Focal adhesion kinase (FAK) mediates tumor cell–intrinsic behaviors that promote tumor growth and metastasis. We previously showed that FAK also induces the expression of inflammatory genes that inhibit antitumor immunity in the microenvironment. We identified a crucial, previously unknown role for the dual-function cytokine interleukin-33 (IL-33) in FAK-dependent immune evasion. In murine squamous cell carcinoma (SCC) cells, specifically nuclear FAK enhanced the expression of the genes encoding IL-33, the chemokine CCL5, and the soluble, secreted form of the IL-33 receptor, called soluble ST2 (sST2). The abundance of IL-33 and CCL5 was increased in FAK-positive SCC cells but not in normal keratinocytes. IL-33 associated with FAK in the nucleus, and the FAK–IL-33 complex interacted with a network of chromatin modifiers and transcriptional regulators, including TAF9, WDR82, and BRD4, which promote the activity of nuclear factor κB (NF-κB) and its induction of genes encoding chemokines, including CCL5. We did not detect secretion of IL-33 from FAK-positive SCC cells; thus, we propose that the increased production and secretion of sST2 likely sequesters IL-33 secreted by other cell types within the tumor environment, thus blocking its stimulatory effects on infiltrating host immune cells. Depleting FAK, IL-33, or sST2 from SCC cells before implantation induced tumor regression in syngeneic mice, except when CD8⁺ T cells were co-depleted. Our data provide mechanistic insight into how FAK controls the tumor immune environment, namely, through a transcriptional regulatory network mediated by nuclear IL-33. Targeting this axis may boost antitumor immunity in patients.

**INTRODUCTION**

Reprogramming the immunosuppressive tumor environment to promote antitumor immunity is a major objective of immunomodulatory therapies currently in clinical use or development. Cancer cells contribute to orchestrating the composition of this environment through driving enrichment of immune cell populations with intrinsic immunosuppressive function, thereby evading the antitumor activity of cytotoxic CD8 T cells. Identification and characterization of key molecular pathways that regulate cancer cell expression of immune modulators, such as chemokines and cytokines, may therefore provide new therapeutic strategies for use in combination immunotherapy.

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that signals downstream of integrins and growth factor receptors to control the malignant phenotype in multiple ways, including by regulating adhesion, migration, proliferation, and survival (1). FAK is frequently increased in abundance in human cancers (2–4) and contributes to skin, mammary, intestinal, and prostate tumorigenesis in mouse models (5–8). A number of small-molecule FAK kinase inhibitors are now in early-phase clinical trials. In addition to its role at the plasma membrane, we previously showed that nuclear FAK controls expression of inflammatory genes in SCC (12), we demonstrated that nuclear FAK controls expression of chemokines and cytokines, including Ccl5 and Tgfb2, that drive increased numbers of regulatory T (Treg) cells in the tumor environment, resulting in protection from an antitumor CD8⁺ T cell response (9).

Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines and is secreted by necrotic epithelial cells and activated innate immune cells or held within the cell as a nuclear factor. Secreted IL-33 binds to its cognate receptor, a heterodimeric complex composed of ST2L [interleukin 1 receptor-like 1 (IL1RL1)] and IL-1 receptor accessory protein (IL-1RAcP), to initiate activation of mitogen-activated protein kinase and nuclear factor κB (NF-κB) (13), wherein IL-33 has potent proinflammatory functions and is considered a “danger” signal. IL-33 released into the tumor environment has been suggested to both inhibit (14) and promote tumor formation (15), indicating that IL-33’s roles in cancer development and progression are unclear. IL-33 can also localize to the nucleus, where it either alternates or represses transcription through association with the transcription factor NF-κB or the NF-κB p65 promoter, respectively (16, 17). The precise mechanisms that underpin IL-33’s regulation of transcription are not well understood.

ST2, a component of the IL-33 receptor complex, can exist as a functional transmembrane receptor (ST2L) or as a shorter secreted decoy receptor [soluble ST2 (sST2)] (13). ST2L is present on the cell surface of several hematopoietic cells, including T cells, macrophages, neutrophils, and myeloid-derived suppressor cells (MDSCs), and activation of downstream signaling can alter cytokine production or immunosuppressive capacity (14, 18). ST2L of host immune cells is required for tumor clearance; hence, it has been linked to the antitumor properties of IL-33 (14). Conversely, sST2 is proposed to function as a decoy receptor for IL-33, suppressing its potent proinflammatory functions, and high serum levels of sST2 have been correlated with poor prognosis in estrogen receptor–positive breast cancer (19). Like IL-33, the precise role of sST2 in cancer remains somewhat controversial.

Here, we found that nuclear FAK is critical for the expression of IL-33 and ST2 in cancer cells. IL-33 was restricted to the nucleus in murine SCC cells, where it acts downstream of FAK to promote...
Ccl5 expression and tumor growth. Mechanistic protein network analyses suggested that IL-33 regulates gene expression by interacting with chromatin modifiers and transcriptional regulators. ST2 was secreted by SCC cells, and it suppressed CD8+ T cell–mediated tumor clearance. Our findings reveal new insight into the molecular mechanisms by which nuclear FAK regulates chemokine expression, placing nuclear IL-33 at the heart of a complex transcriptional network that specifies the antitumor immune response.

RESULTS

Nuclear FAK regulates expression of IL-33 and its receptor ST2

We have previously reported that nuclear FAK regulates the expression of chemokines, including Ccl5, and this is important for driving infiltration of Treg cells into murine SCC tumors, enabling them to evade the antitumor immune response (9). To explore the mechanisms of FAK-dependent chemokine regulation, we analyzed Affymetrix microarray data comparing murine FAK−/− SCC cells with those re-expressing FAK-WT (wild-type) (herein referred to as FAK-WT) to identify genes that are regulated by FAK. In the set of genes that significantly down-regulated after FAK depletion, the only significantly enriched gene ontology term was “extracellular region” (P = 0.049). Using the genes contained within this category, we generated a protein interaction network based on direct physical interactions. The largest connected network was found to contain Ccl5 and the gene encoding the cytokine IL33 (Fig. 1A). Given the link between IL-33 and the regulation of gene expression (16, 17), we investigated whether, and if so how, IL-33 contributed to FAK-dependent transcription of chemokines.

