

RESEARCH ARTICLE

Tissue-specific expression of IL-15RA alternative splicing transcripts and its regulation by DNA methylation

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ABSTRACT. The human IL-15RA gene encoding the alpha chain of the IL-15 receptor is expressed in a variety of immune and non-immune cell types from different tissues, and generates multiple splicing events of functional importance. We aimed to evaluate expression of IL-15RA transcripts generated by alternative usage of transcription start site (Var1 and Var2) and by deletion of exon 3 (Del3), exon 2 (Del2), or both (Del2,3) in different human tissues. Since a CpG island was found near to the IL-15RA gene transcription start site, we also investigated the role of DNA methylation on the expression of IL-15RA full-length and alternative transcripts fragments in peripheral blood mononuclear cells (PBMC). IL-15RA transcription of functional (full-length and del 3) and non-functional (del 2 and del 2,3) variants was detected in many tissues, however, the number of different IL-15RA transcripts variants detected in each tissue did not correlate with the level of gene expression. IL-15RA transcript variants Var1 and Var2 presented similar expression levels in different human tissues. However, we found a distinct expression profile of functional and non-functional IL-15RA transcripts fragments. A preferential expression of transcripts that bind IL-15 compared to IL-15 non-binding transcripts was seen in the tissues investigated. When PBMC cultures were treated with 5-azacitidine (AZA), a DNA methyltransferase inhibitor, we detected a significant increase in IL-15RA copy number. Only alternative exon skipping events of Var1 (Del 2, Del 3 and Del 2, 3) were altered by AZA treatment, which is consistent with the CpG island localization in the regulatory region 5' upstream of the transcription start site of Var1 and not of Var2. Therefore, this work shows a broad expression pattern of functional IL-15RA splicing forms and suggests a regulatory role of DNA methylation in IL-15RA transcript Var1 expression in mononuclear cells.

Keywords: IL-15RA, alternative splicing, DNA methylation, 5-azacitidine

Interleukin-15 (IL-15) is a pleiotropic cytokine of the 4-a-helix bundle cytokine family and exerts profound effects on proliferation, survival and differentiation of distinct cell types and tissues [1]. It is a unique cytokine in several aspects including mechanisms of cytokine synthesis, presentation (trans-endosomal recycling), and signalling events conferred by the trimeric receptor containing the high affinity α chain and the common IL-2 receptor gamma and beta chain (IL-2/IL-15R $\beta\gamma$) [2, 3]. IL-15 receptor alpha (IL-15RA α) is a type I transmembrane protein structurally related to the IL-2R α chain [4, 5]. Human IL-15RA α protein consists of four major domains: a signal peptide, an extracellular domain containing a conserved protein-binding motif (sushi domain, also known as the glycoprotein-I motif or short consensus

repeat), a single membrane-spanning transmembrane stretch, and a rather short cytoplasmic domain which contains N- and O-linked glycosylation sites [4, 6]. The IL-15RA gene is expressed in a variety of immune and non-immune cell types and tissues, including T, B and NK cells, macrophages, thymic and bone-marrow cells, brain, intestine, liver, skeletal muscle, lung, heart and kidney [6], and signalling through the IL-15/IL-15R system has been implicated in some clinical settings. For example, recombinant soluble IL-15RA α inhibit allogenic responses *in vitro* and *in vivo* and enhance cardiac allograft survival in experimental transplant models [7]. Our group has demonstrated that the IL-15RA transcript is up-regulated during cardiac transplant rejection and Chagas infection in humans [8]. In inflammatory

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autoimmune diseases, such as rheumatoid arthritis, neutralizing IL-15 with antibodies has been shown to inhibit pro-inflammatory cytokine production and has been tested in phase I/II clinical trials [9]. Splicing of IL-15RA transcripts is extremely complex and alternative splicing events in the transcripts occur in various combinations altering the molecular properties of the proteins generated [6, 10, 11]. These events include transcription from two start sites generating transcripts (Var 1 and Var 2) with two different first exons and deletions of exon 2 (Del2), exon 3 (Del3), of both (Del2, 3) [6, 11]. Other events have been found in mice, such as deletions of exon 4, exons 3 and 4, and exons 3-5 [10]. Exon 2 and/or exon 3 deletions do not change the reading frame. Also, the exon 2-encoded sushi domain is the essential element involved in IL-15 binding, but exon 3-encoded linker sequence participates in the binding of IL-15 [12]. On the other hand, the exon 7-encoded cytoplasmic domain is dispensable for binding and signalling [11]. However, the exon 7-encoded cytoplasmic domain is essential for the trans-endosomal recycling of the IL-15/IL-15RA complex [3]. Transfection of COS-7 cells with full-length and Del2 IL-15RA transcripts showed that both transcripts were routed to plasma membrane and exposed on the surface as glycosylated proteins. Transcripts that contain exon 2 have also been co-localized with the nuclear membrane, the ER and the Golgi apparatus, but the transcript lacking this exon was found only in the non-nuclear membrane compartments [11]. Natural, soluble IL-15R α is constitutively generated from the transmembrane receptor through a defined proteolytic cleavage, and involves the activity of tumor necrosis factor- α -converting enzyme (TACE/ADAM-17) [13] or as recently showed, two sushi domain isoforms are generated through alternative transcript splicing of exons 3, 4, 5, 6, 7 [14].

The epigenome is comprised of modifiable chromatin and DNA methylation, which are two major regulatory elements that interact to direct gene expression and other biological processes in somatic cells [15]. DNA methylation can regulate gene expression by targeting methyl-CpG binding proteins (MBD) to methylated genes. These MBD proteins then recruit a complex of transcription repressors including histone deacetylase (HDAC) to suppress genes transcription by inactivating chromatin structure [16, 17]. DNA methylation has been implicated in T cell differentiation. For example, changes in the methylation status and chromatin structure in the regulatory regions of the *IL-4* and *IL-13* genes are involved in the differentiation of Th2 cells [18], whereas similar changes affect the *IFN- γ* gene but not the *IL-4* and *IL-13* genes in Th1 cells [19]. It was also shown that DNA methylation is involved in the regulation of lymphocyte activation. In naïve T lymphocytes, the *IL-2* gene was quiescent, with promoter methylation and locus localized in an inactive chromatin. After activation, but before cell division, specific CpGs in the *IL-2* promoter were demethylated, suggesting a catalytic, active, demethylation process during lymphocyte activation [20]. Other molecules selectively expressed in T cell subsets may be similarly regulated by DNA methylation and chromatin structure.

