Transcriptional and epigenetic mechanisms underlying autoimmune diseases

Towards identification of novel therapeutic targets

Janneke Peeters

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Transcriptional and epigenetic mechanisms underlying autoimmune diseases

Towards identification of novel therapeutic targets

Transcriptionele en epigenetische mechanismen
die ten grondslag liggen aan auto-immuunziekten
(met een samenvatting in het Nederlands)

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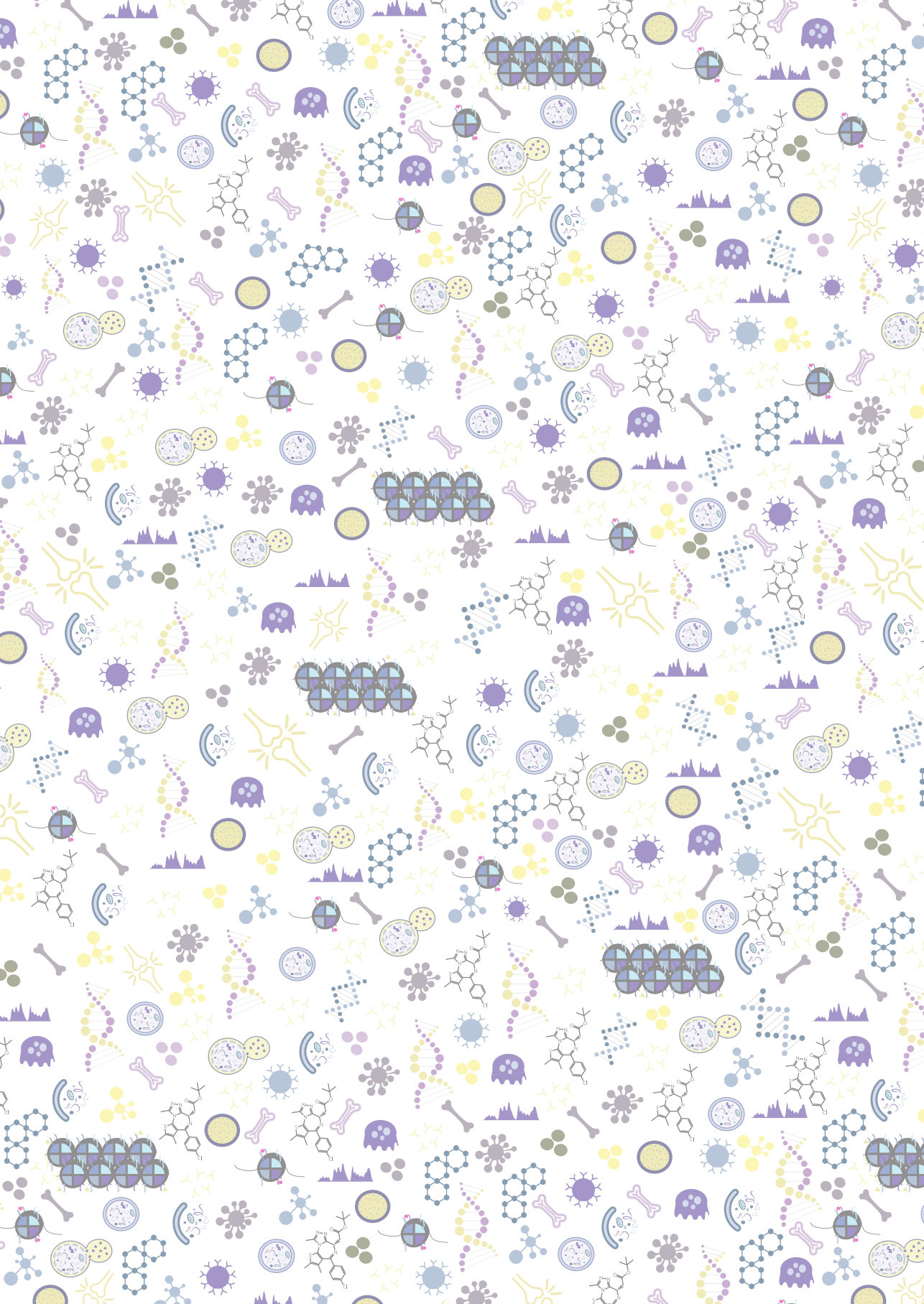
"Alleen kan je niks, je moet het samen doen"

Johan Cruijff (1947 – 2016)



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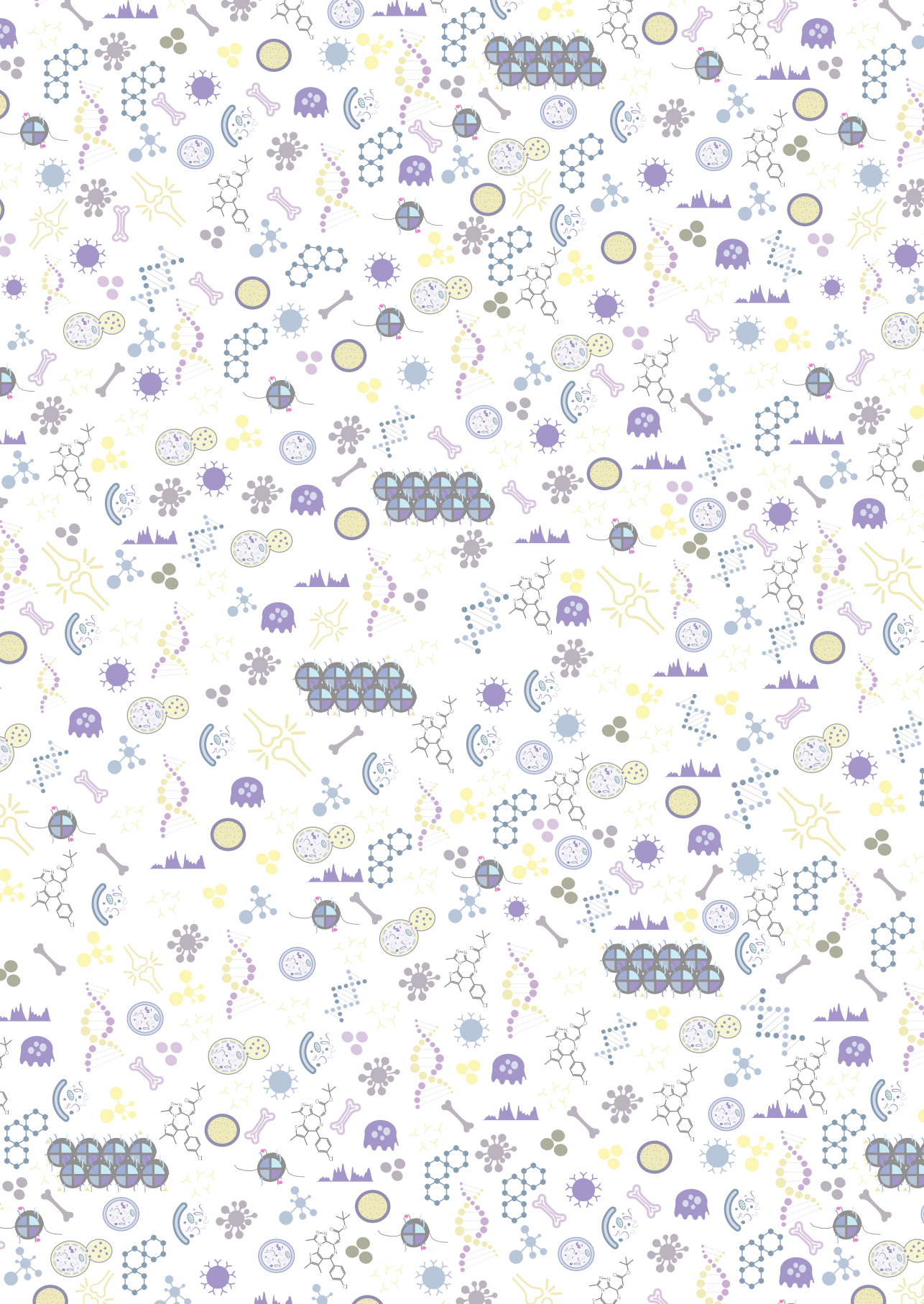

General introduction 1

AUTOIMMUNE DISEASES

Autoimmune diseases are characterized by loss of immunological tolerance, which is defined as the ability of the immune system to prevent itself from mounting an immune response against self-antigens. The prevalence of autoimmune diseases is estimated to range from 7.6–9.4%[1]. In this estimation 29 different autoimmune diseases, such as Crohn's disease (CD) and rheumatoid arthritis (RA), are included. However since more than 80 different diseases are characterized by an autoimmune response the actual prevalence is probably higher, with reports up to 20% in the United States[2]. Self-antigens can be organ-specific, such as pancreas-specific for type 1 Diabetes, or systemically expressed, which is the case for systemic lupus erythematosus (SLE).

ETIOLOGY OF AUTOIMMUNE DISEASES

For monogenic autoimmune diseases, such as immune dysregulation-polyendocrinopathy-enteropathy-X-linked (IPEX) syndrome, the underlying etiology is in general well understood. However, the majority of autoimmune diseases are multifactorial, where a complex interplay between genetics, epigenetics, and environmental factors defines disease outcome[3]. Over the last two decades many genome-wide associates studies (GWAS) have been performed to increase our understanding of the etiology of autoimmune diseases and more than 200 genetic loci have been associated[3]. For many autoimmune diseases a strong genetic association with human leukocyte antigen (*HLA*) genes, encoding for the major histocompatibility complex (MHC) in humans, has been described, likely reflecting the involvement of an altered T cell-mediated antigen response in the development of autoimmunity[3,4]. Also non-HLA-related genes have been linked with multiple autoimmune diseases, such as *protein tyrosine phosphatase non-receptor type 22* (*PTPN22*) which functions both in the innate and adaptive immune system, by enhancing pattern recognition receptor (PRR) signaling or by inhibiting T cell receptor (TCR) signaling, respectively[5]. However, these genetic associations only apply to a small proportion of patients and can also be present in healthy persons. For the majority of GWAS-identified loci the causal single nucleotide polymorphism (SNP) or affected gene(s) have not been identified, making it difficult to extrapolate these findings to a better insight into disease pathogenesis.
The complexity of genetic predisposition for autoimmune diseases is underscored by studies in monozygotic twins where, depending on the type of autoimmune disease, the concordance rate ranges from 11-83%[4]. For example, for RA the concordance rate is 15-30%, while for celiac disease this is 75-83%[4]. It is therefore thought that autoimmune diseases are the result of immune reactions triggered by the complex interaction between several environmental factors in a genetically susceptible individual. Examples of environmental factors associated with autoimmunity are infectious agents, vitamin D, nutrition, smoke, ultraviolet light, hormones and heavy metals[4]. Regarding infectious agents it has been demonstrated that certain pathogen-derived antigens share sequence or structural similarity with self-antigens, a principle referred to as molecular mimicry, and are therefore capable of cross-activating autoreactive T and B

cells[6]. For other environmental factors the exact mechanisms that contribute to autoimmunity remain elusive, but are .probably associated with the alteration of epigenetic modifications.

EPIGENETIC MECHANISMS CONTRIBUTING TO AUTOIMMUNE RHEUMATIC DISEASES

DNA METHYLATION

Epigenetic modifications refer to changes that affect gene expression without altering the genetic code. DNA methylation is an epigenetic mechanism that has been widely studied in autoimmune diseases, especially rheumatic autoimmune diseases. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and involves the addition of a methyl group to a cytosine base that is located 5′ to guanine in a CpG dinucleotide[7]. DNA methylation often occurs at CpG islands, which are stretches of DNA of which at least 50% consists of CpG dinucleotides, which are abundantly present in gene promoter regions. DNA methylation is associated with gene repression and alterations in the DNA methylation pattern, either global or gene-specific, have been reported for several autoimmune diseases[8]. In general, autoimmune rheumatic diseases are associated with DNA hypomethylation, resulting in increased gene expression. For example, in RA synovial fibroblasts (RASF) hypomethylation of promoter regions has been observed in genes associated with pathways involved in cell migration, cell adhesion, and extracellular matrix interactions, likely contributing to the invasive phenotype of these cells[9,10]. Indeed, treatment of healthy SF with the drug 5-azacytidine, which inhibits DNA methyltransferases, induced hypomethylation of these genes and resulted in an activated phenotype[11]. Based on their DNA methylation signature, RASF can be distinguished from osteoarthritis SF (OASF) and RASF isolated from different joints can be discriminated[12]. This demonstrates that DNA methylation patterns are highly disease- and joint-specific and suggests that these DNA methylation differences contribute to distinct pathogenic mechanisms underlying OA and RA. Furthermore, differentially methylated positions within the MHC locus have been associated with RA. Also, gene-specific hypomethylation of (the promoter of) *interleukin 6* (*IL6), C-X-C motif chemokine 12* (*CXCL12*), *interleukin 1 receptor, type II* (*IL1R2*)*, IL10, CD40L,* and *interferon gamma* (*IFNG*) has been reported in RASF or RA patient-derived peripheral blood mononuclear cells (PBMCs)[13–19]. *IL6* hypomethylation correlates with increased IL-6 serum levels, indicating that DNA hypomethylation can directly contribute to disease pathogenesis. For SLE $CD4^+$ T cells a global hypermethylation pattern and hypomethylation of *IL10, IL1R2, ITGAL, CD40LG, PRF1, CD5,* and interferon-inducible genes has been observed[20–27]. Reduced DNA methylation might be the result of decreased DNMT expression, this has indeed been described in several RA and SLE-patient derived cell types, including total PBMCs, T cells, and SF[10,11,20–23,28,29]. For some genes hypermethylation has been observed in RA patient cells, such as for *death receptor 3* (*DR3*) in synovial tissue cells and *cytotoxic T-lymphocyte associated protein 4* (*CTLA4*) in regulatory T cells (Tregs)[30,31]. Hypermethylation of these genes likely contributes to increased resistance of synovial cells to apoptosis and reduces the suppressive Treg function, respectively. Taken together, these data indicate that

selective hypomethylation of pro-inflammatory genes and hypermethylation of anti-inflammatory genes contribute to autoimmunity. Identifying the upstream regulators involved in this selective DNA methylation might provide inside into autoimmune disease pathogenesis.

POST-TRANSLATIONAL MODIFICATION OF HISTONES

Post-translational modification of histones is another epigenetic mechanism involved in the regulation of gene expression, but has been far less extensively studied in autoimmune diseases compared to DNA methylation. In general, methylation of histone proteins is associated with transcriptional repression and acetylation with transcriptional activation by rendering the DNA less or more accessible for transcription factors, respectively[32]. Global histone H3 and H4 hypoacetylation and hypomethylation of histone H3 at lysine 9 (H3K9) has been described in $CD4^+$ T cells from SLE patients[33]. Furthermore, SLE $CD4^+$ T cells display a gene-specific increase of the repressive H3K27me3 mark at the promoter of *hematopoietic progenitor kinase* (*HPK1*) a negative regulator of T cell-mediated immune responses, which is associated with reduced binding of the histone demethylase jumonji domain containing 3 (JMJD3/KDM6B)[34]. Increased H3K27 *HPK1* methylation correlated with decreased *HPK1* expression and therefore might contribute to increased T cell reactivity. H3K27 methylation is amongst others regulated by JMJD3, and correspondingly in the same cells, decreased H3K27me3 and increased JMJD3 binding of the *ITGAL* (CD11a) promoter was observed, illustrating that epigenetic regulation can be highly gene-specific[35]. CD11a is a component of lymphocyte function-associated antigen 1 (LFA-1), and associated with increased T cell activation in SLE pathogenesis. Furthermore, *JMJD3* expression is increased in SLE monocytes, which correlates with decreased H3K27me3 levels[36]. Recently, RASF have been extensively characterized on the epigenomic level and this identified huntingtin-interacting protein-1 (HIP1) as an important protein involved in the aggressive phenotype of RASF, demonstrating the importance of studying epigenetic alterations associated with disease[37]. Increased or decreased expression of histone deacetylases (HDACs), such as HDAC1 and HDAC2, histone acetyltransferases (HATs), such as HAT3A and KAT3B, and lysine methyltransferases, for example KMT1B and KMT6, have been described for RA and SLE[33,38–43]. Altered expression of these enzymes probably contributes to the differences in histone post-translational modifications observed in autoimmune rheumatic diseases. However, many of these enzymes also target non-histone proteins, thus the direct contribution of these findings to altered epigenetic regulation needs to be studied thoroughly[44].

The importance of epigenetic mechanisms in the pathogenesis of autoimmune diseases is underscored by the fact that approximately 90% of disease-associated SNPs are located in regulatory DNA regions, which are highly susceptible to epigenetic regulation[45,46]. Enhancers are regulatory DNA regions and 60% of autoimmune disease-associated SNPs has been demonstrated to localize to enhancer regions[45]. A detailed overview how alterations in the enhancer landscape can contribute to autoimmune arthritis is provided in **Chapter 2**.

Taken together, the involvement of epigenetics in the etiology of autoimmunity and the fact that epigenetic modifications are reversible, suggests that targeting epigenetic regulators could

be a powerful therapeutic strategy for the treatment of autoimmune disease. Indeed, several HDAC inhibitors and inhibitors of enhancer activity have been proven to be successful in *in vitro* experiments using patient material and in several *in vivo* animal models for autoimmune diseases[44,47]. For DNA methylation such studies have not been performed yet as there are presently no small molecules available that can increase DNA methylation. However, it is important to note that epigenetic alterations are highly cell-type specific, suggesting that therapeutic agents might have differential effects on distinct cell types. It is therefore important to select the appropriate tissue or cell type for studies aimed at increasing our understanding of the contribution of epigenetic mechanisms to autoimmune disease pathogenesis.

JUVENILE IDIOPATHIC ARTHRITIS

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic disease in children, with a prevalence ranging from 16-150 cases per 100,000 population[48]. JIA is not a single disease but a generic term that describes several forms of chronic arthritis with an onset before age of 16 years, persisting for more than 6 weeks, and with an unknown origin[49]. Different subtypes of JIA can be distinguished, of which the major types are oligoarticular JIA, polyarticular JIA, and systemic JIA. Oligoarticular JIA refers to arthritis that affects up to four joints within the first 6 months of the disease and usually involves the larger joints, such as the knee and the ankle. Oligoarticular JIA can be self-limiting, but can also extend to more than 4 joints after the first 6 months and is then referred to as extended oligoarticular JIA. Polyarticular arthritis includes 5 or more joints and, besides the larger joints, often smaller joints of the hand and the feet are involved. A minority of polyarticular JIA patients have positive titers for rheumatoid factor (RF), an antibody directed against the Fc part of IgG, which is associated with a worse outcome. Systemic JIA is a severe systemic inflammatory disease, and besides inflammatory arthritis it is characterized by a distinct spiking fever and potential organ involvement, such as skin rash. Systemic JIA is nowadays considered an autoinflammatory disorder instead of an autoimmune disease[50]. Therefore, the rest of this thesis will focus on oligoarticular and polyarticular JIA.

THERAPIES FOR JIA

The first-line therapy for JIA is the administration of nonsteroidal anti-inflammatory drugs (NSAIDs)[48]. These drugs relieve disease symptoms like pain and exhibit mild anti-inflammatory actions, but do not modify the underlying disease process. When only a limited number of (large) joints is affected, NSAIDs will be combined with intra-articular steroid injections, targeting inflammation in the injected joint[49]. Most of the time, maintenance therapy is necessary to control joint inflammation and to prevent damage. First-line maintenance therapy consists of the disease modifying anti-rheumatic drug (DMARD) methotrexate (MTX)[49]. MTX is the most frequently used DMARD in JIA and effective in around 70% of JIA patients[51,52]. However, many (up to 50% of) JIA patients develop daily life disturbing side effects from MTX in the long term, so called MTX intolerance, which is related to MTX-related gastrointestinal adverse effects,

including nausea and abdominal pain[53]. Patients that do not respond to DMARDs, or patients with MTX intolerance resulting in discontinuation of MTX, are subsequently treated with biologicals, which target critical mediators of the inflammatory response involved in JIA, such as tumor necrosis alpha (TNF-α), IL-6, and CD28-CD80/CD86 co-stimulation[54,55]. Targeting TNF-α has been demonstrated to be highly effective in the treatment of JIA[55]. This can be achieved by administration of etanercept, a TNF-receptor-Ig-fusion protein that functions as a decoy receptor for soluble TNF-α, or administration of adalimumab, golimumab and infliximab, monoclonal antibodies that bind to TNF-α. An important difference between the different biologicals targeting TNF-α is their half-life, with etanercept having the shortest half-life (4-5 days)[56]. Whereas the different TNF-α inhibitors have similar response rates with respect to articular inflammation, there is a clear difference with respect to treating extra-articular symptoms, for example JIA-associated uveitis. Other registered biologicals for JIA target the IL-6 pathway, such as the monoclonal antibody tocilizumab that binds to the IL-6 receptor, or inhibit T cell co-stimulation, like abatacept, a soluble fusion protein composed of CTLA-4 and the Fc region of IgG1 that binds to CD80/CD86 and thereby interferes with CD28 binding[57].

Although the development of biologicals has been a major breakthrough in the treatment of JIA, a substantial percentage of patients still does not respond to the current available therapy, only shows partial remission, or becomes resistant during treatment. In addition, patients can relapse after withdrawal of the medication indicating that life-long treatment is necessary, which can potentially lead to severe side effects, such as malignancies and infections, due to general immune suppression[58]. Furthermore, from a pathophysiological point of view, targeting the production of inflammatory mediators might be even more attractive than inhibiting their mechanism of action, the latter referring to the mechanism of action of biologicals. Therefore, there is still a major need for the development of novel therapeutic strategies for the treatment of JIA.

JOINT INFLAMMATION IN JIA

JIA is characterized by severe inflammation of the joints, of which the underlying pathology is still incompletely understood, but involves both the innate and adaptive immune system[49]. Similar to many other autoimmune diseases, the exact initiating event(s) that trigger inflammation remain unknown. In JIA initial inflammation can lead to tissue damage and, as a result, expression of auto-antigens. Auto-antigens derived from extracellular matrix components, for example aggrecan and fibrillin, and from matrix metalloproteinase 3 (MMP3), which can all be found in the synovial compartment, have been reported to induce JIA T cell proliferation *in vitro* and activation of autoreactive $CD4^+$ T cells, leading to the induction of a T cell-driven immune response, which can attribute to chronic joint inflammation[59]. Indeed, one characteristic of joint inflammation in JIA is the synovial infiltration of $CD4^+$ memory cells, displaying high expression of C-C chemokine receptor type 5 (CCR5) and C-X-C Motif Chemokine Receptor (CXCR3)[60–62]. The infiltration of these activated T cells leads to the production of several pro-inflammatory cytokines, such as IFN-γ and IL-17, which stimulates the production of multiple chemokines that

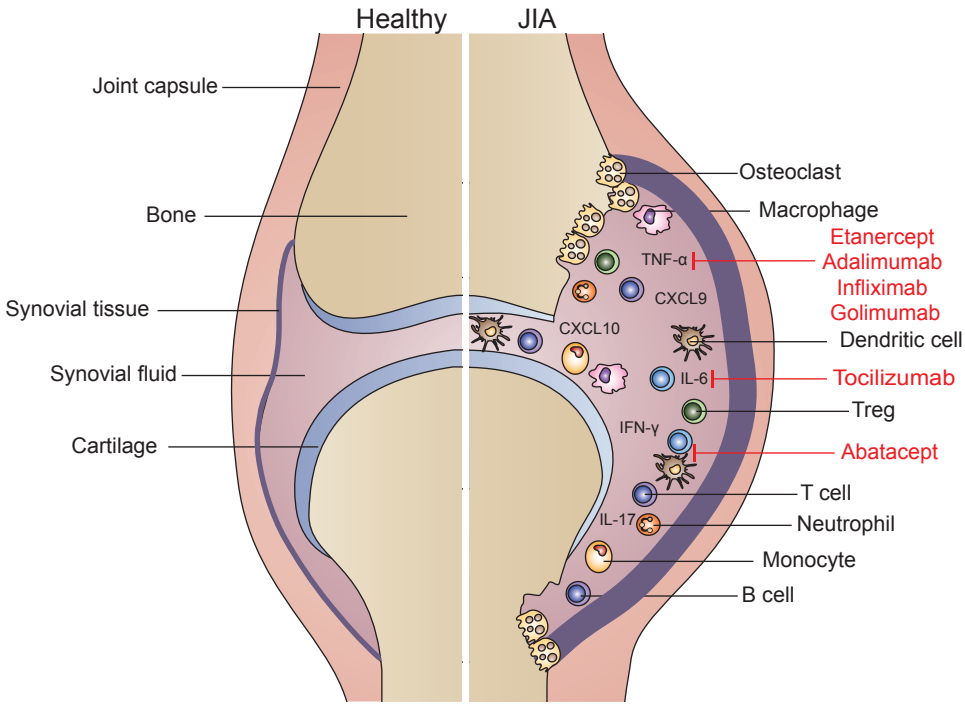

Figure 1. Schematic representation of a healthy and inflamed joint.
Initial inflammation induces tissue damage and the resulting exposure of auto-antigens leads to the infiltration of autoreactive $CD4^+$ T cells within the synovial compartment. Infiltrating T cells produce pro-inflammatory cytokines, such as IFN-γ and IL-17, which activate synovial fibroblasts within the synovial tissue to produce chemokines, for example CXCL10 and CXCL9. This recruits other cells, such as DCs, macrophages, Treg cells, neutrophils, B cells, and monocytes to the site of inflammation. These cells contribute to the inflammatory loop by stimulating synovial fluid production and induce cell proliferation and activation via the production of pro-inflammatory cytokines, for example TNF-α and IL-6. Furthermore, several pro-inflammatory cytokines, such as IL-17 and TNF-α, can stimulate osteoclast differentiation which leads to bone degradation. Etanercept, adalimumab, infliximab, golimumab, tocilizumab and abatacept are biologicals used for the treatment of JIA patients. Etanercept, adalimumab, infliximab, and golimumab are targeting the TNF-α signaling pathway, tocilizumab inhibits IL-6, and abatacept prevents T cell co-stimulation.

activate SF and recruit immune cells, such as neutrophils, monocytes/macrophages, and B cells to the site of inflammation[63]. These cells contribute to the joint inflammation by the production of other pro-inflammatory cytokines, such as TNF-α and IL-6, which further drives inflammation by inducing cell proliferation and activation, and by attracting more cells to the site of inflammation (**Figure 1**)[49,63].

Furthermore, increased or ongoing joint destruction may lead to the expression of novel auto-antigens, which can activate another pool of autoreactive T cells. In this way a self-sustaining feedback loop is established that drives continuous inflammation within the joint.

The pathogenic T cells present within the synovial compartment are predominantly Th1 and Th17 cells[60,64]. Treg cells cells are crucial in maintaining self-tolerance or preventing autoimmune

responses by suppressing Th1 and Th17 cell activation, either by secretion of the anti-inflammatory cytokine IL-10 or through cell-cell contact[65]. It is generally thought that in JIA there is an imbalance between autoreactive Th1/Th17 and Treg cells, leading to loss of immunological tolerance. This imbalance is likely caused by increased resistance of T effector cells to suppression by Treg cells, since JIA inflammatory-site derived Treg cells are functional in allogeneic suppression assays[66,67]. The chronic inflammatory environment likely contributes to the increased resistance phenotype, since IL-6 and TNF-α induce resistance to suppression *in vitro*[66].

