2728

Circulating levels of Resokine, a soluble modulator of the immune system, are upregulated in both experimental cancer models and in patients across multiple tumor types

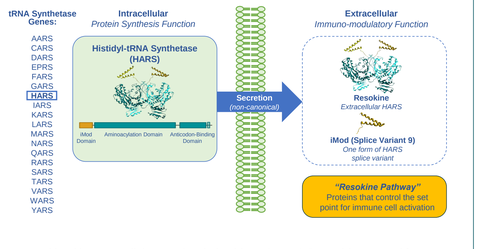
Ryan Adams, Elisabeth Mertsching, Leslie Nangle, Kathy Ogilvie, Steven Crampton, John Bruner, Samantha Tyler, Sanna Rosengren, Andrea Cubitt, David King, John Mendlein

aTyr Pharma, San Diego, CA

Abstract

The Resokine family of proteins are derived from the histidyl tRNA synthetase gene (HARS) via proteolysis or alternative splicing and appear to be important as extracellular modulators of cellular activity. Resokine is a newly identified regulator of immune cell activity, and circulating levels of Resokine in normal individuals may represent a soluble set-point control to modulate T cell activity. Resokine activity is a non-canonical function arising from the tRNA synthetase gene family, and the activity is effected by a 60 amino acid N-terminal domain arising from the gene for histidyl-tRNA synthetase. This domain is present in the full-length protein as well as multiple splice variants that have lost their original tRNA synthetase functionality. Resokine is secreted from cells, including tumor cell lines, and *in vitro* studies have demonstrated that Resokine can inhibit the activation of immune cells. *In vitro*, for example, Resokine addition during T cell activation induced by antibodies to CD3 and CD28, can result in reduced levels of inflammatory cytokines, such as IL-2, interferon gamma, and TNF alpha; inhibition of the up-regulation of cell-surface activation markers, such as CD69, CD40L, and 4-1BB; and inhibition of release of the cytotoxic mediator granzyme B. We have tested levels of circulating Resokine in both mice with syngeneic tumors as well as >300 cancer patients across multiple tumor types. In normal C57BL/6 mice serum levels of Resokine ranged from 70-250pM (n=10) whereas in mice bearing B16F10 tumors, levels were significantly higher (450-3000pM, p<0.001) and correlated with tumor size. Resokine levels in normal human volunteers exhibit a more variable range, from 8pM to >2333pM (n=148), with 18% of individuals having levels <30pM, which was set as the active threshold level based on the concentration required to inhibit T cell activation *in vitro*. In contrast, samples across >300 cancer patients with different tumor types exhibited higher circulating levels with only 4% of individuals having levels below the activity threshold of 30pM. This data is consistent with the hypothesis that tumors secrete Resokine as an additional mechanism to down-regulate immune activity, and suggests further investigation of the utility of Resokine levels as a new biomarker of immune activity in patients.

Resokine: Extracellular Proteins Derived From HARS Gene



- Resokine (the extracellular proteins derived from the HARS gene) is secreted from cells via a non-canonical pathway and circulates naturally in all individuals tested. The N-terminal "iMod" domain consists of amino acids 2-60 from HARS and has structural similarity to 4 alpha–helical bundle cytokines.

iMod Domain: Conservation Among Orthologs

Parent Gene: Human HARS (Histidyl-tRNA Synthetase)

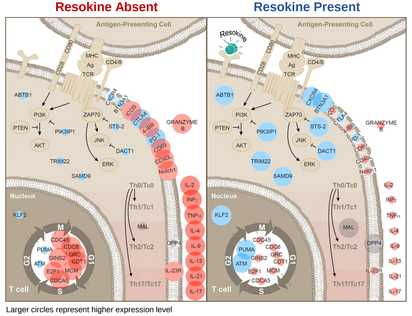
Splice variant 9: Resokine iMod (immuno-modulatory) domain

First evolved	Percent identity to human protein								
~M yrs*	iMod	IL-6	IL-12	IL-17	TNF	IL-13	TGF-β	PD-1	CTLA-4
0.3	100	100	100	100	100	100	100	100	100
25	98	97	96	96	95	94	98	96	97
75	85	41	67	62	79	57	90	59	75
360	70	31	29	49	34	-	50	27	38
450	50	-	29	28	23	-	46	-	-
Parent Gene "HARS" 4,000	-	-	-	-	-	-	-	-	-

*Science 2017; Science 2007; Nature 2002; Nature 2010; Genome Research 2000

- Comparison of sequence alignments between iMod domains in multiple species has shown a high level of evolutionary conservation spanning many millions of years. This type of species conservation is similar or greater to that observed with notable cytokines, growth factors, and checkpoint modulators.