The objective of this study was to evaluate the expression of IL-15RA and its alternative transcripts in different human tissues. Since a CpG island was found near the IL-15RA Var1 and Var2 transcription start site we also decided to investigate the role of DNA methylation on the expression of the full-length and alternative splice forms of IL-15RA in peripheral blood mononuclear cell (PBMC). For this purpose we treated PBMC with 5-azacitidine, a chemical analogue of cytidine, which incorporates into DNA during replication, inhibiting DNA methyltransferase activity and preventing *de novo* methylation of DNA.

METHODS

Bioinformatics

The EST database (dbEST; <http://www.ncbi.nlm.nih.gov>) was used to find IL-15RA Var1 and Var2 using the Basic Local Alignment Search Tool (BLAST) at NCBI and the Human Genome Browser at UCSC (<http://genome.ucsc.edu>). The CpGPLOT program from the European Bioinformatics Institute website <http://ebi.ac.uk/emboss/cpgplot> was utilized to search for the IL-15RA CpG island.

PBMC culture

Peripheral blood mononuclear cells (PBMC) from 13 healthy volunteers were obtained by Ficoll – Hypaque gradient centrifugation (Coppage, 2000). PBMC (3×10^5 /well) were cultured in 96-well plates in RPMI (Sigma), HEPES 2.9 g/L (Sigma) and Garamycin 30 mg/ml (Schering-Plough), with 10% heat inactivated human serum. Non-activated control cells (C) or activated with 100 ng/uL of phytohemagglutinin (PHA) were cultured for 24 h, in triplicate. Thereafter, 1 μ M of DNA methyltransferase inhibitor, 5-azacitidine (AZA-Sigma), was added to half of the wells with PHA-activated cells and the cultures were maintained for an additional 48 h. The number of living cells in culture was determined by a colorimetric method (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, USA), which utilizes tetrazolium (MTS) bio-reduced by cells generating a color product named formazam, the amount of which directly correlates to the number of viable cells in the culture. The absorbance was measured at 490 nm in a spectrophotometer “Labsystems Multiskan MS” (Thermo Labsystems).

RNA samples

Total RNA samples from 13 different healthy human tissues were purchased from Ambion (Austin, TX, USA) and Clontech (Palo Alto, CA, USA): brain, thyroid, kidney, adrenal gland, gallbladder, skeletal muscle, thymus, testes, liver, trachea, prostate, lung and colon, and RNA samples from additional eight tissues, had been previously isolated in our laboratory [21]: duodenum, nerve cell, vaginal mucosa, fallopian tube, tonsil, amygdala, heart and placenta. RNA from peripheral blood mononuclear cells (PBMC) samples was isolated

from the blood of healthy volunteers, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA quality was assessed on an Agilent 2100 Bioanalyzer using RNA 6000 Nano LabChip kit (both from Agilent Technologies, Waldbronn, Germany).

Reverse transcription-polymerase chain reaction

Complementary DNA (cDNA) was prepared with reverse transcription (Fermentas), in a final volume of 50 μ L, using 500 ng total of RNA, from all of the above-mentioned tissues ($n = 20$), with the exception of duodenum and tonsil (because they presented degraded RNA), 400 U of reverse transcriptase, 10 μ L of enzyme buffer, 10 μ L of 10 mM dNTP mixture (all from Invitrogen), 0.2 μ L of oligo(dT) primer, and 50 U of RNase inhibitor (Amersham Biosciences, Uppsala, Sweden). This mixture was incubated at 42°C, for 60 min, and then at 70°C, for 10 min. To control reverse transcription, we amplified a 335 bp fragment of the RNA polymerase II subunit K (POL2K) with primers 5'-GGAAACGCCGGAGTGAGTTT3' and 5'-CTCCCGAAGATAAGGGGGAA-3', as previously described [21]. The cDNA samples were generated by pooling equal volumes of the three transcription reactions.

Quantitative PCR for IL-15RA transcript detection

Real-time PCR reactions of cDNAs and standard curves were performed with SYBR Green I on an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). To prepare a standard curve, we performed a PCR amplification reaction using Mastermix (Promega Corporation) and primers to amplify a fragment between exon 4 and 6 that is common to all IL-15RA isoforms. After separation of the IL-15RA amplicon and the DNA molecular weight ladder (Low mass, Invitrogen) by electrophoresis in ethidium bromide-stained 2% agarose gels, band intensities were captured with the Kodak Digital Science1D program -EDAS 120 system (Eastman Kodak, Rochester, NY, USA). Quantification of the IL-15RA standard amplicon was made using a linear regression based on band intensities of low-mass DNA concentration curve. The copy number was calculated as previously described [22], and was normalized by the amount of RNA Polymerase II chain K gene (POL2K) (ratio between copy numbers of target gene cDNA and of POL2K cDNA). Results were expressed as relative expression of IL-15RA.

