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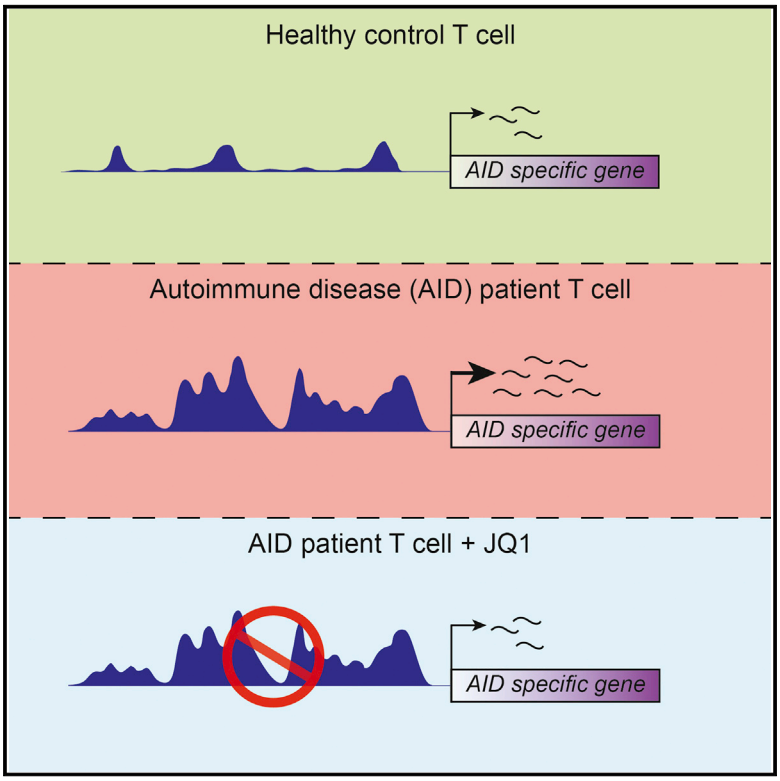
Report

Inhibition of Super-Enhancer Activity in Autoinflammatory Site-Derived T Cells Reduces Disease-Associated Gene Expression

Graphical Abstract



Highlights

- Identification of a disease-specific, inflammation-associated, enhancer signature
- Inflammatory-site-derived cells are epigenetically different from peripheral cells
- Enrichment of disease-related SNPs in disease-associated super-enhancers
- BET inhibition of JIA patient T cells preferentially reduced JIA gene expression

Authors

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In Brief

By defining the active super-enhancer profile of autoimmune disease patients, Peeters et al. identify a disease-specific, inflammation-associated super-enhancer signature. In addition, inhibition of super-enhancer activity, using a BET inhibitor, in autoimmune disease patient-derived cells preferentially reduced disease-associated gene expression. These findings suggest a role for enhancers and super-enhancers in autoimmune diseases and demonstrates the potential use of BET inhibitors for the treatment of such diseases.

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Report

Inhibition of Super-Enhancer Activity in Autoinflammatory Site-Derived T Cells Reduces Disease-Associated Gene Expression

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SUMMARY

The underlying molecular mechanisms for many autoimmune diseases are poorly understood. Juvenile idiopathic arthritis (JIA) is an exceptionally well-suited model for studying autoimmune diseases due to its early onset and the possibility to analyze cells derived from the site of inflammation. Epigenetic profiling, utilizing primary JIA patient-derived cells, can contribute to the understanding of autoimmune diseases. With H3K27ac chromatin immunoprecipitation, we identified a disease-specific, inflammation-associated, typical enhancer and super-enhancer signature in JIA patient synovial-fluid-derived CD4$^+$ memory/effector T cells. RNA sequencing of autoinflammatory site-derived patient T cells revealed that BET inhibition, utilizing JQ1, inhibited immune-related super-enhancers and preferentially reduced disease-associated gene expression, including cytokine-related processes. Altogether, these results demonstrate the potential use of enhancer profiling to identify disease mediators and provide evidence for BET inhibition as a possible therapeutic approach for the treatment of autoimmune diseases.

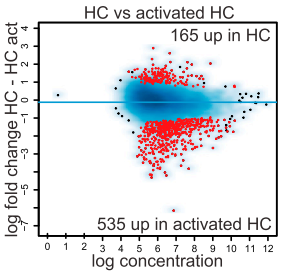
INTRODUCTION

Autoimmune diseases are a heterogeneous group of diseases characterized by loss of immunological tolerance of which the etiology is still largely unknown. Both genome-wide association studies (GWAS) and studies utilizing monozygotic twins have identified genetic susceptibility, but these associations often only apply to a small subset of patients, indicating a role for unknown environmental triggers and suggesting that epigenetic changes might be involved (Meda et al., 2011). Epigenetic regulation of enhancer regions is crucial for regulating cell identity and function. Enhancers are *cis*-regulatory elements in the DNA, typically a few hundred base pairs in size, to which transcription factors and co-factors can bind and control transcription. Active enhancers can be identified based on histone H3 lysine 27 acetylation (H3K27ac) (Creyghton et al., 2010). Very recently, a number of studies have identified extremely large enhancer domains, spanning up to 50 kb, termed super-enhancers (SEs) or stretched enhancers (Hnisz et al., 2013; Parker et al., 2013; Whyte et al., 2013). These SEs were demonstrated to specifically regulate genes associated with cell identity and disease, including oncogenes (Lovén et al., 2013; Whyte et al., 2013). Importantly, BET (bromodomain and extra-terminal domain) inhibitors were demonstrated to impair SE activity and thereby preferentially reduce SE-associated gene expression (Filippakopoulos et al., 2010; Lovén et al., 2013). Interestingly, BET inhibitors, such as JQ1, have been demonstrated to inhibit tumor growth both in vitro and in vivo and are currently tested in several phase 1/2 clinical trials (Delmore et al., 2011; Zuber et al., 2011; https://www.clinicaltrials.gov/).

Various disease-associated SNPs were found to be enriched in enhancers and SEs (Hnisz et al., 2013; Parker et al., 2013). Recently, it was demonstrated that candidate causal variants for 21 autoimmune diseases preferentially mapped to the enhancer profile of several immune cells (Farh et al., 2015). Importantly, these correlation studies were performed using both chromatin immunoprecipitation sequencing (ChIP-seq) and DNase hypersensitivity data from healthy controls (HCs). Comparing the super-enhancer profiles in primary cells derived from patients to HCs, which has not been performed so far, could be even more informative for disease pathogenesis and might reveal potential biomarkers or therapeutic targets.