JOINT DESTRUCTION IN ARTHRITIS

JIA and other forms of chronic arthritis are associated with articular damage and bone erosions when the inflammation is not sufficiently controlled. Bone remodeling is a continuous process depending on the balance between bone production, carried out by osteoblasts, and bone resorption, facilitated by osteoclasts. In the joints of arthritis patients, bone damage is the result of increased bone resorption, but whether this is due to an increased amount of osteoclasts or increased activity of osteoclasts remains unclear[68]. The synovial tissue of RA and JIA patients contains osteoclasts and RA synovial monocytes and macrophages are capable of differentiating into osteoclasts[69–71]. Osteoclasts are multinucleated cells that are formed by fusion of mononuclear myeloid precursor cells, such as monocytes, macrophages, and DCs, that can be found in the peripheral blood and in the bone marrow[72,73]. Osteoclast formation is induced by macrophage colony-stimulating factor 1 (M-CSF/CSF-1), produced by osteoblasts, which upregulates CSF-1 receptor (CSF-1R) and receptor activator of nuclear factor κB (RANK) on the surface of osteoclast precursor cells. Binding of RANK ligand (RANKL), produced by osteoblast, SF or activated T cells, to RANK together with CSF-1 stimulation will further induce osteoclast differentiation and leads to the formation of mature osteoclasts (**Figure 2**)[74]. At the site where mature osteoclasts associate with bone, multiple cell membrane invaginations are formed, a so-called ruffled border. Here, an isolated microenvironment is formed and acids and vesicles containing bone-degrading proteases, such as cathepsin K (CTSK), are secreted across the ruffled boarder, resulting in bone demineralization and bone matrix degradation[75].

Although RANKL is expressed on arthritis-associated T cells within the joint, it remains unclear whether this contributes to enhanced osteoclastogenesis, since T cell-derived IFN-γ, IL-4, and IL-10 inhibit osteoclast formation[76–79]. However, Th17 cells can induce osteoclastogenesis by producing IL-17, which induces RANK expression on osteoclast precursors and RANKL expression on osteoblasts and SF[80–83]. In addition, in a collagen-induced arthritis model it has been demonstrated that a subset of Treg cells within the joint can be converted into Th17 cells with increased osteoclastogenic potential compared to natural Th17 cells[84]. Also, IL-23, an important cytokine for Th17 cell differentiation, is involved in osteoclast formation by expanding the pool of osteoclast precursor cells[85]. Furthermore, IL-17 stimulates innate immune cells, such as macrophages and DCs, and SF to produce pro-inflammatory cytokines, such as IL-6

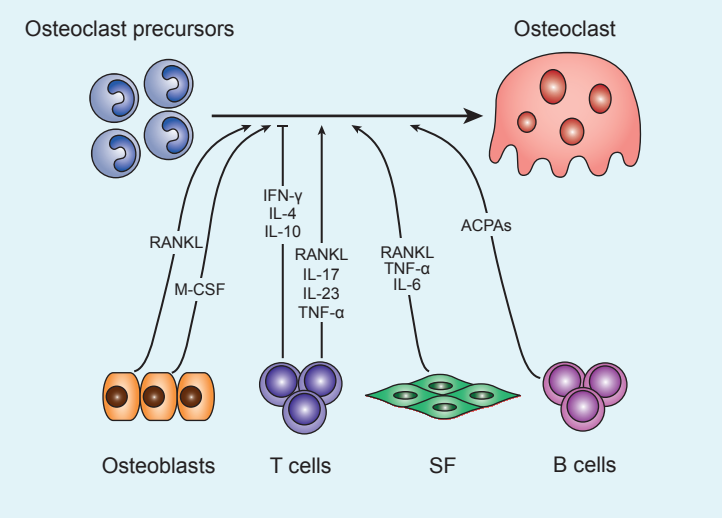

Figure 2. Inflammatory mediators involved in osteoclast differentiation.
Osteoclast precursor cells, for example monocytes, can differentiate towards osteoclasts upon exposure to M-CSF, produced by osteoblasts, and RANKL, produced by osteoblasts, synovial fibroblasts (SF), and T cells. Additionally, several cytokines that can either stimulate or inhibit osteoclasts differentiation. B cells can affect osteoclastogenesis by the production of anti-citrullinated antibodies (ACPAs).

and TNF-α[81]. TNF-α has been demonstrated to induce osteoclastogenesis both *in vitro* and *in vivo* in a RANKL-dependent as well as a RANKL-independent fashion[86–88]. IL-1 family members are critical mediators of TNF-α-induced osteoclastogenesis, by stimulating RANKL expression upon TNF-α exposure in stromal cells[89]. In addition, IL-1 cytokines can induce osteoclast precursor cell differentiation independently of TNF-α[89]. IL-6 also contributes to osteoclast formation by enhancing the interaction between osteoblasts and osteoclasts, which involves regulation of the effects of IL-1 cytokines and TNF-α on osteoclastogenesis[90–92].

Another recently identified mechanism that might contribute to increased osteoclastogenesis in arthritis involves anti-citrullinated protein antibodies (ACPAs). ACPAs are directed against citrullinated peptides, peptides in which the amino acid arginine is converted to the amino acid citrulline by a post-translational modification carried out by protein-arginine deiminase (PAD) enzymes[93]. Osteoclast development and maturation is dependent on PAD enzymes and differentiating osteoclasts express citrullinated proteins on their surface[94]. ACPA addition stimulates osteoclast formation both *in vitro* and *in vivo* and this effect is mediated by stimulation of the release of IL-8, which functions as an autocrine growth factor for osteoclasts and thus enhances osteoclast formation[95]. Additionally, ACPAs can form immune complexes which can stimulate osteoclast activation by binding to Fc receptors on osteoclasts[96].

AUTOPHAGY

One process important for cellular homeostasis and associated with many (autoimmune) diseases is autophagy. Autophagy is a well-conserved degradation pathway in which cytoplasmic content is sequestered and degraded by the lysosome[97,98]. Three types of autophagy can be distinguished: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) In macroautophagy a so-called phagophore or isolation membrane engulfs cytoplasmic proteins or organelles, which after membrane elongation and closure results in the formation of a double-membrane vesicle: the autophagosome. This autophagosome can fuse with a lysosome and lysosomal hydrolases will degrade the cytoplasmic content and inner membrane of the autolysosome. In addition, autophagosomes can fuse with endosomes, multivesicular bodies, and MHC class II-loading compartments[99]. In microautophagy, invaginations of the lysosomal membrane directly engulf cytoplasmic structures. CMA is a selective form of autophagy, in which substrates containing a specific pentapeptide motif are recognized by chaperone proteins, e.g. heat-shock chaperone 70 (HSP70), and translocated into the lysosome via association with lysosomal-associated membrane protein 2A (LAMP2A)[100]. For the rest of this thesis we will focus on macroautophagy, hereafter referred to as autophagy.

AUTOPHAGY INITIATION

Autophagy can be divided into several stages: initiation/nucleation, membrane elongation, membrane completion and maturation, lysosomal fusion, and cargo degradation (**Figure 3**). Starvation-induced autophagy, the best characterized autophagic process, is initiated by the activation of AMP activated protein kinase (AMPK) and the inhibition of mammalian target of rapamycin (mTOR), which are both key sensors off cellular nutrient, energy, and oxygen levels[101]. In nutrient-rich conditions, mTOR is active and phosphorylates UNC-51-like kinase (ULK) 1 or 2 and autophagy-related protein (ATG13), which are part of the ULK complex that furthermore consists of FIP200 and ATG101. Phosphorylation inhibits the interaction between ULK1 and AMPK and renders AMPK and the ULK complex inactive. Upon nutrient starvation, AMPK becomes active and inhibits mTOR, thereby activating the ULK complex, which results in the translocation of the complex to the site of autophagosome formation. The exact origin of the autophagosomal membrane remains unknown, but there are indications that point at subdomains of the ER, *i.e.* omegasomes, the plasma membrane, the nuclear membrane, and the outer mitochondrial membrane[102].

AUTOPHAGOSOMAL MEMBRANE ELONGATION AND MATURATION

The ULK complex recruits the class III phosphatidylinositol 3-OH kinase (PI3K) complex, which consists of the core proteins vacuolar protein sorting 34 (VPS34/PIK3C3), VPS15 (PIK3R4), and Beclin 1, complemented with other subunits, leading to three different PI3K complexes in mammals[103]. ATG14L and UV radiation resistance-associated gene (UVRAG) are mutually exclusive subunits of the PI3K complex, but have both been implicated to be essential for

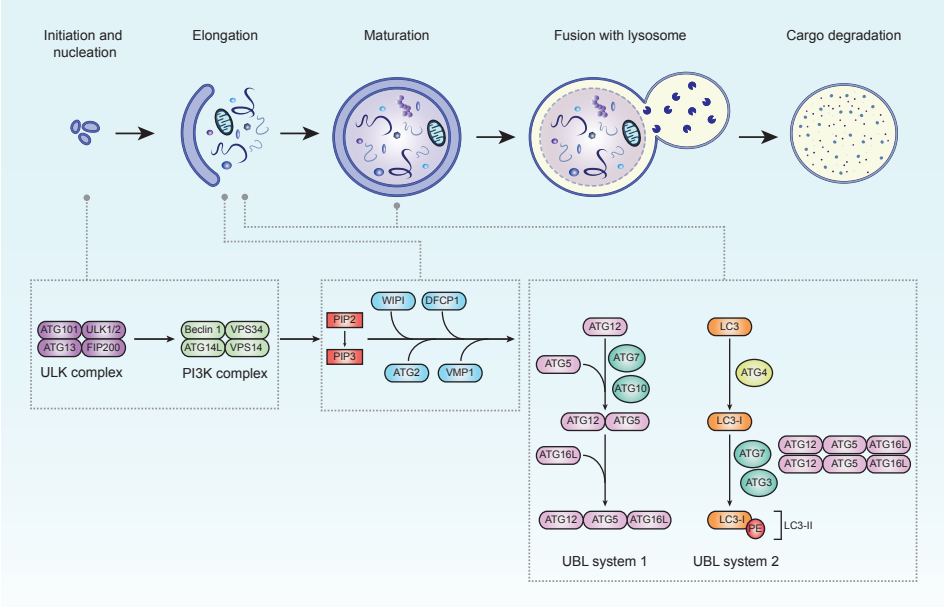

Figure 3. Schematic overview of autophagy and key proteins involved in autophagosome formation. Autophagy-inducing signals, such as nutrient deprivation, initiate the formation of a phagophore, which is mediated by the ULK1 complex (consisting of ULK1/2, FIP200, ATG101, and ATG13). Further nucleation requires the PI3K complex (consisting of Beclin 1, VPS34, VPS15, and ATG14L or another component), which converts PIP2 into PIP3 and thereby recruits other proteins involved in membrane elongation. The resulting double membrane structure engulfs cytoplasmic content for degradation. Further membrane elongation and maturation requires two ubiquitin-like conjugation systems. The first system consisting of ATG7 and ATG10, produces the ATG12-ATG5 conjugate which homodimerizes with ATG16L1. The resulting ATG12-ATG5-ATG16L1 homodimer functions in the second ubiquitin-like conjugation system together with ATG4, ATG7, and ATG13 to generate LC3-PE, which is important for membrane closure and autophagosome formation. The autophagosome can fuse with lysosomal compartments, leading to formation of an autolysosome. Lysosomal hydrolases will degrade the inner membrane and cytoplasmic content of the autophagosome and the degradation products can be used as a source of energy or for the synthesis of macromolecules.

autophagosome formation[104,105]. The UVRAG-containing PI3K complex can recruit Rubicon, which inhibits autophagosome maturation and therefore creates a negative feedback loop[106,107]. The PI3K complex produces phosphatidylinositol-3-phosphate (PI3P), which is an essential signaling molecule in autophagy by recruiting effector proteins, such as double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting (WIPI) family proteins, which are involved in omegasome and autophagosome formation[108,109]. Other proteins implicated to play a role in autophagosome formation are ATG2, vacuole membrane protein 1 (VMP1), and ATG9[110,111].

Two ubiquitin-like (UBL) conjugation systems are important for the membrane elongation and completion, with ATG7 acting as an E1-like enzyme in both UBL systems[112,113]. First, ATG7 activates the ubiquitin-like protein ATG12 and transfers it to the E2-like enzyme ATG10, and subsequently ATG12 becomes conjugated to ATG5. The ATG12-ATG5 conjugate forms a complex

with ATG16L1, homodimerizes and localizes to the expanding autophagosomal membrane. The other ubiquitin-like system starts with the cleavage of the ubiquitin-like mammalian ortholog of Atg8 (LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2, and GABARAPL2; hereafter referred to as LC3), by ATG4 and thereby becomes LC3-I[114]. ATG7 subsequently transfers LC3-I to the E2-like enzyme ATG3, the ATG12-ATG5/ATG16L1 homodimer functions as an E3 ligase and conjugates LC3 to its lipid target phosphotidylethanolamine (PE), thereby forming LC3-II. LC3 is suggested to be important for closure of the autophagosomal membrane[115,116].

SUBSTRATES DEGRADED VIA AUTOPHAGY

Autophagy has long been considered to be a bulk degradation pathway, *i.e.* upon nutrient deprivation cells needs to adapt quickly to maintain energy homeostasis and thus time for substrate selection is limited. Presently, it is clear that autophagy is also important for maintaining cellular homeostasis and is involved in the selective removal of damaged or unnecessary proteins and organelles[117,118]. The removal of distinct organelles has resulted in organelle-specific instances of autophagy, such as mitophagy, reticulophagy, pexophagy and ribophagy, referring to the removal of mitochondria, ER, peroxisomes, and ribosomes, respectively. The specific targeting of cytoplasmic substrates is facilitated by cargo receptors that bind cytosolic substrates as well as LC3 via a conserved LC3-interacting region (LIR), thereby recruiting their cargo to the autophagosome[119]. Many cargo receptors recognize their substrate by poly-ubiquitin chains attached to the substrate surface [120]. Examples of such cargo receptors are sequestosome 1 receptor (SQSTM1/p62), neighbor of BRCA1 gene (NBR1), and nuclear dot protein of 52 kD (NDP52), which bind to ubiquitinated aggregated proteins and defective mitochondria[121]. Differences in the affinity of these cargo receptors for (certain types of) ubiquitin chains, non-ubiquitinated proteins, and different LC3 homologs provides substrate specificity.

AUTOPHAGY REGULATION

Autophagy is regulated within the cytoplasm and within the nucleus. Post-translational modifications, such as phosphorylation, ubiquitination, and acetylation, that occur in the cytoplasm can alter protein-protein interactions or induce differences in subcellular localization[122]. Transcriptional regulation of autophagy, which is especially important for prolonged autophagy, depends on the activation of certain nuclear transcriptional programs. Post-translational regulation of autophagy is a well-studied field, while transcriptional regulation of autophagy remains relatively underexplored. Recent studies have revealed several transcription factors involved in this process and the most important transcriptional regulators will be discussed here (**Table 1**).

TRANSCRIPTIONAL REGULATION OF AUTOPHAGY

Transcription factor EB (TFEB) was one of the first transcription factors identified to be important for autophagy regulation[123]. Under nutrient-rich conditions, TFEB is phosphorylated by mTOR

Table 1. Overview of key transcription factors involved in autophagy regulation.

Transcription Factor	Effect on autophagy	Affected autophagy-associated genes
TFEB	Activating	*ATG4B, ATG9B, ATG16, GABARAPL1, MAP1LC3B, SQSTM1, UVRAG, WIPI*
ZKSCAN3	Inhibiting	*GABARAPL2, ULK1, MAP1LC3B, WIPI2*
FOXO	Activating	*ATG4, ATG5, ATG12, ATG14, BECN1, BNIP3, GABARAPL1, MAP1LC3B, ULK1, PIK3C3*
FXR	Inhibiting	*ATG3, ATG5, ATG7, ATG10, BECN1, GABARAP, GABRAPL1, MAP1LC3A, ULK1, PIK3C3*
CREB	Activating	*ATG3, ATG5, ATG7, ATG10, BECN1, GABARAP, GABRAPL1, MAP1LC3A, ULK1, PIK3C3*
PPARα	Activating	*ATG3, ATG5, ATG7, ATG10, BECN1, GABARAP, GABRAPL1, MAP1LC3A, ULK1, PIK3C3*

ATG: autophagy-related protein, BECN1: Beclin 1, CREB: CRE-binding protein, FOXO: forkhead box O, FXR: farnesoid X receptor, GABARAP:gamma-aminobutyric acid receptor-associated protein, MAP1LC3A/B: microtubule-associated proteins 1A/1B light chain 3A, PIK3C3: phosphatidylinositol 3-kinase catalytic subunit type 3, PPAR-α: peroxisome proliferator-activated receptor alpha, SQSTM1: sequestosome 1 receptor, TFEB: transcription factor EB, ULK: UNC-51-like kinase, UVRAG: radiation resistance-associated gene, WIPI1: WD-repeat domain phosphoinositide-interacting 1, ZKSCAN3: zinc-finger protein with KRAB and SCAN domains 3.

and retained in the cytoplasm[124]. Amino acid and serum starvation inhibits mTOR and thus results in dephosphorylation of TFEB, leading to the translocation of TFEB to the nucleus. Here, TFEB can increase the expression of many autophagy-associated genes, including *UVRAG, WIPI, MAPLC3B, SQSTM1, VPS11, VPS18,* and *ATG9B*, and several genes involved in lysosomal biogenesis. TFEB overexpression is sufficient to increase autophagy, illustrating its critical role in the regulation of autophagy[123]. Another transcriptional autophagy regulator is zinc-finger protein with KRAB and SCAN domains 3 (ZKSCAN3), which inhibits the transcription of multiple autophagy-related genes[125]. ZKSCAN3 is present in the nucleus under homeostatic conditions and shuttles to the cytoplasm upon nutrient starvation, and therefore acts as the transcriptional antagonist of TFEB.

Members of the forkhead box O (FOXO) family of transcription factors are also involved in autophagy regulation. Both FOXO1 and FOXO3 can stimulate autophagy by inducing the expression of various autophagy-related genes, such as *PIK3C3*, *ATG12*, *GABARAPL1*, and via a glutamine synthetase-dependent manner[126,127]. Cytosolic FOXO1 can act as an enhancer of autophagy as well, by interacting with ATG7 and thereby inducing autophagy[128]. FOXO proteins are stress-responsive transcription factors and several other stress-responsive transcription factors have been implicated in the transcriptional regulation of autophagy, *e.g.* tumor suppressor protein 53 (TP53/p53), E2F1, nuclear factor κB (NF-κB), hypoxia-inducible factor 1-alpha (HIF-1α), and c-Jun, which are extensively reviewed elsewhere[129–131]. Recently, also nuclear receptors have been demonstrated to be involved in the transcriptional regulation of autophagy. Under feeding conditions, farnesoid X receptor (FXR) inhibits autophagy-associated gene expression, either directly or indirectly by inhibition of the transcriptional activity of CRE-

binding protein (CREB)[132,133]. Under fasting conditions, peroxisome proliferator-activated receptor alpha (PPAR-α) becomes active and competes with FXR for promoter binding of autophagy genes, indicating that the competition between these two nuclear receptors regulates the autophagic response. CREB induces the transcription of TFEB, and TFEB has been described to promote the expression and activity of PPAR-α, suggesting that TFEB, CREB, FXR, and PPAR-α belong to the same transcriptional network and act in concert to control tight regulation of autophagy[134].

EPIGENETIC REGULATION OF AUTOPHAGY

Besides transcriptional control of autophagy, it is becoming increasingly apparent that epigenetic mechanisms can also contribute to autophagy regulation. Under homeostatic conditions, methyltransferase G9a suppresses genes involved in autophagosome formation, such as *LC3B* and *WIPI1*, by association with their promoters and promoting methylation of H3K9, which is associated with transcriptional repression[135]. Glucose starvation reduces promoter binding of G9a and thus results in autophagy induction. Similarly, methyltransferase enhancer of zeste homolog 2 (EZH2) is involved in the epigenetic repression of several negative regulators of the mTOR pathway, including *tuberous sclerosis complex 2 (TSC2*) and *DEP domain-containing mTOR-interacting protein* (*DEPTOR*), by increasing their H3K27me3 levels[136].

For H4K16ac a strong link with autophagy has been described, mediated by the acetyltransferase human male absent on first (hMOF/KAT8)[137]. Upon induction of autophagy there is a significant downregulation of hMOF expression, and subsequently H4K16 acetylation of autophagy-associated genes is decreased. Since H4K16 deacetylation is associated with gene repression, this results in decreased expression of autophagy-associated genes and a decrease in autophagy, thus providing a feedback mechanism to prevent prolonged autophagy. Acetylated H4K16 functions as a docking site for other proteins involved in transcriptional regulation, for example for bromodomain-containing protein 4 (BRD4)[138]. BRD4 is a negative regulator of autophagy gene expression by recruiting methyltransferase G9a to the chromatin[139]. Since starvation decreases acetylation of H4K16ac, BRD4 will dissociate and G9a will not be recruited, leading to the transcriptional activation of autophagy-associated genes. Another histone mark associated with autophagy regulation is histone H2B monoubiquitination (H2Bub1)[140]. Loss of H2Bub1 induces autophagy, indicating that H2Bub1 functions as a critical epigenetic switch for the regulation of autophagy. Upon starvation, expression of the deubiquitinase USP44 is induced, the resulting decreased H2Bub1 levels lead to the downregulation of genes involved in the positive regulation of NF-κB, an inhibitor of autophagy. The starvation-induced upregulation of USP44 is mediated by DNA methylation, indicating that multiple epigenetic mechanisms act in concert to regulate autophagy.

Cytosolic AMPK has been known for years to be important for autophagy induction, but recently also a role for nuclear AMPK in autophagy has been described[141]. Upon prolonged nutrient deprivation, AMPK protein levels and activity increase in the nucleus leading to FOXO3 phosphorylation. FOXO3 subsequently inhibits S-phase kinase-associated protein 2 (SKP2),

involved in the degradation of coactivator-associated arginine methyltransferase 1 (CARM1). CARM1 stabilization induces autophagy, since CARM1 functions as a co-factor for TFEB and increases H3R17me2 levels of autophagy and lysosomal genes, which induces their transcription. Thus, next to orchestrating the rapid cytoplasmic autophagy response, AMPK is involved in inducing the delayed autophagy response.

Furthermore, a global increase in H4K20me3, a repressive mark, and a global decrease in H3K56ac and H3K4me3, both activating marks, have been observed upon autophagy induction, but whether and how this affects autophagy remains to be determined[142,143]. H4K20me3 and H4K16ac are both involved in the regulation of RNA polymerase II (Pol II) pausing, where H4K20me3 promotes Pol II pausing while H4K16ac facilitates the release of paused Pol II[144]. This suggests that a decrease in H4K16ac and an increase in H4K20me3 upon autophagy induction might inhibit gene expression by promoting Pol II pausing.

AUTOPHAGY AND THE IMMUNE SYSTEM

The most direct involvement of autophagy within the immune system is by autophagy-dependent elimination of bacterial pathogens and neosynthesized viral proteins, where pathogens or viral proteins are engulfed by the autophagosome and degraded after delivery to the lysosome[145,146]. This process is referred to as xenophagy and virophagy, respectively, and has been demonstrated to take place in phagocytic cells as well as non-phagocytic cells. Over the years, it has become increasingly clear that autophagy plays an important role in specific aspects of both the innate and the adaptive immune system[147–150]. Here, we will discuss several important processes in which autophagy is involved. In addition, some autophagy-associated genes have been demonstrate to regulate immune responses in an autophagy-independent manner. These processes are outside the scope of this thesis and will therefore not be discussed.

AUTOPHAGY AND CYTOKINES

Autophagy can modulate cytokine secretion by interfering with numerous pattern recognition receptor (PRR) signaling pathways. Autophagy affects IL-1β and IL-18 production by inhibiting inflammasome activation[151,152]. The inflammasome is a cytosolic multimeric protein complex in myeloid cells that activates caspase-1, which proteolytically cleaves pro IL-1β and pro IL-18, resulting in the secretion of these pro-inflammatory cytokines[153]. The inflammasome is activated by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Inflammasome activation induces the ubiquitination of apoptosis-associated speck-like protein containing a CARD (ASC), an essential adaptor protein of the inflammasome, which is recognized by p62 and subsequently targeted to the autophagosome for destruction, leading to reduced IL-β and IL-18 secretion[152]. In addition, IL-1β secretion is inhibited by autophagy via the sequestration of pro IL-1β in autophagosomes[154]. IL-1β can induce autophagy in macrophages, suggesting that autophagy is involved in a negative feedback loop regulating IL-1β production[155]. The importance of autophagy for IL-1β and IL-18 production is furthermore

underscored by the observation that *Atg16l1*- or *Atg7*-deficient macrophages display an enhanced cytokine response upon Toll-like receptor (TLR) 4 stimulation[156]. In addition, autophagy can modulate the production of type I IFNs. Autophagy stimulates TLR7 and TLR9-mediated production of type I IFNs in plasmacytoid DCs upon sensing of viral single-stranded RNA and microbial DNA, respectively, while it negatively regulates type I IFN production by retinoic acid inducible gene I (RIG-I)-like receptor signaling (RLR) upon stimulation with viral double-stranded RNA[157–159]. Some of the effects of autophagy on cytokine production are mediated by limiting intracellular reactive oxygen (ROS) levels via mitophagy, as ROS is a known inducer of cytokine production[157,160]. Indeed, the caspase 1-independent maturation and production of IL-1α depends on ROS and is increased in autophagy-deficient macrophages[161].

Furthermore, autophagy has been implicated in the negative regulation of IL-12, IL-17, TNF-α, IL-6, CXCL1, and IL-23[161–163]. The relation between autophagy and cytokines is reciprocal, as a plethora of immune-related signaling molecules have been demonstrated to induce autophagy. In general Th1 related cytokines are thought to induce autophagy, *e.g.* TNF-α and IFN-γ, while Th2 cytokines, such as IL-4 and IL-13, negatively affect autophagy levels[164,165]. In addition, more innate-related cytokines, such as type I IFNs and IL-1β, and DAMPs, *e.g.* S100 proteins, ATP, ROS, high mobility group box (HMGB) proteins, and DNA complexes, are also involved in autophagy induction[166,167]. In contrast, the anti-inflammatory IL-10 cytokine negatively affect autophagy levels[165]. This diverse nature of extracellular signaling molecules affecting autophagy suggests that autophagy is regulated in a context and cell type-specific manner. Furthermore, given the predominant inhibitory effect of autophagy on cytokine secretion and the multitude of pro-inflammatory stimuli inducing autophagy, autophagy probably functions as negative-feedback mechanism to control the production of pro-inflammatory mediators.