PCR for IL-15RA transcript variant detection in human tissues

Sequences of primers for amplification of IL-15RA transcript fragments are shown in *table 1*; primer locations are represented in *figure 1A*. To detect IL-15RA alternative transcription start site variants (Var1 or Var2) with exon skipping, we developed a nested PCR as follows. In the first PCR reaction with 18 cycles, forward primers were located in exon 1 of Var1 or Var2 and reverse primer located in the longer cytoplasmic domain of exon 7 [6]. The reaction was performed on 1 μ L of

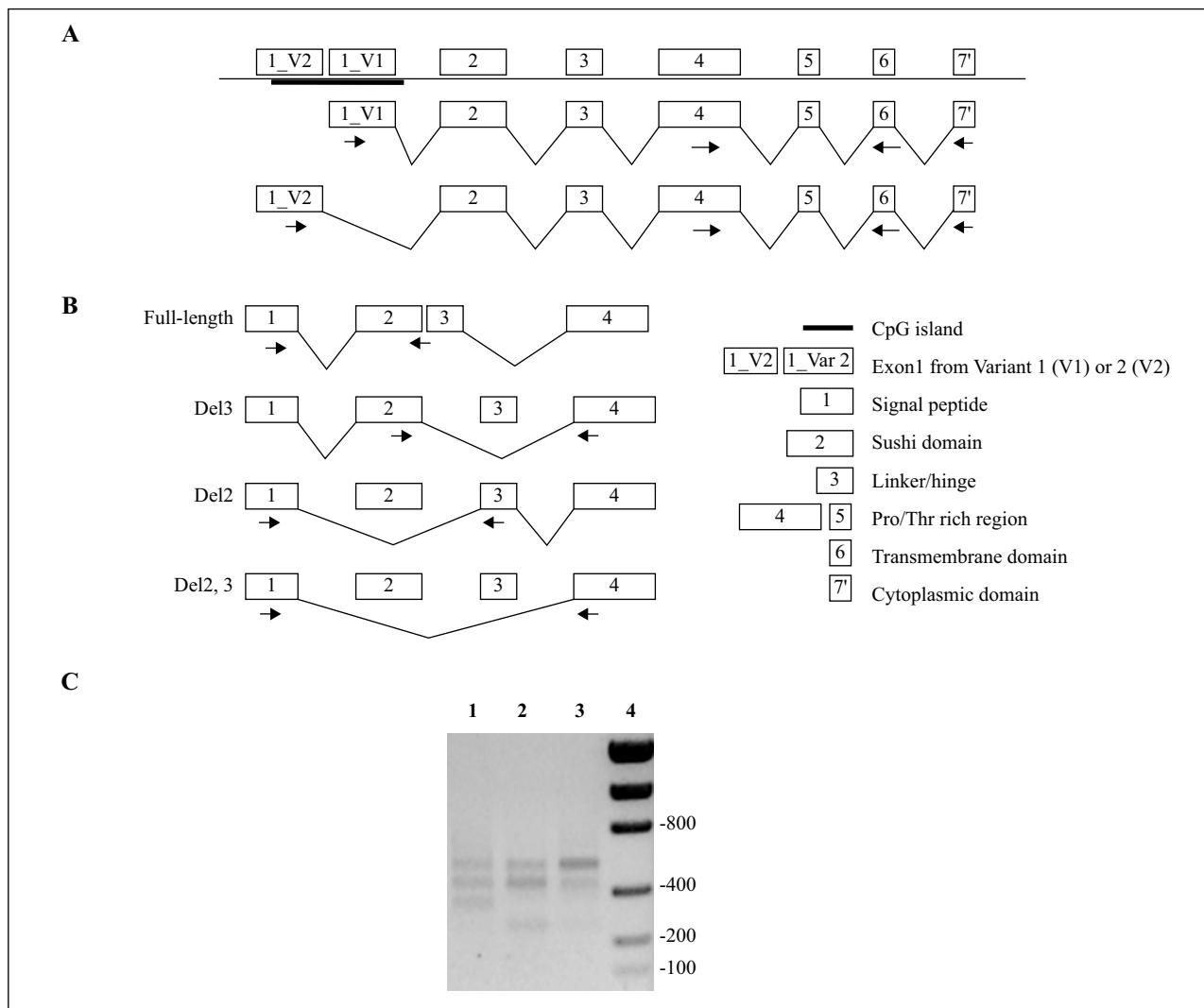
cDNA of each sample, using MasterMix (Promega Corporation). The conditions were 94°C for 45 s, 57°C for 30 s, 72°C for 90 s (PTC-200 Thermocycler; MJ Research, Watertown, MA, USA). To detect IL-15RA transcript fragments in human tissues, we used 1 μ L of the first-PCR reaction product and then amplified it for an additional 35 cycles with primers located in exon 1 and exon 4 (*figure 1B*). Four bands, equivalent to full-length, Del3, Del2 and Del2,3 transcripts fragments (same as *figure 1C*, lane 1, 2 and 3), were quantified and intensities were transformed into log values. BRB Array-Tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) was used to construct clusters using Euclidean distance and average linkage.

Semi-quantitative PCR for IL-15RA transcript variant detection in PBMC

To quantify each IL-15RA transcript fragments in PBMC, before and after AZA treatment, we used different pairs of primers (*figure 1B*) that included exon transcripts (full length) and selective enzymatic depletion for exon skipping transcripts (*figure 1B*). Briefly, 1 μ L of the first PCR product obtained with primers located in exon 1 (from Var1 and Var2) and exon 7 were amplified with primers in exon 1 and in exon 2 and 3 junction for detection of full-length transcripts. Alternatively, 10 μ L of the first PCR product were treated with 1U of the restriction enzymes *Bst*XI and *Bst*EII or both (New England Bio-Labs, Beverly, MA, USA) with recognition sites in exon 2 and exon 3. After depletion of exon 2 and/or exon 3, 1 μ L of the enzymatic reaction was re-amplified (second PCR) for an additional 25-30 cycles with the primers shown in *figure 1B*. For Del3, the primers were located in exons 2 and 4, for Del2, in exons 1 and 3, and, for Del2, 3, in exons 1 and 4 (*figure 1B*). Controls of no-cut re-amplification reactions were performed for all reactions. All PCR products, full-length, Del3, Del2 and Del2,3 were separated by electrophoresis in ethidium bromide-stained 1.5% agarose gels, and band intensities were captured under ultraviolet light with the Kodak Digital Science-DAS 120 system (Eastman Kodak, Rochester, NY, USA). All the products were sequenced to confirm transcript. Variants were recovered from the gel using GFX PCR DNA and the Gel Band Purification kit (Amersham Biosciences). Linear regression curves of band intensities and low-mass DNA concentration were used to quantify IL-15RA transcript fragments. Sequencing was performed with a BigDye Terminator Cycle Sequencing kit (v3.1) on an ABI Prism 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA).

