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Follistatin-like protein 1 modulates IL-17 signaling via IL-17RC regulation in stromal cells

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Abstract

Follistatin-like protein 1 (FSTL-1) possesses several newly identified roles in mammalian biology, including IL-17 driven inflammation, though the mechanism underlying FSTL-1 influence on IL-17 mediated cytokine production is unknown. Using parallel *in vitro* bone marrow stromal cell models of FSTL-1 suppression we employed unbiased microarray analysis to identify FSTL-1 regulated genes and pathways that could influence IL-17 dependent production of IL-6 and G-CSF. We discovered that FSTL-1 modulates *Il17rc* gene expression. Specifically, FSTL-1 was necessary for *Il17rc* gene transcription, IL-17RC surface protein expression and IL-17-dependent cytokine production. This work identifies a mechanism by which FSTL-1 influences IL-17 driven inflammatory signaling *in vitro* and reveals a novel function for FSTL-1, as a modulator of gene expression. Thus, enhanced understanding of the interplay between FSTL-1 and IL-17 mediated inflammation may provide insight into potential therapeutic targets of IL-17 mediated diseases and warrants ongoing study of *in vivo* models and clinical scenarios of FSTL-1-influenced diseases.

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Competing Interests

The University of Pittsburgh has a patent "Immunomodulation of inflammatory conditions utilizing Follistatin-like Protein-1 and agents that bind thereto" (No 8334274) listing RH as an inventor.

Introduction

Follistatin-like protein 1 (FSTL-1) is a 306 amino acid glycoprotein belonging to the SPARC/BM-40/osteonectin family was originally cloned from the osteoblast cell line MC3T3-E1 as a TGFβ inducible molecule[1]. Murine and human FSTL-1 share 91% a.a. sequence homology, with five conserved domains: the eponymous follistatin-like domain, a Kazal serine protease domain, two EF-hand calcium binding domains and a von Willebrand domain, though the functionality of these domains remain unclear. FSTL-1 has been identified in both secreted and intracellular compartments, suggesting differential cell trafficking regulation. Highly expressed in mammalian cells of mesenchymal lineage, several studies have implicated FSTL-1 with functions in a myriad of settings including embryonic development, cardiac and lung dysfunction and repair, as well as tumorigenesis and metastasis and inflammation[2–9]. *In vivo* modeling has been limited as FSTL-1 germline knockout mice display a perinatal lethal phenotype associated with multiple lung, skeletal and urogenital defects. However, murine models have employed neutralizing antibodies, siRNA, adenoviral-gene transfer, partial gene disruption (FSTL-1 hypomorphs) and FSTL-1 transgenic models to begin characterizing *in vivo* functions of FSTL-1[2, 3, 5, 6, 8, 10, 11].

The role of FSTL-1 in inflammation and immunity remains unclear. We had shown that FSTL-1 expression positively correlated with degree of inflammation and joint disease severity in the collagen induced arthritis and Lyme arthritis models[10, 12–14]. Further, FSTL-1 facilitates cytokine production in response to a number of inflammatory signals. However the specific mechanism(s) involved and cell populations of significance are actively being explored. For example, we have previously shown that in macrophages/monocytes, FSTL-1 mediates NLRP3-inflammasome activation[15]. We have also observed that IL-17A in cooperation with TNFα acts on bone marrow stromal cells in an FSTL-1-dependent manner to induce IL-6 and MCP-1 production[13], suggesting that novel mechanisms may be involved in FSTL-1 mediated inflammatory pathways.

Interleukin-17 (IL-17), the eponymous cytokine produced by Th17 cells, signals via the IL-17 receptor complex. IL-17 mediated immune responses are central to both host defense against fungal and bacterial infection as well as autoimmune diseases, including ankylosing spondylitis, psoariasis and psoariatic arthritis[16–24]. IL-17 is not a potent mediator of inflammatory signaling in its own right, but signals potently with a variety of other stimuli including TNFα, FGFR and IFN-γ[25]. Bone marrow stromal cells (BMSC) represent a unique source of multipotent cells important for bone and joint homeostasis. They are also critically important for immune regulation and the host inflammatory response, in part through the IL-17 signaling axis[26–28]. Though FSTL-1 participates in several biologic and inflammatory processes the interaction between FSTL-1 and IL-17 signaling in bone marrow stromal cells remains unknown. We sought to explore the interaction of FSTL-1 and IL-17 mediated inflammatory signaling *in vitro* in order to identify pathways that could be potential targets for therapeutic intervention and further investigation.

We first validated that FSTL-1 mediates IL-17A/TNFα induced cytokine production using parallel strategies of FSTL-1 suppression in two *in vitro* bone marrow stromal cell systems. We then employed microarray analysis of these systems to identify candidate FSTL-1

influenced gene targets. Unexpectedly, we found that the IL-17 receptor C (IL-17RC), an essential subunit of the heterodimeric IL-17 receptor complex, was upregulated by FSTL-1. FSTL-1 KO cells were refractory to IL-17 signaling. Ectopic *Il17rc* expression via plasmid transfection rescued IL-17-stimulated cytokine production in the FSTL-1 KO cells. Further, plasmid *Fstl1* transfection also rescued *Il17rc* gene expression and concomitant IL-17A stimulated cytokine production. Mechanistically, FSTL-1 dependent *Il17rc* transcript abundance was due to *de_novo*_gene transcription and not mRNA stability, identifying for the first time a role for FSTL-1 in transcriptional regulation. Together, these studies confirm that FSTL-1 regulation of IL-17RC is a novel mechanism by which FSTL-1 modulates IL-17 stimulated cytokine production and a potential target for therapeutic intervention in IL-17 mediated diseases.