AUTOPHAGY AND ANTIGEN PRESENTATION

The adaptive immune response relies on the recognition of "non-self"-derived peptides that are presented on major histocompatibility complex (MHC) molecules. Endogenous-derived antigens are generated by the proteasome and usually presented by MHC class I molecules to activate $CD8^+$ T cells. Exogenous material is taken up by antigen presenting cells (APCs) by endocytosis, processed in endolysosomal compartements, loaded on MHC class II molecules that have trafficked from the ER to MHC class II-loading compartments and presented to $CD4^+$ T cells. Autophagy contributes to the presentation of endogenous material on MHC class II molecules by the fusion of autophagosomes containing cytoplasmic material with MHC class II-loading compartments[99,168–171]. This has been demonstrated in several *in vitro* models, but also *in vivo* where specific deletion of *Atg5* in DCs leads to the generation of an impaired $CD4^+$ T cells response after Herpes Simplex Virus (HSV) infection[172]. In addition, stimulation of autophagy by rapamycin, an inhibitor of mTOR signaling, enhances DC antigen presentation and provides a strong T helper cells response upon antigen challenge[170]. Presentation of intracellular material is highly important in thymic epithelial cells (TECs), which present self-antigens on MHC molecules for the induction of central T cell tolerance, which is the negative selection of

autoreactive T cells. Indeed, TECs display high autophagy levels and autophagy deletion in TECs leads to an altered selection of specific MHC-II-restricted T-cell and results in autoimmunity[173,174]. While the role of autophagy in MHC class II presentation is evident, the contribution of autophagy to MHC class I antigen presentation is less clear[99]. Cross-presentation refers to the process that exogenous-derived antigens are loaded on MHC class I molecules and presented to $CD8^+$ T cells. Autophagy directly contributes to cross-presentation by facilitating tumor and virus-derived antigen presentation on MHC class I by a specific subset of DCs, and is indirectly involved in cross-presentation via the direct transfer of antigen-sequestering autophagosomes from antigen-donor cells to APCs[175–179].

AUTOPHAGY AND T CELLS

Besides modulating antigen presentation, autophagy is involved in many other aspects associated with proper T cell function. Thymocyte development and maturation is not altered in mice with either *Atg5*, *Atg7*, *Atg3* or *Vps34* deleted from their T cells, but naïve $CD4^+$ and $CD8^+$ T cell numbers in the spleen are significantly reduced and show impaired proliferation upon stimulation[180–186]. This decreased number of peripheral cells is amongst others due to an increase in apoptosis. Autophagy can directly affect apoptosis by the degradation of cell death-associated proteins, which has been demonstrated in *Beclin 1*–deficient mice, where caspase-8 is found in p62- and ubiquitin-containing protein aggregates in activated T cells[187]. In addition, autophagy indirectly contributes to apoptosis by the removal of mitochondria which keeps ROS levels low. Indeed, autophagy-deficient peripheral T cells are characterized by an increase in ROS levels and increased mitochondrial content[181,182,184,188]. Autophagy can regulate T cell proliferation through selective degradation of the cell-cycle inhibitor cyclin-dependent kinase inhibitor 1B (CDKN1B/p27Kip1), providing a possible explanation for the reduced proliferation rate of autophagy-deficient T cells[189]. Furthermore, autophagy is involved in maintaining ER and calcium homeostasis in T cells, since *Atg7*- and *Atg3*-deficient T cells display an increased ER content, increased ER calcium storage, and an impaired calcium influx[184,190]. Calcium signaling is important for T cell activation and autophagy has indeed been demonstrated to be induced upon T cell activation and autophagy inhibition impairs T cell activation[185,191,192].

Autophagy can modulate T cell receptor (TCR) signaling by regulating of the degradation of the adaptor protein Bcl10, which mediates TCR-to-NF-κB signaling, and Protein Tyrosine Phosphatase Non-receptor Type 1 (PTPN1), which is involved in the upregulation of anergic genes[193,194]. Indeed, autophagy inhibition in T cells induces a hyporesponsive, *i.e.* anergic state, and by preventing the induction of anergy upon TCR stimulation autophagy thus acts as a tolerance-avoidance mechanism. In addition to TCR stimulation, autophagy is induced by common γ-chain cytokines, including IL-2 in a janus kinase (JAK)-dependent manner[195].

Autophagy is also important for the establishment of memory CD8+ T cells, since infection of mice containing *Atg7*-deficient $CD8^+$ T cells with influenza, cytomegalovirus or lymphocytic choriomeningitis virus all resulted in impaired memory T cell formation and a decreased secondary immune response[196–198]. The transition from effector T cells to memory T cells

depends on the metabolic switch from glycolysis to fatty acid oxidation, suggesting that autophagy is involved in this metabolic reprogramming step. Because autophagy is a key mechanism to provide cells with energy substrates, autophagy is likely very important to meet the metabolic demands of T cell activation. For example, autophagy inhibition using lysosomal inhibitors decreases the production of ATP that usually occurs upon T cell activation, probably by reducing glycolysis and inhibiting mitochondrial respiration[185,194]. More specifically, autophagy-mediated degradation of cytosolic proteins has recently been demonstrated to provide T cells with L-cysteine, which activates the mTOR complex that drives the metabolic transitions needed upon T cell activation[199].

Regulatory T cells (Tregs) are characterized by a unique metabolic signature that is distinct from effector T cells, *i.e.* they depend less on glycolysis and more on lipid oxidation[200]. Autophagy levels are increased in Tregs compared to other $CD4^+$ T cells and autophagy has been indicated to be involved in reducing glycolytic activity[201,202]. It is therefore not surprising that decreased Treg stability, survival and cellular function, illustrated by the production of pro-inflammatory cytokines, is observed for autophagy-deficient Tregs[186,202]. Another distinct T cell subset that depends on autophagy is invariant natural killer T (iNKT) cells, which display impaired thymic development and function in the absence of autophagyx[186,203].

Altogether, autophagy plays an important role in many T cell subsets and in various T cell-dependent process. Therefore, manipulating autophagy levels in T cells is not a straightforward manner to alter T cell responses and further studies are necessary to better understand the complex role of autophagy in T cells.

AUTOPHAGY AND B CELLS

B-cell specific depletion of *Atg5* leads to a significant reduction of the numbers of a subset of B cells, due to defective pro- to pre-B cell transition[204]. Additionally, a study using *Beclin 1*-deficient B cells suggests that autophagy is required for the maintenance of early B lymphocyte progenitor cells, while the peripheral B cell compartment is not affected[205]. Together, this demonstrates that autophagy is involved in B cell development. Next to this, autophagy has also been implicated in various B cell effector functions. For example, plasma cell differentiation, either T-cell dependently as well as T-cell independently, and the maintenance of memory B cell requires autophagy[206–209].

AUTOPHAGY AND AUTOIMMUNE DISEASES

Given the role of autophagy in thymic selection and T and B cell activation, it is not surprising that autophagy is linked with autoimmunity[148,210]. Here, we will discuss the role of (altered) autophagy in several autoimmune diseases (**Table 2**).

AUTOIMMUNE DISEASE-ASSOCIATED SNPS IN AUTOPHAGY GENES

GWAS have identified the autophagy-associated genes *ATG16L1*, *NOD2*, and immunity-related

Table 2. Overview of autophagy in autoimmune diseases.

Disease	Role of autophagy
CD	Disease-associated SNPs in *ATG16L1*, *IRGM, NOD2*[211,212]
SLE	Disease-associated SNPs in *ATG5* and *DRAM1*[227–229,232] Increased autophagy levels in naïve CD4$^+$ T cells[238] CD4$^+$ T cells are resistant to autophagy induction[238]
RA	Increased autophagy levels in total CD4$^+$ T cells and SF[242,243] Decreased autophagy levels in naïve CD4$^+$ T cells[244] Increased ATG7 and Beclin 1 expression in osteoclasts[253]
PsA	Loss-of-function mutations in AP1S3 in keratinocytes[248] Increased ATG16L1 expression in DCs[252]
MS	Increased *ATG5* expression[245]

AP1S3: adaptor related protein complex 1 sigma 3 subunit, ATG: autophagy-related gene, ATG16L1: autophagy-related gene 16 L1, CD: Crohn's disease, DCs: dendritic cells, DRAM1: DNA damage-regulated autophagy modulator 1, IRGM: immunity-related GTPase family M protein, MS: multiple sclerosis, NOD2: nucleotide-binding oligomerization domain-containing protein 2, PsA: psoriatic arthritis, RA: rheumatoid arthritis, SF: synovial fibroblasts, SLE: systemic lupus erythematosus, SNP: single nucleotide polymorphism.

GTPase family M protein (*IRGM*) as susceptibility genes associated with Crohn's disease, an inflammatory bowel disease (IBD)[211,212]. ATG16L1, NOD2 and IRGM are all involved in the autophagic clearance of intracellular pathogens[213–215]. DCs from patients containing the CD-associated *ATG16L1* (T300A) risk variant or one of the three *NOD2* risk variants both displayed impaired autophagy, bacterial targeting to lysosomes, and antigen presentation to CD4$^+$ T cells[213,216]. Furthermore, complete or partial loss of *Atg16l1* expression in mice results in enhanced susceptibility to experimentally induced IBD, altered expression of certain inflammatory signaling molecules in intestinal Paneth cells, and increased production of IL-1β and IL-18 by macrophages upon LPS stimulation[156,217,218]. Interestingly, the WD-repeat domain where the T300A mutation in ATG16L1 occurs is not present in yeast Atg16 and is indispensable for classical autophagy in mammals, suggesting that alternative effects of ATG16L1 contribute to CD pathogenesis[219–221]. Indeed, interaction of ATG16L with transmembrane protein 59 (TMEM59) induces a non-canonical form of autophagy and the T300A mutation reduced TMEM59-induced autophagy, resulting in defective xenophagy[222,223]. In addition, the T300A mutation leads to increased susceptibility of ATG16L1 to caspase-3 cleavage, thereby decreasing the protein levels of ATG16L1, which results in defective autophagy and pathogen clearance[224,225]. Recently, a non-canonical form of autophagy has been demonstrated to regulate the release of antimicrobial peptides by Paneth cells, providing a possible explanation for the abnormal secretion of antimicrobial peptide-containing granules observed in hypomorphic *Atg16l1* mice and CD patients[226]. The observation of Cadwell and collegues that infection of *Atg16l1* hypomorphic mice with an enteric norovirus was necessary to develop Paneth cell-related abnormalities suggests that a complex interplay between virus and autophagy triggers intestinal

inflammation and provides an explanation why only a proportion of the people harboring a similar risk allele for CD develop disease[218].

In both Chinese and European populations *ATG5* variants have been identified to confer susceptibility to SLE[227–230]. One of these variants has been studied in detail and is associated with decreased IFN-α and increased IL-10 production[231]. Furthermore, DNA damage-regulated autophagy modulator 1 (DRAM1), a gene involved in the tight regulation of autophagy and apoptosis, has been associated with SLE susceptibility[232]. ATG5 has been demonstrated to be required for apoptotic cell clearance *in vivo*[233]. Since SLE is characterized by defects in apoptotic corpse clearance, it can be hypothesized that genetic variation in ATG5 and DRAM1 might contribute to altered regulation of apoptosis[234–236].

ALTERED AUTOPHAGY LEVELS IN AUTOIMMUNE DISEASE PATIENT-DERIVED CELLS

Autophagy is affected in both T and B cells from SLE patients. In lupus animal models an increased number of autophagosomes has been detected in both T and B cells, which is in line with observations in human SLE patient-derived naïve $CD4^+$ T cells and B cells[209,237,238]. SLE patient-derived T cells are resistant to autophagy induction by IgG serum from SLE patients, while this serum induces autophagy in healthy control T cells[238]. Treatment of SLE patients with rapamycin, a known inducer of autophagy via mTOR inhibition, reduced disease activity and restored T cell activation[239]. Conversely, hydroxychloroquine (HCQ), an inhibitor of autophagy, is effectively being used in the clinic to treat SLE patients[240]. A possible explanation for these discrepancies could be that rapamycin and HCQ affect other process besides autophagy, as they are non-specific manipulators of autophagy. mTOR is involved in the regulation of many cellular process and mTOR activation has been linked to the abnormal activation of SLE T lymphocytes[239,241]. Altogether, this suggests that the role of autophagy in SLE pathogenesis is probably context and cell type-specific and warrants further investigation.

RASF display an increased autophagic flux compared to osteoarthritis SF and autophagy induction in RASF seems to have a dual role: induction of autophagy via ER stress leads to the induction of cell death, while autophagy induction via proteasome inhibition prevents apoptosis[242]. Furthermore, autophagy has been demonstrated to be increased in peripheral blood total $CD4^+$ T cells from RA patients and from mice suffering from collagen-induced-arthritis[243]. However, for naïve $CD4^+$ T cells from RA patients a decrease in autophagy has been described, which is linked to a defective switch in metabolism[244]. In both cell populations altered autophagy levels are linked to differences in susceptibility for apoptosis, as autophagy inhibition increased the proportion of apoptotic cells[243,244]. In addition, HCQ treatment of collagen-induced arthritis mice reduces disease severity, indicating that autophagy contributes to the pathogenesis of RA.

ALTERED EXPRESSION OF AUTOPHAGY-ASSOCIATED GENES IN AUTOIMMUNE DISEASE PATIENT-DERIVED CELLS

Increased *ATG5* expression has been observed in multiple sclerosis (MS) patient-derived T cells and *Atg5* expression strongly correlates with clinical severity in experimental autoimmune

encephalomyelitis (EAE), an animal model for MS[245]. Although autophagy levels have not been measured in these studies, this suggests a link between autophagy and MS. Indeed, mice specifically lacking *Beclin 1* or *Atg7* in their $CD4^+$ cells are resistant to EAE induction[187,194]. The protective effect of defective autophagy in EAE models might be related to the inhibitory effect of autophagy on apoptosis induction, suggesting that autophagy inhibition leads to increased apoptosis of autoreactive T cells. Furthermore, *Atg*7 deficiency in DCs and neutrophils also reduces EAE severity, which is attributed to defective antigen presentation or reduced neutrophil degranulation, respectively[246,247]. Also, HCQ treatment has been demonstrated to reduce EAE-related disease symptoms, underscoring the importance of autophagy in the regulation inflammatory demyelination in mice[194,246]. Whether autophagy fulfills a similar role in the human disease setting needs to be further explored.

For keratinocytes of psoriatic arthritis (PsA) patients altered expression of the autophagy-associated protein adaptor related protein complex 1 sigma 3 subunit (AP1S3) has been described[248,249]. AP1S3 is involved in autophagosome generation from the trans-Golgi network and AP1S3 loss-of-function mutations have been associated with a specific form of PsA[250,251]. AP1S3 deletion in keratinocytes severely impairs autophagy and results in the accumulation of p62, a feature that is also characteristic for keratinocytes of PsA patients[248,249]. Furthermore, autophagy is involved in the negative regulation of inflammatory cytokine expression, such as IL-36 and IL-1β, in keratinocytes. This indicates that decreased autophagy in keratinocytes of PsA patients might contribute to the increased secretion of pro-inflammatory mediators. In addition, increased *ATG16L1* expression has been reported in DCs from PsA patients, but whether and how this affects altered DC responses remains to be investigated[252].

ATG7 and Beclin 1 protein levels are increased in RA osteoclasts, indicative of increased autophagy in these cells[253]. Genetic or pharmacologic inhibition of autophagy reduces osteoclast differentiation *in vitro*[253,254]. Furthermore, $ATG7^{fl/fl}$ *LysM* Cre^+ transgenic mice demonstrate reduced bone destruction in TNFα-induced arthritis, which might be due to the decreased expression of IL-1β and IL-6[253]. Moreover, autophagy is involved in the formation of the osteoclast ruffled boarder and the secretion of bone-degrading enzymes in the resorption lacuna[255].

Altogether, these data illustrate that precise regulation of autophagy and expression of autophagy-associated genes is critical for immune-homeostasis. Furthermore, dysregulation of autophagy or altered expression of autophagy-associates genes can contribute to the development of autoimmunity.

OUTLINE OF THIS THESIS

Autoimmune diseases are complex diseases and the various molecular mechanisms that contribute to autoimmune pathogenesis as still poorly understood. The work described in this thesis is aimed to create insight into molecular mechanisms underlying autoimmune diseases, focusing on epigenetic regulation and autophagy.

In **Chapter 2** the role of enhancers in the pathogenesis of autoimmune arthritis is reviewed and it is discussed whether inhibition of enhancer activity might be a therapeutic strategy for the treatment of autoimmune arthritis. In **Chapter 3** it is demonstrated that autoimmune disease-derived T cells display a distinct enhancer profile compared to healthy control cells and that arthritis-associated SNPs are enriched within these enhancer regions. Furthermore, it is demonstrated that this altered enhancer profile correlates with altered gene expression and that inhibition of enhancer activity results in reduced disease-associated gene expression. In **Chapter 4** monocytes derived from the site of inflammation from autoimmune disease patients were analyzed at the epigenetic and transcriptomic level. The increased epigenetic regulation and expression of osteoclast differentiation-associated genes led to the observation that JIA synovial fluid can induce the differentiation of monocytes towards osteoclasts. In **Chapter 5** autophagy is demonstrated to be increased in JIA synovial fluid-derived T cells and autophagy inhibition reduced the inflammatory phenotype of these cells, suggesting that autophagy contributes to the activated phenotype of JIA synovial fluid T cells. To identify novel regulators of autophagy, genome-wide transcriptomic and epigenomic profiling of nutrient-deprived cells was performed, which is described in **Chapter 6**. EGR1 is identified as a transcriptional regulator of autophagy, since it induces autophagy-associated gene expression and enhances autophagic flux. In **Chapter 7** the findings described in this thesis are discussed, speculating how this contributes to a better understanding of autoimmune disease pathogenesis and whether inhibition of enhancer activity and autophagy inhibition might be a potential therapeutic strategy for the treatment of autoimmune diseases.

Taken together, the studies described in this thesis provide novel insight into transcriptional and epigenetic mechanisms in an autoimmune disease setting and demonstrate that altered enhancer regulation and autophagy is associated with autoimmunity. Furthermore, these findings indicate that targeting these molecular mechanisms might be of interest for the treatment of autoimmune diseases.

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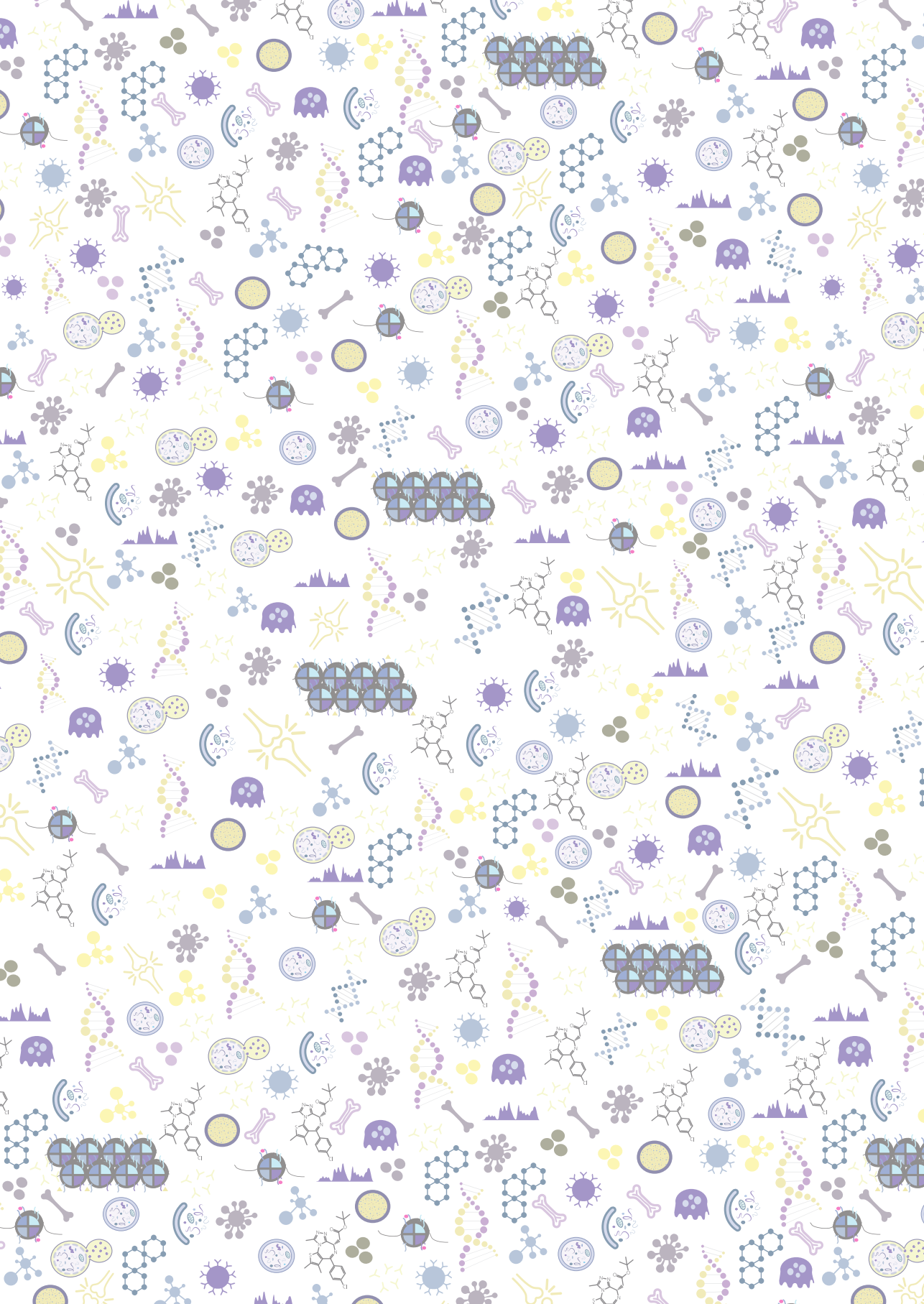

Enhancers in autoimmune arthritis: implications and therapeutic potential

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INTRODUCTION

Genome-wide association studies (GWAS) have identified hundreds of single nucleotide polymorphisms (SNPs) associated with autoimmune diseases (AIDs), including autoimmune arthritis (AA). So far, it has proven difficult to translate these findings into disease understanding and novel therapeutic approaches, as the majority of these SNPs are not located in protein coding regions. Recently, various studies found that a large amount of AID-associated SNPs affect DNA regulatory units, suggesting that altered epigenetic control of transcription is an important process in disease pathogenesis[1,2]. Technical developments have allowed for detailed analysis of the epigenetic profile of disease-associated cells, creating new opportunities to study gene regulation in the context of disease. In this review, we will discuss these advances in the field of epigenetics, focusing on (super-)enhancers associated with AA. Furthermore, we will describe how enhancer profiling of disease-specific cells can contribute to better understanding of disease pathogenesis. Additionally, we will outline strategies that can target enhancer activity and discuss their potential use as therapeutic approaches in the treatment of AA.

GENETIC BASIS OF AUTOIMMUNE ARTHRITIS

The genetic basis of AA has been studied extensively, especially in the last decade. Advances utilizing high-throughput genome sequencing have identified multiple risk variants associated with various rheumatic diseases, including rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), systemic lupus erythematosus (SLE), ankylosing spondylitis (AS), and psoriatic arthritis (PsA)[3–8]. Although these studies provide some important clues about the biological pathways that might be affected, novel insights regarding the molecular function and role in disease pathogenesis remain limited. For example, genome-wide significant loci, including the MHC loci, and regions with suggestive associations can only explain 18% of the risk for JIA[4].

This is mainly because of two different reasons, the first being the difficulty to define which SNP is the disease-causal variant. Disease-associated loci identified by GWAS contain numerous SNPs. This is due to linkage disequilibrium (LD), the non-random association between two alleles of different loci. Therefore, disease-causal variants are often surrounded by neutral or other disease-causal variants, making it difficult to pinpoint the candidate disease-causal SNP(s)[9]. Improvements in identifying disease-causal variants have recently been made by several groups by developing algorithms that take into account either cell type or tissue specific epigenomic information (e.g. probabilistic identification of causal SNP [PICS], EPIGWAS, Risk Variant Interference using Epigenomic Reference Annotation [RiVIERA], Robust Allele Specific QUAntitation and quality controL [RASQUAL]), (predicted) gene function and expression data (Data-driven Expression-Prioritized Integration for Complex Traits [DEPICT]), microRNA (miRNA)–target gene networks (miRNA–target gene enrichment analysis in GWAS [MIGWAS]), or genome-wide information from all SNPs, instead of a restricted SNP set, together with explicit modeling

of LD[2,10–15]. For example, the PICS algorithm demonstrates that only 5% of the SNPs that were originally thought to be disease-causing are actually assigned as being disease-causal variants[2]. This indicates that genetic fine mapping of AID variants will further unravel the genetic basis of autoimmunity.

The second reason that it has been difficult to translate GWAS findings into disease understanding is that approximately 90% of disease-associated SNPs are located outside of protein-coding regions, in regulatory DNA regions, making it difficult to understand which gene(s) are affected and how[1,2]. As these regulatory DNA regions are epigenetically regulated, a better understanding of the epigenetic landscape is needed to understand the contribution of genetic variation to autoimmunity.

ENHANCERS AND SUPER-ENHANCERS

To fit the approximately two-meter-long DNA strand in the nucleus DNA is tightly packed. DNA is wrapped around the histone proteins H2A, H2B, H3, and H4, two of each type, thereby forming a nucleosome, and creating the chromatin structure. The N-terminal tail of histones can be covalently modified. Generally, methylation allows tight packing of nucleosomes, rendering the DNA inaccessible. Acetylation reduces the positive charge of the histone tail, thereby reducing the interaction with the negatively charged DNA, allowing enzymes and transcription factors to bind[16] (**Figure 1**). Regulatory DNA regions are characterized by deoxyribonuclease I (DNase I) hypersensitivity sites (DHS), meaning that in these regions DNA can be cleaved by DNase I, indicating a loose chromatin structure. Of the SNPs located in regulatory DNA regions, the majority localizes to so-called enhancers[10,17]. Enhancers are cis-regulatory DNA elements to which transcription factors and co-factors can bind and are crucial for transcriptional regulation. By recruitment of RNA polymerase II and Mediator complex to the promoter of their target gene, enhancer elements regulate gene expression (**Figure 2A**).

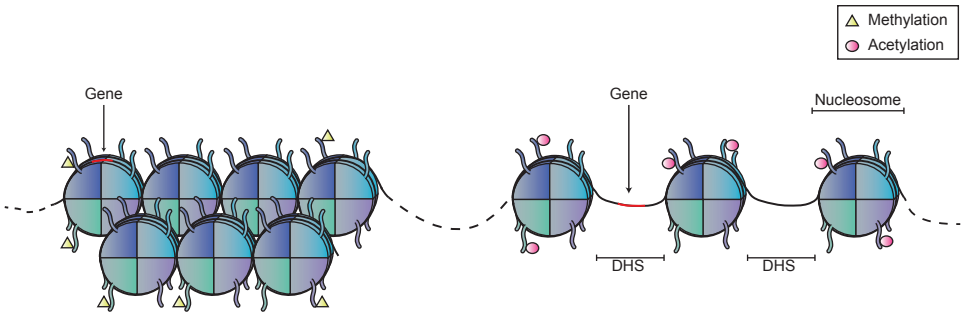

Figure 1. Schematic representation of chromatin structure.
DNA is wrapped around an octamer of histone proteins, together forming a nucleosome. Nucleosomes can be tightly packed, rendering the DNA inaccessible (left), or can have a more loose structure, allowing transcription factors to bind (right).