We first used quantitative real-time polymerase chain reaction (qRT-PCR) to compare IL33 expression in SCC cells expressing FAK-WT, FAK−/−, FAK-nls (a mutant that is largely excluded from the nucleus), and FAK-kd (a kinase-deficient mutant) and found that regulation of IL33 mRNA was dependent on both FAK kinase activity and its nuclear localization (Fig. 1B). Western blotting for IL-33 abundance in whole-cell lysates revealed similar findings at the protein level (Fig. 1C and fig. S1A). We have shown previously that mutation of the nuclear localization signal in FAK does not completely abolish FAK nuclear localization. We believe that this explains why we observed slightly increased abundance of IL-33 in cells expressing this mutant when compared to FAK−/− or FAK-kd SCC cells. Similarly, analysis of SCC cells expressing FAK-Y979F, an autophosphorylation-defective mutant of FAK that is kinase-deficient, showed reduced IL33 expression similar to that observed in FAK−/− SCC cells (fig. S1B). Treatment of FAK-WT cells with the FAK catalytic inhibitor VS4718 inhibited IL-33 abundance on both mRNA and protein levels (Fig. 1, D and E), even with a low (50 nM) concentration of VS4718 (fig. S1, C and D). IL-33 can function both as a nuclear cytokine and a secreted alarmin (20). Using both an anti–IL-33 enzyme-linked immunosorbent assay (ELISA; Fig. 1F) and Western blotting (fig. S1E), we could not detect IL-33 in SCC cell–conditioned media, implying that IL-33 predominantly functions as a nuclear cytokine in SCC cells.

As an extracellular cytokine, IL-33 mediates signaling by binding to the IL-33R complex, composed of ST2 (also termed IL1RL1) and IL-1RAcP (21). Furthermore, both IL-33 signaling and nuclear IL-33 have been shown to regulate ST2 expression (22, 23). The protein encoded by ST2 exists in two forms: (i) as ST2L, a membrane-anchored receptor that activates downstream signaling upon IL-33 engagement, or (ii) as sST2, a secreted soluble decoy receptor that inhibits IL-33 signaling (13). Using qRT-PCR with a primer set that would detect cumulative amounts of mRNA encoding both ST2L and sST2, we found that ST2 expression was greater in FAK-WT cells when compared to FAK−/−, FAK-nls, or FAK-kd cells (Fig. 1G). Thus, FAK’s regulation of ST2 expression is also dependent on both nuclear localization and kinase activity. Using an anti-ST2 ELISA, we found abundant secreted amounts of sST2 in FAK-WT cell–conditioned medium, and this was reduced in media conditioned by FAK−/−, FAK-nls, and FAK-kd cells (Fig. 1H). However, flow cytometry analysis did not detect the presence of ST2L on the surface of FAK-WT and FAK−/− SCC cells (Fig. S1F), indicating that sST2 is the predominantly produced isoform upon ST2 induction in SCC cells. Treatment with the FAK inhibitor VS4718 reduced its expression and secretion (Fig. 1, I and J). Collectively, these results indicate that the kinase activity of FAK in the nucleus is a key regulator of the abundance of both nuclear IL-33 and sST2, thereby influencing both the nuclear and extracellular/alarmin functions of IL-33 signaling.

We next investigated the mechanism by which FAK controls IL33 expression and sST2 abundance in SCC cells. Using an experimentally derived nuclear FAK interactome from FAK-WT cells (9), we used Ingenuity Pathway Analysis (IPA) to identify direct experimentally observed relationships between nuclear FAK-interacting proteins and transcription factors that regulate the expression of IL33 and ST2 (obtained from Qiagen’s DECODE database; fig. S1G). This identified associations with several IL33- and ST2-regulatory transcription factors. We noticed that four of these associations, RUNX1, SP1, NCOA2, and NR3C1, were linked to transcription factors associated with regulating the expression of both IL33 and ST2. We know that FAK associates with SP1 (confirmed in fig. S1, H and I) and RUNX1 (24) and is involved in regulating RUNX1-containing protein complexes, posttranslational modification, and, ultimately, transcription factor function. Small interfering RNA (siRNA)–mediated depletion of both RUNX1 and SP1 suggested that these transcription factors acted together to regulate IL33 abundance (fig. S1, J and K). The precise details of their coordinated activities require further investigation, but nonetheless, our findings identify several connections between FAK-interacting proteins and transcription factors that regulate IL33 and ST2.