Resokine Sets Level for T Cell Activation at pM Concentrations



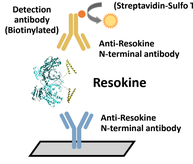
Larger circles represent higher expression level

Cartoon compiled from RNAseq, flow cytometry, and ELISA data (publication submitted)

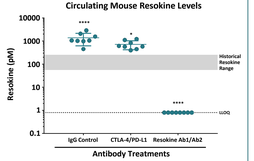
- The presence of Resokine attenuates the activation of T cells stimulated with antibodies against CD3/CD28. Analysis of gene expression profiles from stimulated T cells revealed lowered levels of many immune activation markers of inflammation when treated with picomolar amounts of Resokine.

Resokine Levels Are Elevated in Syngeneic Mouse Cancer Models

Detection of Circulating Resokine by ECLIA



Resokine Levels are Elevated in a B16F10 Melanoma Mouse Model

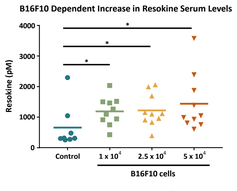


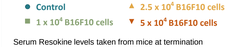
LLOQ, lower limit of quantification

Serum Resokine levels taken from mice at termination based upon tumor volume (days 20-21)

Historical range defines naïve C57BL/6 mice. Comparison to historical means run by 1-way ANOVA.
*p<0.05; ****p<0.0001

Implantation of B16F10 Melanoma Cells in Mice Results in Increased Circulating Mouse Resokine Levels

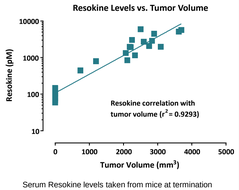




Serum Resokine levels taken from mice at termination based upon tumor volume (days 13-18)

Statistics by Mann-Whitney test; *p<0.05

B16F10 Tumor Volume in Mice Correlates with Increased Resokine Levels



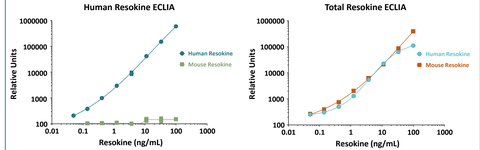
Serum Resokine levels taken from mice at termination based upon tumor volume (days 22-27)

Statistics by linear regression

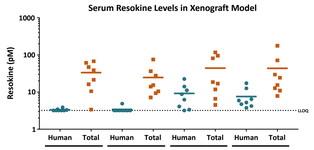
- Detection of Resokine was performed using a standard electrochemiluminescence immunoassay format (ECLIA) with two N-terminal directed monoclonal antibodies against Resokine. A mouse monoclonal capture antibody was coated onto Mesoscale Discovery assay plates and Resokine was detected with a non-competing biotinylated mouse monoclonal antibody. This assay format allows for the detection of full-length Resokine along with all splice variants.
- To study the levels of mouse Resokine in cancer, a B16F10 syngeneic mouse model was utilized. Mice were implanted with B16F10 cells and Resokine levels were measured in mice treated with an IgG control antibody, a combination of anti-CTLA-4/PD-L1 antibodies, or two anti-Resokine antibodies. Levels of Resokine were significantly elevated in mice treated with both IgG control and CTLA-4/PD-L1 antibodies as compared to historical naïve C57BL/6 mice.
- To examine the relationship between tumor volume and Resokine levels, C57BL/6 mice were implanted with increasing numbers of B16F10 cells (1x10e^6, 2.5x10e^6, or 5x10e^6 cells). Circulating serum Resokine levels increased concomitantly with B16F10 cell numbers.
- Circulating mouse serum Resokine levels, in mice implanted with B16F10 tumors, were positively correlated with tumor volume.