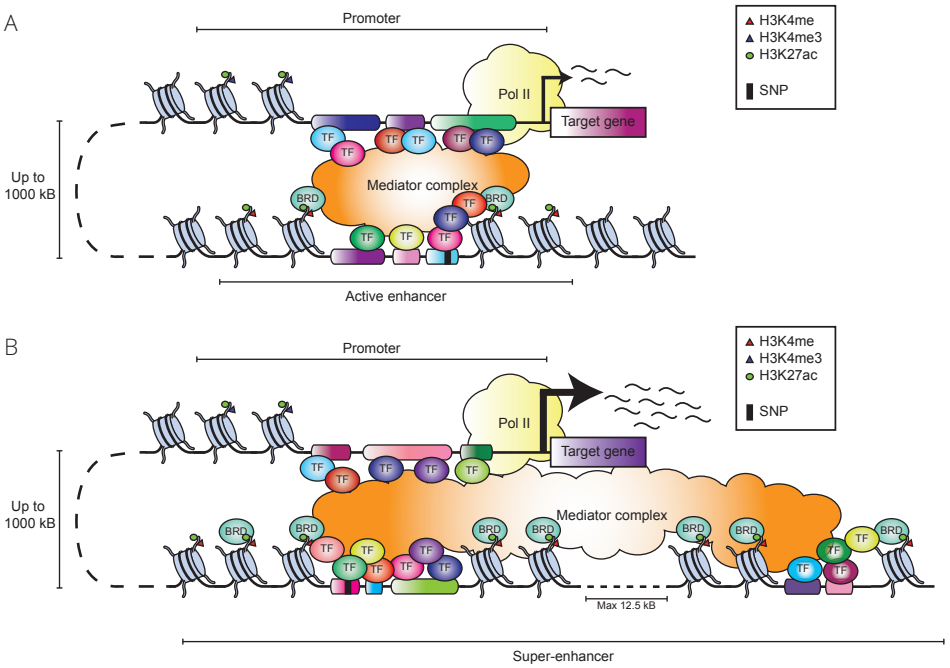

Figure 2. Schematic representation of transcriptional regulation by enhancers and super-enhancers. (**A**) Gene expression of a target gene driven by an active enhancer. Active enhancers are characterized by methylation and acetylation of histone H3, on lysine 4 and 27, respectively. Enhancers facilitate transcription by transcription factor (TF) binding, recruitment and binding of bromodomain and extraterminal proteins, consisting of bromodomain-containing proteins (BRDs), the mediator complex, looping of the DNA, and RNA Polymerase II (Pol II) recruitment. SNPs are enriched in regulatory DNA regions, for example enhancers. Enhancers can be located up to 1000 kB from their target gene. (**B**) Gene expression of a target gene driven by a super–enhancer (SE). SEs are large enhancers characterized by extensive acetylation of histone H3 at lysine 27 and increased binding of the mediator complex and transcription factors. Active enhancers within 12.5 kB of each other can together form a super-enhancer, leading to increased gene expression. Super-enhancers are enriched for SNP localization compared to active enhancers. H3K4me3 = trimethylation of histone H3 at lysine 4.

Enhancers are generally a few hundred base pairs in size, contain multiple transcription factor binding sites, and can be located up to 1000 kb upstream or downstream of the promoter of their target gene[16]. The dispersion of enhancers throughout the genome and the 3D chromatin conformation make it difficult to define which gene a certain enhancer is regulating. Generally, epigenomic studies assume that enhancers regulate the gene whose transcriptional start site is closest to the enhancer. For a more precise understanding of the gene regulated by a certain enhancer, chromosome conformation capture techniques (3C-based technologies) are available which enable capturing of the physical interactions between enhancers and promoters[18]. These technologies indicate that 27-40% of the active enhancers indeed interact with their nearest promoter, suggesting that 3C-based technologies are pivotal for understanding the epigenetic landscape[19,20].

Enhancers that are permissive for transcriptional regulation but are not active, i.e. inactive/poised enhancers, are characterized by mono-methylation of histone H3 at lysine 4, i.e. H3K4me, while active enhancers contain both H3K4me and acetylation of histone H3 at lysine 27, i.e. H3K27ac[21,22]. In any mammalian cell type, tens of thousands enhancers can be found, exceeding the amount of protein-coding genes (~20.000), indicating that a single gene can be regulated by multiple enhancers within one cell and that the enhancer(s) regulating a certain gene can differ between cells[19,23].

In addition, a number of studies have recently identified extremely large enhancer domains, containing clusters of individual enhancers, termed super-enhancers (SEs) or stretched enhancers[24–26]. Active enhancers within 12.5 kb of each other can together form a SE (**Figure 2B**). SEs are identified by extensive H3K4me, H3K27ac, p300 binding, Mediator complex occupancy, and increased DHS and are characterized by increased transcription factor binding. SE-regulated genes are expressed to a higher extent than genes driven by a regular enhancer[26]. This increase in gene expression seems to be due to synergy between individual enhancer constituents within a SE, rather than being the sum of individual enhancers[27–29]. However, as some SE are single enhancers and enhancers within 12.5 kB of each other do not necessarily form a SE, it remains questionable whether SEs are a novel concept in gene regulation, or whether they are more a reflection of the characteristics of their individual enhancer constituents, such as a different sequence composition leading to increased transcription factor binding and increased transcriptional activity[30]. SEs preferentially regulate genes important for cell identity as well as genes associated with disease. For example, in multiple cancer subtypes SEs are associated with tumor oncogenes[24,31]. Altogether, this suggests that understanding the role of (super-)enhancers in immune cell function will help in defining their function in disease pathogenesis. For AA, it seems logical to analyze the importance of (super-)enhancers in cells from both the adaptive and innate immune system.

ENHANCERS IN IMMUNE CELL FUNCTION

The diverse properties of different cell types within the immune system are reflected within their enhancer landscape, demonstrating that enhancers can be highly cell type specific. This is illustrated by the distinct enhancer landscape of naïve $CD4^+$ T cells versus differentiated T cell subsets[32,33]. STAT proteins are pivotal for establishing subset-specific enhancers, with STAT1 and STAT4 being important for the generation of Th1-specific enhancers, while STAT6 shapes Th2-specific enhancers[33]. T cell SEs are preferentially associated with cytokines and cytokine receptors, for instance the T cell subtype-specific genes *Ifng, Il13,* and *Il17a* are associated with a SE in mouse Th1, Th2, and Th17 cells, respectively[34,35]. The strongest SE in $CD4^+$ Th1, Th2, and Th17 cells is linked to *Bach2*, a suppressor of T effector cell differentiation. *Bach2* deletion reduces expression of other T cell SE-associated genes, suggesting the presence of a key regulatory node in T cells driven by BACH2[35]. Also in B cells BACH2 is associated with a SE, highlighting the important role of this gene in adaptive immune cell regulation[24].

The innate immune response, with monocytes and macrophages being important mediators, is characterized by the rapid expression of a subset of genes 0.5-2 hours after stimulation, i.e. primary response genes, and expression of another gene subset 2-8 hours after stimulation, i.e. secondary response genes[36]. These characteristics are reflected within the enhancer landscape of innate cells. Namely, TLR4 stimulation leads to the acquirement of H3K27ac by some poised enhancers, reduction or complete loss of certain enhancers, and reduction of the strength of a subset of enhancers already present in unstimulated conditions [37,38]. These epigenetic alterations are rapid and thus likely to be associated with expression of early primary response genes. TLR4 stimulation also induces the formation of approximately thousand *de novo* enhancers. As *de novo* enhancer formation takes time and involves nucleosome remodeling, which is known to be required for secondary response gene expression, these enhancer are linked to secondary response genes[39].

In addition to LPS, the proinflammatory cytokine tumor necrosis factor (TNF), via activation of nuclear factor κB (NF-κB), can shape the enhancer repertoire[40]. This occurs mainly via *de novo* SE formation and drives proinflammatory gene expression. Since some of these proinflammatory genes are cytokines, this implies a (positive) feedback loop where cytokines can affect the enhancer profile in an autocrine and paracrine fashion. Similar effects of the (local) microenvironment on the enhancer landscape are described for tissue-resident macrophages. For example, transplantation of macrophages into distinct tissues of recipient mice leads to the acquirement of an enhancer profile comparable to that of recipient tissue-resident macrophages[41,42]. Taken together, these studies illustrate that the microenvironment drives selection and function of enhancers and thereby regulates cellular identity and plasticity. This implies that it might be crucial to analyze the enhancer profile of cells directly after isolation from their tissue, as this reflects their enhancer landscape within the microenvironment. Especially for AA, where the microenvironment is one of the main drivers of the inflammatory cellular phenotype, this is important to take into account.

ENHANCER REGULATION IN AUTOIMMUNE ARTHRITIS

Given the critical role of (super-)enhancers for proper immune cell function, it is almost inevitable that they play a significant role in AA as well. However, the role of enhancers in immune-related diseases has hardly been investigated. The importance of enhancers for autoimmunity is underscored by the enrichment of AID-associated SNPs in enhancer regions of immune cells of healthy controls (HCs), with the highest enrichment in SEs compared to regular enhancers[2,24,25,35]. For example, RA-associated non-coding SNPs are 3.2x more enriched in SEs and 2.2.x more in regular enhancers compared to other DNA regulatory regions. In addition, disease-associated variants preferentially map to (super-)enhancers that are specific for disease-relevant cell types[24,25,35]. For instance, the majority of RA-associated SNPs map to T cell, NK cell, and B cell (super-)enhancers[43]. For SLE, SNPs are predominantly located within B cell SEs, underscoring their important role in SLE pathogenesis[24]. It might therefore be informative to

map disease-associated SNPs to the (super-)enhancer profile of different immune cell (subsets) for not so-well characterized diseases, as this can reveal cell type(s) involved in the disease.
Since approximately 90% of disease-associated SNPs are located in non-coding regions and 60% of these SNPs map to enhancers, disease-associated SNPs might affect gene transcription[1,2]. Indeed, SNPs present in DHS are four times more likely to have an effect on gene expression compared to SNPs located outside DHS[44]. Nonetheless, due to the presence of multiple SNPs in LD in a risk locus and the difficulty to define which of these SNPs is causal for the disease, it is difficult to determine exactly the transcriptional effect of a SNP within a regulatory region. The effect of genetic enhancer variants on transcription is thought to be relatively small, with reports ranging from 1.3 – 2 fold difference in target gene expression[45]. Although modest, these differences can play important roles in disease pathogenesis.
For example, disease-associated SNPs are enriched at expression quantitative trait loci (eQTLs), which are genomic regions containing DNA variants that affect gene expression and that thus might alter the immunophenotype[46]. A large proportion of eQTL SNPs is present around the transcription start site, and therefore likely to affect the promoter region[47]. So far, eQTL studies have mostly been focused at promoter biology, but enhancer eQTL data is starting to emerge. For example, eQTL SNPs are enriched in DHS and regions characterized by active histone marks, with a strong enrichment for H3K27ac regions 5 to 100 kB upstream from the transcription start site, suggesting enrichment within enhancer regions[44]. A more recent study, looking at SNPs associated with heritable differences in peripheral blood gene expression, reports that ~9% of the eQTL SNPs is located within promoters and ~14% within enhancers, suggesting that enhancer eQTL SNPs are important to take into account[2]. However, considering that 60% of non-coding variants map to enhancers, this also suggests that a large proportion of enhancer SNPs do not map to eQTLs, and it remains to be investigated if and how these variants affect gene expression. There are some suggestions, for example for RA and SLE, that these diseases cannot be explained by one SNP, but that genetic variants within clusters of enhancers present at risk loci together affect gene expression and therefore confer to disease susceptibility, but this needs to be further investigated[48].
For autoimmune vitiligo, three SNPs are located within a MHC class II SE, which also corresponds to an eQTL for HLA-DR and HLA-DQ expression. The presence of these SNPs correlates with increased HLA-DR and HLA-DQ surface expression and increased cytokine production, illustrating how SNP localization in a specific genomic region can contribute to the development of autoimmunity[49]. Similarly, two SLE-associated SNPs are located in an enhancer element downstream of the *TNFAIP3* promoter[50]. *TNFAIP3* encodes for A20, an inhibitor of NF-κB signaling. The presence of these two variants impairs NF-κB binding to the enhancer, thereby reducing promoter-enhancer interaction, leading to reduced A20 expression[50]. This results in increased NF-κB signaling and thus supports a causal role for the SNP pair in SLE pathogenesis. For AS a possible disease-causal SNP has been identified within an enhancer between *IL23R* and *IL12RB2*[51]. The presence of this SNP corresponds with reduced H3K4me of the enhancer region in $CD4^+$ T cells, impaired binding of nuclear proteins to the SNP-containing DNA region, and

reduced reporter activity. However, expression of *IL23R* and *IL12RB2* is not affected, although the frequency of Th1 cells, which express *IL12RB2*, is altered. A possible explanation for this discrepancy could be that the SNP-containing enhancer is not regulating *IL23R* and *IL12RB2* expression but has another, yet unidentified, target gene affecting Th1 cell numbers. 3C-based technologies could help in unraveling the biological effect of this particular SNP and other disease-associated SNPs that have so far not been ascribed a molecular function.

A confounding factor in linking GWAS data with epigenomics data, is that the epigenetic data being used are predominantly based on HC cells and might not represent the epigenetic landscape of disease-relevant, patient-derived cells. Additionally, for most diseases it is more informative to profile cells from the affected tissue than cells from the peripheral circulation. Indeed, JIA (super-)enhancer profiling of synovial-derived $CD4^+$ T cells revealed a different profile compared to peripheral blood HC cells[52]. Similar observations have been made in SLE-derived monocytes[53]. eQTL mapping is generally also performed using HC samples, however, recently eQTLs were mapped using a RA cohort[54]. This revealed that RA GWAS hits are enriched in RA-identified eQTLs compared to HC eQTLs and that RA eQTLs are enriched in enhancer regions. Furthermore, combination of this eQTL data with GWAS and epigenomics data identified novel disease-relevant genes[54]. Consistent with these observations, arthritis-associated SNPs are more enriched in JIA SEs compared to HC SEs[52].

These findings underscore the importance of utilizing patient-derived cells and illustrate that integrating multiple datasets can be more informative than a single dataset in identifying AA-associated molecular mechanisms. In addition, comparing the enhancer profile of different AIDs can be useful in unraveling AID pathogenesis. This is of particular interest since AID-associated SNPs are profoundly enriched in JIA T cell SEs, while this is not the case for non-AID-associated SNPs[52]. Correspondingly, it has been reported that AIDs have shared risk loci, suggesting overlap in disease pathogenesis[2]. In addition, enhancer data may also define distinct pathogenic processes between diverse types of AA. For example, RA synovial fibroblasts (RASF) are distinguishable from osteoarthritis-derived SF based on DNA methylation and transcriptome data[55]. Furthermore, these data can also discriminate between RASF isolated from different joints. These observations suggest that joint-specific epigenetic signatures exist and thus indicate that molecular pathways affected in the disease might differ from one joint to another. Epigenetic profiling can be used to identify these differences and more importantly these observations raise the question whether epigenetic knowledge can be translated into novel therapeutic strategies for the treatment of AA.

INHIBITION OF ENHANCER ACTIVITY IN RELATION TO AUTOIMMUNE ARTHRITIS

The observations that AA-associated SNPs are preferentially located in enhancers and that SEs are associated with disease-relevant genes strongly suggest that enhancers directly contribute to disease pathogenesis. Therefore, there is a growing body of interest in the development of

therapeutic strategies aimed at targeting (super-)enhancer activity. Enhancer regions are critically dependent on chromatin regulators (e.g. reader proteins recognizing histone modifications), which recruit transcription factors and other proteins to facilitate transcription initiation and elongation. Important reader proteins at enhancers are bromodomain and extra-terminal (BET) proteins, consisting of bromodomain-containing protein 2 (BRD2), BRD3, BRD4, and bromodomain testis-specific protein (BRDT), each containing two bromodomains (Figure 2)[56]. The bromodomain allows BET proteins to bind to acetylated histone and non-histone proteins. BRD4 is most extensively studied and is present at promoters and active enhancers, with increased localization at SEs[31,57]. Binding of BET proteins to acetylated transcription factors might contribute to their preferential localization in (super-)enhancer regions[58].

Recently, a wide range of small molecule inhibitors of BET proteins has been developed (**Table 1**)[59]. The therapeutic potential of BET inhibitors has been demonstrated in a numerous *in vitro* and *in vivo* tumor models, where BET inhibitors inhibit the expression of different oncogenes, including c-*myc*[58,60]. Inhibition of c-*myc* is linked to the disruption of c-*myc*-associated SE activity[31]. This is probably related to the high levels of BRD4 present at SEs and the transcriptional dependency thereof and thus contributes to the preferential inhibition of SE-driven gene expression by BET inhibitors.

BET inhibitors are highly effective in shaping the adaptive immune response. For example, the BET inhibitors JQ1 and I-BET762 significantly impair differentiation of naïve $CD4^+$ T cells into T effector cell subsets, both *in vitro* and *in vivo*[61,62]. Furthermore, BET inhibition of differentiated $CD4^+$ T cells has a profound effect on cytokine production, such as IL-17. BET inhibition also impairs B cell function by inhibiting Ig class switch recombination and B cell expansion and proliferation[63,64]. Additionally, BET-inhibitors can affect innate immune responses. For instance, JQ1, I-BET762, and I-BET151 all inhibit proinflammatory cytokine production by LPS-stimulated monocytes and macrophages *in vitro* and are effective in different *in vivo* animal models[65–69]. Genes affected by I-BET762 in LPS-stimulated macrophages belong to the secondary response genes. Given the epigenetic differences underlying the expression of these genes, this suggests that I-BET762, and possibly other BET inhibitors as well, preferentially acts on *de novo* enhancers[68]. This corresponds with the observation that *de novo* SEs, induced by TNF-α and LPS, are highly susceptible to BET inhibition, resulting in decreased SE-mediated gene expression[37,40].

Inflammation-associated diseases are characterized by proinflammatory mediators present at the site of inflammation, suggesting that BET inhibition might be a way to specifically target cytokine-induced, and thus disease-associated, (super-)enhancers. Indeed, JQ1 treatment of JIA synovial-derived $CD4^+$ T cells preferentially reduces expression of disease-associated genes, with the majority being involved in proinflammatory and cytokine-related processes[52,70]. Correspondingly, I-BET151 treatment of RASF suppresses the production of matrix metalloproteinases (MMPs) and cytokines after stimulation with TNF-α, IL-1b or TLR ligands, resulting in reduced proliferation and chemo-attractant properties[71]. Similar observations are obtained by genetically silencing BET proteins in RASF[72].

Table 1. Overview of small molecules affecting enhancer activity.

Small molecule	Target	Effect on the immune system
CP1-0610	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1	Not assessed
DRB	CDK9	Inhibition of T cell priming under Th2 and Th17 conditions[62]
I-BET151	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1 and BD2	Inhibition of proinflammatory gene expression in LPS-stimulated monocytes and macrophages[66,69] Inhibition of inflammatory genes and matrix degrading enzymes in RASF[71] Suppression of inflammatory-induced arthritis, TNF-induced bone resorption, and EAE[66,74]
I-BET762	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1 and BD2	Inhibition of macrophage and $CD4^+$ T cell cytokine production[62,67,68] Suppression of EAE[62]
JQ1	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1 and BD2	Inhibition of proinflammatory cytokine production in macrophages and T cells[61,65,75] Inhibition of DC maturation[86] Inhibition of T cell differentiation[61] Inhibition of Ig class switch recombination and mitogenesis in B cells[63,64] Inhibition of cytokine production in JIA $CD4^+$ T cells[52,70] Suppression of CIA, EAE, psoriasis and endotoxic shock in mouse models[61,65,72,73]
LY294002	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1	Inhibition of inflammation in LPS-stimulated PBMCs[87]
MS417	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1 and BD2	Inhibition of HIV-associated kidney disease[88]
Olinone	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1	Not assessed
OTX015	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1 and BD2	Disruption of HIV-1 latency[89]
PC579	CDK9	Suppression of CIA[80]
PC585	CDK9	Suppression of CIA[80]
PFI-1	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1 and BD2	Inhibition of IL-1β-induced inflammation in airway epithelial cells[90]
RVX-208	BRD2, BRD3, BRD4, BRDT Bromodomain: BD2	Not assessed
RX-37	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1 and BD2	Not assessed
TEN-010	BRD2, BRD3, BRD4, BRDT Bromodomain: BD1 and BD2	Not assessed
THZ1	CDK7	Not assessed
Tofacitinib	JAK	Inhibition of RA-associated risk genes in HC $CD4^+$ T cells[35] Inhibition of TLR-induced cytokine gene expression in macrophages[67]

CIA: collagen-induced arthritis, DC: dendritic cell, EAE: experimental autoimmune encephalomyelitis, HC: healthy control HIV: human immunodeficiency virus, JIA: Juvenile Idiopathic Arthritis, RA: Rheumatoid Arthritis, RASF: Rheumatoid Arthritis synovial fibroblasts, PBMCs: peripheral blood mononuclear cells.

These data indicate that BET inhibition can be a putative therapeutic approach for the treatment of AIDs. This has been tested using several *in vivo* AID models. For instance, JQ1, I-BET151, or I-BET762 treatment of experimental autoimmune encephalomyelitis (EAE; an animal model for multiple sclerosis), and an *in vivo* psoriasis model, significantly reduces the onset and severity of disease symptoms[61,62,66,73]. These results are associated with the preferential suppression of IL-6 and IL-17 production and Th17 cell numbers. The protective effect of BET inhibitors has also been explored in AA mouse models. For example, in serum-induced and collagen-induced arthritis (CIA) models JQ1 and I-BET151 dramatically reduce disease progression and plasma and serum cytokine levels[61,72,74]. Furthermore I-BET151 inhibits the differentiation of monocytes towards osteoclasts *in vitro* and reduces TNF-α-induced bone resorption *in vivo*. This is in agreement with observations that JQ1 inhibits receptor activator NF-κB ligand (RANKL)-induced upregulation of osteoclast-associated genes[75]. Since osteoclast formation and bone resorption are related to AA, this implies a dual role for BET inhibitors in the treatment of AA. As TNF-α and IL-6 can induce osteoclast differentiation, the effect of BET inhibition on osteoclast differentiation is likely to result from a combination of direct as well as indirect mechanisms of action, the latter via inhibition of TNF-α and IL-6 production by cells in the synovial compartment[76]. Of note, three SNPs associated with RA are present in BRD2[77]. It might be informative to study the effects of these SNPs on regulation of gene transcription, disease, and susceptibility to BET inhibitors.

In addition to BET inhibitors, some other compounds could be used to inhibit enhancer activity, for instance cyclin-dependent kinase (CDK) inhibitors specific for CDK7 or CDK9. CDK7 and CDK9 are both implicated in transcription initiation and elongation by phosphorylation of RNA Polymerase II[78]. CDK7 inhibition, via the small molecule THZ1, affects SE-driven gene expression in Myc-dependent tumor models[79]. A recent study provides evidence for the use of CDK inhibitors in AIDs as well, as CDK9 inhibition delays disease onset and reduces disease symptoms in a CIA model[80]. Whether this effect is mediated via (preferential) inhibition of SEs is not investigated and remains to be further explored. Comparison of the effect of CDK9 inhibition and BET inhibition has revealed some differential effects on T cell priming, suggesting that different inhibitors of enhancer activity can have distinct properties[62]. Therefore, the therapeutic potential of these compounds might differ between different disease types, probably reflecting differences in the underlying disease mechanisms.

The observed enrichment of STAT proteins at T cell (super-)enhancers prompted researchers to investigate the effect of the Janus kinase (JAK) inhibitor tofacitinib, which is also approved for the treatment of RA as second-line therapy. *In vitro* treatment of T cells with tofacitinib dramatically alters gene expression, with a more drastic effect on genes driven by SEs compared to regular enhancers[35]. Since the majority of RA-associated SNPs are located within T cell SEs, tofacitinib treatment of RA T cells could result in selective targeting of RA-associated risk genes, underscoring its potential for the treatment of RA. Considering the (high) levels of acetylation and methylation associated with (super-)enhancer activity, it could be speculated that targeting enzymes affecting acetylation and methylation of specific histone sites might be an alternative

way to impair enhancer activity. For example, GSK-J4, an inhibitor of the H3K27me3 demethylases KDM6A and KDM6B impairs the LPS-induced inflammatory response in both HC and RA patient-derived macrophages[81]. Furthermore, GSK-J4 suppresses *in vitro* Th17 differentiation and has been demonstrated to suppress EAE via the induction of tolerogenic dendritic cells[82,83]. A possible explanation for this observation could be that decreased demethylation of H3K27 in enhancer regions renders these enhancers inactive, reducing the likelihood of acquiring acetylation and thus becoming active. Therefore, the immunomodulatory effect of GSK-J4 could be mediated via reduction of enhancer activity, but whether this is actually the case needs to be further explored.

FUTURE PERSPECTIVE

In this review, we have discussed the recent advances in the field of epigenetics, focusing on enhancers, and their implications for AA. Epigenetic regulation of immune cells is essential for proper cell function and therefore crucial for mounting an appropriate immune response. It is becoming more and more evident that alterations related to (super-)enhancers are linked to autoimmunity, including AA.

