mRNA stability and translation of IL-17 cytokines and receptors. This software was used in this study based on the following rationales: *first*, this software includes conserved and poorly conserved miRNAs; *second*, it individually ranks microRNA-target mRNA binding efficacy; and *third*, this software is the most widely used target prediction program(82-84). Of note, some microRNAs that we predicted for targeting the mRNAs of IL-17 cytokines and receptors are well-characterized. Our data showed that miRNA-21 is found to target human IL-17RD. Previous reports showed that miRNA-21 has been validated as a bona fide oncogene, which decreases apoptosis, promotes survival and proliferation. In addition, one of the potential targets of miRNA-21 is IL-12p35, a subunit of IL-12, suggesting that miRNA-21 may regulate inflammation and type 1 T helper cell (Th1) polarization(85). Previous report also showed that miRNA-221/222 inhibits angiogenesis(86). Our results showed that miRNA-221/222 may inhibit human IL-17RB expression, implying that IL-17RB promotes angiogenesis. Moreover, previous report showed that miRNA-146 regulates several inflammatory pathways as part of a negative feedback(85). Our results suggest that miRNA-146 may inhibit human IL-17A expression, which correlated well with the previous studies. Finally, previous report showed that miRNA-155 promotes regulatory T cell development and suppresses excessive inflammation(85). Our results suggest that miRNA-155 may inhibit human IL-17RB expression, which correlated well with the previous studies. Taken together, our results correlated well with the previous reports for some well-characterized microRNAs with the function in regulating inflammation. In addition, eight human microRNAs, that target IL-17 cytokines and receptors, also targeted cardiovascular disease molecule mRNAs, inflammatory molecule mRNAs, and cancer-related mRNAs. In conclusion, as shown in our working model (Figure 6), our results suggest that microRNAs and other mechanisms regulate the expression, structure, and translation of IL-17 cytokines and receptors. These findings provide an insight into pathogenic processes of cardiovascular disease, inflammation, and cancers.

7. ACKNOWLEDGEMENTS

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