IL-33 is required for FAK-dependent expression of a subset of chemokines

Nuclear IL-33 has been linked to regulation of gene expression in several model systems (16, 17). To determine whether nuclear IL-33 was required for FAK-dependent chemokine expression, we depleted IL-33 in FAK-WT SCC cells using both short hairpin RNA (shRNA; Fig. 2, A and B, and fig. S2A) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (fig. S2B). IL-33 has previously been linked to the regulation of Ccl5 (23), and we have shown that FAK-dependent Ccl5 expression regulates the antitumor immune response (9). We found that IL33 and Ccl5 expression was increased in SCC cells when compared to primary keratinocytes (Fig. 2, C and D), correlating with nuclear FAK in SCC cells, and that IL-33 was required for Ccl5 expression in FAK-WT SCC cells (Fig. 2E and fig. S2C). A previous study in endothelial cells (23) demonstrated that IL-33 silencing increased sST2 expression. Here, we found that IL-33 silencing reduced sST2 abundance (fig. S2D). Although we have used different readouts (mRNA versus protein), it is likely that the regulation of ST2 by IL-33 may be context-dependent. To investigate further the requirement for IL-33 in FAK-dependent
Fig. 1. Nuclear FAK regulates expression of IL-33 and its receptor ST2. (A) Gene ontology enrichment analysis (cellular component terms) on the significantly down-regulated set of genes in the FAK−/− SCC transcriptome relative to the wild-type [WT; percentage false-positives (pfp) < 0.05]. Genes annotated with the over-represented term (extracellular region; Benjamini-Hochberg–corrected hypergeometric test) were used to seed a protein interaction network based on direct physical interactions (gray lines). Color of each node (circle) is proportional to the log-transformed fold change in gene expression. The largest connected graph component is displayed. (B and C) Abundance of IL-33 at the mRNA level [(B); by qRT-PCR] and protein level [(C); by Western blot] in FAK-WT, FAK−/−, FAK-nls (a mutant that is largely excluded from the nucleus), and FAK-kd (a kinase-deficient mutant) SCC cells. (D and E) Abundance of IL33 mRNA [(D); by qRT-PCR] and IL-33 protein [(E); by Western blot] in FAK-WT SCC cells treated with control (dimethyl sulfoxide (DMSO)) or VS4718 (250 nM; for 24 hours). Western blot additionally assessed in FAK−/− SCC cells for reference. (F) Analysis of enzyme-linked immunosorbent assay (ELISA) for IL-33 in conditioned media from FAK-WT, FAK−/−, FAK-nls, and FAK-kd SCC cells. (G) qRTPCR analysis of ST2 expression in FAK-WT, FAK−/−, FAK-nls, and FAK-kd SCC cells. (H) Analysis of ELISA for sST2 in conditioned media from FAK-WT, FAK−/−, FAK-nls, and FAK-kd SCC cells. (I) qRT-PCR analysis of ST2 expression in FAK-WT SCC cells treated with control (DMSO) or VS4718 (250 nM; for 24 hours). (J) Analysis of ELISA for sST2 in conditioned media from FAK-WT SCC cells treated with control (DMSO) or VS4718 (250 nM; for 24 hours). Data are means ± SEM. n = 3 experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by Tukey’s corrected one-way analysis of variance (ANOVA) (B, G, and H) or two-tailed unpaired t test (D, I, and J).
chemokine expression, we performed NanoString PanCancer Immune Profiling of FAK-WT/pLKO, FAK−/−, and FAK-WT IL-33–shRNA SCC cells. Hierarchical clustering of log-transformed fold changes, relative to control cells, identified a subset of chemokines coregulated by FAK and IL-33 (Fig. 2F), including CCL5. Furthermore, overexpression of IL-33 in FAK−/− SCC cells increased Ccl5 expression (Fig. 2G), suggesting that IL-33 is sufficient to promote Ccl5 expression. Therefore, FAK regulates IL-33, which in turn can mediate FAK-dependent chemokine expression.

IL-33/ST2 axis supports tumor growth by suppressing the immune response

IL-33 is expressed by several cell types within the tumor environment, and secreted IL-33 can have both pro- and antitumor effects (14, 15). ST2L, the IL-33 receptor, is present on various immune cells, including Treg cells, macrophages, and CD8+ T cells, and activation of ST2L signaling can alter their phenotype and/or function. For example, ST2L-positive Treg cells exhibit a more potent immunosuppressive function than ST2L-negative Treg cells (25), implying that IL-33–ST2L
signaling could enhance the suppressive activity of \( T_{\text{reg}} \) cells, thereby promoting tumor growth. In contrast, activation of ST2L on cytotoxic CD8\(^+\) T cells enhances the cells’ cytotoxic function (18), resulting in an improved antitumor immune response. Therefore, it is possible that FAK-dependent regulation of the decoy receptor sST2 could have pro- or antitumor effects.

Having established that nuclear IL-33 regulates chemokine expression, including Ccl5, we next assessed the effects of IL-33 depletion on SCC tumor growth. Therefore, \( 1 \times 10^6 \) FAK-WT, FAK\(^{-/-}\), and FAK-WT/IL-33\(-/-\)-shRNA1 cells were injected subcutaneously into Friend leukemia virus B strain (FVB) mice (the syngeneic host strain), and tumor growth was monitored. FAK-WT tumors exhibited exponential growth until they reached defined end points by which time the mice had to be sacrificed (see Materials and Methods). In contrast, FAK\(^{-/-}\) SCC tumors grew until about day 7, after which they stalled and underwent complete regression (Fig. 3A, left graph), as we have also reported previously (9). IL-33 depletion from FAK-WT cells resulted in six of eight tumors exhibiting a period of growth followed by complete regression, in a similar manner to FAK\(^{-/-}\) tumors, whereas two of eight tumors showed a growth delay (Fig. 3A). Similar studies using CRISPR/Cas9 to deplete IL-33 from FAK-WT cells confirmed the requirement for IL-33 in supporting SCC tumor growth (Fig. 3B). Thus, IL-33 is required to support tumor growth and permit immune evasion, likely by regulating transcription of vital chemokines, including Ccl5.

To next probe the role of sST2 in tumor growth, we generated sST2-depleted FAK-WT SCC cell lines using shRNA (Fig. 3C) and injected \( 1 \times 10^6 \) FAK-WT, FAK-WT/pLKO (shRNA control), FAK-WT/ST2-shRNA1, and FAK-WT/ST2-shRNA2 SCC cells into syngeneic FVB mice. We found that most tumors (six of eight for shRNA1 and five of eight for shRNA2) exhibited a period of growth followed by complete regression (Fig. 3D) like FAK\(^{-/-}\) tumors, albeit with different kinetics. To address how ST2 depletion might result in tumor regression, we profiled the immune cells in FAK-WT tumors to determine the quantity of ST2L on the cell surface. We found that after 12 days of growth, FAK-WT tumors have an extensive immune cell infiltrate accounting for about 60% of the viable cell population (Fig. 3, E and F). Using a range of surface and intracellular markers (table S1), we identified activated CD8\(^+\) T cells, activated CD4\(^+\) T cells, \( T_{\text{reg}} \) cells, neutrophils, and macrophages as the major immune cell populations that displayed surface-bound ST2L (Fig. 3, G and H). Because IL-33 engagement with ST2L expressed on activated CD8\(^+\) T cells can enhance cytotoxic function and drive increased expression of the effector cytokine interferon-\( \gamma \) (IFN-\( \gamma \)) (14), we hypothesized that tumor regression in response to depletion of ST2L from SCC cancer cells was likely CD8\(^+\) T cell–dependent. To test this, we used CD8-depleting antibodies (Abs) and found that depletion of CD8\(^+\) T cells was sufficient to completely rescue the growth of FAK-WT/ST2-shRNA SCC tumors (Fig. 3I), implying that ST2L from the tumor cells plays an important role in suppressing CD8\(^+\) T cell–mediated antitumor immunity. Consistent with previous results (9), we also observed enhanced growth of FAK-WT tumors upon depletion of CD8\(^+\) T cells, implying that even the FAK-WT cells remain under negative pressure from the CD8\(^+\) T cell–mediated immune response.