Retrieval of RNA sequencing (RNA-seq) data for IL-15RA

Illumina sequencing read counts aligned to the hg18 human genome for adipose and lymph node tissues were obtained from [23] (GEO accession number GSE12946), and for CD4 $^{+}$ T cells from [24] (GEO accession number GSE16190). Transcript levels of the short and long isoforms of the IL-15RA gene were quantified by counting the number of read counts that mapped to the

**Figure 1**

Schematic representation of the IL-15RA gene and detection of its transcript variants.

A) Structural genomic organization and a CpG island (black line) localization in the IL-15RA gene. Representation of exon 1 of IL-15RA alternative transcript start sites corresponding to variants 1 and 2 (1_Var1 and 1_Var2). Forward and reverse primers utilized for quantification of the common region of all transcripts by real time PCR and amplification of transcript variants Var1 and Var2 are indicated by black arrows.

B) Localization of primers for detection of IL-15RA transcript variants (full-length, Del3, Del2 and Del2, 3). Forward primers located in exon 1 were also utilized in the first PCR reaction for Var1 and Var2 (shown in **A**). Full-length reverse primer was located in exon 2 and 3 junction, and reactions for alternative splicing events (Del3, Del2 and Del2, 3) were performed with primers indicated by arrows after treatment of the first PCR product with endonucleases with recognition sites in exon 2 and exon 3 for elimination of full-length transcript (Del3, BstEII enzyme; Del2, BstXI enzyme; Del2,3, both enzymes).

C) A photograph of agarose gel after electrophoresis of Var2 amplified products obtained by nested PCR, after amplification of first PCR products, with primers located in exon 1 (forward) and exon 4 (reverse) without treatment with endonucleases. This reaction was performed to detect all IL-15RA transcripts in different human tissues. Lane 1: three fragments of 541, 442, 306 bp corresponding to full-length, del3 and del2; lane 2: three fragments of 541, 442 and 247 bp corresponding to full-length, del3 and del2,3; lane 3: two fragments of 541 and 442 bp corresponding to full-length and del3; lane 4: low molecular mass ladder (Invitrogen).

first exon of each of these isoforms and normalizing read counts by exon size and total number of mapped reads in the library to obtain normalized expression RPKM (number of reads per kilobase of exon model per million mapped reads [25] values of isoforms).

DNA extraction and IL-15RA CpG island methylation analysis

Genomic DNA was prepared from PBMC samples using the phenol/chloroform protocol [26], and then subjected to sodium bisulfite treatment [27]. Identification of the

IL-15RA CpG island was accomplished using the human genome sequence corresponding to the promoter region of the transcription start site (TSS) of the IL-15RA gene. We identified the RefSeq number based on the GenBank accession and submitted the gene sequence to the Blat Search Genome at the UCSC Genome bioinformatics website <http://genome.ucsc.edu>. We selected a 2,000 bp sequence extending from the 5' upstream region to 1,000 bp downstream region of the TSS. The sequence was submitted for analysis to the CpGPlot program from the European Bioinformatics Institute website <http://ebi.ac.uk/emboss/cpgplot>. A typical CpG island

was identified as a 200 bp sequence that had a C+G content of 50% and a value of > 0.6 for the ratio (CpG observed)/(CpG expected) [28]. The selected region of 434 bp starting from nucleotide - 536 to - 102 of Var1 or starting from nucleotide + 70 to + 504 of Var2, was amplified from bisulfite-treated DNA samples using a nested PCR amplification protocol. The first set of primers included a sense primer 5' - AGA GGT GAA GTA TTG TGG - 3' and an antisense primer 5' - CAC AAC CAC CCC TAT C - 3'. The second set of primers included a sense primer 5' - GGG AGT AAA GTT GTA GTA GGA - 3' and an antisense primer 5' - AAC CCA AAA CCT TAC CCA - 3'. The PCR conditions for both sets of primers were as follows: one round of 94°C for 12 min, 94°C for 3min, 48°C for 3 min, 72°C for 2 min, five cycles of 94°C for 3min, 50°C for 3 min, 72°C for 2 min, and 35 cycles of 94°C for 1min, 52°C for 1 min, 72°C for 2 min. The same conditions and annealing temperatures were used for the nested reaction. Amplified products were purified using the Qiaquick Gel Extraction Kit (Qiagen) and cloned into a T-vector cloning vector (Promega Corporation). Random positive colonies were screened for the correct amplicon using PCR. Six selected clones from six individuals were sequenced using the vector's universal forward/or reverse primers. DNA sequencing reactions were performed using Big Dye Terminator technology (Applied Biosystems) on an ABI 3100 sequencer (Applied Biosystems) according to the manufacturer's instructions.

Statistical analyses

The Spearman correlation coefficient was used to determine any correlation between variables. IL-15RA transcript fragments expression levels in PHA-activated and AZA-treated PBMC were compared using the Wilcoxon matched pairs test, with $p < 0.05$ considered significant.

RESULTS AND DISCUSSION

Expression of IL-15RA transcripts in different human tissues

We first determined the total level of IL-15RA gene expression in several human tissues, using a quantitative PCR with primers between exons 4 and 6 (dashed line arrows in *figure 1A*) flanking a region common to all known human IL-15RA transcripts and most of the ESTs found in databases (<http://genome.ucsc.edu/>; <http://www.ncbi.nlm.nih.gov/>). As shown in *figure 2A*, the IL-15RA transcript was detected in many of the tissues investigated, which is in agreement with previous reports [6, 11, 29, 30]. This broad expression of the IL-15RA gene suggests a wide spectrum of IL-15 activity beyond the immune system. The comparison of IL-15RA expression levels in different tissues observed in the present study (*figure 2A*) with those from a published microarray data set [31] containing 16 tissues in common with our study, revealed a significant correlation ($p = 0.002$; Spearman $r = 0.70$) (data not shown).