Results

Follistatin-like protein 1 suppression by shRNA reduces IL-17/TNFα mediated cytokine production

To examine the role of FSTL-1 in bone marrow stromal cells, we targeted *Fstl1* mRNA expression using shRNA in the ST2 bone marrow stromal cell line following IL-17A/TNFα stimulation, as previously described[13]. Using this approach, we observed reduced *Fstl1* mRNA abundance with or without IL-17A stimulation. Additionally we found that IL-17A stimulation increased *Fstl1* mRNA suggesting that FSTL-1 was, at least in part, inducible by the IL-17 receptor signaling axis (Fig. 1A). Knockdown with shRNA caused a significant reduction in secreted FSTL-1 protein both at baseline and following IL-17A stimulation (Fig 1B). To assess the effect of *Fstl1* mRNA suppression on IL-17A driven cytokine production, we evaluated the IL-17A dependent cytokines *Csf3*/G-CSF and *Il6*/IL-6. Again, we observed that IL-17A stimulated *Csf3*/G-CSF was reduced in ST2 cells transfected with shRNA targeting the *Fstl1* mRNA compared to control shRNA (Fig. 1C and 1D). Additionally, IL-17A stimulated *Il6*/IL-6 production was substantially reduced in the FSTL-1 knockdown ST2 cells compared to control shRNA (Fig. 1E and 1F).

Follistatin-like protein 1 suppression by gene deletion reduces IL-17/TNFα mediated cytokine production

To further validate the effect of FSTL-1 in IL-17A/TNFα driven cytokine production, we employed a parallel approach to attenuate FSTL-1 production in primary bone marrow stromal cells. As previously described[29], BMSCs from FSTL-1 knockout (KO) embryonic mice had markedly reduced *Fstl1* transcription and protein secretion (Fig. 2A and 2B) with and without stimulation in wild-type BMSCs. The IL-17A/TNFα stimulated production of *Csf3*/G-CSF was significantly reduced in FSTL-1 KO BMSCs (Fig. 2C and 2D), as was *Il6*/IL-6 (Fig. 2E and 2F). Collectively, these data reveal that IL-17 receptor signaling is attenuated in the context of *Fstl1* suppression.

Microarray and gene expression analysis of the role of FSTL-1 in IL-17 signaling

To evaluate potential pathways and gene targets underlying these FSTL-1 mediated events, we used an unbiased approach to evaluate gene expression by Affymetrix microarray. We assessed gene expression in ST2 cells and primary BMSCs, comparing control (wild-type)

cells in which FSTL-1 was attenuated (ST2 shFSTL-1 or FSTL-1 KO, respectively) under baseline conditions and following stimulation with IL-17A/TNFα. We reasoned that *bona fide* candidate targets of FSTL-1 mediated IL-17A cytokine production would be differentially regulated in both the ST2 cell system and primary BMSCs under both conditions. To determine FSTL-1 regulated target genes *independent* of IL-17A stimulation we identified transcripts upregulated (Fig. 3A) or downregulated (Fig. 3B) under unstimulated and IL-17A/TNFα-stimulated conditions. Validating this workflow construct, *Fstl1* was downregulated in ST2 cells under unstimulated (4.99 fold, p=0.018) and IL-17A stimulated (3.1 fold, p=0.0095) conditions. *Fstl1* was also downregulated in unstimulated (17.99 fold, p=0.033) and stimulated (23.29 fold, p=0.021) BMSCs, as expected. Indeed, 46 genes were upregulated (Fig. 3A) and 23 genes were downregulated (Fig 3B) in this dataset. Notably, we observed that *Il17rc* expression was downregulated in the ST2 unstimulated (1.1 fold, p=0.034) and IL-17A stimulated (1.34 fold, p=0.0086) system as well as in the primary BMSC unstimulated (2.34 fold, p=0.0057) and stimulated (2.39 fold, p=0.0336) system. These data suggest that *Il17rc* expression may be a significant mediator of FSTL-1-dependent IL-17A cytokine regulation.

FSTL-1 attenuation is associated with reduced IL-17RC mRNA and protein expression

To verify the microarray finding that *Il17rc* is decreased in an FSTL-1 dependent manner, we assessed its expression by qRT-PCR for this transcript in both ST2 cells (Fig. 4A) and primary BMSCs (Fig. 4B). These results indeed confirmed that *Il17rc* transcript was downregulated in the absence of FSTL-1. We then examined whether the decrease in transcript abundance was associated with reduced surface expression of IL-17RC protein by FACS. A significantly higher percentage of WT cells expressed the IL-17RC protein compared to FSTL-1 KO cells (Fig. 4C). Additionally, WT cells had a significantly greater mean fluorescence intensity (MFI) compared to FSTL-1 KO BMSCs (Figs. 4D, E). Together, these data demonstrate that attenuation of FSTL-1 results in reduced *Il17rc* transcription and IL-17RC protein surface expression.

Ectopic Il17rc expression restores IL-17-dependent signaling in FSTL-1 KO cells

Having confirmed that IL-17RC was decreased in an FSTL-1 dependent manner, we next sought to determine whether complementation with IL-17RC could rescue this effect. Accordingly, FSTL-1 KO BMSCs were transfected with a plasmid expressing murine *Il17rc*. This approach yielded increased *Il17rc* (Fig. 5A), and transfection of *Il17rc* partially rescued *Il6* (Fig. 5B) and *Csf3* (Fig. 5C) production following IL-17A/TNFα stimulation in FSTL-1 BMSCs at or above the level of WT control-transfected cells. These data demonstrate that IL-17RC complementation in the FSTL-1 KO BMSCs is capable of partial rescue of IL-17A stimulated cytokine suppression.

Ectopic Fstl1 expression rescues Il17rc expression and cytokine production in FSTL-1 KO cells

To investigate the relationship between FSTL-1 and IL-17RC regulation, we transfected FSTL-1 KO BMSCs with *Fstl1* treated with or without IL-17A/TNFα stimulation (Fig. 6A). Indeed, *Fstl1* transfection significantly increased *Il17rc* transcript abundance (Fig. 6B), confirming a causal relationship between *Fstl1* expression and *Il17rc* modulation. Moreover,

Fstl1 complementation conferred increased production of *Csf3* and *Il6* (Figs 6C and 6D) upon stimulation, confirming that FSTL-1 regulated IL-17RC expression is sufficient to rescue IL-17 stimulated cytokine production.