Juvenile idiopathic arthritis (JIA) is a generic term to describe all types of chronic arthritis with an unknown cause that have their onset before the age of 16 years. JIA is a multifactorial autoimmune disease associated with the accumulation of various immune cells, including activated CD4$^+$ memory/effector T (Tmem/eff) cells, in the joint synovial fluid (SF) (Prakken et al., 2011). The early onset of this disease and the possibility to obtain cells

A



B

Selected genes associated with increased H3K27ac signal in activated HC

Chemokine (receptors)	Interleukin (receptors)	CD markers	Associated with T cell activation
CCR3	*IL10RA*	*CD2*	*ITK*
CCR4	*IL1R1*	*CD247*	*STAT1*
CCR7	*IL2RA*	*CD28*	*STAT3*
	IL4R	*CD40LG*	*IRF4*
	IL6R	*CD82*	*TNFSF14*

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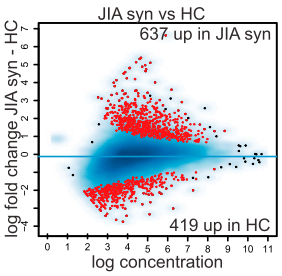


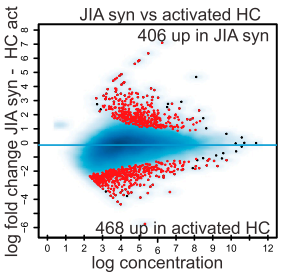
E

Selected genes associated with increased H3K27ac signal in JIA syn

Chemokine (receptors)	Interleukin (receptors)	CD markers	Associated with T cell activation
CCL3	*IL10RA*	*CD2*	*CTNNB1*
CCR1	*IL17REL*	*CD27*	*KLF6*
CCR4		*CD58*	*ITGB1*
CXCR3		*CD74*	*IRF4*
CXCR4		*CD80*	*TNFRF4*

F

G



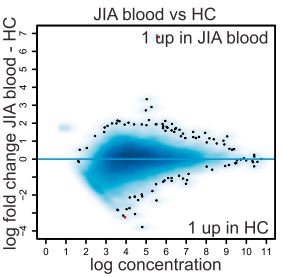
H

Selected genes associated with increased H3K27ac signal in JIA syn

Chemokine (receptors)	Interleukin (receptors)	CD markers	Associated with T cell activation
CCR1	*IL10RA*	*CD2*	*CTLA4*
CCR4	*IL17C*	*CD47*	*SSH*
CXCR6	*IL17REL*	*CD74*	*TNFRSF13B*
		CD80	

I

J



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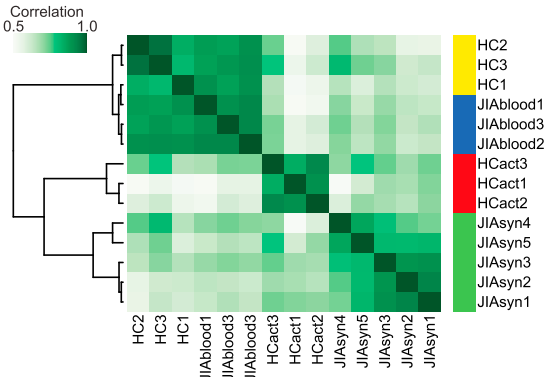

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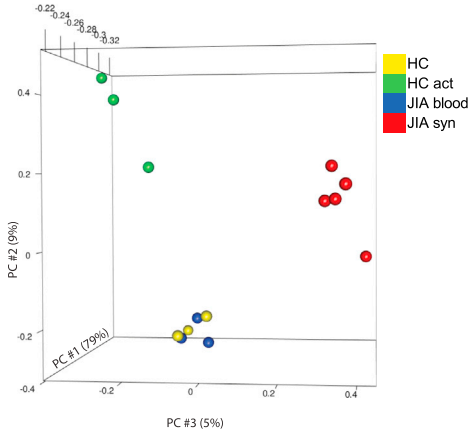

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directly from the site of inflammation, i.e., the joints, make JIA exceptionally well suited as a model for studying autoimmune diseases in humans.

In this study, we identified a set of JIA-associated active enhancers and SEs in primary Tmem/eff cells. To assess the regulatory function of these active enhancers and super-enhancers, RNA sequencing was performed to identify genes that are differentially expressed in patient cells derived from the autoinflammatory site. Inhibition of typical enhancer and super-enhancer activity preferentially inhibited disease-specific gene expression, implicating a role for typical enhancer and super-enhancer activity in disease pathogenesis. Since the first clinical trials utilizing inhibitors of typical enhancer and super-enhancer activity in cancer treatment have already started, this study might pave the way for BET inhibition as a possible treatment for autoimmune diseases.

RESULTS

Enhancer Profiling in Memory/Effector T Cells Reveals JIA-Associated Enhancers

We aimed to define the active enhancer profile in primary $CD4^+$ T cells from autoimmune disease patients, for which we used oligoarticular JIA as a model. $CD4^+$ T cells are an important contributor to JIA pathogenesis and can be divided into naive T cells ($CD4^+CD45RA^+CD45RO^-$) and Tmem/eff cells ($CD4^+CD45RA^-CD45RO^+$) (Prakken et al., 2011). Of these subtypes, the Tmem/eff cells are involved in inflammation and proinflammatory cytokine production and are therefore the most relevant for disease pathogenesis. We indeed observed that specifically in the inflamed joints of JIA patients T cells were almost totally composed of Tmem/eff cells (Figure S1A). Enhancer profiling of naive T cells and Tmem/eff cells, using publically available H3K27ac ChIP-seq data (Schmidl et al., 2014), revealed that the H3K27ac signal of ~2,000 enhancers was significantly different between naive and Tmem/eff cells, which associated with genes that regulate T cell activation (Figures S1B–S1F). SE profiling demonstrated that most SEs were shared between both subtypes, although various SEs were found to be specific for Tmem/eff cells and correlated with leukocyte activation (Figures S1G–S1K). Since naive and Tmem/eff cells are epigenetically different, we focused our further analysis solely on sorted $CD4^+$ Tmem/eff cells.