**Nuclear IL-33 interacts with an extensive network of transcriptional regulators**

Having established an important role for nuclear IL-33 in the regulation of chemokine expression and tumor growth, we addressed the molecular mechanisms that may underpin IL-33’s regulation of chemokine transcription. Using cellular fractionation, we prepared cytoplasmic, nuclear, and chromatin extracts from FAK-WT and FAK\(^{-/-}\) SCC cells as we have done before (9). Our results show that IL-33 is largely chromatin-associated (Fig. 4A), in agreement with previous studies that identified IL-33 as a histone 2A (H2A)/2B–binding protein (26). To investigate the functional significance of IL-33 association with chromatin, we made use of a proteomics technique called “BioID” (27). First, we generated an IL-33 protein fused to the 35-kDa *Escherichia coli* biotin protein ligase BirA (IL-33–BirA). Next, we expressed either IL-33–BirA or BirA alone in FAK-WT cells, from which endogenous IL-33 was deleted (FAK-WT/IL-33–CRISPR SCC cells, Fig. 4, B and C), and cultured these in the presence of biotin for 24 hours. This resulted in the biotinylation of proximal interacting proteins, enabling their purification and identification by mass spectrometry (MS). To ensure identification of robust interactions, we applied stringent criteria, including that (i) proteins must be present in all three biological replicates, (ii) proteins must have greater than threefold enrichment when compared to BirA only control, and (iii) fold enrichment must be statistically significant (\( P < 0.05 \)). With these criteria, we identified 105 proteins that associated with IL-33. Gene ontology analysis of both biological and cellular processes [Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics database] represented within the data set identified significantly enriched terms associated with chromatin organization and transcriptional regulation (fig. S3, A and B). Further analysis of the nuclear IL-33 interactome using IPA identified a connected network of proteins that were implicated in regulation of transcription, chromatin remodeling, and nucleosome disassembly (Fig. 4D). There were several members of the Baf-type complex (also known as SWI/SNF), the PTW/PP1 phosphatase complex, and the transcription factor transcription factor IID (TFIID) complex, establishing a link between nuclear IL-33 and the core transcriptional machinery. We extracted a complete list of genes belonging to these complexes from the gene ontology database AmiGO and used IPA to reconstruct all three complexes on the basis of known physical interactions (fig. S3, C to E).

We next contextualized the BioID proteomics–informed IL-33 nuclear interactome onto these networks (highlighted in pink in fig. S3, C to E) and identified potential interactions linking IL-33 to key members of these complexes. Using streptavidin pulldown after incubation of IL-33–BirA fusion protein–expressing FAK-WT/IL-33–CRISPR SCC cells in the presence of biotin, we confirmed the interactions of IL-33 with WDR82, SMARCC1, and TAF9 (Fig. 4E). We found that IL-33 depletion in FAK-WT cells resulted in loss of WDR82 from the chromatin fraction (Fig. 4F), implying that IL-33 is required to stabilize the association of WDR82 with chromatin. We find this interesting because WDR82 is a key component of complexes associated with chromatin modification, such as the PTW/PP1 phosphatase complex (28) and the Set1A/Set1B histone H3-Lys4 (H3K4) methyltransferase complex (29). Therefore, IL-33 likely plays a key role in scaffolding complexes required to modulate chromatin structure and permit transcription.

**IL-33 interacts with and enhances regulators of Ccl5 expression**

As mentioned above, we previously showed that CCL5 secreted by FAK-WT SCC cells drives the infiltration of immunosuppressive \( T_{\text{reg}} \) cells into SCC tumors, shifting the CD8\(^+\) T cell/\( T_{\text{reg}} \) ratio in favor of tumor tolerance (9). Thus far, we have found that IL-33 is both necessary and sufficient to drive Ccl5 expression downstream of FAK (Fig. 2, E and G). To define how IL-33 contributes to regulation of...
Ccl5 expression, we mapped the nuclear IL-33 interactome onto a network of proteins associated with predicted Ccl5 transcription factors (Fig. 4G). This revealed that IL-33 interacts with transcription factors and transcriptional regulators that affect expression of Ccl5. Notably, three factors belonging to the TFIID complex were found to interact with nuclear IL-33, including TAF9, a protein that we found previously to form complexes with FAK (9). To explore the connection between FAK, IL-33, and Ccl5 transcription, we mapped the nuclear FAK interactome onto the set of Ccl5 regulatory proteins found to interact with nuclear IL-33 (Fig. 4G). This identified proteins...
in common between the nuclear FAK and nuclear IL-33 interactomes, suggesting that these proteins may be part of the same molecular complexes that regulate Ccl5 expression. Using streptavidin-coated beads, we isolated the IL-33 BioID fusion protein and confirmed that FAK and IL-33 exist in complex under steady-state conditions (Fig. 4H). Hence, we conclude that FAK binds to IL-33, and together, they complex with key Ccl5-regulatory transcription factors, to coregulate chemokine gene expression.
Besides TFIID, many of the IL-33–interacting partners converge on regulators of NF-κB, suggesting that NF-κB may be central to FAK and IL-33 regulation of chemokine expression. Notably, we showed that IL-33 interacts with the bromodomain protein BRD4 and the histone deacetylase HDAC1 (histone deacetylase 1; Fig. 4H). BRD4 is a member of the bromodomain and extraterminal domain (BET) family of transcriptional coactivators and elongation factors that recruit chromatin remodeling factors, including the SWI/SNF complex (30), to the promoters of genes via recognition of polyacetylated histone tails (31). Because IL-33 binds to both BRD4 and members of the Baf-type (SWI/SNF) complex, we hypothesize that IL-33 plays a role in formation of this complex at actively transcribing genes. It is known that BRD4 directly binds to acetylated p65 NF-κB, which leads to enhanced NF-κB transactivation activity (32), together suggesting a role for BRD4 in inflammatory transcriptional signaling. To support these conclusions, we treated FAK-WT SCC cells with the BET family inhibitor JQ1. This resulted in reduced Ccl5 expression (Fig. 4I), implying a role for BET family proteins in the regulation of chemokine expression. In contrast, HDAC1 has been shown to negatively regulate NF-κB transcriptional activity via a direct interaction with p65 (RelA) (33). We used the HDAC inhibitor vorinostat and identified a clear induction of Ccl5 expression upon HDAC inhibition (Fig. 4J). Collectively, our data support a model, whereby FAK binds to IL-33, which is a central component of a network of transcriptional regulators associated with the dynamic regulation of NF-κB–dependent chemokine transcription.