For a better understanding of enhancers in the context of AA, it is crucial to perform enhancer profiling of disease-relevant, patient-derived cells. As enhancers are highly cell type specific, it is pivotal that the enhancer landscape is defined in specific cell types and not in a mixed population of cells, as this will affect interpretation of the results. Since patient material is often limited, one of the challenges will be to obtain a sufficient number of cells. Another important factor to take into account is patient stratification, as distinct disease subtypes can have different enhancer landscapes. From another perspective, enhancer profiling could actually assist in patient classification into existing disease subtypes and may be used for the identification of novel disease subtypes. For example, in the case of JIA, where the designation of disease subtypes is still on clinical ground, this might be useful. Furthermore, it is important to know exactly what the target gene(s) of each enhancer are. Integration of chromosome conformation information with GWAS data will help in ascribing a molecular function to GWAS hits. This will contribute to better insight into the (shared) pathogenesis of AA and might lead to the identification of novel therapeutic targets. In addition, enhancer profiling might unravel novel biomarkers that could be used to predict therapeutic responses. This information could be extremely relevant when aiming for more personalized or stratified treatment regimens for AA. The increased localization of SNPs in enhancer regions and the observations that proinflammatory cytokines can shape the enhancer repertoire suggest that AA-associated enhancer alterations can be a cause as well as consequence of the disease and it might be difficult to distinguish one from another. Furthermore, this indicates that (self)regulatory feedback loops can contribute to disease pathogenesis and thus suggests that it is important to disrupt these regulatory loops (**Figure 3**). This can be achieved by inhibition of (super-) enhancer activity, e.g. by BET-inhibitors. Compared to treatment with biologicals, BET-inhibitors

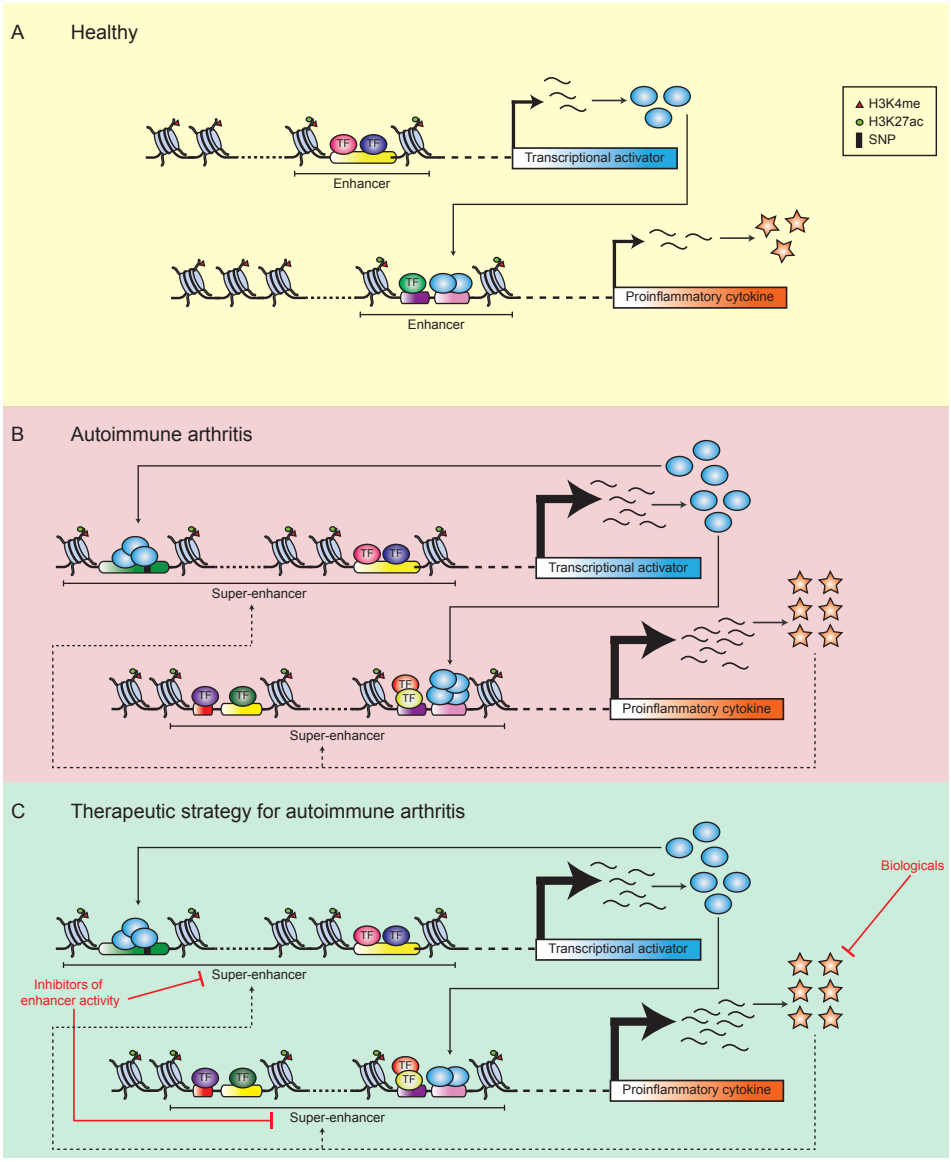

Figure 3. Schematic representation of the role of (super-)enhancers in autoimmune arthritis.
(**A**) In a healthy situation, enhancers regulate gene expression of proinflammatory cytokines and transcriptional activators, leading to controlled gene transcription. (**B**) In autoimmune arthritis, SEs contribute to proinflammatory cytokine expression. Proinflammatory cytokines can shape the (super-)enhancer repertoire, thereby creating self-regulatory loops and stimulating expression of transcriptional activators. Transcriptional activators can function in a regulatory feedback loop by regulating their own expression and that of proinflammatory cytokines. (**C**) Inhibitors of enhancer activity can inhibit (super-)enhancers, thereby reducing proinflammatory gene expression. Biologicals can inhibit the mechanism of action of proinflammatory cytokines, thereby disrupting the regulatory feedback loop.

might be favorable, as they preferentially inhibit the expression of numerous disease-associated genes at once and inhibit the production of proinflammatory cytokines instead of inhibiting or blocking them after production. It could also be argued that combination therapy of BET inhibitors and biologicals might be an even more powerful strategy, as biologicals will prevent (super-)enhancer formation by the inflammatory environment and thereby contribute to disruption of the regulatory feedback loop.

Although BET inhibitors seem highly effective in *in vivo* AA models, it remains to be established whether they will be as efficacious in a human AID setting. So far, the use of BET-inhibitors in clinical trials for the treatment of severe cancers seems to be promising and with limited side effects, such as thrombocytopenia, gastrointestinal adverse effects, and fatigue[84,85]. However, it needs to be determined whether these side effects are acceptable for AA patients. In line with this, development of compounds with a higher selectivity for individual BET proteins, bromodomains, or novel targets will allow for more selective modulation of (super-)enhancer activity and thus for a more specific therapeutic application.

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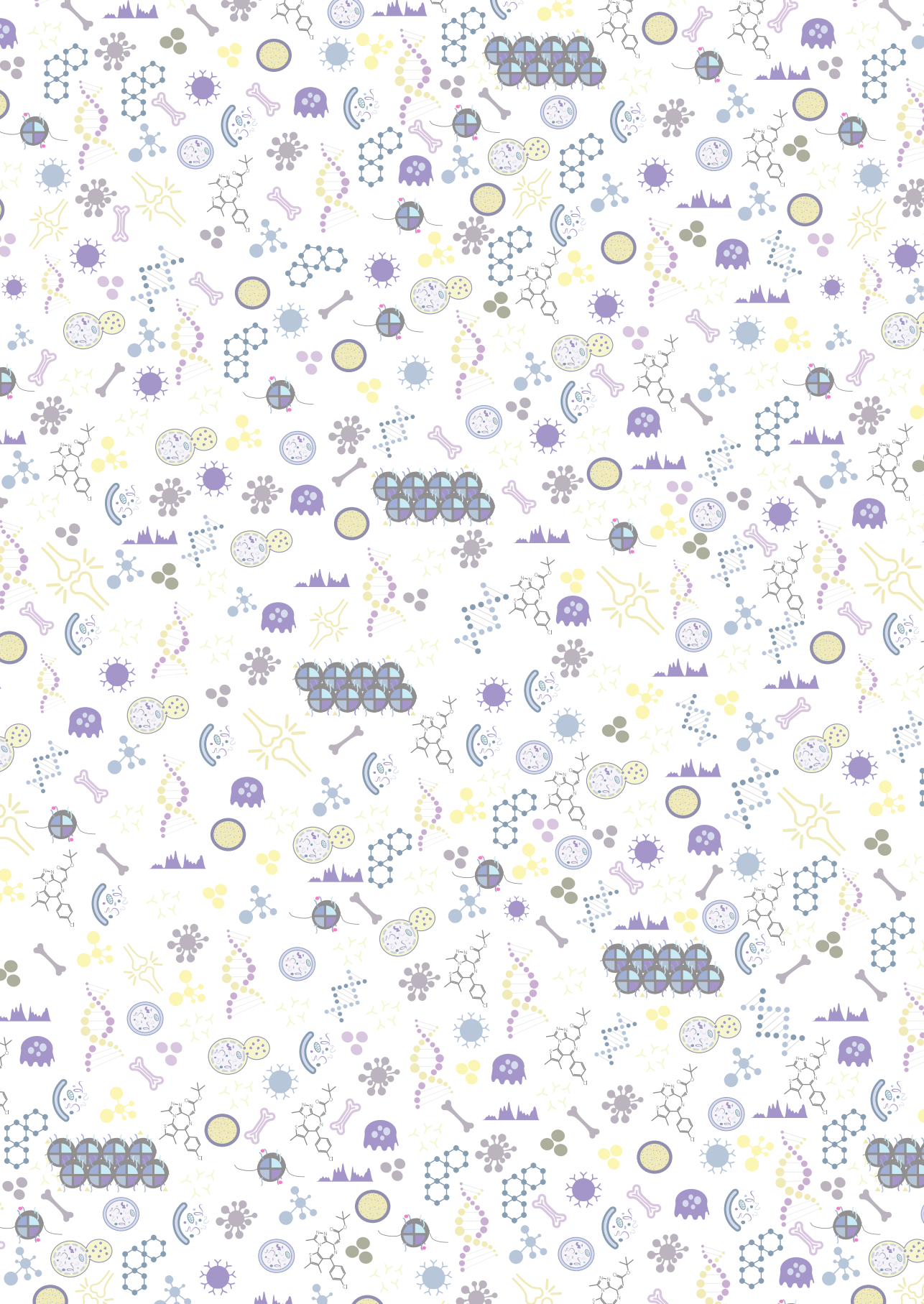

Inhibition of (super-)enhancer activity in autoinflammatory site-derived T cells reduces disease-associated gene expression

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ABSTRACT

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, (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[1]. 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)[2]. Very recently, a number of studies have identified extremely large enhancer domains, spanning up to 50 kilobases, termed super-enhancers (SEs) or stretched enhancers[3–5]. These SEs were demonstrated to specifically regulate genes associated with cell identity and disease, including oncogenes[5,6]. Importantly, BET (bromodomain and extra-terminal domain) inhibitors were demonstrated to impair SE activity, and thereby preferentially reduce SE-associated gene expression[6,7]. 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[8–10].
Various disease-associated single nucleotide polymorphisms (SNPs) were found to be enriched in enhancers and SEs[3,4]. Recently, it was demonstrated that candidate causal variants for 21 autoimmune diseases preferentially mapped to the enhancer profile of several immune cells[11]. 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)[12]. The early onset of this disease and the possibility to obtain cells 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 unique set of JIA-associated active enhancers and SEs in primary Tmem/eff cells. To assess the regulatory function of these active (super-)enhancers RNA sequencing was performed to identify genes that are differentially expressed in patient cells derived from the autoinflammatory site. Inhibition of (super-)enhancer activity preferentially inhibited disease-specific gene expression, implicating a role for (super-)enhancer activity in disease pathogenesis. Since the first clinical trials utilizing inhibitors of (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.

MATERIAL AND METHODS

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 minutes incubation at 37 degrees 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 16h with human T-activator CD3/CD28 dynabeads (1 cell: 3 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, 60s cycle time, 8 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 75bp single-end on Illumina NextSeq500 sequencer (Utrecht DNA sequencing facility). The ChIP-seq data presented in this study have been deposited in NCBI's GEO and are accessible through GEO Series accession number GSE71596 (linked to GSE71597).

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 (1 cell: 3 beads) (Life Technologies) and cultured for 16h 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 4h 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 75bp single-end on Illumina NextSeq500 (Utrecht DNA sequencing facility). The RNA-seq data presented in this study have been deposited in NCBI's GEO and are accessible through GEO Series accession number GSE71595 (linked to GSE71597).

MULTIPLEX IMMUNOASSAY

Multiplex analysis (xMAP; Luminex) was performed on supernatant derived from JIA patient-derived cells treated for 16h 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 using the Benjamini-Hochberg procedure. ChIP-seq regions with a significantly different H3K27ac signal were defined using a FDR<0.05. Significance of JQ1(+) treatment on inflammatory cytokine production was determined using paired student t-tests. The significance of SDF-1 expression in the SF compared to expression in the PB was determined using an unpaired student t-test.

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 naïve T cells ($CD4^+CD45RA^+CD45RO^-$) and Tmem/eff cells ($CD4^+CD45RA^-CD45RO^+$)[12]. 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 comprised of Tmem/eff cells (**Supplemental Figure 1A**). Enhancer profiling of naïve T cells and Tmem/eff cells, using publically available H3K27ac ChIP-seq data[13], revealed that the H3K27ac signal of ~two thousand enhancers was significantly different between naïve and Tmem/eff cells which associated with genes that regulate T cell activation (**Supplemental Figure 1B-F**). 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 (**Supplemental Figure 1G-K**). Since naïve 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 (**Supplemental Figure 2A**). 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 (**Figure 1A and Supplemental Figure 2B**). Genes associated with enhancers with a significantly higher H3K27ac signal in activated HC cells indeed correlated with T cell activation (**Figure 1B and 1C**). More than one thousand enhancers significantly differed in H3K27ac signal between HC cells and JIA SF-derived cells, of which the majority was increased in JIA patients (**Figure 1D and Supplemental Figure 2C**). Genes associated with these enhancers comprised of many CD markers, chemokine- and interleukin-receptors, and genes associated with lymphocyte activation (**Figure 1E and 1F**). The H3K27ac signal of over 800 enhancers differed between *in vitro* activated HC cells 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 (**Figure 1G-I and Supplemental Figure 2D**). 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 (**Figure 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 (**Supplemental Figure 3A-L**). In conclusion, these data defined an active enhancer signature of 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[11,14]. 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 was increased compared to that of typical enhancers (**Supplemental Figure 4A**). Various HC and JIA-specific SEs could also be identified (**Figure 2B and Supplemental Figure 4B**). 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 in JIA patients strongly correlated with regulation of apoptosis and T cell activation, indicating that

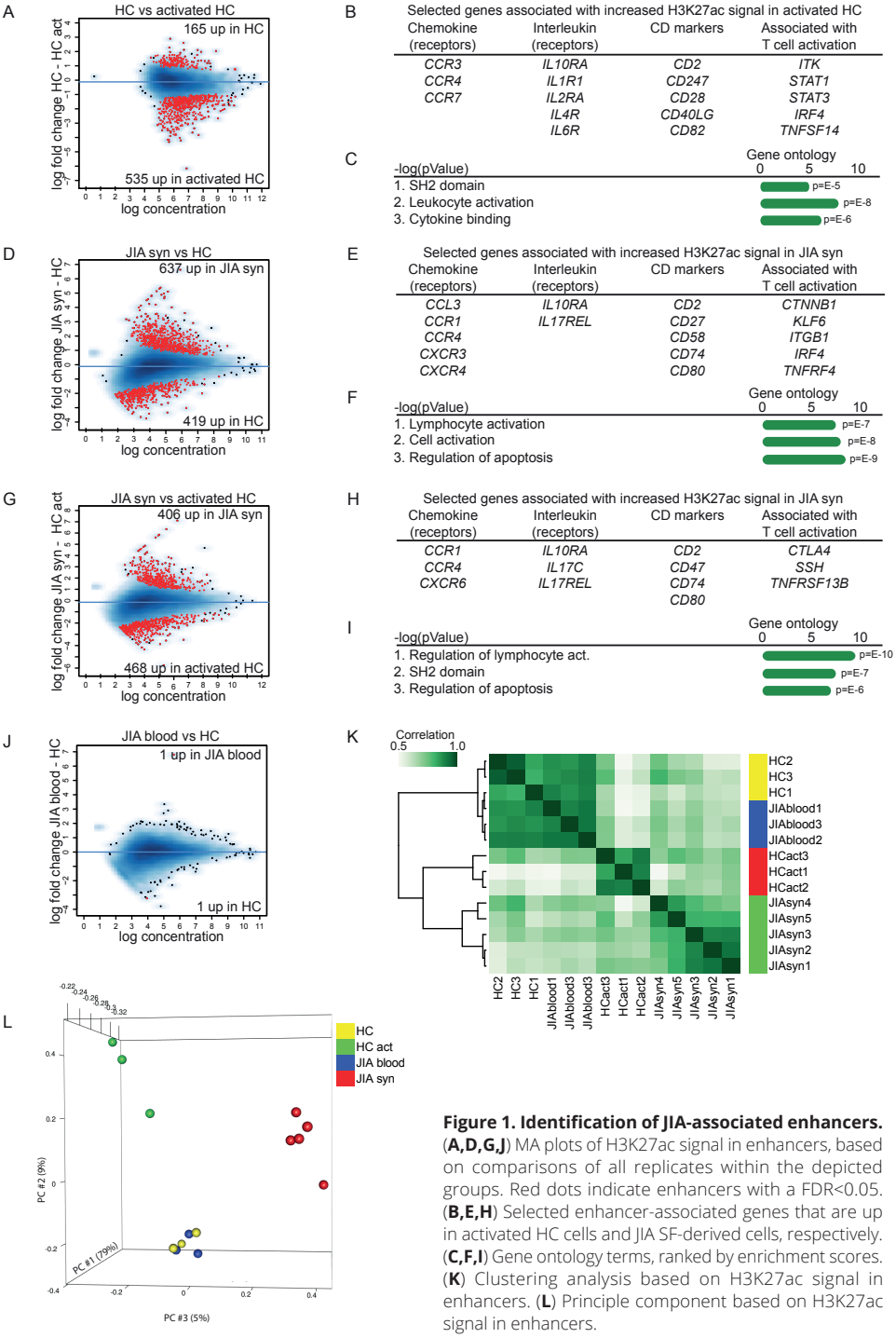

Figure 1. Identification of JIA-associated enhancers. (**A,D,G,J**) MA plots of H3K27ac signal in enhancers, based on comparisons of all replicates within the depicted groups. Red dots indicate enhancers with a FDR<0.05. (**B,E,H**) Selected enhancer-associated genes that are up in activated HC cells and JIA SF-derived cells, respectively. (**C,F,I**) Gene ontology terms, ranked by enrichment scores. (**K**) Clustering analysis based on H3K27ac signal in enhancers. (**L**) Principle component based on H3K27ac signal in enhancers.

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 (super-)enhancers (**Supplemental Table 1**). As demonstrated in **Figure 2C** and **Figure 2D**, SNPs were enriched in both typical enhancers and SEs, in the latter case to the highest extent. Importantly, enrichment of 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 (**Supplemental Table 4C**). 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 (**Supplemental Figure 4D**). 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[15]. 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 7 peaks were significantly different between JIA patients and HC (**Supplemental Figure 3E**). 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 (**Figure 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[16]. 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 (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.

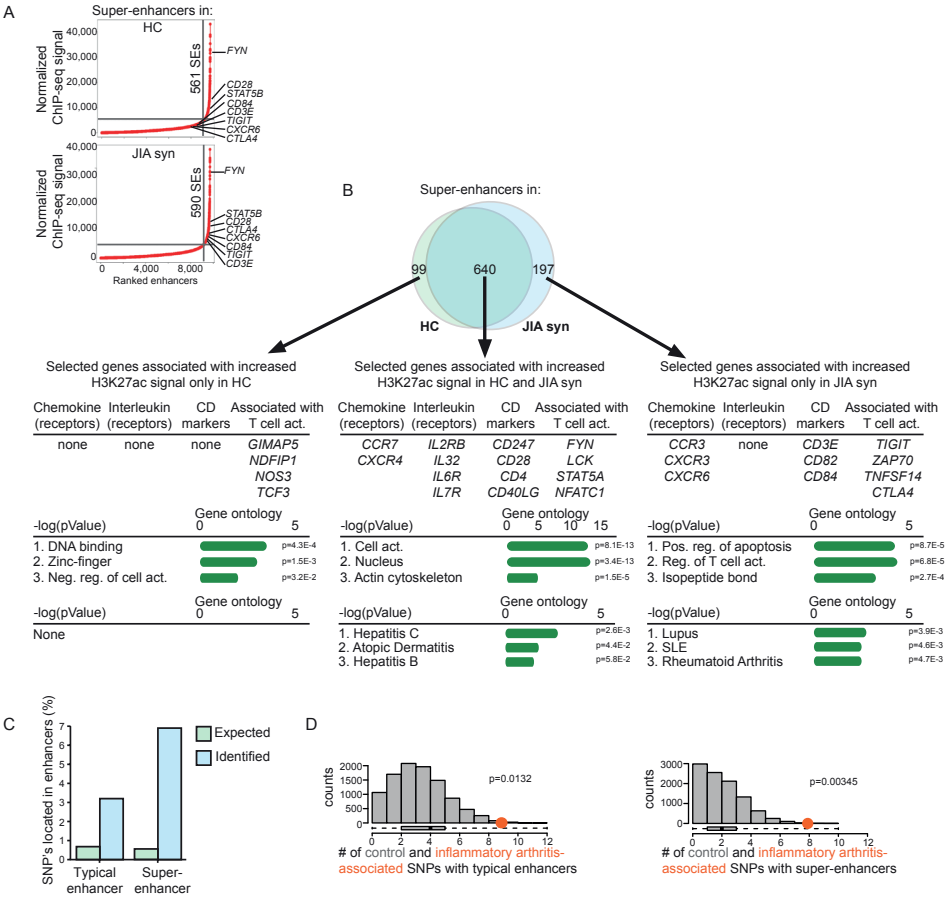

Figure 2. Identification of JIA-associated SEs.
(**A**) Normalized distribution of H3K27ac. Representative examples are depicted. (**B**) Venn diagram of all SEs identified in CD4$^+$CD45RO$^+$ T cells from HC PB (3 samples) and JIA synovium (5 samples), and their associated genes and gene ontology for 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 (grey bars). P values were calculated with binominal cumulative distribution function.

More than 900 genes were differentially expressed between both groups, of which most were increased in JIA (**Figure 4A and 4B and Supplemental Table 2**). Correspondingly to our (super)-enhancer analysis, these genes comprised of chemokine and interleukin receptors, CD markers, and genes associated with T cell activation and defense responses (**Figure 4C and 3D**). 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 (**Supplemental Figure 5A and 5B**). Since it was demonstrated that SE activity (and typical enhancer activity to a lesser extent) can be repressed

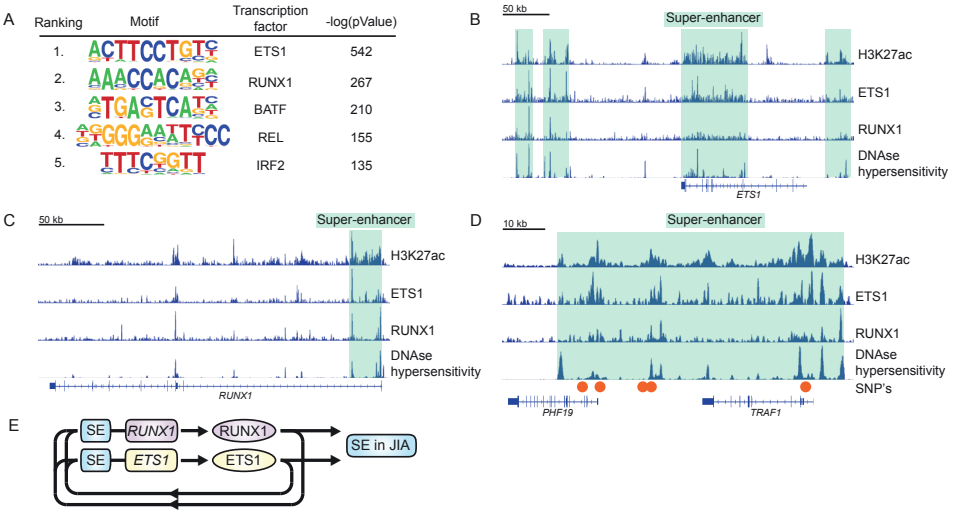

Figure 3. ETS1 and RUNX1 binding motifs are enriched in JIA-associated SEs.
(**A**) Transcription factor binding motifs enriched in JIA SEs. (**B**,**C**) Gene track for *ETS1* and *RUNX1* displaying ChIP-seq signals for H3K27ac, ETS, RUNX1, and DNAse hypersensitivity sites. Each light green shaded area represents an individual SE. (**D**) Gene tracks for *PHF19* and *TRAF1* showing ChIP-seq signals for H3K27ac, ETS1, RUNX1, 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.

with BET-inhibitors, we hypothesized that BET inhibition could reduce expression of JIA-associated genes in JIA patient T cells[6]. JIA patient-derived CD4$^+$ Tmem/eff cells were treated with the BET-inhibitor JQ1(+) or an inactive control JQ1(-), and RNA sequencing was performed (**Figure 4E and 4F and Supplemental Table 3**). The expression of both SE- and typical enhancer-associated genes was reduced by JQ1 treatment (**Supplemental Figure 5C**). Similarly as the genes upregulated in JIA, genes downregulated by JQ1(+) treatment in JIA-derived cells correlated with a "defense response" (**Figure 4G and 4H and Supplemental Figure 5D**). 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 (**Figure 4I and Supplemental Figure 5E**). 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 (**Supplemental Figure 5F-H**). 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.

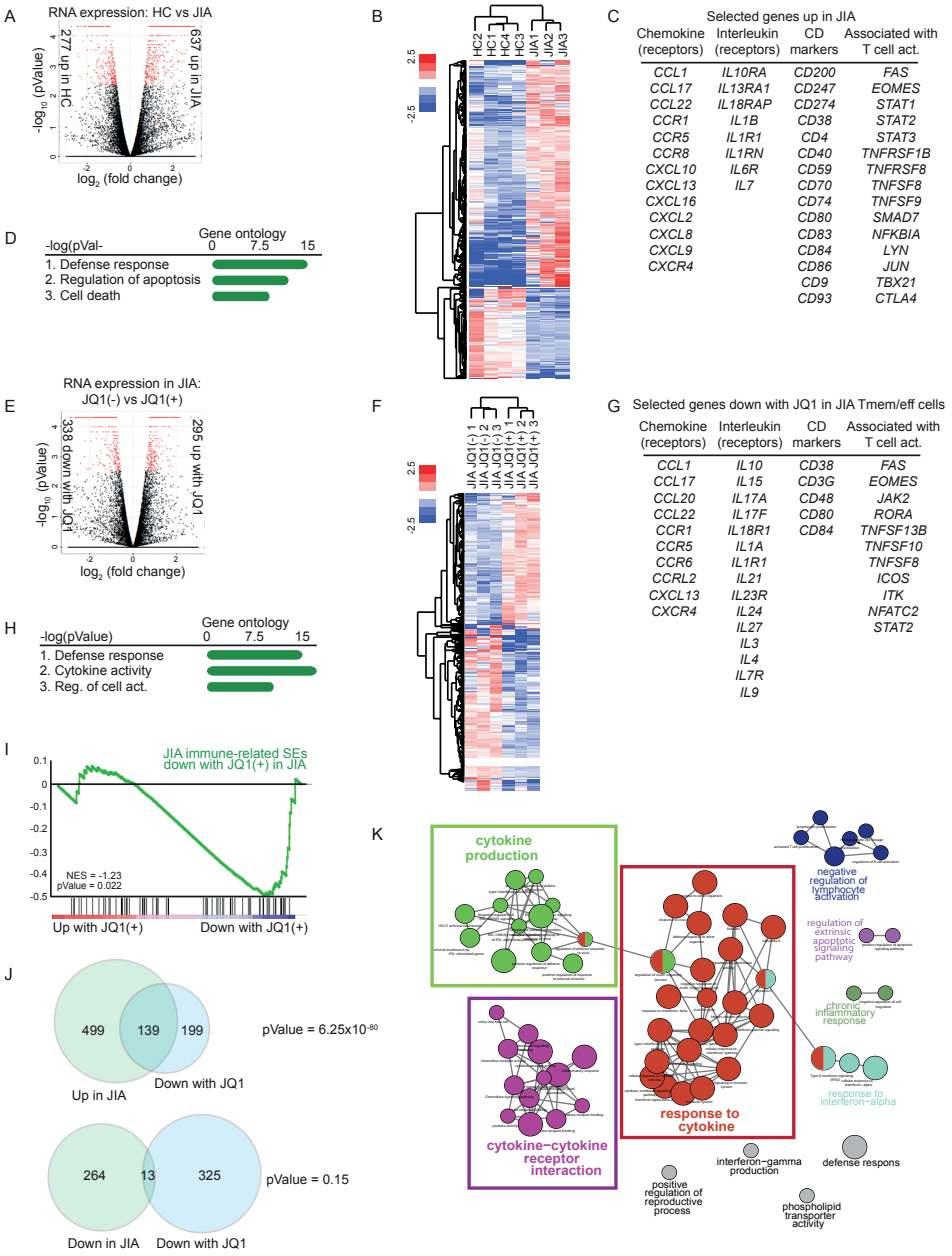

Figure 4. BET-inhibition preferentially inhibits JIA-associated gene expression.
(**A**) Volcano plot of genes differentially expressed between HC PB-derived and JIA patient SF-derived CD4$^+$CD45RO$^+$ T cells. Red dots indicate genes with a FDR<0.05. (**B**) Heatmap of genes significantly different 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. (**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

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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 familywise 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.