Fstl1 influences Il17rc transcript abundance via transcriptional regulation

To better understand the mechanism by which FSTL-1 influences mRNA transcript abundance and subsequent protein surface expression, we stimulated WT and FSTL-1 KO BMSCs under conditions that inhibit new RNA synthesis (ActinomycinD), and separately using a nascent RNA labeling approach. To assess whether *Fstl1* regulates mRNA stability of *Il17rc*, we incubated WT and FSTL-1 KO cells with 5ug/ml ActinomycinD, a potent inhibitor of gene transcription, followed by RNA isolation at various timepoints (Figs. 7A, B). Before ActinomycinD treatment and all subsequent timepoints, there was a reduction of *Fstl1* and *Il17rc* mRNA in FSTL-1 KO BMSCs. However, the rate of decay of *Il17rc* transcript was similar for both WT and FSTL-1 KO BMSCs (Fig. 7C), suggesting that the half-life of *Il17rc* transcript abundance was not FSTL-1 dependent.

To assess if FSTL-1 regulated *de novo* gene transcription, adherent WT and FSTL-1 KO cells were labeled with 5-ethynyl uridine, which incorporates only into newly synthesized (nascent) RNA. We then examined nascent RNA at various timepoints post-labeling (Fig 7 D–F). This approach revealed that *Hprt, Fstl1* and *Il17rc* transcripts were actively synthesized in BMSCs within 6 hours showing similar nascent *Hprt* transcription between genotypes. However, FSTL-1 KO cells had decreased nascent *Fstl1* and *Il17rc m*RNA identifying that newly synthesized *Il17rc* transcripts were reduced in the absence of FSTL-1. Together, these studies suggest that FSTL-1 influences *Il17rc* transcript abundance by regulating gene transcription but not RNA stability.

Fstl1 expression correlates with Il17rc expression in the CD45-negative bone marrow population

To evaluate if these findings *in vitro* were also observed *in vivo*, we sought to evaluate *Fstl1* and *Il17rc* transcription in a bone marrow stromal cell population. We harvested bone marrow cells from femur flushes of wild-type C57Bl//6 mice and FSTL-1 Hypomorphic mice. FACS-sorted CD45-negative cells (predominately bone marrow stromal cells) (CITE) underwent gene expression analysis for *Fstl1*, *Il17rc* and *Il17ra* (an IL-17 Receptor complex herterodimeric partner of IL-17RC). In this heterogenous CD45- population FSTL-1 Hypomorphs showed slightly reduced expression of (A) *Fstl1*, (B) *Il17rc* and (C) *Il17ra* compared to WT controls. However, Pearson's correlation analysis demonstrated a strong positive correlation between (D) *Fstl1* and *Il17rc* ($r=0.9512$, $R^2=0.9048$, $p<0.0001$) but no correlation between (E) *Fstl1* and *Il17ra* ($r=-0.1982$, $R^2=0.0393$, $p=0.5162$), suggesting that the *in vivo* murine system likely reflects our *in vitro* observations.

Discussion

FSTL-1 is a molecule of emerging biological importance with recent studies identifying a role in tissue repair and development, novel mechanisms of transcriptional regulation and inflammation and host defense. While some of these pathways have begun to be defined, the

role of FSTL-1 in IL-17 elicited gene production is unknown. Nonetheless, advances in understanding of IL-17/Th17 driven inflammation have provided targets for therapeutic intervention, including small molecule inhibitors and monoclonal antibodies aimed at IL-17 driven host responses, including autoimmune joint disease and psoariasis[30–34]. Therefore, understanding the mechanism underlying the influence of FSTL-1 on IL-17 inflammatory responses may spur development of novel therapies aimed at this disease pathway. Here, we identified the intersection of FSTL-1 and IL-17 using bone marrow stromal cells, a clinically relevant model system. Herein, we report that *in vitro* FSTL-1 modulates IL-17 mediated cytokine production by regulating *Il17rc* gene expression. Indeed, FSTL-1 appears to be necessary and sufficient to regulate *Il17rc* expression, and that complementation of IL-17RC rescues IL-17 driven cytokine production in the context of FSTL-1 deficiency.

This observation is the first to show that FSTL-1 regulates gene transcription, a cytokine receptor, opening a new avenue of investigation into the molecular mechanism by which FSTL-1 performs this function. Disco-interacting protein 2 homolog A (DIP2A) functions as an FSTL-1 receptor in mediating FSTL-1 dependent Akt phosphorylation in endothelial cells, cardiomyocytes and a rat ischemic stroke model[11, 35]. Other studies have suggested that FSTL-1 mediates multiple DAMP/PAMP stimulated inflammation pathways involving TLR4/CD14 and NF-κB perhaps via phosphorylating multiple intermediates[36, 37]. We have shown that FSTL-1 localizes to mitochondria in macrophages yielding increased NLRP3 inflammasome activation and IL-1β secretion[15]. FSTL-1 also plays a critical role in organogenesis as a TGFβ-inducible factor interacting with BMP signaling/Smad phosphorylation, as well as influencing cell cycling[3, 8, 15, 29, 38, 39]. It remains to be determined the common factors shared among these systems, as regulation of *Il17rc* gene expression fails to explain the diverse functions of FSTL-1 in mammalian biologic systems. Conversely, none of the above pathways have been implicated in *Il1rc* gene regulation, suggesting that additional cellular functions of FSTL-1 have yet to be identified.

Strikingly, there is very little information regarding the molecular mechanisms regulating *Il17rc* gene transcription. The methylation status of *Il17rc* has been associated with macular degeneration in human studies, though the precise role of *Il17rc* epigenetic regulation in this disease remains an area of active investigation[40, 41]. Additionally, multiple transcription factor binding sites have been identified within *Il17rc* exons suggesting that several independent pathways may influence its expression. Furthermore, while IL-17RC is highly expressed in bone, joint, prostate, liver and lung, IL-17RC is selectively and minimally detected in the leukocyte compartment suggesting that cell specific expression is tightly regulated[42–47]. Notably, several splice variants of *Il17rc* are expressed in a tissue specific manner, and factors influencing their regulation are unknown[47, 48]. Thus, further study of the FSTL-1 regulation is likely to yield enhanced understanding of pathways influencing in IL17RC expression and possibly function.