**DISCUSSION**

Nuclear FAK is emerging as an important regulator of gene expression in cancer cells, controlling transcriptional networks that influence multiple cellular functions. For example, FAK is reported to interact with the transcription factors p53 and GATA4, resulting in their inactivation with effects on cell survival (34). We have shown that nuclear FAK regulates expression of chemokines and cytokines, including Ccl5, likely via interactions with transcription factors and transcriptional regulators (9). Here, we show that both IL-33 and sST2 are transcriptionally regulated by nuclear FAK in a kinase-dependent manner and that FAK interacts with transcription factors and transcriptional regulators linked to control of expression of IL33 and ST2.

Depletion of IL-33 abundance in FAK-WT cells revealed that it is vital downstream of FAK in the regulation of chemokine expression, including that of Ccl5. We have previously identified a CCL5–CCR1/3/5 paracrine signaling axis between SCC FAK-WT cells and tumor-infiltrating T<sub>reg</sub> cells and have shown that CCL5 depletion results in FAK-WT tumor regression as a result of reduced tumor-infiltrating T<sub>reg</sub> cells (9). Our tumor growth studies here revealed that IL-33 depletion caused FAK-WT tumor regression, presumably because of IL-33–dependent regulation of Ccl5 and other chemokines. Thus, IL-33 regulates proinflammatory gene programs downstream of FAK that we have shown to play an important role in defining the tumor-immune environment, affecting SCC tumor growth and survival.

sST2 functions as a decoy receptor that is secreted into the tumor environment, leading to competitive inhibition of IL-33–ST2 autocrine and paracrine signaling (13). Although IL-33 is not secreted by SCC cells, it can be secreted by macrophages and neutrophils (35), influencing the function of immune cell populations. ST2L is also present on activated CD8<sup>+</sup> T cells and natural killer cells, and IL-33 stimulation can increase IFN-γ expression (14) and cytotoxic activity (18). We identified ST2L on several immune cell types in the SCC tumor environment, including activated CD8<sup>+</sup> T cells. Depletion of sST2 in FAK-WT tumors largely resulted in CD8<sup>+</sup> T cell–dependent tumor regression, implying that sST2 contributes to inhibition of CD8<sup>+</sup> T cell–mediated immunity.

**Fig. 5.** Nuclear FAK regulates IL-33/ST2 signaling to control the antitumor immune response. Model of the mechanism. Nuclear FAK regulates IL33 expression (“1”) through interaction with transcription factors (TFs) and transcriptional regulators (TRs). Nuclear FAK and IL-33 cooperate to drive expression of Ccl5 and sST2 (“2” and “3,” respectively) through interaction with transcription factors and transcriptional regulators. Ccl5 and sST2 are secreted from SCC cancer cells, promoting immune evasion. We have previously reported a CCL5–CCR1, 3, and 5 paracrine signaling axis between FAK-WT SCC cells and tumor-infiltrating regulatory T (T<sub>reg</sub>) cells that contributes to immune evasion. We propose that sST2 contributes to immune evasion through competitive inhibition of IL-33/ST2 signaling on cytotoxic CD8<sup>+</sup> T cells (“4”), resulting in tumor tolerance.
Studies using an ST2 knockout mouse have shown that host ST2 signaling is required for tumor regression to occur in response to overexpression of a secreted form of IL-33 (14). Therefore, we conclude that the presence of ST2 enables FAK-abundant tumors to benefit from nuclear IL-33 while countering the potential antitumor effects of secreted IL-33 (Fig. 5).

Nuclear IL-33 has been linked to regulation of transcription previously (16, 17), although the precise mechanisms underpinning this are unknown. We confirmed that nuclear IL-33 interacts with chromatin, supporting its role in transcriptional regulation, and BioID proteomics identified proximal interactions with components of the Baf-type (SWI/SNF), PTW/PP1 phosphatase, and TFIID complexes. This implies that identified proximal interactions with components of the Baf-type (SWI/SNF) promote transcriptional regulation, and BioID proteomics was absent from chromatin after depletion of IL-33 from FAK-WT cells, showing that IL-33 is required to stabilize the WDR82 chromatin complex. WDR82 is a component of the PTW/PP1 phosphatase complex and is involved in regulating chromatin structure (28). It is also a core component of the mammalian Set1A/Set1B histone H3K4 methyltransferase complex that is associated with regulating H3K4 trimethylation, a key step in transcriptional activation (36).

Finally, we identified BRD4 and HDAC1 positive (37) and negative (33) regulators of NF-xB activity, respectively, as previously unknown nuclear IL-33–interacting proteins. BRD4 is also required for recruitment of the SWI/SNF complex to active promoters (30), and we have shown that IL-33 binds to several components of this complex. Therefore, IL-33 likely binds to BRD4, HDAC1, and other chromatin modifiers to control the dynamic expression of NF-xB target genes, such as Cc7 and other chemokines. Collectively, our data suggest that IL-33 acts to regulate chromatin organization and FAK-dependent transcription, promoting a proinflammatory gene program that enables evasion of the antitumor immune response.