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 which 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 (**Figure 5B-E**). 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**)[17]. 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 novel insight into the mechanisms of various diseases[3,5,6,18]. Enhancer regions can be targeted through the use of BET-inhibitors, enabling the therapeutic treatment of aberrant enhancer and SE regions[6,7,9]. 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[8,9].

Based on genetic and epigenetic mapping of HCs it has been shown that, for a number of autoimmune diseases, the majority of causal variants maps to immune cell enhancers[11]. 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 *in vitro* activated PB-derived HC cells may not 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[15]. Although Tmem/eff cells are considered to be one of the major

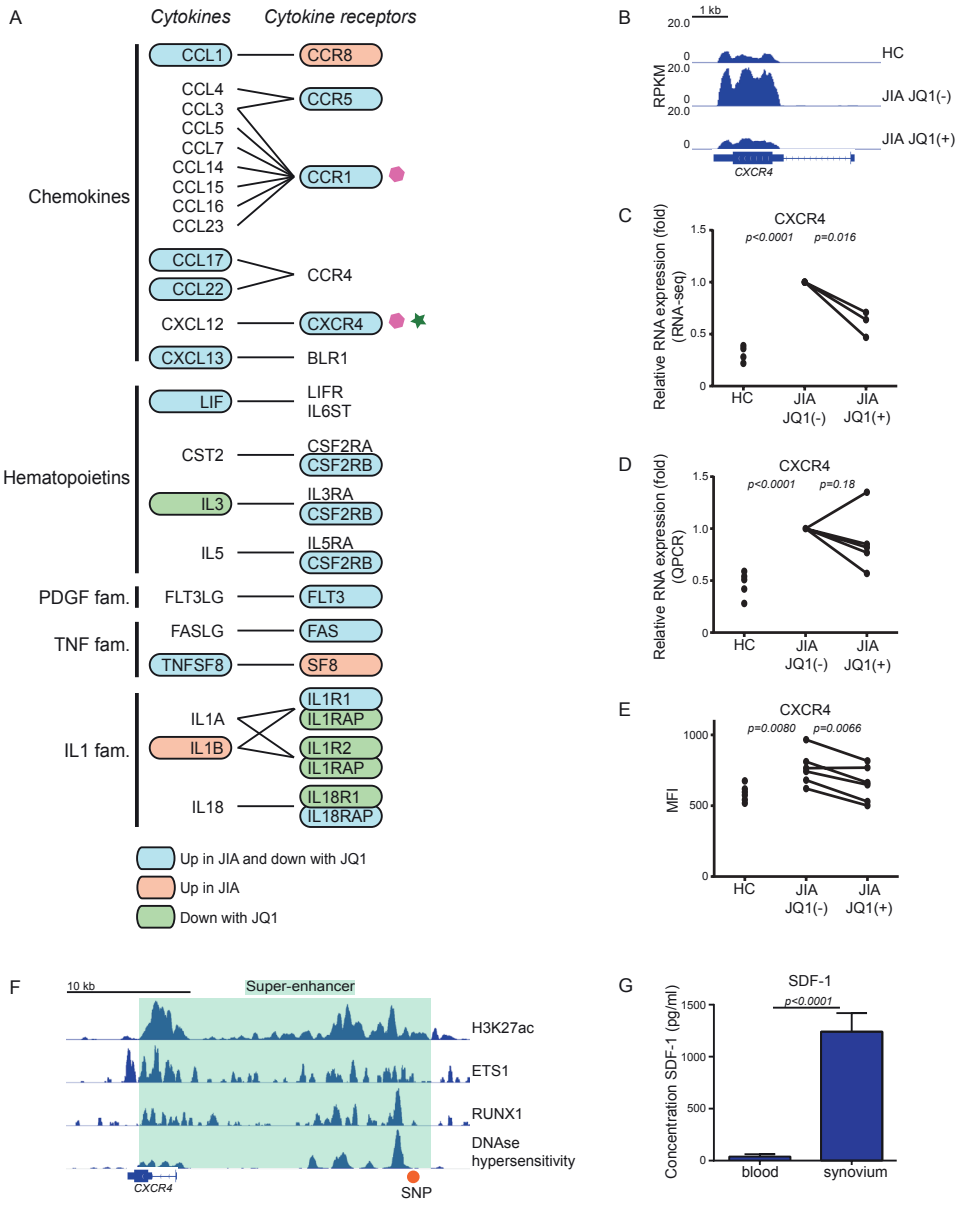

Figure 5. CXCR4 is associated with JIA and its expression is inhibited by BET-inhibitors.
(**A**) Overview of cytokines and cytokine receptors up in JIA and/or down with JQ1(+). Genes associated with an enhancer with significantly different H3K27ac occupancy (pink polygon) and SE-asssociated genes are (green star) depicted. (**B**) RNA sequencing gene track for *CXCR4* for HC or JIA patient-derived CD4⁺ Tmem/eff cells treated with JQ1(-) or JQ1(+). (**C,D**) Relative RNA expression of *CXCR4* in CD4⁺ Tmem/eff cells treated with JQ1(-) or JQ1(+), data 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 a paired student t-test.

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[19]. Interestingly, our data demonstrates 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[20–22]. 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[6,7]. 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 16h with JQ1, while others observed a preferential effect on SE activity after 6h[6]. 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[23]. 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 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[24,25]. Furthermore, *in vitro* treatment of RA patient-derived fibroblasts with I-BET151 reduced their inflammatory and proliferative properties[26]. Currently, BET-inhibitors are being explored as novel therapeutics in the treatment of a variety of human cancer types[8,9]. 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 anti-tumor 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[27]. 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.

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SUPPLEMENTAL INFORMATION

ANALYSIS OF CHIP-SEQUENCING DATA

Analysis method #1

Sample demultiplexing and read quality assessment was performed using BaseSpace (Illumina) software. Reads with quality score of Q>30 were used for downstream analysis. Reads were mapped to the reference genome (hg19) with Bowtie 2.1.0[28] using default settings. SAM files were converted to BAM files using samtools version 0.1.19. Peaks were subsequently called using MACS-2.1.0[29]. Enriched regions were identified compared to the input control using MACS2 callpeak --nomodel --exttsize 300 --gsize=hs -p 1e-9. The mapped reads were extended by 300bp and converted to TDF files with igvtools-2.3.36 and were visualized with IGV-2.3.34[30]. Differential binding analysis was performed using the R package DiffBind version 1.8.5[31]. In DiffBind read normalization was performed using the TMM technique using reads mapped to peaks which were background subtracted using the input control. Differentially bound sites with an FDR < 0.05 were used for further analysis. Enhancer gene associations were determined as the nearest TSS to the center of the enhancer and super-enhancer locus. BEDtools v2.17.0 was used for general manipulation of peak bed-files[32]. Clustering analysis was performed on all significantly different peaks between any of the groups (~2300 peaks). Publicly available datasets were downloaded from the NCBI SRA archive. The SRA files were converted to FASTQ using the sratoolkit-2.4.1 and samples were subsequently processed as described above.

Analysis method #2

Peaks were called using Cisgenome 2.0[33] (–e 150 -maxgap 200 –minlen 200). Peak coordinates were stretched to at least 2000 base pairs and collapsed into a single list. Overlapping peaks were merged based on their outmost coordinates. Only peaks identified by at least 2 independent datasets were further analyzed. Peaks with differential H3K27ac occupancy were identified using DESeq[34] (padj < 0.05 and absolute log2FoldChange > 0.6). Clustering analysis was performed on all significantly different peaks between any of the groups.

Analysis method #3

Reads were mapped onto hg38 using Bowtie (v1.1.0)[28] with the following options: -l 45 -n 1 --phred33-quals --best --strata --chunkmbs 512 -m 1 -S. SAM files were converted to BAM files, sorted and indexed using Samtools (v0.1.19). Peaks were subsequently called using MACS2 (v2.1.0)[29] with the following options: callpeak --nomodel --extsize 400 --llocal 100000 -g hs -p 1e-5. The number of reads within peaks was used to Read-Per-Million-normalize the generated treat_pileup.bdg files, which were used to create TDF files with igvtools (v2.3.36). All peaks were extended to a minimum of 2000 basepairs and enriched regions within 1000 basepairs of a transcriptional start site were excluded (extracted from RefSeq gene list from UCSC Table Browser). Comparison and modification of enhancer files was done with Bedtools (v2.20.0) and custom Python scripts[32].

By comparing all peak files and merging overlapping peaks, a non-redundant enhancer list was made. Read counts within these regions were used for differential expression (DE) analysis and clustering. Duplicate reads within a sample were removed.
DE analysis was done using edgeR (v3.6.8)[30] for R (v3.1.2), which uses a TMM method to normalize read counts. Differentially expressed regions were calculated using the functions: estimateCommonDisp(), estimateTagWiseDisp(), exactTest(). FDR < 0.05 was used to define significant differentially expressed regions.
Clustering was done on Z-Normalized RPKM values generated from the read counts. Samples were divided over 2 groups according to sequencing run, and Z-Normalized within their group. A PCA plot was generated using the default PCA function in R. For the correlation heatmap, a pairwise Pearson correlation matrix was made after which heatmap.2() (gplots v2.14.2) was used with Euclidean distance and complete linkage to create a dendrogram.

Identification of super-enhancers

Super-enhancers were identified by employing the ROSE algorithm[5] using a stitching distance of the MACS2 called peaks of 12.5kb, peaks were excluded that were fully contained in the region spanning 1000bp upstream and downstream of an annotated TSS (-t 1000). The H3K27ac signal was corrected for background using the input control and subsequently ranked by increasing signal. Super-enhancer gene associations were determined as the nearest TSS to the center of the enhancer and super-enhancer locus. BEDtools v2.17.0 was used for general manipulation of peak bed-files[32]. Motif enrichment analysis was performed using the HOMER software v4.7 (findMotifsGenome.pl; hg19; -size 200). Prior to motif enrichment analysis, regions of open chromatin delineated by DNAse hypersensitive regions were identified for each H3K27ac region[16] (GSM665839).

Enrichment of SNPs

Enrichment of SNPs in regulatory regions was performed as described previously[35]. In brief: a variant falling within peak coordinates was considered as overlapping variant. Random matched variant sets were generated from variants present on Human Omni1S genotyping chip (Illumina). Random variant selection was matched for similar minor allele frequency and distance to the closest gene. Linkage Disequilibrium (LD) information was accessed from HapMap[36]. To determine the significance of overlap of inflammatory arthritis-associated SNPs with typical enhancers and super-enhancers, the p-value was calculated using binominal cumulative distribution function b(*x; n, p*) using R pbinom() function as described previously[37].

ANALYSIS OF RNA SEQUENCING DATA

Read quality assessment and demultiplexing was performed analogous to the ChIP-seq libraries. The Tophat2 pipeline was used for analysis of RNA-seq reads[38]. Reads were mapped to the human reference genome (hg19) using TopHat v2.0.9 (tophat2 -p 8 --library-type fr-firststrand -G hg19.gtf). Subsequently transcripts were assembled using CuffLinks v2.2.1 with the reference

gene annotation (hg19) as a guide. The transcriptomes were merged using Cuffmerge after which differential gene expression analysis was performed using Cuffdiff. Reads were quartile normalized (--library-norm quartile) and reads mapping to rRNA or tRNA were masked from the quantification using the -M option. Similarly quartile normalized count and FPKM tables were generated using Cuffnorm. The compatible R package CummeRbund was used for quality assessment of the RNA-seq data and figure generation[39]. Gene expression heatmaps were generated using the FPKM tables generated by Cuffnorm, which were expressed as fold change relative to the average and clustered using the software Cluster 3.0 (using city-block clustering and log transformation). Visualization of the heatmaps was performed by Java TreeView software version 1.1.6. The DAVID gene ontology database (http://david.abcc.ncifcrf.gov/) was used for functional annotation of differentially expressed genes and ChIP-seq bound regions. Since JQ1 treatment affects RNA expression on a global level, the relative decrease in expression of SE and typical enhancer-associated genes was quantified by normalizing counts against stable mRNAs as actin. Gene set enrichment analysis (GSEA, Broad Institute) was used to determine the enrichment of JIA super-enhancer-associated genes in JQ1-downregulated genes and super-enhancer associated genes. Cytoscape (ClueGo plugin) was used to generate functionally grouped networks of genes[40].

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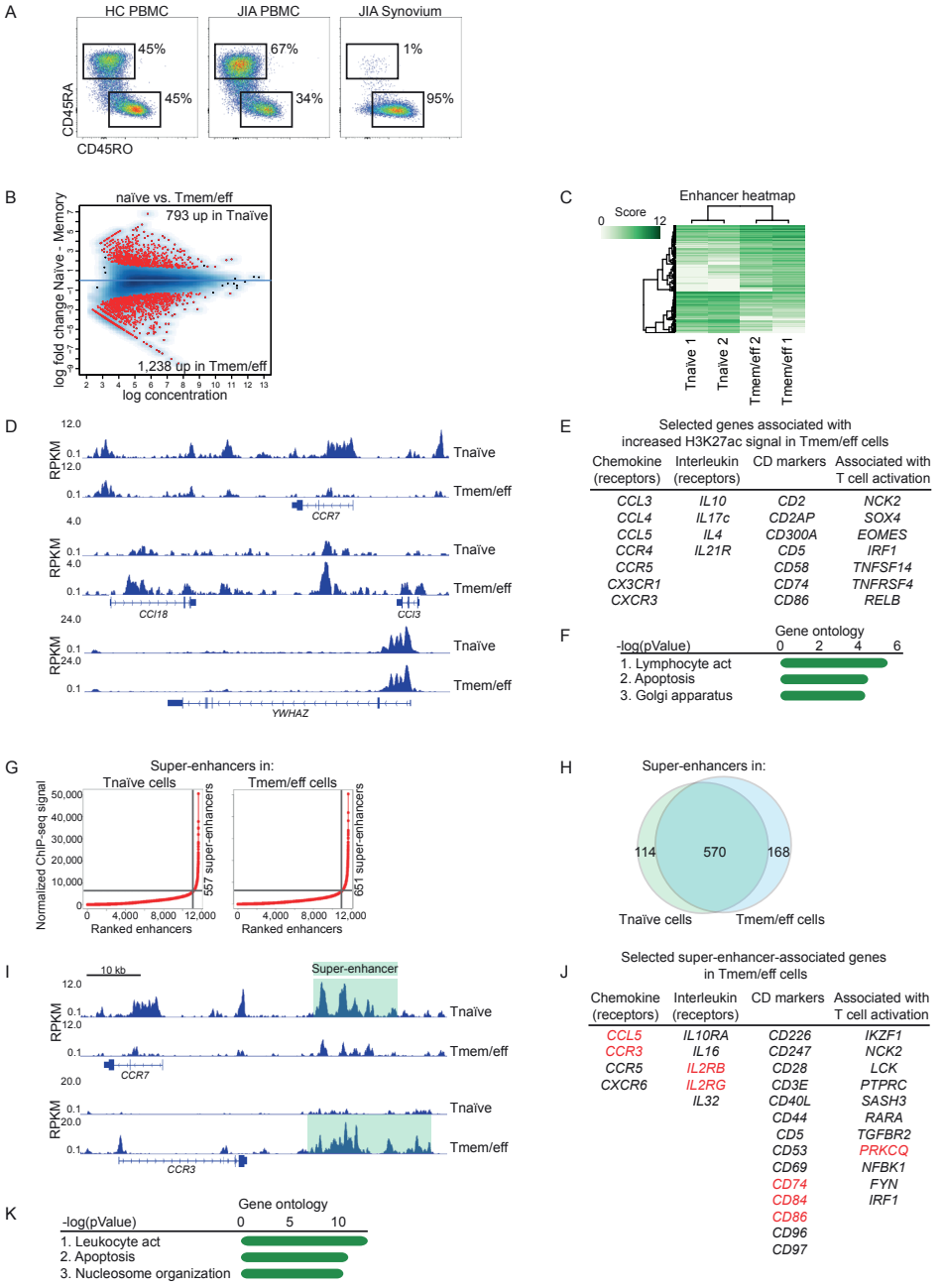

Supplemental Figure 1. Different active enhancer repertoire in CD4⁺ memory/effector and naïve T cells. (**A**) FACS plots of CD4⁺CD45RO⁺ and CD4⁺CD45RA⁺ cell populations within HC and JIA patient PB or SF-derived samples. (**B**) MA plots depicting H3K27ac signal in enhancers identified in CD4⁺ naïve and memory/effector T cells (2 replicates each). Red dots indicate enhancers with significant different H3K27ac occupancy (FDR<0.05). (**C**) Heatmap of H3K27ac signal in individual samples (only enhancers with significantly different H3K27ac occupancy

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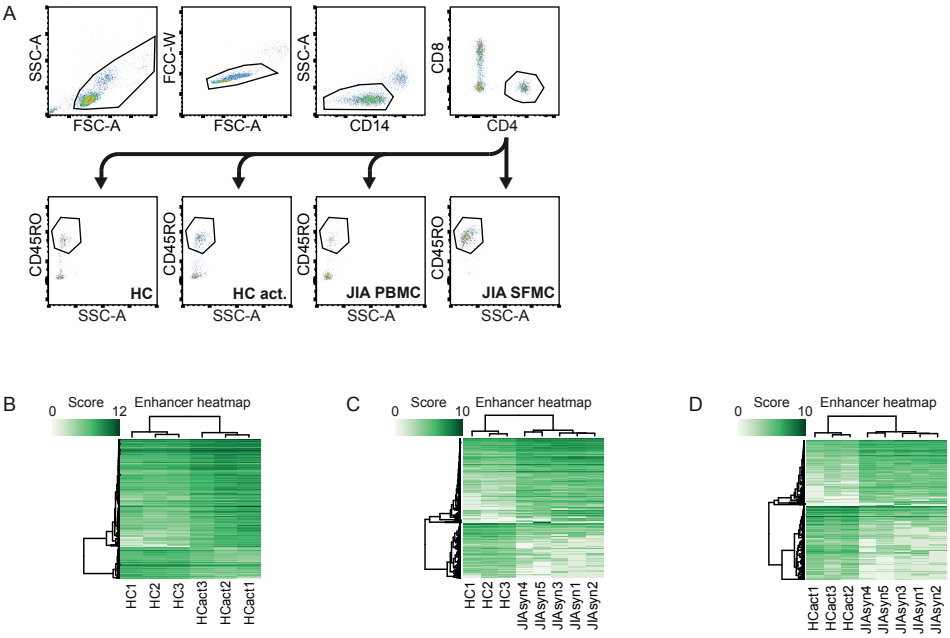
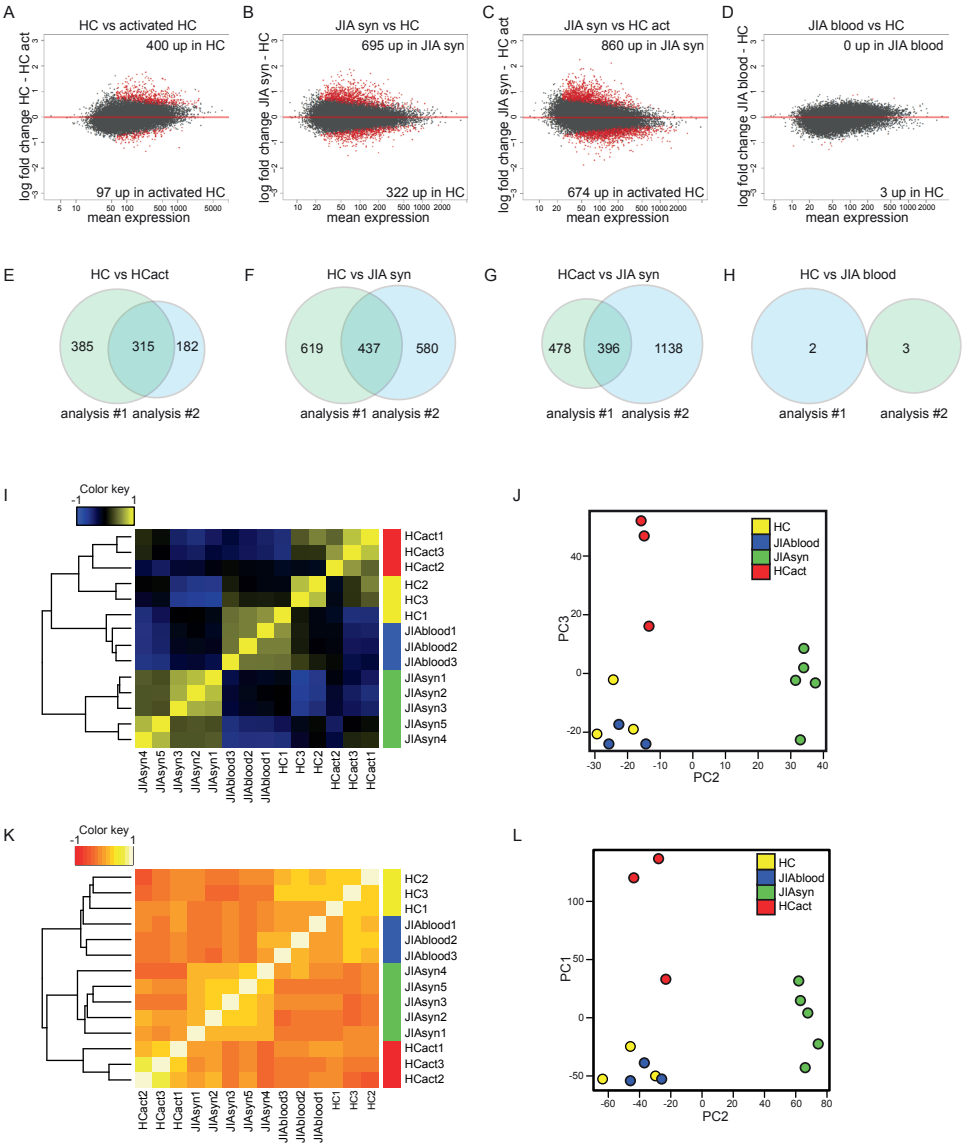
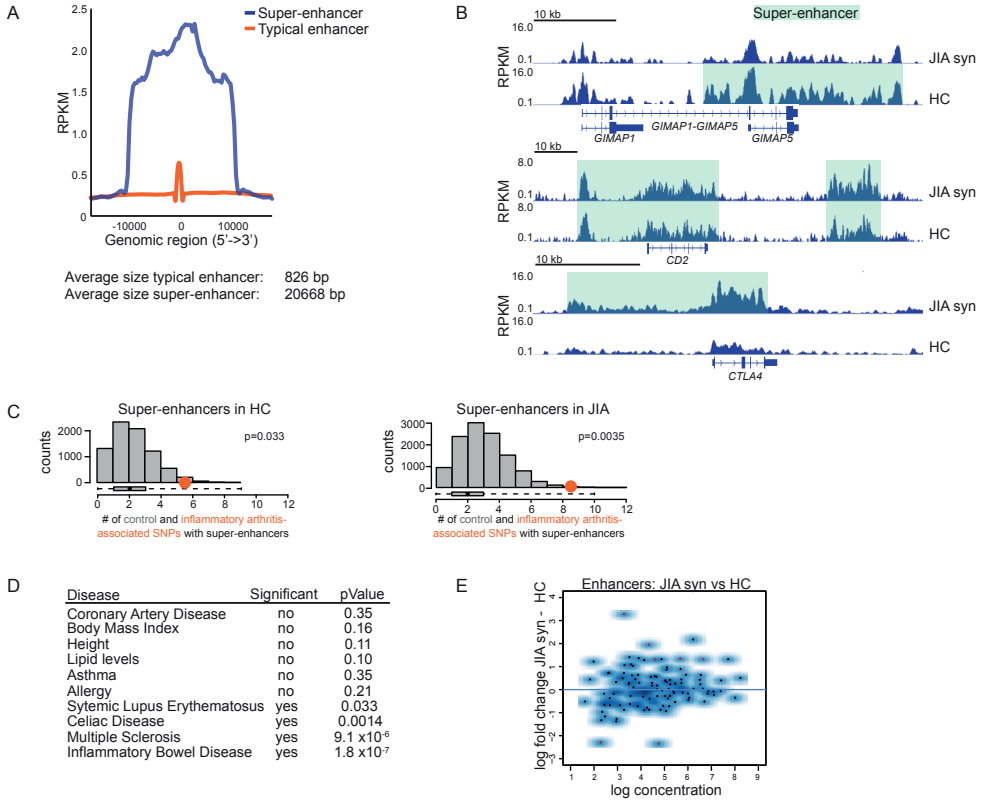
between naïve and memory/effector cells are shown). (**D**) Normalized H3K27ac signal tracks in the vicinity of *CCR7*, *CCL18, CCL3,* and *YWAHZ*. (**E**) Selected genes in the vicinity of enhancers with a significantly increased H3K27ac signal in Tmem/eff cells. (**F**) Gene ontology terms and their p-value related to genes associated with enhancers that were up in Tmem/eff cells. (**G**) Representative example of the normalized distribution of H3K27ac signal across naïve and Tmem/eff cell stitched enhancers, with super-enhancers indicated. (**H**) Venn diagram, based on all replicates, illustrating the super-enhancer overlap for naïve and Tmem/eff cells. (**I**) H3K27ac signal tracks displaying a super-enhancer in the vicinity of *CCR7* and *CCR3*. (**J**) Selected genes within the vicinity of a super-enhancer in Tmem/eff cells. Genes marked in red contained a super-enhancer exclusively present in Tmem/eff cells. (**K**) Gene ontology terms and their p-value related to genes associated with a super-enhancer in Tmem/eff cells.



Supplemental Figure 2. Enhancer profiling in CD4+ memory/effector T cells.
(**A**) FACS strategy for the isolation of $CD4^+CD45RO^+$ T cells derived from the PB of HCs, either *in vitro* activated utilizing anti-CD3/CD28 dynabeads or not, and derived from the PB or SF from JIA patients. (**B-D**) Heatmap of H3K27ac signal of significantly different enhancers.

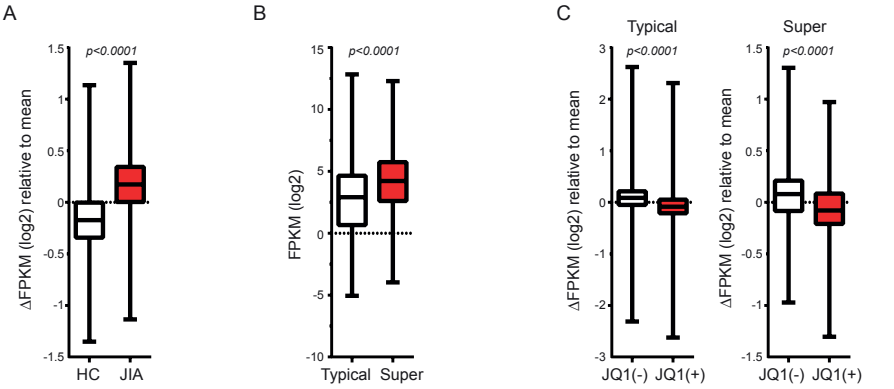


Supplemental Figure 3. Independent validation of ChIP-seq data analysis.
(**A-D**) MA plots depicting H3K27ac signal in enhancers. Red dots indicate enhancers with significantly different H3K27ac occupancy ($p<0.05$). (**E-H**) Venn diagrams displaying the overlap between two independent analysis methods determining enhancers with differential H3K27ac signal. (**I**) Unsupervised clustering analysis of HC, activated HC, PB-derived JIA patient cells and, SF-derived JIA patient Tmem/eff cells based on enhancer profiles (Method #2). (**J**) Unsupervised principal component analysis of HC, activated HC, PB-derived JIA patient, and SF-derived JIA patient memory/effector T cells based on H3K27ac enhancer occupancy (Method #2). (**K**) Unsupervised clustering analysis of HC, activated HC, PB-derived JIA patient cells and, SF-derived JIA patient Tmem/eff cells based on H3K27ac enrichment. (Method #3). (**L**) Unsupervised principal component analysis of HC, activated HC, PB-derived JIA patient, and SF-derived JIA patient Tmem/eff cells based on enhancer profiles (Method #3).