The number of different IL-15RA transcript fragments in each tissue (black diamonds in *figure 2A*) did not show any correlation with the level of IL-15RA gene expression ($r = 0.10$, $p = 0.64$). Although we detected many different transcripts in some of the tissues with high IL-15RA expression (e.g., lung and placenta), and few transcripts in some of the tissues with low IL-15RA-expressing tissues (e.g., gallbladder, brain and thyroid), we also observed high IL-15RA-expressing tissues, as colon and testis, presenting only three or four different transcripts, and low-expressing tissues, such as skeletal muscle and adrenal gland, exhibiting more than five different IL-15RA transcripts fragments (*figure 2A*). These results suggest a tissue-specific regulation of the expression of alternative IL-15RA transcripts. It also suggests that differential splicing does not correlate with total IL-15RA mRNA abundance in the cell.

Because alternative splicing in IL-15RA can generate transcripts with different functional activity [11], we searched for exon 2 and exon 3 skipping variants of IL-15RA (Del2, Del3, and Del2, 3), each of them with alternative usage of the transcription start site (Var1 and Var2). For this, we first performed a PCR with primers specific either for Var1 or for Var2 (black arrows; *figure 1A*) followed by a second PCR with primers specific to full-length, Del3, Del2 or Del2, 3 transcripts (*figure 1B, C*, lanes 1, 2, and 3). Similarities in expression levels of different IL-15RA isoforms across many tissues were investigated by cluster analysis. We performed this analysis only in tissues with a good RNA quality index ($RQI > 10$), because the normalization of semi-quantitative values (band intensities) by copy number values of the control gene is not accurate in samples with degraded RNA. As shown in the dendrogram of *figure 2B*, IL-15RA transcript variants Var 1 and Var 2 have overall similar expression across human tissues because all four transcripts (full-length, Del3, Del2 or Del2, 3) clustered together, independently of the expression of Var1 or Var2: this suggests a common regulatory mechanism for both alternative transcription start sites. On the other hand, we detected a distinct expression pattern of functional (full-length and Del3) and non-functional (Del2 and Del2, 3) IL-15RA transcript fragments. In fact, we observed a preferential expression of transcripts that bind IL-15 (full-length and Del3) compared to IL-15 non-binding transcripts (Del2 and Del2, 3) in the tissues investigated (*figure 2B*).

Using publicly available data for the RNA-seq, we next compared expression levels of Var1 and Var2 of the IL-15RA gene in three human tissues (lymph node, CD4+ T lymphocytes and adipose tissue) that had measurable levels of expression [23-25]. Because Var1 and Var2 have a completely different exon 1, it is possible to measure their absolute levels by counting the number of sequences corresponding to either exon in RNA-seq results. We found that Var1 was more frequent in immune tissues (lymph node and CD4+ cells) than Var2, but both variants had similar expression in adipose tissue (*figure 2C*). These results in lymph node and CD4+ cells are concordant with the data in PBMC obtained by PCR where Var1 also had higher levels than Var2 (*figure 2B*).

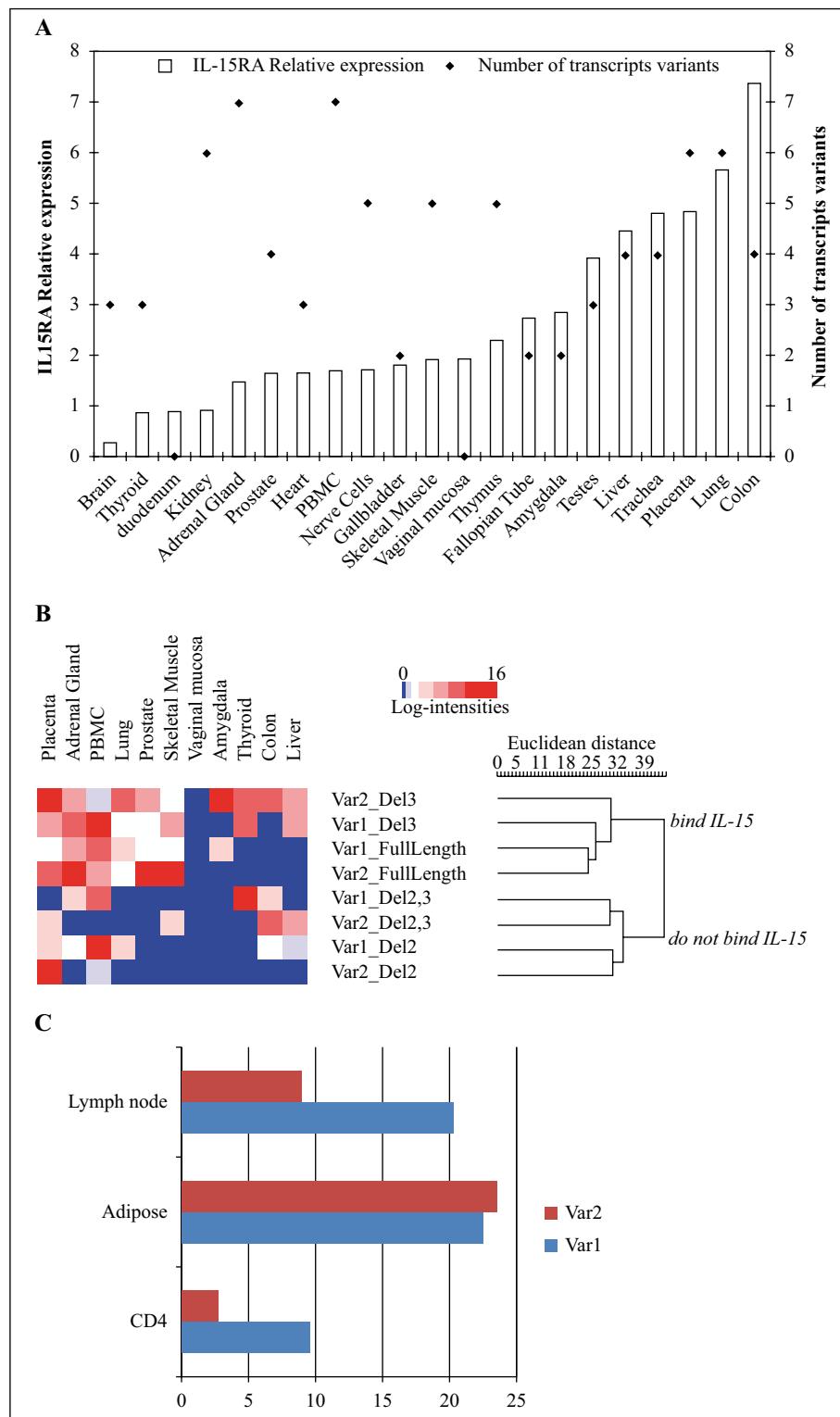


Figure 2

Expression of IL-15RA transcripts in human tissues.