MATERIALS AND METHODS

Cell lines

Isolation and generation of the FAK SCC cell model is described in detail by Serrels et al. (12). Briefly, SCC cells were induced in K14CreER FAKfl/fl mice on an FVB background using the dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate two-stage skin chemical carcinogenesis protocol and cells isolated. After treatment with 4-hydroxytamoxifen, a FAK-null (FAK−/−) cell clone was isolated, and retroviral transduction was used to stably reexpress FAK-WT and FAK mutant proteins. Phoenix Ecotropic cells were transfected with pWZL (Hygro) FAK using Lipofectamine 2000 (Thermo Scientific) as per the manufacturer’s instructions. Twenty-four hours after transfection, cell culture supernatant was removed, filtered through a 0.45-μm Millex-HA filter (Millipore), diluted at a 1:1 ratio with normal SCC growth medium, supplemented with polybrene (Millipore) to a final concentration of 5 μg/ml, and added to SCC cells for 24 hours. Cells were subjected to two rounds of lentiviral infection before selection in puromycin (2 μg/ml).

shRNA-mediated ST2 and IL-33 knockdown

To generate lentiviral particles, 2 × 10⁶ human embryonic kidney 293FT cells were transfected with a mixture of 2 μg of shRNA [RMM4534-EG77125 (IL-33) and RMM4534-EG17082 (ST2/IL11R); GE Healthcare], 0.5 μg of MDG2, and 1 μg of PAX2 plasmid DNA using Lipofectamine 2000 (Thermo Fisher Scientific) as per the manufacturer’s guidelines. Forty-eight hours after transfection, medium was removed and filtered through a 0.45-μm Millex-AC filter (Millipore) and mixed at a 1:1 ratio with normal SCC growth medium, supplemented with polybrene (Millipore) to a final concentration of 5 μg/ml, and added to SCC cells for 24 hours. Cells were subjected to two rounds of lentiviral infection before selection in puromycin (2 μg/ml).

siRNA-mediated knockdown of SP1 and RUNX1

To knockdown SP1 and RUNX1, FAK-WT SCC cells were transfected in 10 nmol of nontargeting siRNA SMARTPool, SP1 siRNA SMARTPool, or Runx1 siRNA SMARTPool (all from Dharmacon, siGenome), or a combination of 10 nmol of SP1 and RUNX1 siRNA SMARTPool using HyPerFect Transfection Reagent (Qiagen) as per the manufacturer’s guidelines. Transfection was performed in serum-free medium for 24 hours, after which the medium was replaced with normal growth medium. Protein lysates were collected 48 hours after transfection.

IL-33 CRISPR/Cas9 construct generation

Guide sequence oligonucleotides (forward: TTTCTTGGCTTTATA-TATCTTTGTG GAAGACGAAACACCGTTCTAGA AGA TCCGTTGGAT and reverse: GACTAGCTTATTTAACTGC-TATTTTCTAGCTCTAAACATCCACGGGAATTCTAGGAC) including Eco RI restriction overhangs and a protospacer adjacent motif (PAM) sequence targeting exon 3 within IL33 were annealed using Phusion High-Fidelity Polymerase [New England Biolabs (NEB)] according to the manufacturer’s guidelines. Ten micromolar of each oligonucleotide was mixed with 25 mM MgCl₂, 2.5 mM deoxynucleotide triphosphate, 5X Phusion High-Fidelity Buffer, and Phusion Polymerase to a final volume of 50 μl. Cycling conditions were 98°C (30 s), 30 cycles of 98°C (10 s), 55°C (30 s), and 72°C (20 s), followed by a final incubation at 72°C (20 s). The final PCR product was analyzed by agarose gel electrophoresis. Annealed oligonucleotides were ligated into the gRNA_cloning vector (pCR-Blunt II-TOPO), a gift from G. Church (Addgene plasmid no. 41824). gRNA_cloning vector DNA (1.5 μg) was digested with 1.5 units of Afl II restriction enzyme (NEB) according to the manufacturer’s guidelines and incubated for 1 hour at 37°C. Four nanograms of annealed oligonucleotides was ligated with 30 ng of linearized gRNA_cloning vector using Gibson Assembly Master Mix (NEB) according to the manufacturer’s guidelines and incubated for 1 hour at 50°C. Constructs were transformed into DH5α chemically competent cells and selected on agar plates containing kanamycin (50 μg/ml). To identify positive colonies, DNA was prepared and digested with Eco RI restriction enzyme (NEB).

IL-33 CRISPR/Cas9 transfection and single-cell clone expansion

SCC-FAK-WT cells were seeded on a 100-mm tissue culture dish and grown until ~70% confluent. Cells were cotransfected with 3 μg of hCas9 and 3 μg of gRNA_cloning vector IL33–specific guides, using
Lipofectamine 2000 (Thermo Scientific) according to the manufacturer’s guidelines. IL-33 CRISPR knockout clones were isolated by dilution cloning; once cells reached confluence, they were trypsinized and resuspended in 10 ml of growth media. To ensure single-cell suspension, cells were passed through a cell strainer (Thermo Scientific), and 5 μl of the cell suspension was plated into a 15-cm plate. Several colonies were picked and expanded, and knockout of IL-33 was confirmed by quantitative PCR (qPCR) and Western blot. IL-33 primers used were forward: GGATCCGATTTTCGAGAGCTTAAACAT and reverse: GCGGCCGCATGAGACCTAGAATGAAGT. All primer sequences are detailed in table S2.

**Immunoblotting**

Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM tris-HCl at pH 7.4, 150 mM sodium chloride, 5 mM EGTA, 0.1% SDS, 1% NP-40, and 1% deoxycholate) supplemented with a protease and phosphatase inhibitor cocktail (mini Complete ULTRA Protease tablet and phosSTOP tablet from Roche). Lysates were clarified by high-speed centrifugation (16,000g for 15 min at 4°C). Protein concentration was measured using a Micro BCA Protein Assay (Thermo Scientific), and 10 to 20 μg of total protein were supplemented with 2X SDS sample buffer (tris (pH 6.8), 20% glycerol, 5% SDS, β-mercaptoethanol, and bromophenol blue) and boiled at 95°C for 5 min. Samples were separated by polyacrylamide gel electrophoresis using 4 to 15% Mini-PROTEAN TGX gels (Bio-Rad), proteins transferred to nitrocellulose, blocked [5% bovine serum albumin (BSA) in PBS–Tween 20 (BSA/PBS-T)], and probed with either anti-IL-33 (R&D Biosystems), anti-γ-caryaldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology), anti–phospho-FAK (Y397; Cell Signaling Technology), anti-FAK (Cell Signaling Technology), anti–WDR82 (Abcam), anti-TPAF9 (Abcam), anti-SMARC C1 (Abcam), anti-BRD4 (Abcam), anti-HP1 α/β (Cell Signaling Technology), anti-SP1 (Abcam), anti-Runx1 (Cell Signaling Technology), or anti-HDAC1 Abs (Cell Signaling Technology; all 1:1000 in 5% BSA/PBS-T). Bound Ab was detected by incubation with anti-rabbit, anti-mouse, or streptavidin-conjugated horseradish peroxidase secondary Ab (Cell Signaling Technology) and visualized using the Bio-Rad ChemiDoc MP Imaging System.