We aimed to determine whether the active enhancer profile of JIA patients is aberrant compared to HCs. To define active enhancers in primary Tmem/eff cells, H3K27ac ChIP sequencing was performed on sorted $CD4^+CD45RO^+$ T cells derived from the PB of HCs, either in vitro activated or not, to define general T cell activation-associated enhancers, and $CD4^+CD45RO^+$ T cells derived from the PB or SF from JIA patients (Figure S2A). Comparing the active enhancer profile of HC cells with that of in-vitro-activated HC cells revealed 700 enhancers with significant differential enrichment for H3K27ac (Figures 1A and S2B). Genes associated with enhancers with a significantly higher H3K27ac signal in activated HC cells indeed correlated with T cell activation (Figures 1B and 1C). More than 1,000 enhancers significantly differed in H3K27ac signal between HC cells and JIA SF-derived cells, of which the majority was increased in JIA patients (Figures 1D and S2C). Genes associated with these enhancers comprised many CD markers, chemokine and interleukin receptors, and genes associated with lymphocyte activation (Figures 1E and 1F). The H3K27ac signal of over 800 enhancers differed between HC cells activated in vitro and SF-derived JIA patient cells, indicating that there are many JIA-specific changes and that JIA is not merely the cause of enhanced T cell activation (Figures 1G–1I and S2D). Remarkably, when PB-derived JIA patient samples were compared to HC cells only two significant differences were detected, indicating that the oligoarticular JIA-specific signature is localized in the joints and that the Tmem/eff cells in the blood are relatively similar to HCs (Figure 1J).

Although clustering analysis did separate all four groups, principle component analysis (PCA) demonstrated that cells from the blood of HCs and patients cluster together, while Tmem/eff cells from patient SF and activated HCs clustered separately (Figures 1K and 1L). The raw data were independently validated utilizing different analysis methods, in which a large degree of overlap in the differentially expressed enhancers was found. Both clustering and the PCA using RPKM-based and *Z*-score-based analysis methods showed similar results, indicating the robustness of the data (Figures S3A–S3L). In conclusion, these data defined an active enhancer signature in JIA patient-derived synovial Tmem/eff cells, thereby revealing enhancers that are specifically associated with the joint inflammation in JIA.

JIA SE Signature Correlates with the Disease

Since SEs in HCs have been demonstrated to be enriched for SNPs associated with autoimmune diseases, we next aimed to define the SE profile in primary Tmem/eff cells from JIA patients (Farh et al., 2015; Vahedi et al., 2015). SEs in both patient and HC cells were identified based on our H3K27ac ChIP-seq data from primary $CD4^+$ Tmem/eff cells (Figure 2A). As expected, the average size and H3K27ac signal of SEs were increased compared to that of typical enhancers (Figure S4A). Various HC and JIA-specific SEs could also be identified (Figures 2B and S4B). GO-terms related to SE-associated genes exclusively present in HCs or SE-associated genes shared between HC and JIA linked to (negative) regulation of cell activation and general homeostatic processes. In contrast, SEs exclusively present

Figure 1. Identification of JIA-Associated Enhancers

(A, D, G, and J) MA plots of H3K27ac signal in enhancers, based on comparisons of all replicates within the depicted groups. Red dots indicate enhancers with a FDR <0.05.

(B, E, and H) Selected enhancer-associated genes that are up in activated HC cells and JIA SF-derived cells, respectively.

(C, F, and I) Gene ontology terms, ranked by enrichment scores.

(K) Clustering analysis based on H3K27ac signal in enhancers.

(L) Principle component analysis based on H3K27ac signal in enhancers.

See also Figure S1, S2, and S3.