These studies are currently limited by the lack of knowledge regarding molecular interactions between FSTL-1 and transcription factors or epigenetic regulators capable of modulating *Il17rc* gene expression, and studies to identify these aspects are needed to inform the cellular function(s) of FSTL-1. Additionally, as our work examines *in vitro* mechanisms of FSTL-1/IL-17 interaction, further work exploring the role of FSTL-1 in *in*

vivo IL-17/Th17 models of inflammation and clinical disease processes is warranted to clarify the significance of our findings. While FSTL-1 is expressed in heart, lung, neuron and bone amongst others tissues, the cell and tissue specific expression of FSTL-1 and the relationship to complex inflammatory diseases has yet to be explored.

In summary, this study identified a new function for FSTL-1, gene transcription modulation. Specifically FSTL-1 mediated *Il17rc* regulation is a mechanism by which FSTL-1 mediates IL-17 driven cytokine production *in vitro* in bone marrow stromal cells. This discovery represents a significant advance in our understanding of IL-17 biology, and ongoing mechanistic investigation of FSTL-1 function in this pathway may prove valuable for the design of therapies aimed at modulating Th17/IL-17 driven diseases. Additionally, this work suggests that further FSTL-1 focused investigation into innate and adaptive immune responses may yield new therapeutic targets, as the precise function of FSTL-1 in different inflammatory processes remains elusive.

Methods

Bone marrow stromal cell culture

The murine ST2 bone marrow stromal cell line has been utilized extensively for the *in vitro* study of IL-17 signaling[18, 49]. Cells were stably transduced with murine FSTL1 short hairpin RNA (shRNA) or a control lentivirus shRNA (Santa Cruz Biotechnology) according to the manufacturer's protocol and clonally selected, as previously described[13]. Primary bone marrow stromal cells (BMSCs) were isolated from FSTL-1 KO embryos as previously described[29]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS) for ST2 cells and 15% FBS for BMSCs. In 12 wells, we plated 100,000 cells/well followed by stimulation with either 8ng/ml IL-17A (R&D 7956-ML) and 2ng/ml TNFα (R&D 410-MT-010) or media alone in duplicate; for microarray studies each group contained n=3. Cells were then harvested for RNA at 6 hours post-stimulation or collection of supernatant 24 hours post-stimulation. Randomization was included in treatment design prior to each experiment. Each experiment depicted was performed at least two times to ensure reproducibility.

RNA and microarray analysis

Total RNA was isolated from cells using the RNeasy kit (Qaigen), and cDNA was synthesized using iScrip 8t (Bio-Rad) according to the manufacturers instructions. Gene expression was analyzed by qPCR performed using primer/probe pairs for *Hprt, Fstl1, Il6, Csf3, Il17rc* (Applied Biosystems) in the C1000 Touch/CFX96 Real-Time System (Bio-Rad). RNA quality was verified using the 2100 Bioanalyzer (Agilent) before undergoing microarray analysis performed on ST2 cells using Mouse WG8.0 and BMSCs using Mouse WG6 2.0 Expression Bead Chip (Affymetrix) at the Genomics and Proteomics Core Laboratories, University of Pittsburgh. We defined differential expression as significant by setting threshold levels of ≥1.1 fold-change and $p<0.05$ by t-test with Bonferroni correction. Gene lists meeting these criteria were cross-analyzed for common FSTL-1 dependent differentially expressed genes.

Protein secretion analysis

Supernatant protein concentrations were determined for FSTL-1 by ELISA, as previously described[13], and for IL-6 and G-CSF using the xMAP multiplex system (Millipore) according to the manufacturer's instructions.

FACS Analysis

WT and FSTL-1 KO cells were stained for IL-17RC surface expression using APC-conjugated anti-IL-17RC (R&D FAB2270A) or APC-IgG control (R&D IC108A). Cells were trypsinized, washed in 0.5%FBS in PBS, centrifuged at low speed, Fc blocked with CD16/32 and stained with APC-conjugated anti-mouse IL-17RC or APC-conjugated isotype control. Cells were sorted using an LSR II flow cytometer, and data was analyzed using FlowJo software (TreeStar).

Il17rc and Fstl1 plasmid transfection

Murine *Il17rc* (Addgene 46864), *Fstl1* (Origene MR204305) and empty vector pCMV6-Entry control (Origene PS 100001) plasmids were transformed in and grown in Mix and Go *E. coli* (Zymo Research) in 5ml Luria-Bertani broth containing selection antibiotic and prepared using the QIAprep Spin Miniprep kit (Qiagen 27106), per manufacturer's instructions. FSTL-1 KO BMSCs were transfected with plasmid by electroporation using the Neon Transfection System (ThermoFischer Scientific), per manufacturer's instructions, with transfection settings of 30ms at 1400V for 1 pulse. Cells were cultured in triplicate in complete DMEM + 15% FBS and 12–18 hours post-transfection treated identically as described above.

mRNA regulation studies

To assess mRNA stability, WT and FSTL-1 KO cells were incubated with 5ug/ml ActinomycinD, to inhibit gene transcription, followed by RNA isolation at various timepoints post-ActinomycinD treatment. At each time, untreated cells were used as a normalizing control to assess rate of decay. Gene expression was assessed as described above.

To assess nascent mRNA systhesis, WT and FSTL-1 KO BMSCs were cultured for 18hours to allow adherence. Using the Click-iT Nascent RNA Capture Kit (ThermoFisher Scientific Cat# C10365), cells were labeled for various lengths of time (0, 1, 2, 4, and 6 hours) with 150uM 5-ethyl Uridine (EU). Cells were harvested using TRIzol reagent and half of this volume was used to isolate total RNA, while the remainder was used as input for the nascent RNA capture, which was followed according to the manufacturer's protocol to recover the labeled, newly-synthesized mRNA. Labeled mRNA underwent reverse transcription using iScript in addition to total cell RNA as described above. Nascent mRNA for each transcript was normalized to total *Hprt* for relative abundance analysis.