**Chromatin preparation**

Cells (1.5 × 10^6) were plated in 100-mm dishes and, after 24 hours, adhered to the tissue culture dish were washed two times in ice-cold PBS and lysed in RIPA lysis buffer as above. Protein concentration was measured using Micro BCA Protein Assay, and protein quantities were used for normalization of ELISA values.

**qRT-PCR and NanoString**

RNA was prepared from cells using the Qiagen RNeasy mini kit as per the manufacturer’s instructions, inclusive of deoxyribonuclease (DNase) digestion. Final concentration of RNA was measured using a NanoDrop (Thermo Scientific). For qRT-PCR, 5 μg of total RNA was converted to complementary DNA (cDNA) using the SuperScript II cDNA Synthesis Kit (Thermo Fisher Scientific). For qRT-PCR, 0.25 μl of 10 μM qPCR primers to a final reaction volume of 10 μl. IL-33 primers used were forward: GGATCCGATTTTCGAGAGCTTAAACAT and reverse: GCGGCCGCATGAGACCTAGAATGAAGT. ST2 primers were used forward: GCGGGAATGGAAGACTA and reverse: AAGCAAGCTGAAACGGAAT. Cd5 primers used were forward: CCCTCAACCATCATCCICTACT and reverse: CCCTCAACTGAGACGAACAGCA. qRT-PCR was performed on a StepOne Plus qRT-PCR instrument (Applied Biosystems). PCR conditions were as follows: 95°C (3 min), followed by 40 cycles of 95°C (5 s), 60°C (10 s), and 72°C (10 s). Melt curve analysis was performed after each qPCR reaction. Data were analyzed using the delta-delta cycle threshold (ddCT) method, and expression was calculated relative to GAPDH. For NanoString analysis, 150 ng of RNA was labeled with gene-specific bar codes as per the manufacturer’s instructions and quantified using a NanoString nCounter DX. NanoString analysis was carried out by the Newcastle NanoString nCounter analysis service. Analysis was performed using nSolver analysis software (NanoString).

**Generation of IL-33-BirA–expressing SCC cell line**

IL-33 cDNA was amplified by PCR using gene-specific primers (forward: GCGGCCGCAATGAGACACCCTAAATATTG and reverse: TGGTCCGATCCCATTTTCGAGAAGTTAAACAACGAAATATTG) and subcloned into the Not1/BamH1 sites of pcDNA3.1-IL-33 DNA template was mixed with 10 μM of each primer in PfuUltra Hotstart master mix (Stratagene) and subjected to PCR with cycle conditions as follows: 98°C (30 s; 30 cycles), 98°C (10 s), 60°C (30 s), and 72°C (1 min), followed by a final incubation at 72°C (10 min). After PCR, samples were gel-purified (Qiagen Gel Purification Kit) and eluted in 30 μl of nuclease-free water (NFW). The purified PCR product was incubated with 1 μl of BamH1 restriction enzyme, 1 μl of Not1 restriction enzyme, 5 μl of Buffer 3.1 (all from NEB) to a total of 20 μl, and incubated at 37°C.
for 2 hours. One microgram of pQCXIN-BirA-Myc was incubated with 1 μl of BamH1 restriction enzyme, 1 μl of NotI restriction enzyme, and 5 μl of Buffer 3.1 to a total of 20 μl and incubated at 37°C for 1 hour. After digestion, both the PCR product and digested vector were gel-purified and eluted in 30 μl of NFW. To ligate the digested IL-33 PCR product into the BamH1/NotI sites of pQCXIN-BirA-Myc, a 1:4 ratio of digested vector to PCR product was used and incubated with 0.5 μl of T4 DNA ligase (NEB), along with DNA ligase buffer in a final reaction volume of 10 μl, and incubated for 2 hours at room temperature (RT). DH5α chemically competent cells (50 μl) (Life Technologies) were transformed with 5 μl of ligation reaction according to the manufacturer’s guidelines and plated in agar plates containing ampicillin (100 μg/ml). Resulting colonies were checked for successful ligation using a diagnostic digest with BamH1 and NotI restriction enzymes and, if positive, sequenced. Phoenix Ecotropic cells were transfected with empty vector–BirA or IL-33–BirA using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer’s guidelines. Forty-eight hours after transfection, cell culture supernatant was removed, filtered through a 0.45-μm Millex-HA filter (Millipore), diluted at a 1:1 ratio in normal SCC cell culture medium, supplemented with polybrene (5 μg/ml), and added to SCC FAK-WT IL-33–CRISPR cells for 24 hours. A total of two rounds of infection were performed to generate each cell line before selection in G418 (400 μg/ml).

Proteomic analysis of the IL-33 interactome using BioID

Cells were incubated with 50 μM biotin (Sigma-Aldrich) in complete cell culture medium at 37°C for 24 hours; washed two times in ice-cold PBS, and lysed in RIPA lysis buffer as above. Cell lysates were sonicated using a Bioruptor (Diagenode) (30-s pulses with 30-s intervals over 5 min) and cleared by high-speed centrifugation (16,000g for 15 min at 4°C). Protein concentration was measured using Micro BCA Protein Assay (Thermo Scientific), and 1 mg of total cell lysate was incubated with 50 μl of streptavidin-C1 Dynabeads (Life Technologies) overnight at 4°C with agitation. Beads were washed using a magnetic tube rack three times with ice-cold RIPA buffer and two times with ice-cold PBS. Captured proteins (from experiments performed in biological triplicate) were subjected to on-bead proteolytic digestion, desalting, and liquid chromatography–tandem MS as described previously (38). Mean label-free MS intensities were calculated for each biological replicate. Peptide and protein false discovery rates were set to 1%. Proteins enriched from SCC-FAK-WT IL-33–CRISPR-IL-33–BirA cells by at least threefold when compared to SCC-FAK-WT IL-33–CRISPR-EV–BirA (P < 0.05) were considered specific. Proteomics analysis of nuclear FAK protein complexes is described in (9). All other protein interaction network analysis was performed using IPA (Qiagen). The IL-33 MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRoteomics IDEntifications (PRIDE) database repository (39) with the data set identifier PXD007698.