Resokine Levels Are Elevated in Xenograft Mouse Cancer Models

Species-Specific Resokine Detection by ECLIA

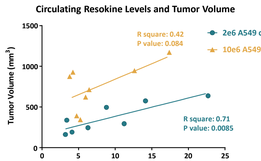


Circulating Human Resokine is Detectable in A549 Mouse Xenograft



Serum Resokine levels taken from mice at termination (day 42)

Human Resokine Levels Correlate With Tumor Volume in Xenograft Model

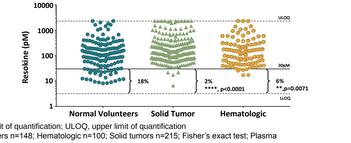


Statistics by linear regression

- Two Resokine immunoassays were designed with non-competing antibodies to detect specifically human Resokine (Human Resokine) or both human and mouse Resokine (Total Resokine). ECLIA were performed on a Mesoscale Discovery QuickPlex instrument. Limits of quantification were established for each assay as indicated in subsequent figures.
- Implantation of tumors from the human lung adenocarcinoma cell line, A549, into athymic mice (nu/nu) resulted in circulating levels of human Resokine in mouse serum. Human Resokine levels were undetectable in naïve and control treated mouse serum but were clearly detectable in mice implanted with 2 x 10^6 or 10 x 10^6 A549 cells after 42 days. Levels of total Resokine slightly increased consistent with the addition of human Resokine to the serum.
- Circulating human Resokine levels in mice were plotted against tumor volumes from implanted A549 cells. Tumor volumes correlated, in both A549 dosed cohorts, with human Resokine in mouse serum.

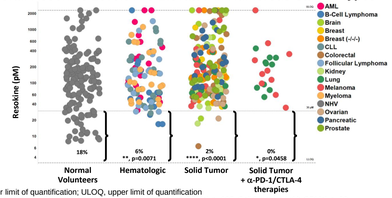
Resokine Levels Are Elevated in Human Cancer Patients

Resokine Levels Are Elevated in Cancer Patient Plasma



LLOQ, lower limit of quantification; ULOQ, upper limit of quantification
Normal Volunteers n=148; Hematologic n=100; Solid tumors n=215; Fisher's exact test; Plasma

Overall Resokine Levels Are Elevated in Numerous Cancer Types



LLOQ, lower limit of quantification; ULOQ, upper limit of quantification
Normal Volunteers n=148; Hematologic n=100; Solid tumors (no CPMs) n=195; Solid tumors (with CPMs) n=20; Fisher's exact test; Plasma

- Plasma samples from human cancer patients were obtained commercially from Conversant Bio (Huntsville, AL). Plasma samples from healthy human volunteers also came from Conversant Bio as well as Innovative Research (Novi, MI) and some additional donated samples.
- Human plasma samples from patients with solid tumors (n=215) or hematologic (n=100) cancers are less likely to have reduced circulating Resokine levels as compared to normal healthy volunteers (n=148). A population of normal healthy volunteers contains 18% of samples below 30pM of Resokine in circulation. Conversely, only 2% of solid tumor patients (p<0.001) and 6% of hematologic patients (p=0.0071) have levels below 30pM.
- Characterization of 15 types of cancer revealed a uniform distribution of Resokine among the various subtypes. Approximately 20 individual patients were obtained across 15 different cancer types and circulating Resokine levels were assessed. All cancers tested showed a similar pattern of elevated circulating Resokine. Lung and melanoma cancer patients treated with checkpoint modulating therapies (anti-PD-1 or CTLA-4) were further stratified from the pool of cancer patients. No differences in Resokine levels were observed in these patients as compared to those without checkpoint modulator treatment.

- Is enhanced Resokine secretion used by tumors an additional mechanism to down-regulate anti-tumor immune responses?
- We have previously demonstrated that these levels of circulating Resokine are sufficient to modulate T cell activity. Thus, we hypothesize that the enhanced release of Resokine from tumor cells may further increase the threshold stimulation required to generate an active immune response. This may represent an additional mechanism by which tumor cells regulate immune responses.

Conclusions

- Resokine proteins are extracellular proteins derived from the HARS gene, including full-length HARS and a number of splice variants.
- Resokine proteins contain an N-terminal domain, termed the iMod domain, which has immunomodulatory activity both *in vitro* (inhibition of T cell activation) and *in vivo*.
- Levels of circulating mouse Resokine were shown to be elevated in a syngeneic B16F10 mouse tumor model and that these levels correlated with tumor volume.
- Characterization of human Resokine in an A549 lung adenocarcinoma xenograft mouse model revealed the presence of circulating human Resokine derived from A549 cells and that these levels correlated with tumor volume.
- Resokine levels were elevated in human cancer subjects with low Resokine levels (<30pM). These levels were nearly absent in 15 cancer subtypes as compared to normal healthy controls.

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