Bone marrow population analysis

C57Bl/6 and FSTL-1 Hypomorphic (on C57Bl/6 background) mice were housed in accordance with the approved University of Pittsburgh School of Medicine Institutional

Animal Care and Use Committee protocol[12]. The bone marrow compartment of male and female wild-type and FSTL-1 Hypomorhic mice was isolated by femur flush protocol[50]. Isolated bone marrow cells were resuspended in sterile phosphate-buffered saline and counted using trypan blue stain and a hemocytometer. The cells were first blocked using anti-mouse CD16/CD32 (eBioscience) and subsequently surfaced stained in the presence of anti-mouse CD45 antibody (clone 30-F11, BD Bioscience). CD45-positive and CD45-negative cell populations were then sorted on a FACSAria via FACSDiva software (Becton-Dickinson) into phosphate-buffered saline. The sorted cell populations were pelleted and resuspended in RNA Lysis Buffer (Zymo Research). RNA was processed for gene expression analysis as previously described. These experiments were performed twice with n=3 or n=4 per group each time. Data shown include cells from all animals in the two experiments.

Statistical analysis

Investigators were not blinded to treatment, but were blinded to individual/group during data analysis. All statistics were performed using GraphPad Prism 6. Briefly, all data are presented with mean ± SEM. Studies comparing two groups were analyzed by two-sided student's t-test. Studies comparing more than two groups were analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons. *Ex vivo* gene expression correlation was determined using Pearson's correlation coefficient analysis. All statistical analyses considered $p<0.05$ significant.

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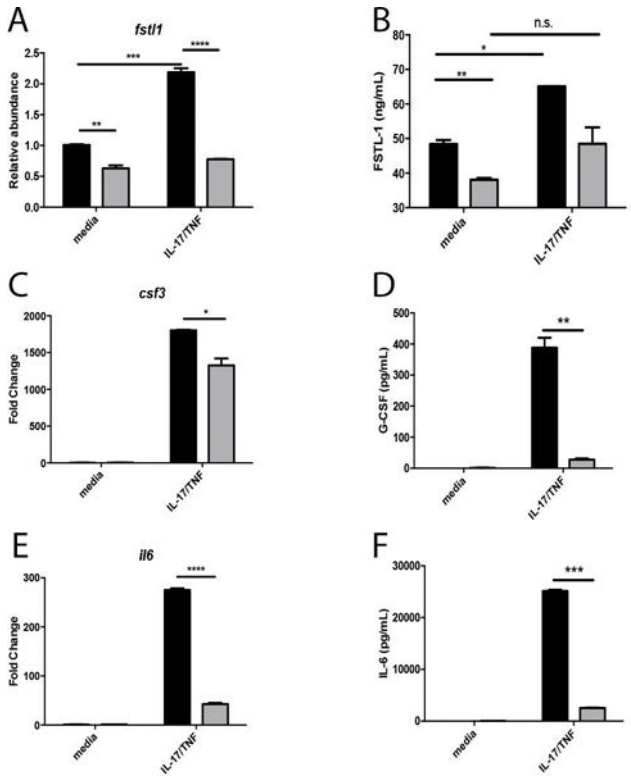

Figure 1. Follistatin-like protein 1 suppression by shRNA reduces IL-17/TNFα mediated cytokine production

In the ST2 cell line (A) *Fstl1* transcript abundance and (B) FSTL-1 protein production increased following IL-17/TNFα stimulation, but was reduced in cells transfected with shRNA targeting *Fstl1* (gray bars) compared to control shRNA (black bars) IL-17/TNFα dependent cytokine production is reduced by *Fstl1* inhibition as determined by (C, E) transcript induction and protein secretion (D, F). ** p<0.01, ***p<0.001, ****p<0.0001

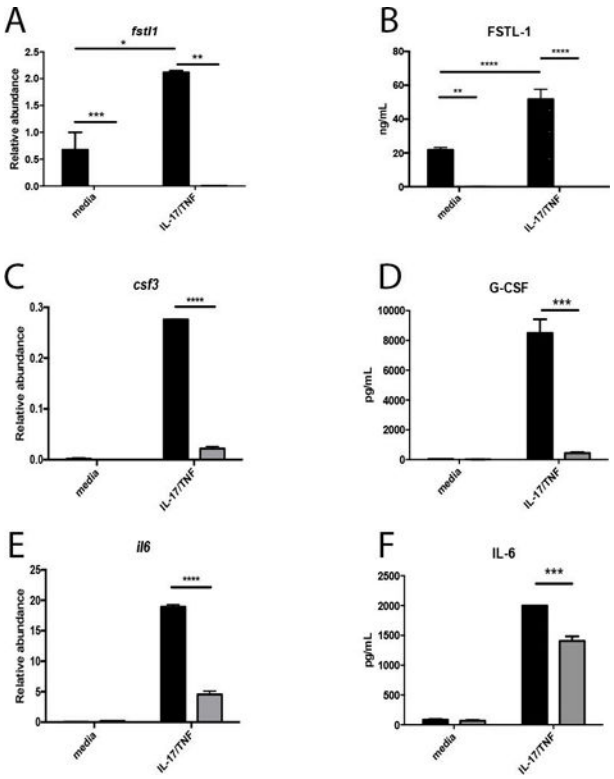

Figure 2. Follistatin-like protein 1 suppression by gene deletion reduces IL-17/TNFα mediated cytokine production

In primary bone marrow stromal cells (A) *Fstl1* transcript abundance and (B) FSTL-1 protein production increased following IL-17A and TNFα stimulation, but was reduced in cells from FSTL-1 KO mice (gray bars) compared to wild-type littermate control mice (black bars) IL-17/TNFα dependent cytokine production is reduced by Fstl1 inhibition as determined by (C, E) transcript induction and protein secreton (D, F) *p<0.05. ** p<0.01, ***p<0.001, ****p<0.0001