Interaction network analysis

Genes differentially expressed in FAK−/− SCC cells compared to FAK-WT SCC cells were extracted from microarray data (9) using rank product analysis. Significantly differentially expressed genes [P < 0.0005, percentage false-positives (ppf) < 0.05] were subjected to gene ontology enrichment analysis using DAVID Bioinformatics Resources (version 6.8) (40). Genes annotated with overrepresented top-level cellular component terms as determined by Benjamini-Hochberg–corrected hypergeometric test (P < 0.05) were used to seed a protein interaction network based on direct physical interactions constructed using the GeneMANIA plugin (version 3.4.1; mouse interactions) in Cytoscape (version 3.3.0) (41). The largest connected graph component was clustered using the Allegro Spring–Electric force–directed algorithm (Allegro Viva).

Hierarchical cluster analysis

Unsupervised agglomerative hierarchical clustering was performed on the basis of Euclidean distance or Pearson correlation computed with an average-linkage or complete-linkage matrix using R or Cluster 3.0 (C Clustering Library, version 1.50) (42). Clustering results were visualized using R or Java TreeView (version 1.1.6) (43) and MultiExperiment Viewer (version 4.8.1) (44).

Inhibitor treatment

All experiments involving animals were carried out in accordance with the UK Coordinating Committee on Cancer Research guidelines by approved protocol (Home Office Project License no. 60/4248). SCC cells (1 × 10^6; defined earlier) were injected into both flanks of FVB mice, and tumor growth was measured twice weekly using calipers. Animals were sacrificed by cervical dislocation when tumors reached maximum allowed size (1.5 cm in diameter) or when signs of ulceration were evident. Group sizes ranged from three to five mice, each bearing two tumors, and tumor volume was calculated in Excel (Microsoft) using the formula π/6 * x^3. Statistics and graphs were calculated using Prism (GraphPad).

CD8+ T cell depletion

Anti-mouse CD8 (clone 53-6.7) and appropriate isotype control Abs (rat immunoglobulin G 2a) were purchased from BioXcel. T cell depletion was achieved after intraperitoneal injection of 150 μg of depleting Ab (same for all Abs) into female age-matched FVB mice for three consecutive days and maintained by further intraperitoneal injection at 7-day intervals until the study was terminated. SCC FAK-WT (1 × 10^6) or FAK−/− cells were injected subcutaneously into both flanks 6 days after initial Ab treatment, and tumor growth was measured twice weekly as described above.

Fluorescence-activated cell sorting analysis of immune cell populations

Tumors established after injection of 1 × 10^6 SCC cells into both flanks of FVB mice were removed at day 12 into RPMI (Sigma-Aldrich) supplemented with 10% FBS (Life Technologies). Tumor tissue was mashed into a pulp using a scalpel and resuspended in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with collagenase D (2 mg/ml) and DNase1 (200 U/ml; Roche). Samples were incubated for 30 min at 37°C with agitation, pelleted by centrifugation at 1600 rpm for 5 min at 4°C, resuspended in 5 ml of red blood cell lysis buffer (Pharm Lysing Buffer; Becton Dickinson) for 10 min at 37°C, pelleted by centrifugation at 1600 rpm for 5 min at 4°C, resuspended in PBS, and mashed through a 70-μm cell strainer. The resulting single-cell suspension pellet was pelleted by centrifugation at 1600 rpm for 5 min at 4°C and resuspended in PBS. This step was repeated a further time, and the
resulting cell pellets were resuspended in 100 μl of PBS containing e506 fixable viability dye (1:1000 dilution) and transferred into the well of a round-bottom 96-well plate. Samples were incubated at 4°C for 30 min. Cells were then pelleted by centrifugation at 1600 rpm for 5 min at 4°C and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% FBS and 0.1% sodium azide). This step was repeated for a total of three times. Cell pellets were resuspended in 100 μl of Fc block [1:200 dilution of Fc Ab (eBioscience) in FACS buffer] and incubated for 15 min at RT. One hundred microliters of Ab mixture [1:200 dilution of Abs except anti-FoxP3, which was used at 1:100 (listed in fig. S3) in FACS buffer] was added to each well, and the samples were incubated for 30 min in the dark at 4°C. The plate was then centrifuged at 1600 rpm for 5 min at 4°C, and the cells were resuspended in FACS buffer. This step was repeated three times. Samples were analyzed using a BD Fortessa FACS Analyser. Data analysis was performed using FlowJo software. All Abs were from eBioscience. Statistics and graphs were calculated using Prism (GraphPad).

SUPPLEMENTARY MATERIALS

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Fig. S1. Identification of common upstream regulators of IL33 in mammalian cell lines using CRISPR-Cas9 screening.

Table S1. Primer sequences.

REFERENCES AND NOTES

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IL-33 and ST2 mediate FAK-dependent antitumor immune evasion through transcriptional networks

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FAK directs tumor immune evasion
Tumors are adept at escaping the immune system's surveillance or suppressing its activity. The kinase FAK is implicated in immune escape mechanisms. Serrels et al. found that FAK activates a transcriptional network that induces —and is then further mediated by—the nuclear abundance of interleukin-33 (IL-33) in tumor cells. In a mouse model of squamous cell carcinoma, FAK–IL-33 complexes boosted the production and secretion of two key factors in immunosuppression: the chemokine CCL5, which stimulates immunosuppressive regulatory T cells, and soluble ST2, a decoy receptor for cytotoxic T cell–stimulatory IL-33. Blocking these FAK-mediated signals may help the patient's immune system find and kill tumors.

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