A) Total levels (white columns, left Y axis) of IL-15RA gene expression in one sample from 21 tissues quantified by real-time PCR, using primers flanking regions common to all IL-15RA transcripts, normalized by Pol2K gene expression. Only one PBMC sample was used as a representative of this tissue. Error bars were not seen because we did not observe a great variation (less than 25%) in triplicate samples of real-time reactions. On the right Y axis, the number of IL-15RA transcript variants detected in each tissue (black diamonds) by semi-quantitative PCR amplification.

B) Clustering of transcript variants based on the level of their expression in different tissues represented by color-map (log-intensities of bands obtained by semi-quantitative PCR).

C) Normalized number of RNA sequences of IL-15RA transcript variant 1 (Var1) and 2 (Var2) in lymph node, adipose and CD4+ T lymphocytes.

It is now recognized that signaling *via* the IL-15/IL-15R system modulates not only T cell functions (T lymphocyte development and homeostasis, and memory CD8+ T-cell and NK cell development, maintenance, expansion and activities), but also the functions of other numerous cell populations. It was demonstrated that expression of IL-15RA in hepatocytes, bronchial epithelial cells and colon epithelial cells was related to the ability of IL-15RA to recruit TRAF2, an anti-apoptotic molecule that rescues these cells from apoptosis [32]. Also, IL-15RA expression by distinct cell types controls lymphoid subset development and integrates immune and non-immune cell types and tissues [33]. These findings, combined with our results regarding the ubiquitous expression of IL-15 receptor, suggest an important role of IL-15 in the interaction of the immune system with numerous other tissues.

Expression of IL-15RA transcripts in PBMC after treatment with 5-azacitidine (AZA), a DNA methyltransferase inhibitor

Because a CpG island has been found near the IL-15RA transcription start site, we decided to investigate the role of DNA methylation in the expression of IL-15RA transcripts. For this, we induced proliferation of PBMC from healthy volunteers with phytohemagglutinin (PHA). This mitogen activates cells by CD3, so, after stimulation we observed a major population of proliferating T cells. After 24 h, we treated the cultures with a DNA methyltransferase inhibitor, 5-azacitidine, a chemical analogue of cytidine that incorporates into DNA during replication, inhibiting DNA methyltransferase activity and preventing *de novo* methylation of DNA after DNA replication. As expected [13], we observed a significant ($p < 0.001$) increase in the overall copy number of the IL-15RA transcript after polyclonal mitogen-(PHA) stimulation of PBMC, compared with non-stimulated (C) cells (figure 3A). In the cells treated with AZA after PHA stimulation, we detected a further increase in the IL-15RA copy number as compared to the AZA non-treated group (figure 3B, $p < 0.01$), suggesting the involvement of DNA methylation in the regulation of IL-15RA gene expression.

Next we investigated which IL-15RA transcript fragment variants were transcriptionally regulated by AZA treatment. As observed in figure 3C, only alternative exon skipping events of Var1 were altered by AZA treatment. Del2 was significantly altered ($p < 0.03$) and Del3 and Del2, 3 showed a trend ($p < 0.1$) towards increased expression after AZA treatment. Var1 and Var2 full-length, as well as alternative exon skipping events of Var2 Del3, Del2 and Del2,3 were not altered by AZA treatment (figure 3C). Although we observed a similar expression pattern for Var1 and Var2 in different tissues, suggesting some common regulatory mechanisms for both variants, in PBMC, only Var1 might be altered by DNA demethylation. This is consistent with the fact that a CpG island is located 5' upstream of the transcription start site of Var1 but not of Var2 (figure 1).

IL-15RA CpG island methylation analysis by sodium bisulfite sequencing

We next sought to assess directly the methylation status of the putative CpG island found in IL-15RA gene. This island has 1529 bp and contains the regulatory 5' upstream region and first exon of Var1 and a part of exon 1 of Var2 (figure 1A). We studied a region of 434bp containing 46 CpG dinucleotides, which spans positions -536 to -102 from the start site of Var1 or +70 to + 504 from the start site of Var2 (figure 4A). We analyzed the methylation pattern of six independent alleles (six clones) before (-) and after (+) AZA treatment in six samples of PBMC (S1 to S6) (figure 4B). Overall, we observed methylation of a small number of CpG sites (CpG 3, 5, 13, 15, 19, 22, 37, 41 and 44) that, as expected, all demethylated after AZA treatment. The number of methylated CpG dinucleotides varied from 1 to 3 per individual. As previously demonstrated by Bruniquel & Schwartz (2003), methylation of a quite restricted subset of CpG sites in the IL-2 promoter was sufficient to inhibit transcription of this gene [20]. It is possible that methylation of only a small number of CpG sites might be involved in the regulation of IL-15RA gene expression. Thus, we looked to see if there was a correlation between the number of methylated CpG sites and change in expression (treated AZA – untreated) of IL-15RA transcript variants. As shown in figure 4C, we observed a trend for a correlation between the number of methylated CpGs and difference in expression for Var1 full-length isoform after AZA treatment ($r = 0.57$ and $p = 0.1$, figure 4C) but not for Var2 ($r = 0.2$ and $p = 0.3$). These results further suggest that only Var1 may be affected by DNA methylation, which could be regulating the accessibility of its promoter.