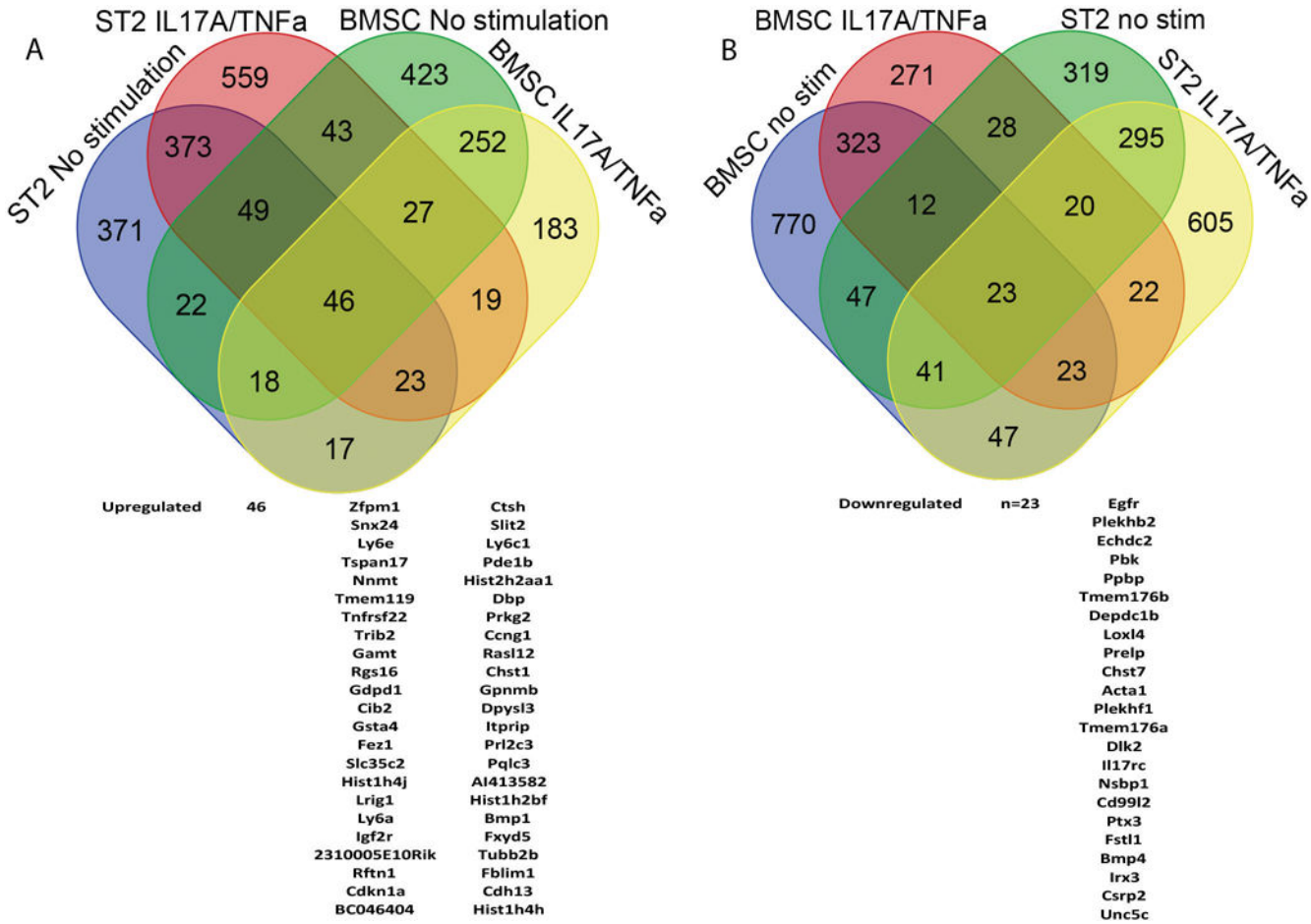

Figure 3. Microarray and pathway analysis of the role of FSTL-1 in IL-17 signaling

cDNA from ST2 shControl and shFSTL-1 cells, as well as primary WT and FSTL-1 KO BMSCs, with and without IL-17A/TNFa stimulation underwent Microarray analysis. Differential expression (≥1.1 fold-change) for FSTL-1 influenced targets were identified for each condition by t-test with Bonferroni correction ($p<0.05$) of non-transformed data. (A) Forty-six symmetrically upregulated and (B) twenty-three downregulated genes in both cell systems with and without IL-17A/TNFa stimulation are displayed schematically in a Venn diagram corresponding to Table 1.

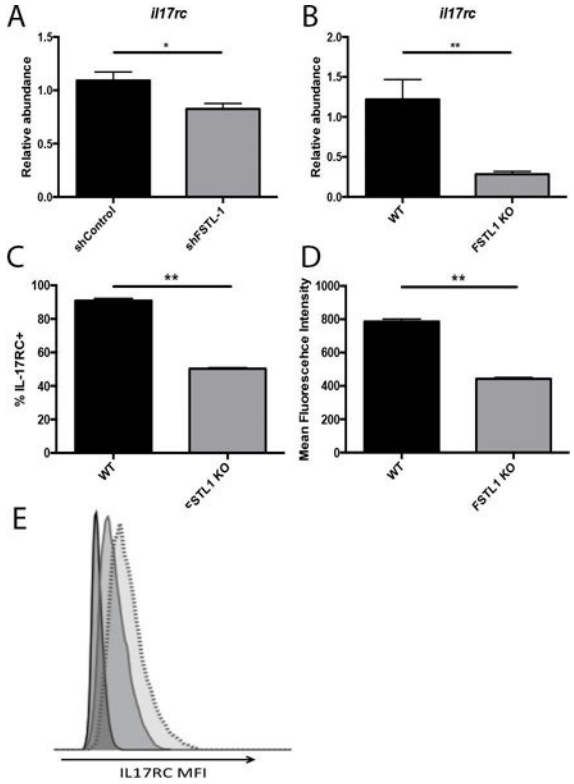

Figure 4. Follistatin-like protein 1 attenuation is associated with reduced IL-17RC mRNA and protein expression

Il17rc transcript abundance is reduced in (A) ST2 cells with shRNA FSTL-1 suppression as well as (B) primary BMSCs from FSTL-1 KO mice when compared to controls. Surface expression of IL-17RC is reduced as measured by FACS determined by (C) %IL17RC+ and (D) mean fluorescence intensity as noted by (E) representative histograms of isotype (dark grey), FSTL-1 KO (light grey) and WT BMSCs (dashed line). *p<0.05, **p<0.01.