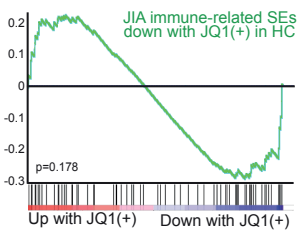
Disease	Significant	pValue
Coronary Artery Disease	no	0.35
Body Mass Index	no	0.16
Height	no	0.11
Lipid levels	no	0.10
Asthma	no	0.35
Allergy	no	0.21
Sytemic Lupus Erythematosus	yes	0.033
Celiac Disease	yes	0.0014
Multiple Sclerosis	yes	9.1×10^{-6}
Inflammatory Bowel Disease	yes	1.8×10^{-7}

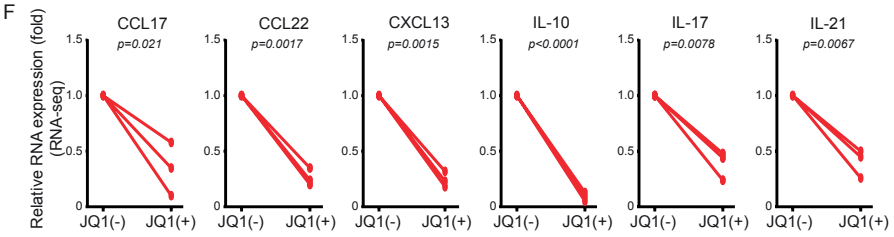
Supplemental Figure 4. Super-enhancer profiling in memory/effector T cells.
(**A**) Metagenes of H3K27ac density across typical enhancers and super-enhancers in Tmem/eff cells. Metagenes are centered on the enhancer region. (**B**) H3K27ac enrichment tracks of *GIMAP1GIMAP5*, *CD2*, and *CTLA4* for HC PB-derived and JIA patient synovium-derived Tmem/eff cells. (**C**) Number of inflammatory arthritis-associated SNPs (red dot) overlapping with super-enhancer regions compared with 10,000 random SNP sets (grey bars). P values were calculated with binominal cumulative distribution function. (**D**) Enrichment analysis of non-autoimmune disease and autoimmune disease-associated SNPs in JIA-associated SEs. P values were calculated with binominal cumulative distribution function. (**E**) MA plots of H3K27ac peaks associated with genes linked to asthma in JIA synovium and HCs. Red dots indicate enhancers with a FDR<0.05.

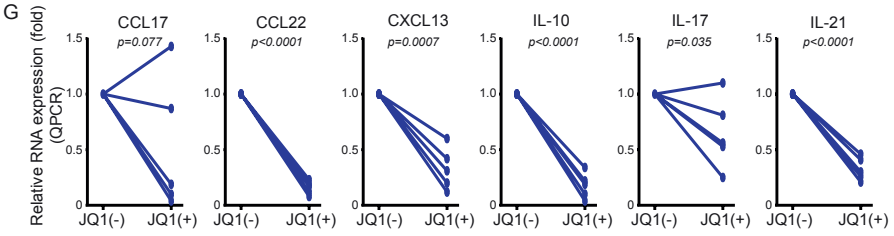


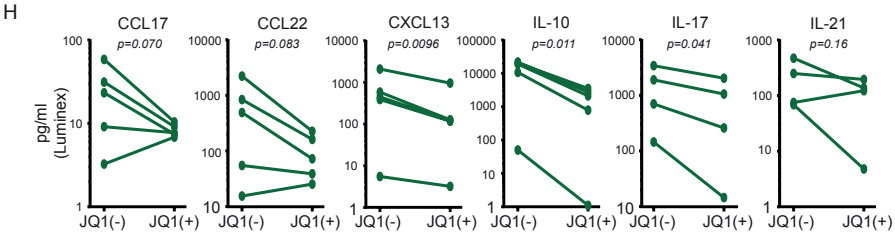
D

Pathways downregulated with JQ1(+) in JIA	FDR
1. Defensins (Reactome)	<0.001
2. Type I Diabetes Mellitus (KEGG)	0.0023
3. Cytokine pathway (Biocarta)	0.0443









► **Supplemental Figure 5. BET inhibition of JIA Tmem/eff cells reduces inflammatory cytokine production and secretion.**
(**A**) Individual normalized Δ FPKM (log2) values of genes associated with a JIA-specific SE in HC and JIA samples relative to the mean of both HC and JIA. (**B**) FPKM (log2) values of genes in JIA samples either associated with a single typical enhancer or a SE. (**C**) Pathways downregulated in JIA patient-derived Tmem/Teff cells upon JQ1(+) treatment based on gene set enrichment analysis. (**D**) Δ FPKM (log2) values of single typical enhancer and SE-associated genes in JIA relative to the mean of both JQ1(-) and JQ1(+) treated samples. (**E**) Gene set enrichment analysis for immune-related JIA super-enhancer-associated genes and genes affected by JQ1 treatment in HCs. P value was determined using the familywise error rate procedure. (**F,G**) Relative mRNA expression of CCL17, CCL22, CXCL13, IL-10, IL-17, and IL-21 in JIA patient SF-derived Tmem/eff cells treated with 300 nM JQ1(-) or JQ1(+) for 16h, data obtained by RNA sequencing (F) or qPCR (G). (**H**) Concentration (pg/ml) secreted CCL17, CCL22, CXCL13, IL-10, IL-17, and IL-21 by JIA patient SF-derived Tmem/eff cells upon 16h treatment with 300 nM JQ1(-) or JQ1(+) (five replicates). For all analyses p values were calculated using a paired student t-test.

Supplemental Table 1. Overview of inflammatory arthritis-associated SNPs.
To assess the enrichment of inflammatory arthritis-associated SNPs within JIA typical enhancers and super-enhancers SNPs described in the articles listed in this table were used.

Disease	SNP reference
Juvenile idiopathic arthritis	Thompson, S. D. *et al. Arthritis Rheum.* 64, 2781–91 (2012) Hinks, A. *et al. Arthritis Rheum.* 60, 258–63 (2009) Behrens, E. M. *et al. Arthritis Rheum.* 58, 2206–7 (2008)
Psoriatic arthritis	Ellinghaus, E. *et al. J. Invest. Dermatol.* 132, 1133–40 (2012) Hüffmeier, U. *et al. Nat. Genet.* 42, 996–9 (2010)
Rheumatoid arthritis	Jiang, L. *et al. Arthritis Rheumatol. (Hoboken, N.J.)* 66, 1121–32 (2014) Orozco, G. *et al. Arthritis Rheumatol. (Hoboken, N.J.)* 66, 24–30 (2014) Okada, Y. *et al. Nature* 506, 376–81 (2014) Negi, S. *et al. Arthritis Rheum.* 65, 3026–35 (2013) Myouzen, K. *et al. PLoS Genet.* 8, e1002949 (2012) Hu, H. J. *et al. Exp. Mol. Med.* 43, 613–21 (2011) Eleftherohorinou, H. *et al. Hum. Mol. Genet.* 20, 3494–506 (2011) Terao, C. *et al. Hum. Mol. Genet.* 20, 2680–5 (2011) Freudenberg, J. *et al. Arthritis Rheum.* 63, 884–93 (2011) Padyukov, L. *et al. Ann. Rheum. Dis.* 70, 259–65 (2011) Kochi, Y. *et al. Nat. Genet.* 42, 515–9 (2010) Stahl, E. A. *et al. Nat. Genet.* 42, 508–14 (2010) Gregersen, P. K. *et al. Nat. Genet.* 41, 820–3 (2009) Raychaudhuri, S. *et al. Nat. Genet.* 40, 1216–23 (2008) Julià, A. *et al. Arthritis Rheum.* 58, 2275–86 (2008) Plenge, R. M. *et al. N. Engl. J. Med.* 357, 1199–209 (2007) Trapnell, C. *et al. Nat. Protoc.* 7, 562–78 (2012) Wellcome Trust Case Control Consortium. *Nature* 447, 661–78 (2007)

Supplemental Table 2. Expression of genes significantly different in JIA patients compared to HC and associated with a SE in JIA.
Normalized FPKM values are shown for Tmem/eff cells derived from each individual HC or JIA patient.

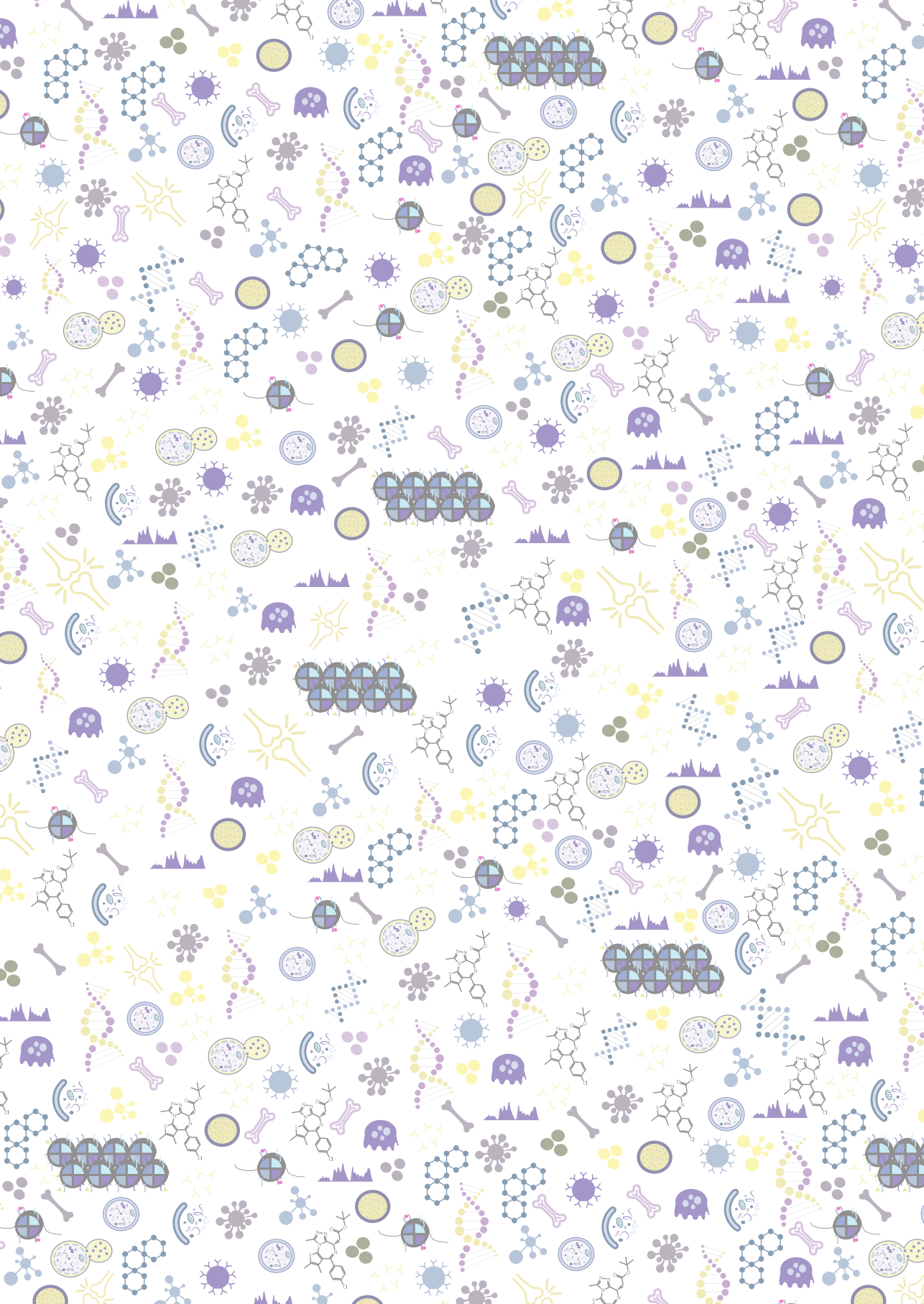
Gene	HC1	HC2	HC3	HC4	JIA1	JIA2	JIA3
ADA	0,430322	0,306425	0,936448	0,539342	1,330889	2,04519	1,307326
IL6R	0,557337	0,641394	1,324537	0,90888	2,643424	1,835825	3,304989
CD4	1,108946	0,658624	2,035277	1,286638	2,423688	3,823414	3,528209
SOD2	3,29279	5,220359	4,453559	3,070305	15,69261	26,15703	36,42352
ADORA2A	0,794161	0,608092	0,939579	1,071356	1,602473	1,635216	2,584962
CD247	1,623401	0,662893	2,224397	1,906938	2,901482	2,99708	4,162117
ATP2B4	0,206509	0,199036	0,432241	0,189974	0,954527	0,938894	0,898873
RAB11FIP1	3,336313	3,137664	3,872092	2,774364	7,844381	8,188755	7,207671
LDLRAD4	0,106623	0,088273	0,111097	0,112757	0,320093	0,219775	0,178624
CXCR4	1,163357	1,099309	1,380842	0,844595	4,307159	1,703855	7,568948
LSP1	0,48672	0,059995	0,901128	0,645257	1,297964	1,071306	2,28503
CTLA4	18,853	32,08488	36,2023	26,5808	87,95205	51,99629	52,9116
ELMSAN1	0,431471	0,289504	0,540063	0,4063	0,783571	1,000236	0,85613
TRABD2A	0,417175	0,238168	0,238987	0,378758	0,092544	0,113668	0,21404
PTPRJ	0,454812	0,346686	0,553518	0,411437	1,052933	0,852143	1,329269
INPP4B	3,398766	4,164952	2,429531	2,95487	2,144187	2,113559	2,458995
FOSB	0,12544	0,036876	0,258745	0,205544	0,10063	0,088503	0,051623
GLRX	0,518491	0,77964	0,730924	1,01422	4,895686	2,494016	1,767993
RASGRP1	1,893113	2,329992	2,079043	1,767373	4,026855	3,447273	3,97789
PIK3IP1	0,123886	0,113287	0,165176	0,234212	0,437854	0,135178	0,661324
CCND3	3,791965	1,65286	2,797488	3,384689	1,674034	1,804409	1,522174
PLS3	0,030377	0,04978	0,010449	0,021827	0,213292	0,801176	0,192001
PIK3R5	0,131438	0,102636	0,259927	0,170843	0,410603	0,519194	0,740241
SNX20	0,458916	0,347708	0,45281	0,403779	0,850351	0,905999	0,799443
ANTXR2	0,245093	0,330113	0,268353	0,239305	0,439362	0,542805	1,196658
CAPN2	0,769995	0,596563	0,76492	0,79586	1,936512	1,52751	2,178818
ANXA6	5,38204	1,62973	6,727966	6,480457	10,70546	10,93816	12,19841
CD84	0,67811	0,805855	0,668482	0,472999	2,146662	1,862947	1,574841
ACTG1	40,2343	24,00362	32,644	35,50927	27,06122	19,11465	26,66245
SELPLG	0,325062	0,059259	0,682257	0,568193	1,148595	0,690688	1,084571
HLA-C	1,78544	0,034307	0,092626	0,172751	7,715668	0,280771	2,559344
GNG2	1,062271	1,657582	1,284184	0,823965	3,218425	2,557122	2,229187
ZFP36L1	3,496313	3,483724	3,593601	2,463206	6,775466	8,658918	5,269975
SAMSN1	6,180008	7,583901	5,699269	4,426227	8,743881	13,32681	20,646
C12orf79	3,786658	4,808617	4,213171	3,297128	8,660464	6,776383	7,494866
P2RX4	0,393297	0,258225	0,556607	0,54929	1,657873	1,322693	0,980833
PTPN1	2,340169	1,895636	17,43903	2,131972	3,926944	3,420867	4,190389

Gene	HC1	HC2	HC3	HC4	JIA1	JIA2	JIA3
GPR132	0,153551	0,037556	0,300164	0,260642	0,412398	0,638678	1,267988
EHD1	0,133628	0,023986	0,227877	0,295976	0,393475	0,779581	1,278543
FAM107B	3,576654	5,587336	4,341666	3,677343	8,507524	6,472394	11,45002
CYTH1	1,853835	1,728007	2,930523	2,798898	5,513853	4,381658	7,930052
CCR7	0,890702	0,524009	1,762631	1,454631	2,79273	4,139909	9,667924
IL10RA	0,623572	0,375563	1,112825	1,015709	2,460464	2,596755	4,241477
IRAK2	0,133932	0,088315	0,168165	0,098716	0,72011	0,760462	1,18686
HMGA1	2,067021	0,228573	1,866576	2,896719	0,77872	0,708925	1,027143
IL7R	1,129924	1,471967	1,054073	1,251421	0,630775	0,677628	1,03516
IRF1	3,087508	0,841009	3,299508	2,961217	6,570971	7,447107	8,145004
ISG20	0,197519	0,026241	0,170037	0,39313	1,212406	0,452888	2,097293
ITGA5	0,22875	0,044959	0,257849	0,250405	0,734692	0,621	0,622193
ZFP36	0,678512	0,704541	0,980641	1,048881	2,265207	2,233486	3,402432
IQGAP1	3,100064	3,168203	2,675646	2,366053	4,984786	5,473736	5,923764
KAT2B	1,239845	1,950728	1,262001	1,390853	3,95488	2,788372	4,012754
SOCS3	1,964116	1,398427	3,286827	3,025421	5,488392	6,140477	9,018304
PTK2B	1,29943	0,501717	1,745574	1,595799	2,355783	3,284446	3,40145
DUSP1	0,394356	0,320693	0,522649	0,492244	0,71995	3,363206	6,496834
DUSP5	2,56464	1,823352	2,991983	2,548926	3,879501	5,659282	10,46846
LCP2	2,049153	1,988907	2,4557	2,157972	4,534663	3,984895	4,067494
NR4A2	0,859785	0,740536	1,170187	1,098152	0,811805	0,7663	0,196005
ANP32A	2,523985	2,731469	2,248618	2,335836	1,653298	1,861908	1,396959
ARL6IP5	1,959454	2,002939	2,044259	1,489581	5,152457	3,056834	5,112175
PNRC1	0,778657	1,088421	1,216761	0,962923	2,508333	4,34585	6,960618
STAT1	5,337765	6,634849	4,228646	5,005401	11,13213	10,37851	12,04227
TBX21	2,57132	1,839267	2,842029	2,859065	6,364582	8,093022	6,159045
RASSF2	1,121766	0,869262	1,14629	0,884944	1,35348	1,528463	3,020246
SYNE2	0,324513	0,524168	0,333292	0,327148	1,208514	0,80384	1,072143
ASH1L	0,646396	0,719023	0,630871	0,528992	0,843134	3,531441	0,801349
NFKBIA	21,56384	16,15317	24,88921	18,97641	43,72652	47,30155	57,9036
PANK2	5,486247	1,281852	0,921987	0,8519	1,319981	1,122369	1,468083
DUSP16	0,536786	0,802791	0,979345	0,831792	1,947225	1,588539	1,897325
APOL6	0,412748	0,61565	0,420144	0,395235	1,297855	0,767199	1,836929
LBH	4,823467	2,274686	6,165377	3,509971	10,82379	11,40919	9,883622
FAM179A	0,077397	0,050752	0,120694	0,10846	0,177322	0,564637	0,209902
LINC00152	9,774219	3,252992	8,163945	6,196366	31,06034	9,383723	7,193257
SOD2	0,030716	0,039175	0	0,031562	0,009577	0,046635	0,023616
ADA	0,430322	0,306425	0,936448	0,539342	1,330889	2,04519	1,307326

Supplemental Table 3. Expression of genes significantly affected by JQ1(+) and associated with a SE in JIA. Normalized FPKM values are shown for Tmem/eff cells derived from each individual JIA patient, treated with or without JQ1(+).

Gene	JIA1	JIA2	JIA3	JIA1 + JQ1	JIA2 + JQ1	JIA3 + JQ1
RPLP1	28,98617	19,34464	25,37108	34,44531	67,55468	39,73011
OGDH	1,104717	0,884506	1,094471	1,702142	1,946904	1,831348
ARHGAP25	1,628009	1,500706	1,05279	0,995233	1,1391	0,532616
CXCR4	4,307159	1,703855	7,568948	2,6437	1,356491	3,672881
CD82	1,671588	2,076611	1,396637	2,101801	4,45876	3,261473
TGFBR2	3,310086	2,69364	4,354689	2,242398	2,256103	2,404821
FAM102A	0,075975	0,101119	0,121198	0,267127	0,262991	0,348015
LFNG	0,234453	0,142727	0,258289	0,719193	0,683581	0,796408
ARHGAP9	1,194741	0,74357	0,644854	0,33284	0,275248	0,194384
KDM6B	0,314562	0,231884	0,206026	0,394554	0,58292	0,372577
TMC6	0,151414	0,056893	0,077444	0,222261	0,349683	0,348299
TSPAN14	0,334282	0,313235	0,355749	0,416815	0,592025	0,644267
RASGRP1	4,026855	3,447273	3,97789	2,664302	2,058054	1,815693
ARHGEF3	1,549374	1,522145	0,919903	2,749378	2,31961	1,687865
INPP4A	0,2736	0,35694	0,314443	0,626025	0,76618	0,620652
NFATC2	0,896778	0,97009	0,920626	0,605489	0,634107	0,577954
IFFO2	0,368285	0,270683	0,275636	0,676227	0,605151	0,489859
FAIM3	0,868406	0,546803	0,379731	0,229654	0,13393	0,092785
PATL2	0,141019	0,063958	0,09819	0,041096	0,006308	0,02415
CD84	2,146662	1,862947	1,574841	0,581569	0,58553	0,710405
LIMS1	1,283377	1,400862	1,380426	0,844314	0,699165	0,666002
FYB	0,990178	0,72664	0,916876	0,444577	0,414637	0,419398
ZFP36L1	6,775466	8,658918	5,269975	15,91091	19,87419	7,344102
SAMSN1	8,743881	13,32681	20,646	4,534113	5,876111	7,887142
LMNA	1,504531	0,754623	0,729341	1,698565	2,334449	2,722875
GPR132	0,412398	0,638678	1,267988	0,548166	1,408267	2,192099
EHD1	0,393475	0,779581	1,278543	0,475047	1,432679	2,322505
IQGAP2	2,670906	3,146523	2,54126	1,660878	1,525751	1,666259
GLIPR2	0,35701	0,466978	0,589982	0,68038	0,795184	1,381
CCR7	2,79273	4,139909	9,667924	5,041752	12,47357	17,22229
CRIP1	0,525963	0,256124	0,518816	0,818932	1,199864	1,327074
AIM1	1,950084	2,728064	4,014615	1,260004	1,479886	2,214223
ARF6	2,562212	2,08895	2,584842	3,937194	3,492295	3,594068
HMGA1	0,77872	0,708925	1,027143	1,530033	1,892236	1,806223
IL7R	0,630775	0,677628	1,03516	0,499191	0,145546	0,43212
JUNB	0,674188	0,280882	0,49212	0,846137	1,264582	1,299788
LTB	0,260404	0,356266	0,179806	0,542358	0,565763	0,49819
MYH9	2,970282	2,4319	2,622549	3,871132	5,200773	4,56892
MAP2K3	0,742259	0,863314	0,67038	1,427201	2,33108	1,875525
PTPN6	2,739161	2,173749	2,050404	0,892384	0,888017	0,959831

Gene	JIA1	JIA2	JIA3	JIA1 + JQ1	JIA2 + JQ1	JIA3 + JQ1
RAC2	4,312406	3,501521	3,836314	6,563556	7,43086	6,069016
RORA	1,053628	0,91689	1,234956	0,719523	0,637841	0,680124
ST6GAL1	2,277866	1,48905	3,21883	0,812527	0,993179	1,115508
SKI	0,113318	0,132607	0,139073	0,221946	0,278548	0,199477
SLC7A5	6,687112	3,906469	5,579877	8,245305	11,98451	8,663668
HIST2H2BE	0,308685	0,276747	0,138083	0,375965	0,52405	0,613051
SOCS1	0,209315	0,180645	0,239911	0,381819	0,587021	0,340471
TNFSF14	7,197601	5,649596	2,163605	12,93214	11,86713	6,315973
DUSP2	12,08244	5,849349	7,214965	15,99634	16,53344	16,24666
PDCD1	0,774058	0,468881	0,6853	0,766055	2,273453	1,284041
FOS	0,734658	0,401256	0,278788	1,42686	1,065985	0,746018
ITK	6,014726	4,083997	5,587652	3,183055	1,950859	2,564469
CD7	0,308709	0,168848	0,370714	0,275479	1,051271	0,88572
HEXIM1	0,341873	0,294164	0,281023	0,528715	1,107269	0,491504
ZFP36L2	0,419728	0,29006	0,602593	0,994106	0,5941	0,65332
PHLDA1	1,242179	0,871052	0,874501	3,669335	3,548455	2,438023
ICOS	38,87475	33,61151	28,18285	28,27186	22,867	12,3156
TBK1	1,997608	1,826397	2,112885	1,170384	1,014477	1,324211
SERTAD1	0,622663	0,48185	0,507075	1,506509	1,629678	1,489562
RGCC	14,33894	17,45716	6,169037	26,54517	29,21713	14,89068
RASSF2	1,35348	1,528463	3,020246	0,773576	1,082834	2,001995
ZHX2	0,51781	0,445998	0,422601	0,166114	0,31724	0,231389
TRAT1	1,742582	1,972655	3,525177	0,659745	0,77107	1,869388
MKNK2	0,526444	0,525797	0,904185	0,890472	1,655712	2,071419
WHSC1L1	2,232736	2,175896	1,842166	1,121726	1,138474	0,908808
PAG1	0,392278	0,437036	0,661577	0,888417	0,894548	0,915607
ASH1L	0,843134	3,531441	0,801349	0,977779	0,64699	0,729626
SASH3	1,986029	1,614229	1,872872	0,803775	0,868049	0,768664
RHOF	1,689248	1,55292	1,496464	2,455446	3,754818	3,3921
HIST1H2BD	1,530056	1,737305	1,080964	3,604816	5,658225	5,196772
COTL1	2,067631	1,649654	1,680892	2,492622	3,490983	2,67817
DUSP16	1,947225	1,588539	1,897325	1,040622	0,686843	0,627736
APOL6	1,297855	0,767199	1,836929	0,753002	0,324914	0,587949
SH3BGRL3	3,87775	3,817903	3,723164	5,623794	8,761603	6,832186
GALM	2,436025	2,23777	2,729174	0,724731	0,943133	1,314706
TIGIT	0,975579	0,544882	1,148579	0,356361	0,267226	0,514088
FAM179A	0,177322	0,564637	0,209902	0,080264	0,12928	0,1053
MALAT1	18,48124	21,7963	20,88071	34,27242	39,12131	34,8568
RPLP1	28,98617	19,34464	25,37108	34,44531	67,55468	39,73011
OGDH	1,104717	0,884506	1,094471	1,702142	1,946904	1,831348



Epigenetic and transcriptomic analyses of inflammatory site-derived monocytes indicate a role in bone degradation

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Manuscript in preparation

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ABSTRACT

The pathogenesis of autoimmune diseases is a multifactorial process, meaning that a complex interplay of genetics, epigenetics, and environmental factors defines disease outcome. This is illustrated by the observation that approximately 60% of the single nucleotide polymorphisms (SNPs) associated with autoimmune diseases are localized within regulatory DNA regions, enhancers. To increase our understanding of epigenetic alterations contributing to autoimmune diseases, we sought to characterize the active enhancer profile of monocytes derived from the synovial fluid (SF) of Juvenile Idiopathic Arthritis (JIA) patients, a well-suited model for studying autoimmune disease. Utilizing H3K27ac chromatin immunoprecipitation, we observed that the active enhancer repertoire of inflammatory site-derived monocytes is distinct from peripheral blood-derived monocytes and is enriched for arthritis-associated SNPs. Furthermore, this active enhancer profile contributes to disease-associated gene expression. Transcriptional and epigenetic analyses of inflammatory site-derived monocytes indicated that osteoclast differentiation could be affected within the synovial compartment. Indeed, we demonstrated that JIA SF potentiates the differentiation of monocytes towards osteoclasts. Altogether, these data provide insight into epigenetic regulation within autoimmune disease patient-derived cells and indicate that inhibition of enhancer activity might be a novel therapeutic strategy for the treatment of JIA and other forms of autoimmune rheumatic diseases.