It has been previously shown that differences in promoter structure may cause differences in alternative splicing of the transcript [34]. Modulation of alternative splicing by the promoter can be via the regulation of Pol II elongation or processivity [35]. Human IL-15RA promoter and regulatory regions have not been extensively explored. A functional NFkB binding site in the IL-15RA promoter region was characterized in Tax-activated HTLV-I-infected T cell lines [36]. Yet, we do not know about transcription activators sites in promoter region that could regulate elongation steps of pol II and alternative splicing events in the IL-15RA gene. Recent studies have shown that chromatin structure could affect alternative splicing. Treatment with Trichostatin A, a potent inhibitor of histone deacetylation, favors skipping of alternative exons, presumably because hyperacetylation of core histones facilitates the passage of the transcribing polymerase [35]. Another study reveals a new role for a chromatin-remodeling factor in alternative splicing. The mechanism of action is independent of its chromatin-remodeling activity and involves the regulation of pol II elongation by qualitative changes in the phosphorylation status of RNA pol II [34]. Here, we show that DNA methylation is one of regulatory events that may lead to differential transcription of IL-15RA variants in PBMC, probably by affecting binding of transcriptional factors. A very recent study has demonstrated that methylation contributes to

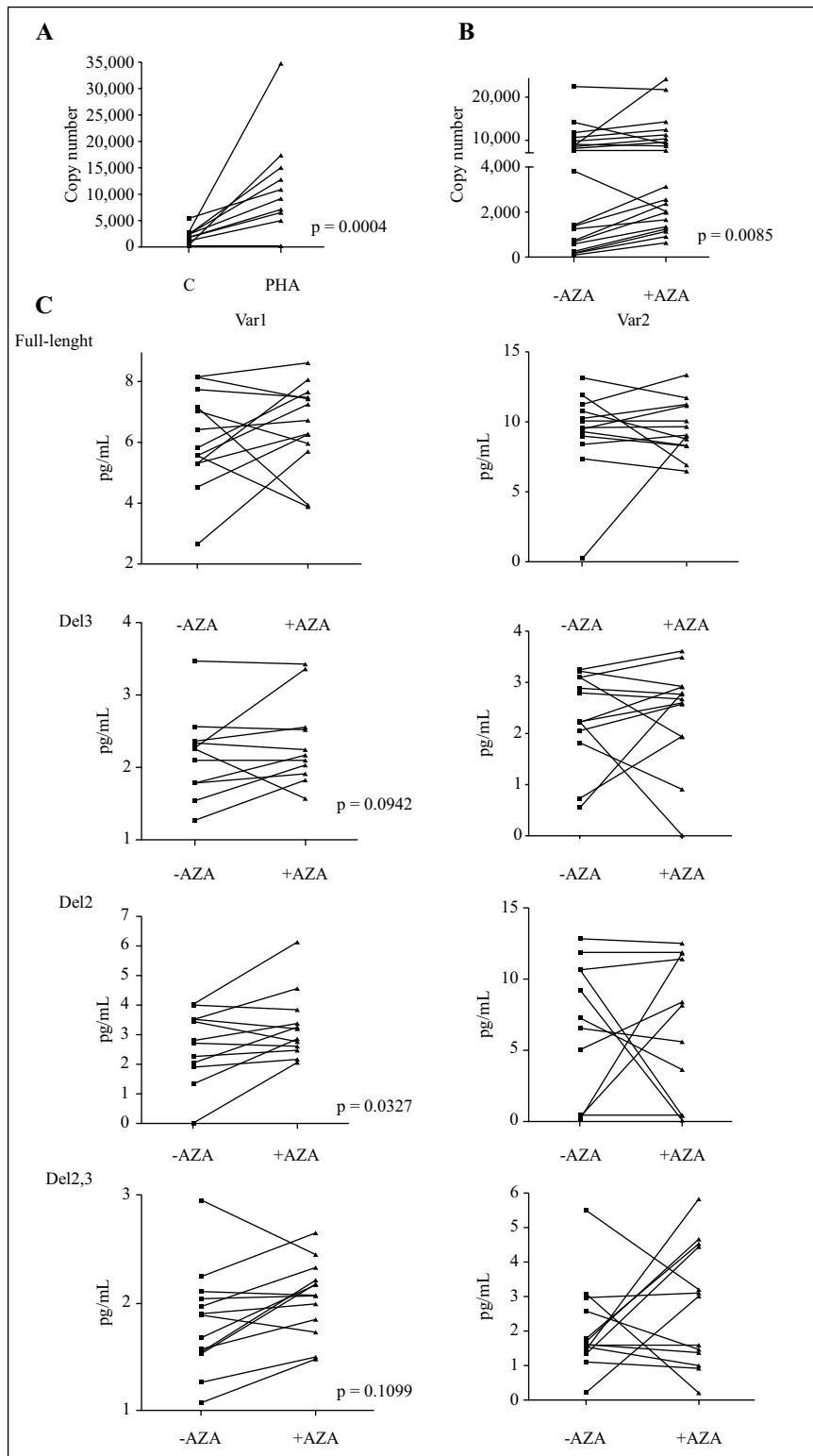


Figure 3

Expression of IL-15RA transcript fragments in PBMC.

A) Total levels of IL-15RA (copy number) in non-stimulated (C) and phytohemagglutinin (PHA) stimulated cultures of peripheral blood mononuclear cells (PBMC) from 13 healthy volunteers.

B) Total levels of IL-15RA (copy number) in PHA-stimulated cultures of PBMC from 26 healthy volunteers with and without addition of azacitidine (AZA). The treatment of these cells with AZA shows an extent of 22% (on average) in their viability.

C) Levels of IL-15RA transcript variants (Var1 and Var2) and alternative splicing (full-length, Del3, Del2 and Del2, 3) expression (ng/uL of cDNA), in PBMC from 13 healthy volunteers, after PHA stimulation, without and with addition of azacitidine (AZA). The Wilcoxon paired test was used for the statistical analysis in A, B and C.

ns : not significant.