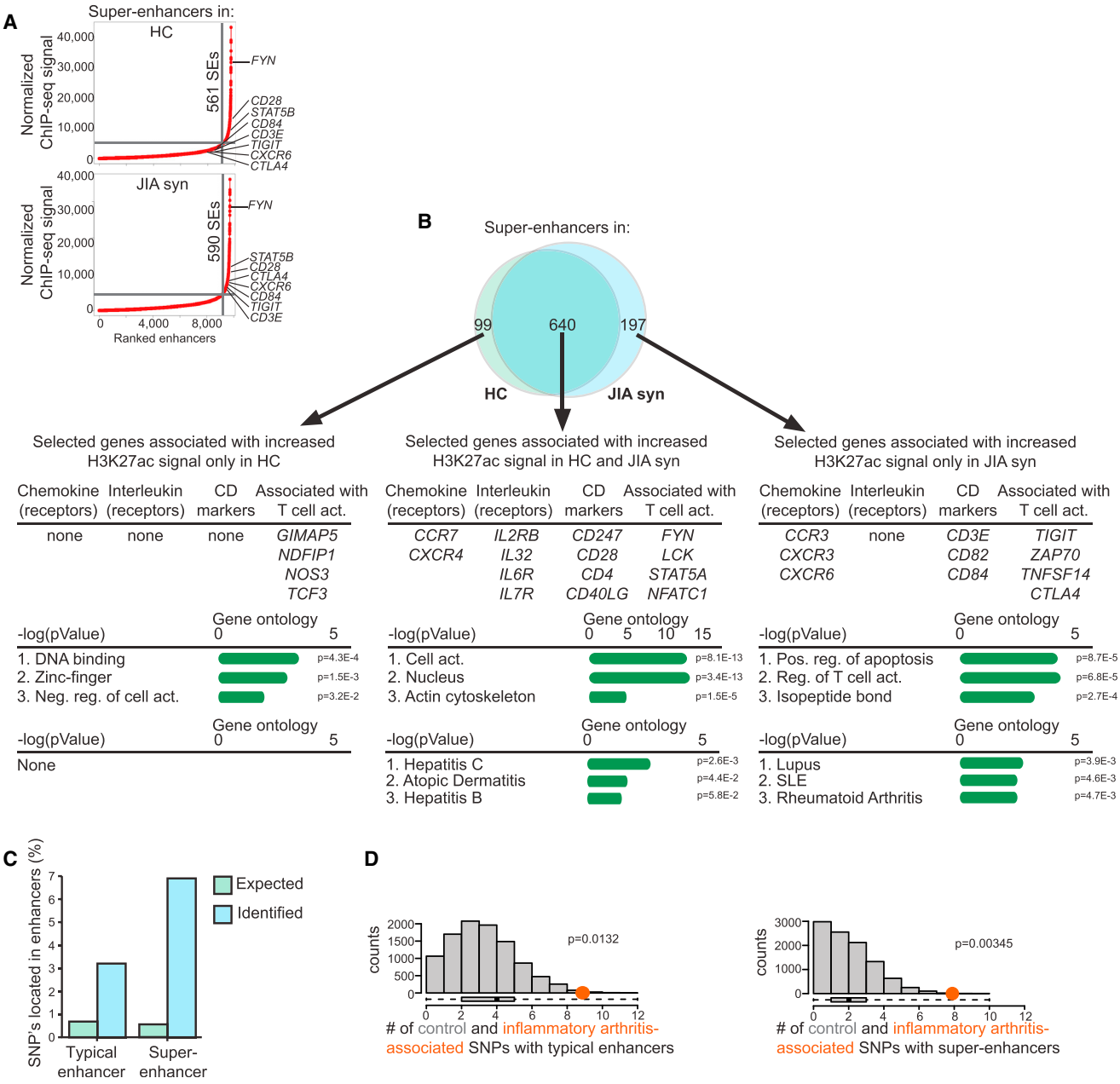

Figure 2. Identification of JIA-Associated SEs

(A) Normalized distribution of H3K27ac. Representative examples are depicted.

(B) Venn diagram of all SEs identified in CD4$^+$CD45RO$^+$ T cells from HC PB (three samples) and JIA synovium (five samples) and their associated genes and gene ontology for biological processes (ranked on enrichment score) and diseases (ranked on p value).

(C) Percentage of expected (based on size) and identified JIA/RA-associated SNPs located in typical enhancers and SEs of JIA patients.

(D) Number of inflammatory arthritis-associated SNPs (red dot) overlapping with the regulatory regions identified in patient cells compared with 10,000 random SNP sets (gray bars). p values were calculated with binominal cumulative distribution function. See also Figure S4.

in JIA patients strongly correlated with regulation of apoptosis and T cell activation, indicating that SE-driven gene expression might contribute to JIA pathogenesis. Correspondingly, JIA-associated SEs correlated with various autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis, while this is not the case for SE-associated genes shared between HC and JIA (Figure 2B). To further assess the relevance of JIA SEs, we assessed the enrichment of inflammatory arthritis-associated SNPs in JIA typical enhancers and super-enhancers (Table S1). As demonstrated in Figures 2C and 2D, SNPs were enriched in both typical enhancers and SEs, in the latter case to the highest extent. Importantly, enrichment of

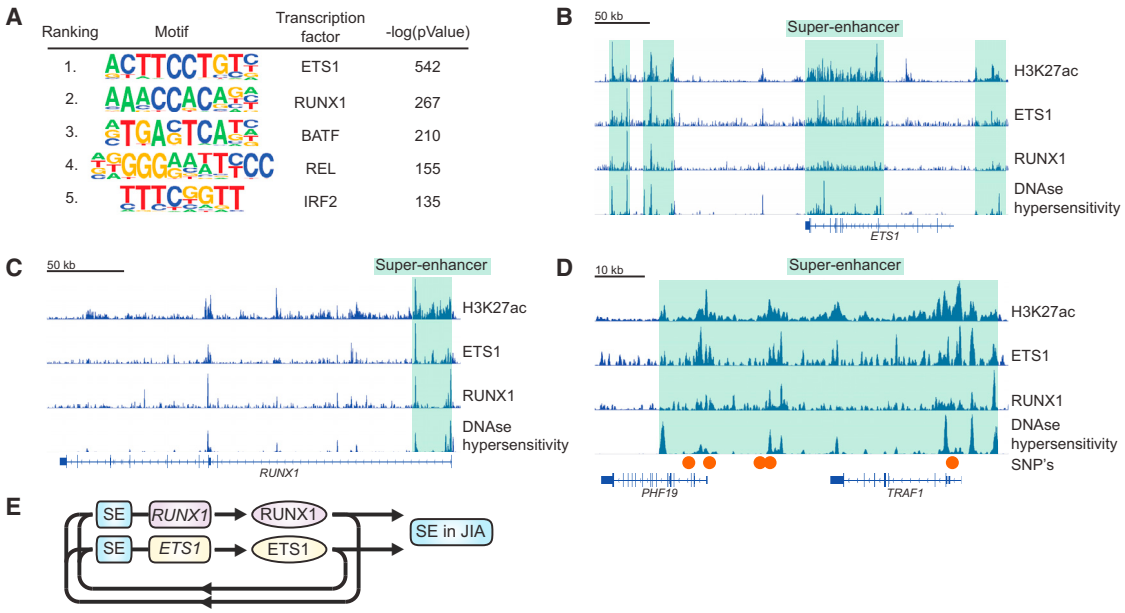

Figure 3. ETS1 and RUNX1 Binding Motifs Are Enriched in JIA-Associated SEs

(A) Transcription factor binding motifs enriched in JIA SEs.

(B and C) Gene track for *ETS1* and *RUNX1* displaying ChIP-seq signals for H3K27ac, ETS, RUNX1, and DNase hypersensitivity sites. Each light-green-shaded area represents an individual SE.

(D) Gene tracks for *PHF19* and *TRAF1* showing ChIP-seq signals for H3K27ac, ETS1, RUNX1, and DNase hypersensitivity sites and JIA/RA-associated SNPs located in these gene regions.

(E) Schematic model illustrating the role of ETS1 and RUNX1 in SE-driven gene expression in JIA.

these SNPs was significantly increased in JIA SEs compared to HCs, supporting the relevance of SEs for disease pathogenesis and the relevance to utilize primary patient cells for these analyses (Figure S4C). To investigate whether the SE profile of JIA patient-derived Tmem/eff cells can be extrapolated to other autoimmune diseases, we assessed whether SNPs associated with various diseases are preferentially located in JIA-associated SEs. We observed that SNPs associated with autoimmune diseases, such as SLE and IBD, were significantly enriched in JIA-associated SEs, while this is not the case for non-autoimmune disease-related SNPs (Figure S4D). To assess this in a more direct manner, we compared our H3K27ac data with published data derived from T cells from asthma patients for which the disease etiology is dissimilar, although partly mediated by T cells (Seumois et al., 2014). Analysis of all H3K27ac peaks associated with the 111 genes that are associated with asthma revealed that in our data for only 31 genes H3K27ac peaks could be identified. Quantitative analysis of the 137 peaks, which associated with these 31 genes revealed that only seven peaks were significantly different between JIA patients and HC (Figure S3E). Collectively, this suggests that the epigenetic profile of JIA patient-derived T cells is distinguishable from the epigenetic profile of another T cell-mediated non-autoimmune diseases.