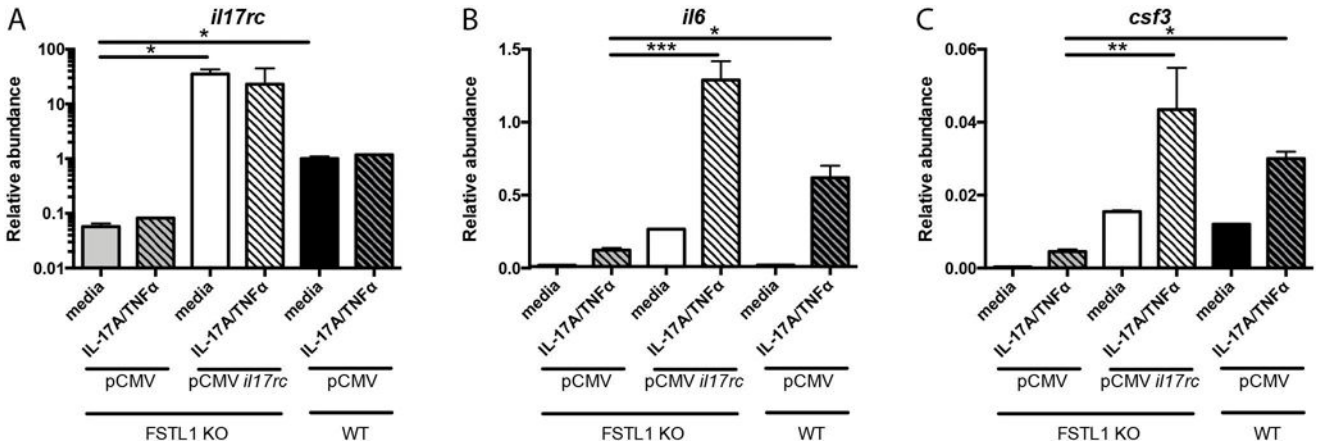

Figure 5. Ectopic Il17rc expression rescues cytokine production in FSTL-1 KO cells

FSTL-1 KO BMSC transfection with pCMV-*il17rc* rescues *Il17rc* transcript abundance (A). pCMV-*Il17rc* complementation in FSTL-1 KO BMSCs rescues IL-17A stimulated transcript levels of (B) *Il6* and (C) *Csf3*. * $p<0.05$, ** $p<0.01$, ** $p<0.001$

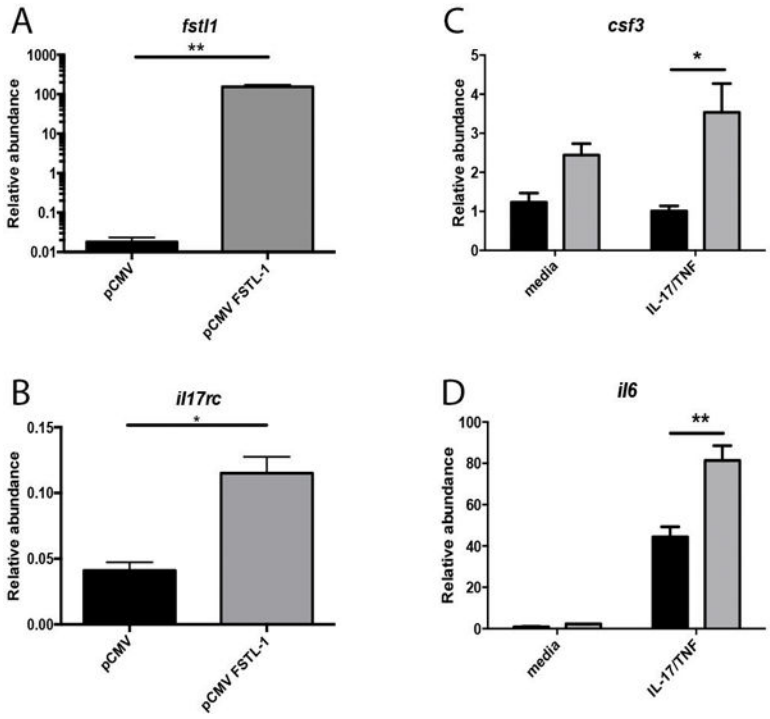

Figure 6. Ectopic Fstl1 expression rescues Il17rc expression and cytokine production in FSTL-1 KO cells

FSTL-1 KO BMSC transfection with pCMV-*fstl1* rescues (A) *Fstl1* and (B) *Il17rc* transcript abundance. pCMV-*Fstl1* complementation in FSTL-1 KO BMSCs rescues IL-17A/TNF stimulated transcript levels of (C) *Il6* and (D) *Csf3*. *p<0.05, ** p<0.01