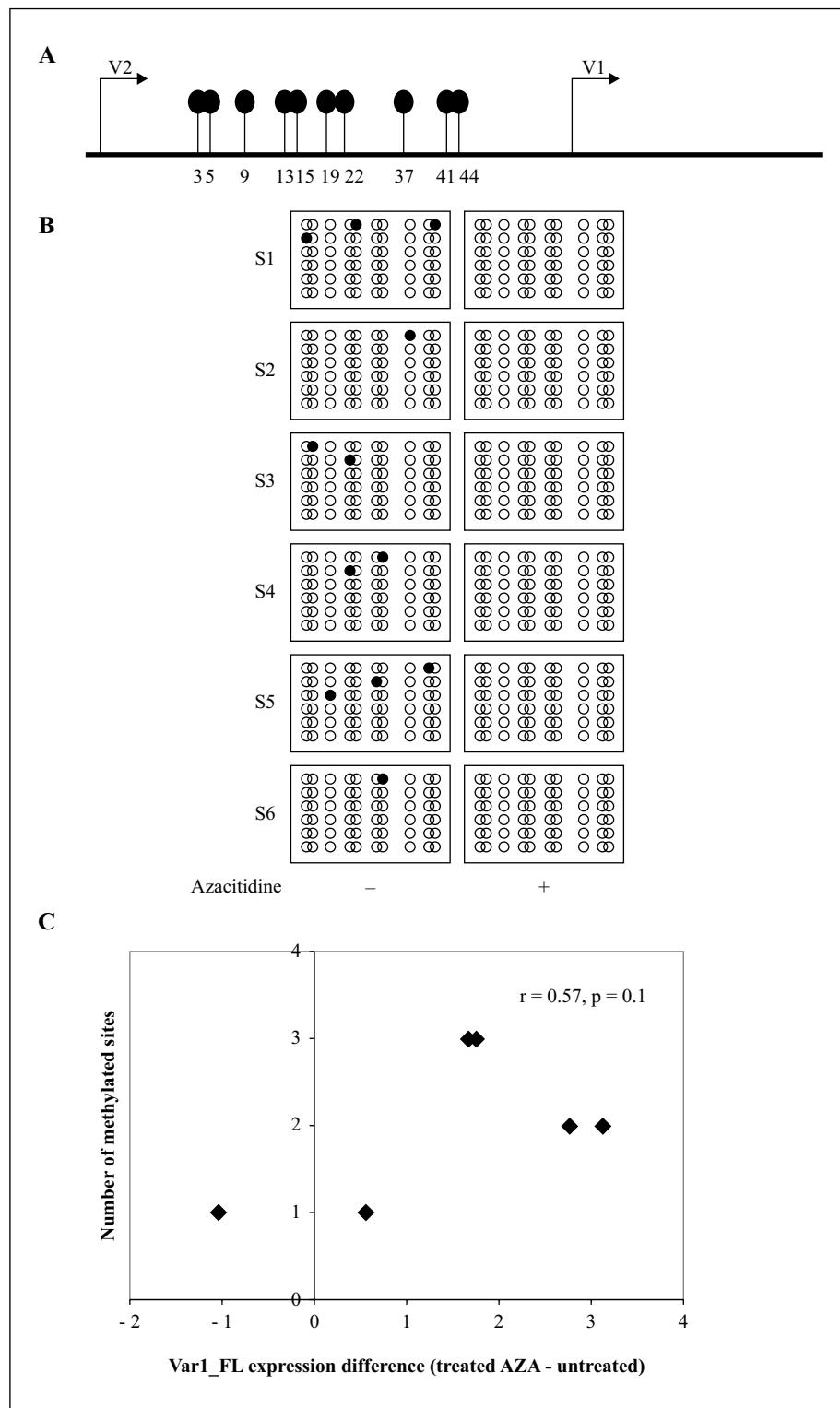


Figure 4

Cytosine methylation of the IL-15RA CpG island from PBMC.

A) Transcription start site of variant 1 (V1) and 2 (V2) and representation of 10 CpG dinucleotides demethylated after AZA treatment (CpGs 3, 5, 9, 13, 15, 19, 22, 37, 41 and 44).

B) CpG dinucleotides from six clones before (−) and after (+) azacitidine treatment of six PBMC samples (S1 to S6). Open circles represent non-methylated CpG and closed circles, methylated CpGs.

C) Correlation between the number of methylated sites and expression difference (treated AZA - untreated) of IL-15RA transcript variant Var1 full-length isoform (FL). Spearman correlation coefficient was used to determine any correlation between variable numbers of methylated site and expression difference.

Table 1
Primer localization, sequences, size and annealing temperature

| Localization | Sequence | Size | Tm |
|--------------------------------|--------------------------------|------|------|
| Exon1 (Var1_NM_002189) | 5' tctccggcgctgtact 3' | 18pb | 61°C |
| Exon1 (Var2_NM_172200) | 5' cgtactgttgcactcttc 3' | 23pb | 61°C |
| Exon4 (forward) | 5' tcagacaacagccaagaactggga 3' | 24pb | 64°C |
| Exon 6 (reverse) | 5' cagcgctcagccacaca 3' | 18pb | 62°C |
| Exon7 (reverse) | 5' cagacgcttccactgagca 3' | 20pb | 62°C |
| Junction exon2 e3 (reverse) | 5' aaccaggcagggtcttaat 3' | 21pb | 58°C |
| Exon2 (forward) | 5' ttgaaacaaggccacgaatgtc 3' | 21pb | 60°C |
| Exon3 (reverse) | 5' cccctggctcgttactgt 5' | 19pb | 59°C |
| Exon4 (reverse) | 5' tgccgtggaggactcatgact 3' | 22pb | 65°C |

the generation of alternative transcripts in the brain that are expressed in a tissue- and cell type-specific manner [37].

In conclusion, our data indicate that IL-15RA alternative transcription can be regulated in a tissue-specific manner with preferential expression of IL15-binding isoforms. DNA methylation seems to be a mechanism that contributes to IL-15RA transcript diversity in human blood mononuclear cells.

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