Enrichment of ETS1 and RUNX1 Binding Motifs in JIA-Associated SEs

In order to identify transcription factors that play a potential role in JIA pathogenesis, the enrichment of transcription factor DNA-binding motifs was assessed in JIA SEs. Motif-enrichment analysis revealed the highest enrichment of ETS1 and RUNX1 binding motifs (Figure 3A). Interestingly, *ETS1* and *RUNX1* themselves were found to be SE-associated genes in JIA (Figures 3B and 3C). ChIP-seq analysis revealed that in $CD4^+$ Tmem/eff cells ETS1 and RUNX1 also bind these SEs at DNase hypersensitivity sites in these regions (Bernstein et al., 2010). Furthermore, several other SEs in JIA were occupied by ETS1 and RUNX1, which also contained SNPs associated with JIA/RA, as illustrated in Figure 3D. Therefore, ETS1 and RUNX1 seem to function in a regulatory feedback loop, not only by regulating their own gene expression, but also by controlling various JIA SEs (Figure 3E).

BET Inhibition Preferentially Inhibits JIA-Specific Gene Expression

To assess the regulatory function of typical enhancers and super-enhancers, RNA expression of primary $CD4^+$ Tmem/eff cells derived from the SF of JIA patients was compared to cells derived from the PB of HCs. More than 900 genes were differentially expressed between both groups, of which most were increased in JIA (Figures 4A and 4B and Table S2). Correspondingly to our super-enhancer analysis, these genes comprised chemokine and interleukin receptors, CD markers, and genes associated with T cell activation and defense responses (Figures 4C and 4D). In addition, genes associated with a JIA-specific SE are more abundantly expressed in JIA compared to HC, and genes associated with a SE are higher expressed in JIA than genes associated with a single typical enhancer (Figures S5A and S5B). Since it was demonstrated that SE activity (and typical enhancer activity to a lesser extent) can be repressed with BET inhibitors, we

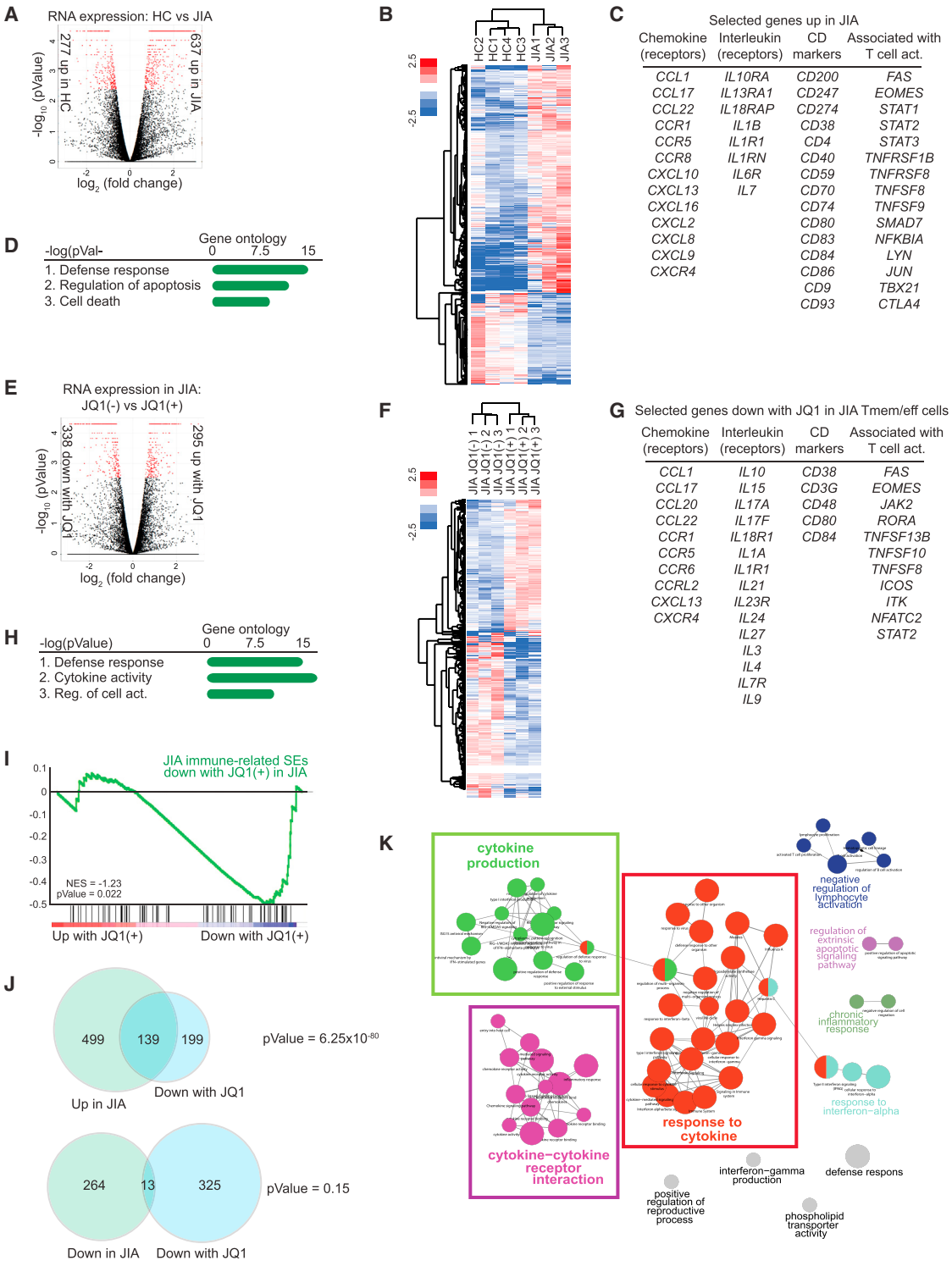

Figure 4. BET Inhibition Preferentially Inhibits JIA-Associated Gene Expression

(A) Volcano plot of genes differentially expressed between HC PB-derived and JIA patient SF-derived $CD4^{+}CD45RO^{+}$ T cells. Red dots indicate genes with a FDR <0.05.

(B) Heatmap of genes significantly different in expression between HCs and JIA patients.

(C) Selected genes that are up in JIA patients.