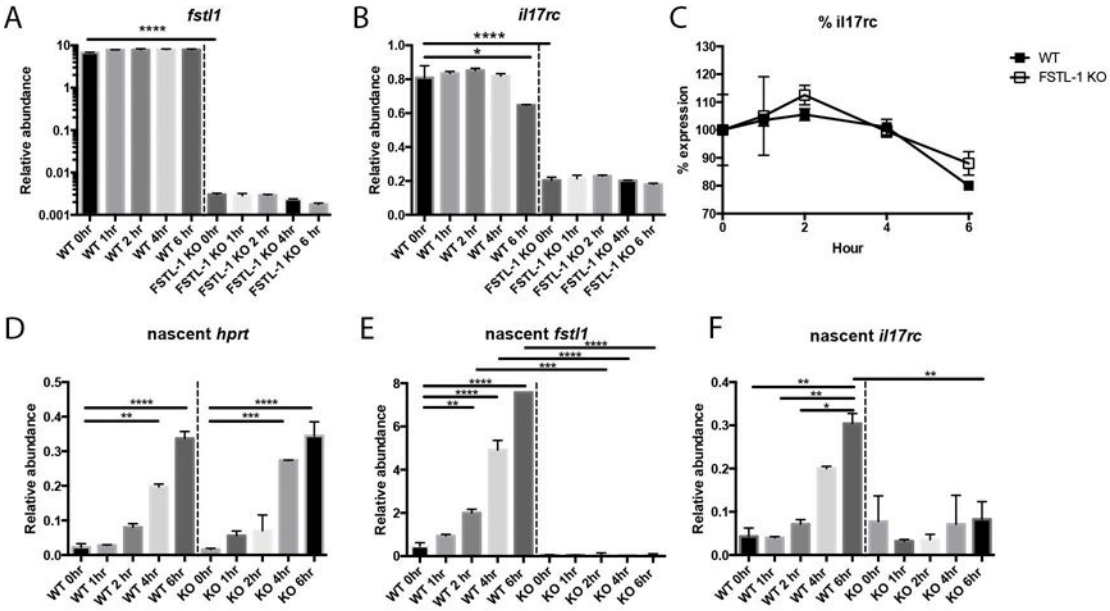

Figure 7. FSTL-1 influences *Il17rc* transcript abundance via transcriptional regulation

Following ActinomycinD treatment, FSTL-1 KO cells had reduced (A) *Fstl1* and (8) *Il17rc* transcript abundance before ActinomycinD treatment and at 1, 2, 4 and 6 hours post-treatment compared with WT BMSCs. Linear regression of *Il17rc* transcript abundance (C) at various timepoints following ActinomycinD treatment showed similar slopes for WT and FSTL-1 KO BMSCs (−3.254±1.200 and −2.457±1.429, p=0.6748). Newly synthesized mRNA (5-EU labeled) was similar for (D) *Hprt*, but reduced for (E) *Fstl1* and (F) *Il17rc*, transcripts in FSTL-1 KO cells compared to WT * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001

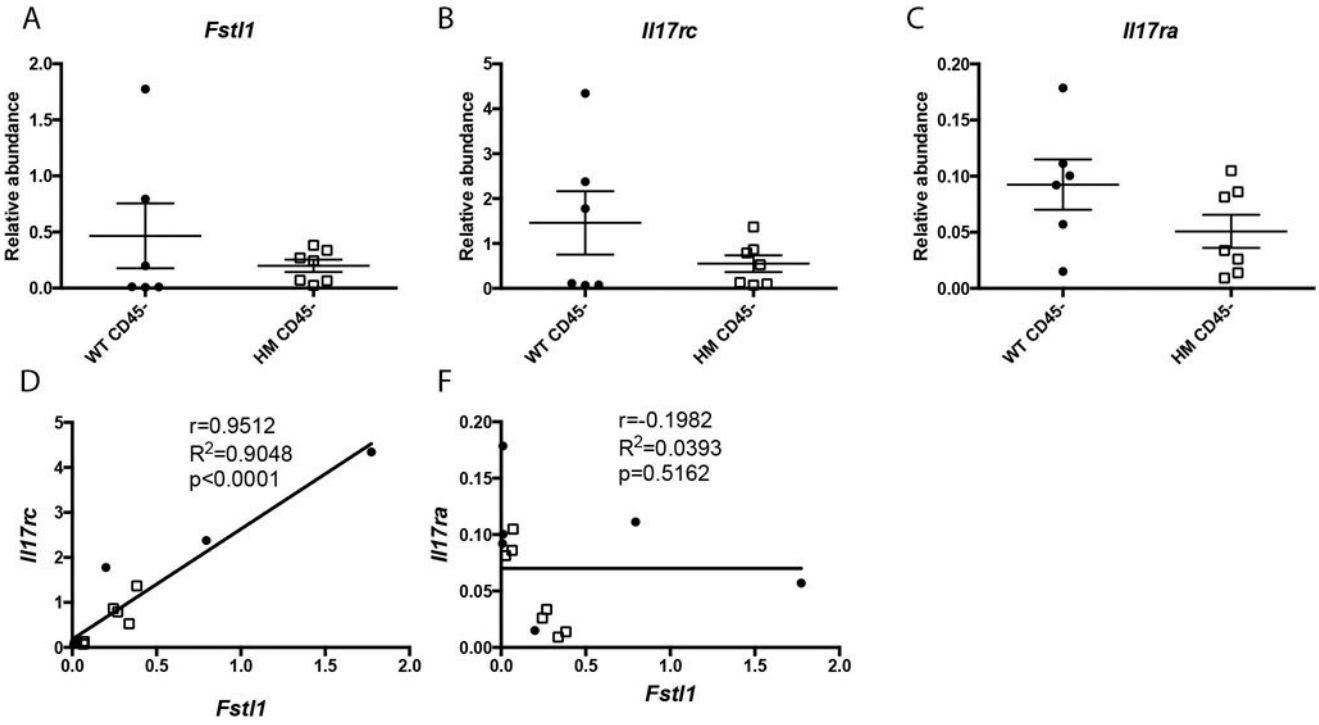

Figure 8. *Fstl1* expression correlates with *Il17rc* expression in the CD45 negative bone marrow population

CD45 negative cells from wild-type and FSTL-1 Hypomorphic mouse bone marrow had slightly reduced (A) *Fstl1*, (B) *Il17rc* and (C) *Il17ra* expression. Correlation analysis revealed highly significant association between (D) *Fstl1* and *Il17rc* (r=0.9512, R2=0.9048, p<0.0001) but not (E) *Fstl1* and *Il17ra* (r=−0.1982, R2=0.0393, p=0.5162).