(D) Gene ontology terms related to genes that are up in JIA patients.

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hypothesized that BET inhibition could reduce expression of JIA-associated genes in JIA patient T cells (Lovén et al., 2013). JIA patient-derived $CD4^+$ Tmem/eff cells were treated with the BET inhibitor JQ1(+) or an inactive control JQ1(–), and RNA sequencing was performed (Figures 4E and 4F; Table S3). The expression of both SE- and typical enhancer-associated genes was reduced by JQ1 treatment (Figure S5C). Similarly as the genes upregulated in JIA, genes downregulated by JQ1(+) treatment in JIA-derived cells correlated with a "defense response" (Figures 4G and 4H and S5D). This cellular process was not affected in HC cells that were treated with JQ1(+) (data not shown). Genes associated with immune-related JIA SEs were significantly downregulated upon JQ1(+) treatment in JIA patients, while this was not the case in HCs (Figures 4I and S5E). Importantly, genes that were upregulated in JIA were significantly downregulated by JQ1(+) treatment, while the expression of genes that were downregulated in JIA was not significantly affected by JQ1(+) treatment (Figure 4J). Genes that were upregulated in JIA and downregulated by JQ1(+) mainly associated with cytokine production and cytokine responses (Figure 4K). Indeed, cytokine production in patient Tmem/eff cells was decreased upon JQ1(+) treatment (Figures S5F–S5H). Taken together, these data demonstrate that BET inhibition in JIA patient cells can preferentially inhibit JIA-specific gene expression, resulting in a downregulation of proinflammatory markers.

CXCR4 Is Associated with JIA and Its Expression Is Inhibited by BET Inhibitors

Since cytokine (receptors) were predominantly present in JIA-associated genes that were affected by BET inhibition, we analyzed all cytokine/cytokine receptors that were upregulated in JIA and/or downregulated with JQ1(+) together with their known receptor/ligand (Figure 5A). CXCR4 was the only cytokine receptor that was upregulated in JIA, inhibited by JQ1(+) treatment, and associated with both a SE in JIA and an enhancer with significantly different H3K27ac occupancy in JIA patients compared to HCs (Figures 5B–5E). Although *CXCR4* is also associated with a SE in HCs, JQ1(+) treatment of HC cells did not significantly decrease *CXCR4* expression, which might be related to the observed difference in gene expression between HC and JIA patients (data not shown). In addition, a JIA-associated SNP is located in the SE of *CXCR4* (Figure 5F) (Finkel et al., 2013). Additionally, expression of the ligand for CXCR4, stromal-derived factor 1 (SDF-1), was significantly increased in the SF of JIA patients compared to peripheral blood plasma (Figure 5G). Collectively, these data suggest that CXCR4 is involved in JIA pathogenesis and validate the relevance of targets identified by our approach.

DISCUSSION

Recent studies have indicated that enhancer and SE landscapes can mark cell-type- and disease-specific genomic regions, providing insight into the mechanisms of various diseases (Hnisz et al., 2013; Lovén et al., 2013; Mansour et al., 2014; Whyte et al., 2013). Enhancer regions can be targeted through the use of BET inhibitors, enabling the therapeutic treatment of aberrant enhancer and SE regions (Filippakopoulos et al., 2010; Lovén et al., 2013; Zuber et al., 2011). Until now, identification and targeting of disease-specific enhancers has largely been restricted to the field of tumor biology and only slightly been extended to autoimmune diseases (Delmore et al., 2011; Zuber et al., 2011).

Based on genetic and epigenetic mapping of HCs, it has been shown that, for a number of autoimmune diseases, the majority of causal variants map to immune cell enhancers (Farh et al., 2015). Correspondingly, using primary patient cells we found a profound enrichment of disease-related SNPs in enhancers and SEs, illustrating the importance of these non-coding genomic regions for disease pathogenesis. In addition, we demonstrated that epigenome analysis in primary patient material is more informative than epigenome analysis in HCs for such SNPs enrichment analyses. It must be noted that PB-derived HC cells activated in vitro may not be the best control for JIA SF-derived cells; however, obtaining enough cells from the SF of HCs for such analyses is not feasible. The observed paradigm does not solely apply to JIA, as recently it has been demonstrated for asthma that typical enhancers are associated with disease and are specific for certain T helper cell types (Seumois et al., 2014). Although Tmem/eff cells are considered to be one of the major contributors to JIA, other immune cells, such as monocytes, also play a role in this disease. Hence, profiling of additional immune cell subsets could provide a broader understanding of JIA pathogenesis.

It remains to be determined whether aberrant enhancer profiles are causally related to disease pathogenesis or merely the consequence of the local proinflammatory environment. It has recently been demonstrated that activation of NF-κB by the proinflammatory cytokine TNF-α results in an altered enhancer landscape, partially by SE formation (Brown et al., 2014). Interestingly, our data demonstrate that JIA-associated SEs are highly enriched for ETS and RUNX1 binding motifs, suggesting that similarly to NF-κB these transcription factors might be involved in altering the enhancer landscape in JIA. ETS and RUNX1 can be activated by proinflammatory signals, such as TNF-α, IL-1, and TGF-β, which are among the cytokines present in the SF (de Jager et al., 2005; Klunker et al., 2009; Redlich et al.,

(E) Volcano plot of genes in JIA patient synovium-derived $CD4^+CD45RO^+$ T cells differentially expressed upon JQ1(+) treatment. Red dots indicate genes with a FDR <0.05.
(F) Heatmap of genes significantly different in expression between JQ1(–)- and JQ1(+)-treated JIA patient cells.
(G) Selected set of downregulated genes as a result of JQ1(+) treatment.
(H) Gene ontology terms related to genes that were down upon JQ1(+) treatment, ranked by enrichment scores.
(I) Gene set enrichment analysis for immune-related JIA SE-associated genes affected by JQ1 treatment in JIA patient-derived cells. p value was determined using the family-wise error rate procedure.
(J) Overlap between genes downregulated upon JQ1(+) treatment and genes either up- or downregulated in JIA patients compared to HCs.
(K) Functionally grouped network of genes upregulated in JIA compared to HC, and downregulated with JQ1(+).
See also Figure S5.

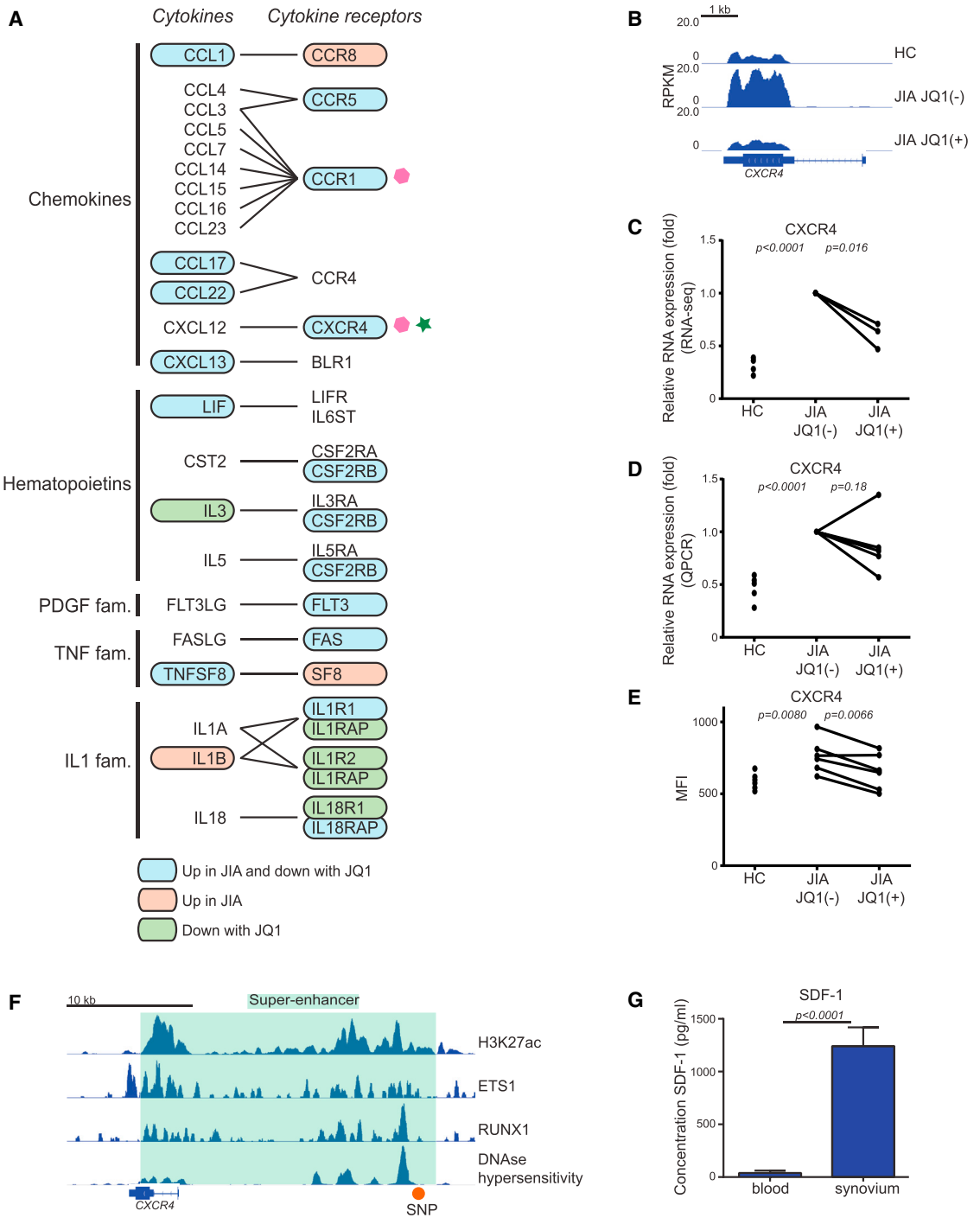

Figure 5. CXCR4 Is Associated with JIA and Its Expression Is Inhibited by BET Inhibitors

(A) Overview of cytokines and cytokine receptors up in JIA and/or down with JQ1(+). Genes associated with an enhancer with significantly different H3K27ac occupancy (pink polygon) and SE-associated genes (green star) are depicted.

(B) RNA sequencing gene track for *CXCR4* for HC or JIA patient-derived $CD4^+$ Tmem/eff cells treated with JQ1(–) or JQ1(+).

(C and D) Relative RNA expression of CXCR4 in $CD4^+$ Tmem/eff cells treated with JQ1(–) or JQ1(+); data were obtained by RNA sequencing (C) or qPCR (D).

(E) Mean fluorescent intensity of CXCR4 in $CD4^+$ Tmem/eff cells treated with JQ1(–) or JQ1(+).

(F) Gene track for *CXCR4* displaying ChIP-seq signals for H3K27Ac, ETS1, RUNX1, and DNase hypersensitivity sites. SNP in *CXCR4*-associated SE is indicated.

(G) Concentration (pg/ml) SDF-1 in the PB serum and SF of JIA patients. p values were determined using an unpaired or paired Student's t test.

2001). Furthermore, ETS and RUNX1 also bind SEs associated with various proinflammatory cytokines. This suggest that SE-mediated regulatory feedback loops may exist, maintaining the expression of proinflammatory mediators in autoimmune diseases. Thus, alterations in the enhancer profile could both be a cause and a consequence of disease pathogenesis.

In order to effectively target disease-associated processes in autoimmune diseases, it is critical to break these regulatory feedback loops. This could be achieved by dual targeting of the proinflammatory environment in combination with specific inhibition of enhancer activity, through BET inhibition (Filippakopoulos et al., 2010; Lovén et al., 2013). We demonstrated that BET inhibition in primary JIA patient cells resulted in the altered expression of 632 genes. Although JQ1 is described to preferentially act on SEs, we observed that JQ1 treatment affected typical enhancers and SEs in a comparable fashion. The reason for this might be that we treated JIA patient cells for 16 hr with JQ1, while others observed a preferential effect on SE activity after 6 hr (Lovén et al., 2013). This prolonged treatment possibly also results in secondary effects, which correlates with our observation that the expression of various genes is increased after JQ1 treatment. In addition, it has been reported that the BET inhibitor I-BET preferentially acts on de novo enhancers (Nicodeme et al., 2010). As proinflammatory cytokines can alter the super-enhancer landscape, disease-associated super-enhancers are likely to be such de novo super-enhancers. This correlates with our observation that JQ1 reduces disease-associated gene expression, regardless of preferential inhibition of SE-associated genes. JQ1 treatment resulted in inhibition of immune-related SEs and preferential downregulation of JIA-associated gene expression. These data suggest that BET inhibition might be a powerful therapeutic tool for the treatment of JIA and possibly other autoimmune diseases. Indeed, it has been shown that BET inhibition in collagen-induced arthritis and experimental autoimmune encephalomyelitis mouse models significantly ameliorated disease (Bandukwala et al., 2012; Mele et al., 2013). Furthermore, in vitro treatment of RA-patient-derived fibroblasts with I-BET151 reduced their inflammatory and proliferative properties (Klein et al., 2014). Currently, BET inhibitors are being explored as therapeutics in the treatment of a variety of human cancer types (Delmore et al., 2011; Zuber et al., 2011). This raises the question whether the immunological effects of BET inhibitors should be taken into account when assessing their use as anti-cancer drugs, as it might also impair the antitumor response. It remains to be investigated whether distinct BET inhibitors or other inhibitors of enhancer activity, such as Cdk7 inhibitors, have similar effects on immune cells (Chipumuro et al., 2014). Moreover, global effects of systemic JQ1 treatment on the immune system need to be considered.

Taken together, we here demonstrate the presence of an autoimmune disease-associated super-enhancer profile. Inhibiting super-enhancer activity utilizing BET inhibitors preferentially inhibited the expression of disease-associated genes, which comprised a proinflammatory signature. Collectively, these data can provide insights into JIA pathogenesis and suggest the use of BET inhibitors for the treatment of autoimmune diseases.

EXPERIMENTAL PROCEDURES

Collection of SF and PB Patient Samples

Fourteen oligoarticular JIA patients were included in this study who at the time of sampling all had active disease and underwent therapeutic joint aspiration allowing SF collection. PB was drawn at the same moment via vein puncture or intravenous drip. Informed consent was obtained from all patients either directly or from parents/guardians when the patients were younger than age 12 years. The study procedures were approved by the Institutional Review Board of the University Medical Center Utrecht (UMCU; METC nr: 11-499c) and performed according to the principles expressed in the Helsinki Declaration. Hyaluronic acid was broken down in SF samples by 30 min incubation at 37°C with Hyaluronidase (Sigma). Synovial fluid mononuclear cells (SFMCs) and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and were used after freezing in FCS (Invitrogen) containing 10% DMSO (Sigma-Aldrich).

ChIP Sequencing

Healthy control (HC) PBMCs, either activated for 16 hr with human T-activator CD3/CD28 Dynabeads (one cell: three beads) (Life Technologies) or not activated and JIA PBMCs and SFMCs, were thawed and $CD4^+CD45RO^+$ cells were sorted by flow cytometry. For each sample, cells were crosslinked with 2% formaldehyde and crosslinking was stopped by adding 0.2 M glycine. Nuclei were isolated in 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 1% Triton X-100 and lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.3% SDS. Lysates were sheared using Covaris microTUBE (duty cycle 20%, intensity 3, 200 cycles per burst, 60-s cycle time, eight cycles) and diluted in 20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% X-100. Sheared DNA was incubated overnight with anti-histone H3 acetyl K27 antibody (ab4729; Abcam) pre-coupled to protein A/G magnetic beads. Cells were washed and crosslinking was reversed by adding 1% SDS, 100 mM NaHCO3, 200 mM NaCl, and 300 μg/ml proteinase K. DNA was purified using ChIP DNA Clean & Concentrator kit (Zymo Research), end-repair, a-tailing, and ligation of sequence adaptors was done using Truseq nano DNA sample preparation kit (Illumina). Samples were PCR amplified, checked for the proper size range and for the absence of adaptor dimers on a 2% agarose gel and barcoded libraries were sequenced 75 bp single-end on Illumina NextSeq500 sequencer (Utrecht DNA sequencing facility).

RNA Sequencing

$CD4^+CD45RO^+$ cells were sorted by flow cytometry from HC PBMCs and JIA-patient SFMCs, activated with human T-activator CD3/CD28 Dynabeads (one cell: three beads) (Life Technologies) and cultured for 16 hr in RPMI Medium 1640 + GlutaMAX supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (all obtained from Life Technologies), and 10% heat-inactivated human AB-positive serum (Invitrogen) at 37°C in 5% CO_2 in the presence of 300 nM JQ1(+) or JQ1(–) (ApexBio). Next, cells were treated for 4 hr with 100 ng/ml phorbol 12-myristate 13-acetate (Enzo Life Sciences) and 1 μg/ml ionomycin (Enzo Life Sciences). Supernatant was harvested and stored at −80°C for multiplex analysis, and total RNA was extracted using the RNAeasy kit (QIAGEN). Sample preparation was performed using TruSeq stranded total RNA with ribo-zero globin sample preparation kit (Illumina), and samples were sequenced 75 bp single-end on Illumina NextSeq500 (Utrecht DNA sequencing facility).

Multiplex Immunoassay

Multiplex analysis (xMAP; Luminex) was performed on supernatant derived from JIA patient-derived cells treated for 16 hr with 300 nM JQ1(–) or JQ1 as described previously (de Jager et al., 2005).

Statistical Analysis

For ChIP-seq and RNA-seq analysis, p values were adjusted with the Benjamini-Hochberg procedure. For ChIP-seq regions with a significantly different H3K27ac signal were defined using a false discovery rate (FDR) <0.05. Significance of JQ1(+) treatment on inflammatory cytokine production was determined with paired Student's t tests. The significance of SDF-1 expression in

the SF in comparison to expression in the PB was determined with an unpaired Student's t test.

ACCESSION NUMBERS

The ChIP-seq data presented in this study have been deposited in NCBI's Gene Expression Omnibus (GEO) database under GEO: GSE71596 (linked to GSE71597). The RNA-seq data presented in this study have been deposited in NCBI's GEO database under GEO: GSE71595 (linked to GSE71597).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.046.

AUTHOR CONTRIBUTIONS

J.G.C.P., S.J. Vervoort, and J.v.L. designed and performed experiments, analyzed, interpreted data, and wrote the paper. S.C.T. and M.P.C. independently validated ChIP-seq data. S.d.R., S.J. Vastert, and F.v.W. provided patient material. G.M. performed synovial fluid experiments. M.M. independently validated ChIP-seq data and assessed SNP enrichment in enhancers. E.E.S.N., B.J.P., and P.J.C. contributed crucial reagents and critically reviewed the manuscript.

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