INTRODUCTION

Enhancers are *cis*-regulatory DNA regions, generally a few hundred base pairs in size, that are pivotal for the spatio-temporal regulation of gene expression by recruiting RNA polymerase II, transcription factors, and co-factors, such as the histone acetyltransferase p300 or the mediator complex[1]. Enhancer regions are characterized by methylation of histone H3 at lysine 4 (H3K4me) and contain H3K27 acetylation in their active status[2,3]. The relevance of enhancers for cell function is underscored by the fact that several monogenic diseases are caused by mutations in genes involved in establishing, maintaining or regulating enhancers[4]. Furthermore, approximately 60% of the single nucleotide polymorphisms (SNPs) associated with autoimmune diseases are localized in enhancer regions[5,6]. In addition, disease-associated variants preferentially map to enhancer regions specific for disease-relevant cell types[7–9]. For example, SNPs associated with rheumatoid arthritis (RA) and ulcerative colitis are enriched in enhancers in T cells and colon tissue, respectively[9]. This suggests that the regulatory effect of enhancer-located SNPs is context specific. It is therefore becoming increasingly clear that characterize of the epigenome of disease-relevant cell types aids to our understanding of how an autoimmune disease-associated genotype can lead to a phenotype.

JIA is one of the most common autoimmune diseases in children and characterized by chronic inflammation of the joints[10,11]. JIA is a well-suited model to study autoimmune disease pathogenesis, as cells can be directly obtained from the inflammatory site. Autoimmune diseases are hallmarked by activated monocytes[12]. Indeed, the activation markers CD80, CD86, and HLA-DR are upregulated on monocytes obtained from the synovial fluid (SF) of JIA patients[13]. In addition, levels of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6), cytokines known to be produced by monocytes, are increased within the SF, indicating that monocytes within the SF display a hyperactive phenotype[14–16]. For JIA SF-derived T cells, T cells obtained from asthma patients, and systemic lupus erythematosus (SLE) patient-derived monocytes differences in the enhancer profile have been described[17,18]. Furthermore, disruption of enhancer-mediated transcription, using the bromodomain and extra-terminal domain (BET) inhibitor JQ1, in JIA SF T cells reduced disease-associated gene expression, indicating that enhancers contribute to disease pathogenesis[19,20]. SLE-associated histone quantitative trait loci (hQTLs), i.e. genetic variants that induce alterations of the amount of histone post-translational modifications, present within enhancer regions are significantly enriched within SLE autoimmune disease risk haplotypes[21]. Besides, these enhancer hQTLs affected gene expression to a higher extent than non-histone QTLs. Recently, RA fibroblast-like synoviocytes (FLS) have been extensively characterized on the epigenomic level, leading to the identification of a novel pathway contributing to the aggressive phenotype of RA FLS[22]. Altogether, these data suggest that a comprehensive understanding of the epigenome of disease-relevant cell types can greatly contribute to increased insight into disease pathogenesis.

To get a better understanding of the epigenomic alterations contributing to autoimmune diseases and to further explore the role of monocytes in the pathogenesis of JIA, we characterized

the active enhancer profile of JIA patient-derived monocytes. We demonstrate that the enhancer landscape of peripheral blood (PB)-derived monocytes from JIA patients is relatively comparable to healthy control monocytes, while SF-derived monocytes display an altered enhancer profile. These enhancer regions are enriched for arthritis-associated SNPs and correlate with disease-associated gene expression. Furthermore, analysis of the pathways associated with the transcriptomic and epigenomic changes in JIA SF-derived monocytes indicated a role for JIA SF monocytes in osteoclast differentiation. Indeed we found that JIA SF potentiates osteoclast differentiation. Altogether, these data indicate that epigenetic and transcriptomic analysis of autoimmune disease patient-derived cells can point towards (novel) pathways involved in disease pathogenesis.

MATERIALS AND METHODS

COLLECTION OF SYNOVIAL FLUID (SF) AND PERIPHERAL BLOOD (PB) SAMPLES

Nine oligoarticular JIA patients were included in this study who at the time of sampling all had active disease. PB and SF samples were obtained at the same moment, either via vein puncture or intravenous drip and therapeutic joint aspiration, respectively. 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: 11-499/C) and performed according to the principles expressed in the Helsinki Declaration. SF samples were treated with hyaluronidase (Sigma-Aldrich) for 30 min, 37°C. To obtain cell-free plasma and SF samples, samples were centrifuged; supernatants were collected, and stored at -80ºC. Synovial fluid mononuclear cells (SFMCs) and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density gradient centrifugation (GE Healthcare) and were frozen and stored at -80°C until further use.

CHIP-SEQUENCING AND ANALYSIS

HC PBMCs, JIA PBMCs and SFMCs were thawed and $CD14^+$ cells were sorted by flow cytometry using a BD FACS Aria II (BD Biosciences). Cells were crosslinked with 2% formaldehyde (Sigma-Aldrich) and after 10 min crosslinking was stopped by adding 0.15 M glycine. Nuclei were isolated in 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 and 0.3% SDS. Lysates were sheared using Covaris microTUBE (duty factor 20%, peak incident power 105, 200 cycles per burst, 480 sec cycle time) and diluted in 20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100. Sheared DNA was incubated overnight with anti-histone H3 acetyl K27 antibody (ab4729; Abcam) pre-coupled to protein A/G magnetic beads (Pierce). Samples were washed and crosslinking was reversed by adding 1% SDS, 100 mM NaHCO3, 200 mM NaCl, and 300 ug/ml proteinase K (Invitrogen). DNA was purified using ChIP DNA Clean & Concentrator kit (Zymo Research), endrepair, a-tailing, and ligation of sequence adaptors was done using Truseq nano

DNA sample preparation kit (Illumina). Samples were PCR amplified and barcoded libraries were sequenced 75 bp single-end on Illumina NextSeq500. Peaks were called using Cisgenome 2.0 (–e 150 -maxgap 200 –minlen 200)[23]. Peak coordinates were stretched to at least 2000 base pairs and collapsed into a single list. Overlapping peaks were merged based on their outmost coordinates. Only peaks identified by at least 2 independent datasets were further analyzed. Peaks with differential H3K27ac occupancy were identified using DESeq (padj<0.1)[24]. Enriched H3K27ac regions that were more than 1 kB away from a transcription start site (TSS) were considered as enhancers. Genes were considered to be associated with an enhancer if their TSS lies within 20 kB of the center of the enhancer region.

RNA-SEQUENCING AND ANALYSIS

For analysis of JQ1 sensitive genes, HC PBMCs were thawed and $CD14^+$ cells were sorted by flow cytometry using a BD FACS Aria II (BD Biosciences). Subsequently, $CD14^+$ cells were cultured o/n in the presence of 100 ng/mL LPS (Invivogen) and treated with 300 nM JQ1(-) or JQ1(+) (ApexBio). Total RNA was extracted using the RNAeasy kit (Qiagen), sample preparation was performed using TruSeq stranded total RNA with ribo-zero globin (Illumina) and samples were sequenced 75 bp single-end on Illumina NextSeq500 (Illumina Inc.; Utrecht DNA Sequencing Facility).

For analysis of gene expression in JIA SF monocytes, HC PBMCs and JIA SFMCs were thawed, $CD3^+$ cells were depleted by human anti-CD3 microbeads (Milteny Biotec) according to the manufacturer's instructions and $CD14^+CD16^+$ cells were sorted by flow cytometry using a BD FACS Aria III (BD Biosciences). Total RNA was extracted using Trizoll, mRNA was isolated using Poly(A)Purist MAG kit (Life Technologies) and additionally purified with a mRNA-ONLY Eukaryotic mRNA Isolation Kit (Epicentre). Transcriptome libraries were then constructed using SOLiD total RNA-seq kit (Applied Biosystems) and sequenced using 5500 W Series Genetic Analyzer (Applied Biosystems) to produce 40-bp-long reads.

Reads were aligned to the human reference genome GRCh37 using STAR version 2.4.2a. Picard's AddOrReplaceReadGroups (v1.98) was used to add read groups to the BAM files, which were sorted with Sambamba v0.4.5 and transcript abundances were quantified with HTSeq-count version 0.6.1p1 using the union mode. Subsequently, reads per kilobase million reads sequenced (RPKMs) were calculated with edgeR's RPKM function. Differentially expressed genes were identified using the DESeq2 package with standard settings. Genes with absolute padj<0.05, with a base mean ≥ 10 and a log2 FC ≥1 or ≤ -1 were considered as differentially expressed and used for further analysis.

SNP ENRICHMENT

Enrichment of SNPs in regulatory regions was performed as described previously[25]. In brief: a variant falling within peak coordinates was considered as overlapping variant. Random matched variant sets were generated from variants present on Human Omni1S genotyping chip (Illumina). Random variant selection was matched for similar minor allele frequency and distance to the

closest gene. Linkage Disequilibrium (LD) information was accessed from HapMap[26]. To determine the significance of overlap of inflammatory arthritis-associated SNPs with enhancers, the p-value was calculated using binominal cumulative distribution function b(x; n, p) using R pbinom() function as described previously[27].

GENE ONTOLOGY ENRICHMENT ANALYSIS AND GENE SET ENRICHMENT ANALYSIS (GSEA)

Gene ontology (GO) enrichment analysis was performed using the ToppFunn tool of ToppGene Suite[28]. GO terms with an adjusted (Benjamini-Hochberg procedure) p-value <0.05 were considered being enriched. Gene set enrichment analysis was performed using GSEA pre-ranked[29]. Significance of the enrichment was calculated based on 1000 cycles of permutations and the normalized enrichment score (NES) and the p-value (FDR) are annotated. Genes belonging to the annotated KEGG pathway osteoclast differentiation are listed in Supplemental Table 1.

MONOCYTE ISOLATION AND DIFFERENTIATION TOWARDS OSTEOCLASTS

Heparinized blood samples were collected from healthy volunteers, from which informed consent was obtained. PBMCs were isolated by Ficoll-Paque density centrifugation (GE Healthcare Lifesciences). CD14+ cells were isolated by magnetic-activated cell sorting (MACS) using human CD14 microbeads (Milteny Biotec) according to the manufacturer's instructions. Monocytes were cultured in minimal alpha essential medium (αMEM; Life Technologies) supplemented with 10% HyClone (Thermo Scientifcic), 100 U/ml penicillin (Gibco), and 100 mg/ml streptomycin (Gibco) in the absence or presence of 10% plasma or SF. 1.5 x 106 cells were seeded on plastic or on bovine cortical bone slices (0.5 mm thick) in 96-well plates. The cells were cultured for 3 days with 25 ng/mL human recombinant M-CSF (R&D Systems). After 3 days the concentration of M-CSF was reduced to 10 ng/mL and 2 ng/mL RANKL (R&D Systems) was added. Cultures were maintained at 37°C and 5% CO2 and medium was refreshed every 3-4 days. Cells were cultured between 17 – 21 days.

TRAP STAINING

After 17 days, osteoclast cultures were washed with PBS, fixed for 10 min with 4% formaldehyde (Sigma-Aldrich), washed with PBS, and stored in PBS at 4°C until further use. Cells were stained for TRAP using the acid phosphatase leukocyte kit (Sigma-Aldrich) according to manufacturer's instructions, but using a solution containing a 3x higher tartrate concentration. Nuclei were visualized using 4',6-diamidino-2-fenylindool (DAPI). Cells were analyzed using a BX-60 microscope (Olympos) equipped with a DFC450C camera (Leica) and LAS 4.7 software (Leica). Pre-defined areas of each condition, with 3 culture replicates per condition, were photographed. Multinucleated $TRAP^+$ cells with three or more nuclei were considered osteoclasts and expressed as the percentage of osteoclasts compared to the total amount of cells.

BONE RESORPTION

After 21 days, bone slices were washed with H_2O and stored at 4°C until further use. Bone slices were sonicated for 30 min in 10% NH_3OH on ice, washed with H_2O, incubated for 10 min in water saturated alum, washed with H_2O and stained with coomassie brilliant blue (Pharmica). Resorption pits were visualized using a BX-60 microscope (Olympos) equipped with a DFC450C camera (Leica) and LAS 4.7 software (Leica). Pre-defined areas of each bone slice, with 2 culture replicates for each condition, were photographed. Resorption areas were analyzed using Fiji and expressed as the percentage of total bone surface area measured[30].

STATISTICAL ANALYSIS

For ChIP-seq and RNA-seq analysis, p-values were adjusted with the Benjamini-Hochberg procedure and a false discovery rater (FDR) ≤ 0.1 and (FDR) ≤0.05 was considered significant for ChIP-seq and RNA-seq, respectively. ΔFPKM for enhancer-associated genes and osteoclast differentiation-associated genes was analyzed using Wilcoxon-matched pairs signed rank test. Osteoclast percentages were analyzed using repeated measures one-way ANOVA. All analyses were performed using GraphPad Prism (GraphPad Software).

RESULTS

IDENTIFICATION OF ACTIVE ENHANCERS IN JIA MONOCYTES

To identify the active enhancer profile of JIA patient-derived cells, we performed H3K27ac chromatin immunoprecipitation-sequencing (ChIP-seq) on $CD14^+$ cells sorted from HC peripheral blood mononuclear cells (PBMCs), JIA PBMCs, and JIA synovial fluid mononuclear cells (SFMCs). Principal component analysis (PCA) of the three different groups demonstrated that distinct clusters can be observed for HC, JIA PB-derived monocytes, and JIA SF-derived monocytes. HC and JIA PB-derived monocytes cluster relatively close to each other, suggesting that the variance between those two groups is relative low (**Figure 1A**). Indeed, when comparing H3K27ac regions between HC and JIA blood-derived monocytes, of the 24341 H3K27ac regions detected, only 158 were significantly decreased and 317 were significantly increased (**Figure 1B**). JIA SF-derived monocytes clustered separately from both HC and JIA blood-derived monocytes, indicating that SF-derived monocytes have an altered H3K27ac pattern. In agreement with this, there are 3425 significantly decreased and 3291 significantly increased H3K27ac regions observed when comparing JIA SF monocytes to JIA PB monocytes (**Figure 1C**). Since we specifically were interested in active enhancers, we focused on enriched H3K27ac regions that were more than 1 kB away from any transcription start site, thus excluding promoter regions. Genes associated with an increased enhancer in JIA SF were associated with (transcriptional) regulation of the inflammatory immune response and comprised of many cytokines, chemokines, and cytokine and chemokines receptors (**Figure 1D-F**). Similar results were obtained by comparing H3K27ac regions between HC monocytes and JIA SF-derived monocytes (**Supplemental Figure 1A-C**). In contrast, no enrichment for biological processes was observed for genes associated with an

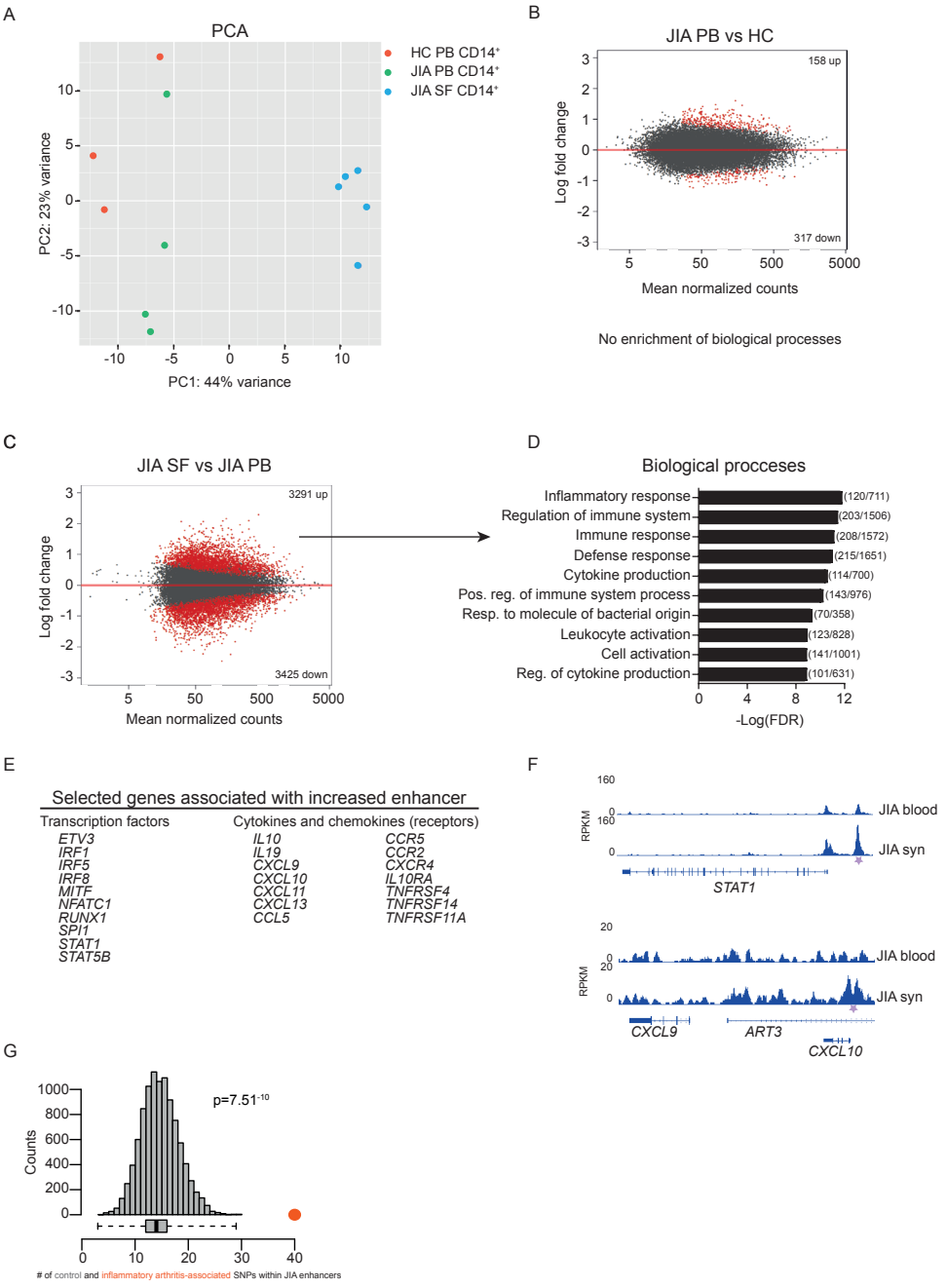


Figure 1. Identification of enhancers in JIA monocytes.
(A) Principal component analysis (PCA) of top 5000 H3K27ac regions within HC (n=3), JIA peripheral blood (n=4) and JIA SF-derived (n=5) monocytes. (**B**) MA plot of differences in H3K27ac signal between HC and JIA PB-derived monocytes. Red dots indicate H3K27ac regions with a FDR<0.1. (**C**) MA plot of differences in H3K27ac signal between

>>>

JIA PB-derived and JIA SF-derived monocytes. Red dots indicate H3K27ac regions with a FDR<0.1. (**D**) Top 10 biological processes associated with genes that are associated with a significantly increased enhancer in JIA SF-derived monocytes. (**E**) Selected genes associated with an increased enhancer in JIA SF-derived monocytes. (**F**) H3K27ac enrichment tracks of *STAT1* and *CXCL9* for JIA PB-derived and SF-derived monocytes. Enhancer regions are indicated with a purple star. (**G**) Number of inflammatory arthritis-associated SNPs (orange dot) overlapping with JIA enhancers compared to 10,000 random SNP sets (grey bars).

increased enhancer in JIA PB monocytes compared to HC monocytes, suggesting that these enhancer-driven genes are probably not involved in disease-associated processes. To further asses the relevance of JIA enhancers, we assessed the enrichment of inflammatory arthritis-associated SNPs within JIA enhancers compared to random SNPs. Arthritis-associated SNPs are enriched within JIA enhancers, suggesting that these regulatory regions are involved in disease pathogenesis. Collectively, these data demonstrate that JIA SF-derived monocytes display a distinct enhancer profile which might be associated with their activated phenotype.

The active enhancer profile of JIA monocytes correlates with disease-associated gene expression
To determine whether the active enhancer profile of JIA SF monocytes was reflected at the gene expression level, RNA-sequencing was performed on HC PB-derived and JIA SF-derived monocytes. This revealed that many genes are differentially expressed between HC and JIA SF monocytes (**Figure 2A**). The genes upregulated in JIA SF-derived monocytes are involved in regulation of the (innate) immune system and overlapped with genes associated with autoimmune diseases (**Figure 2B and 2C**). Gene set enrichment analysis demonstrated that genes associated with an increased enhancer are enriched within the genes upregulated in JIA, indicating that an increased enhancer signal contributes to increased gene expression in JIA SF monocytes (**Figure 2D**). In agreement with this, genes associated with an increased enhancer in JIA are more abundantly expressed in JIA SF monocytes compared to HC monocytes (**Figure 2E and 2F**). Since the upregulated genes in JIA are likely to be associated with disease pathogenesis, this indicates that enhancers contribute to disease-associated gene expression. To further study the contribution of active enhancers to disease-associated gene expression, we analyzed whether disease-associated gene expression is reduced by the bromodomain and extraterminal domain (BET) inhibitor JQ1, which acts as an inhibitor of enhancer-mediated transcription[20]. Treatment of HC monocytes with JQ1 significantly affected gene expression, with the most pronounced effect on inhibition of gene expression (**Supplemental Figure 2A and 2B**). Gene set enrichment analysis demonstrated that the genes upregulated in JIA are enriched within the genes affected by JQ1 in HC monocytes, suggesting that expression of these genes heavily depends on enhancer regulation and can be preferentially inhibited by JQ1 (**Figure 2G**). Altogether, these data indicate that the altered enhancer landscape in JIA SF-derived monocytes contributes to disease-associated gene expression.

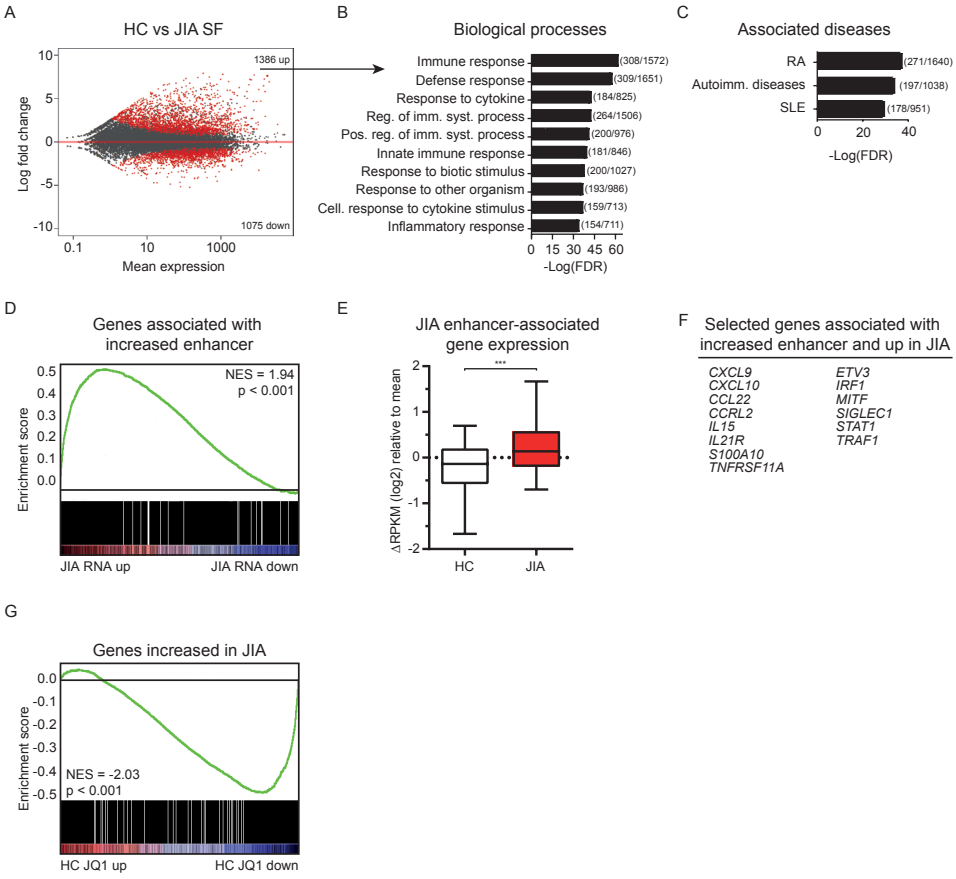


Figure 2. JIA enhancers contribute to disease-associated gene expression.
(**A**) MA plot of genes differentially expressed between HC and JIA SF-derived monocytes. Red dots indicate genes with a FDR<0.05. (**B**) Top 10 biological processes associated with genes that are significantly upregulated in JIA SF-derived monocytes. (**C**) Top 3 diseases associated with genes that are significantly upregulated in JIA SF-derived monocytes. (**D**) Gene set enrichment analysis for genes associated with an increased enhancer in JIA and genes differentially expressed in JIA SF monocytes. (**E**) Boxplot with 5%-95% whiskers displaying ΔFPKM (log2) values of genes associated with an increased enhancer in JIA relative to the mean of both HC and JIA. (**F**) Selected genes associated with an increased enhancer and increased expression in JIA SF-derived monocytes. (**G**) Gene set enrichment analysis for genes increased in JIA monocytes and genes affected by JQ1 treatment in HC monocytes.

EPIGENETIC AND TRANSCRIPTIONAL ANALYSES OF INFLAMMATORY SITE-DERIVED MONOCYTES SUGGEST THAT OSTEOCLAST DIFFERENTIATION IS AFFECTED IN THE SYNOVIAL COMPARTMENT

Analysis of the top 10 pathways related to genes associated with an increased enhancer in JIA and downregulated by JQ1 revealed, amongst others, enrichment of the osteoclast differentiation pathway (**Figure 3A and Supplemental Table 1**). Osteoclasts are bone-degrading cells and since autoimmune arthritis, such as JIA, is characterized by bone degradation we further investigated this pathway in our datasets. Comparison of the top 25 pathways significantly associated with

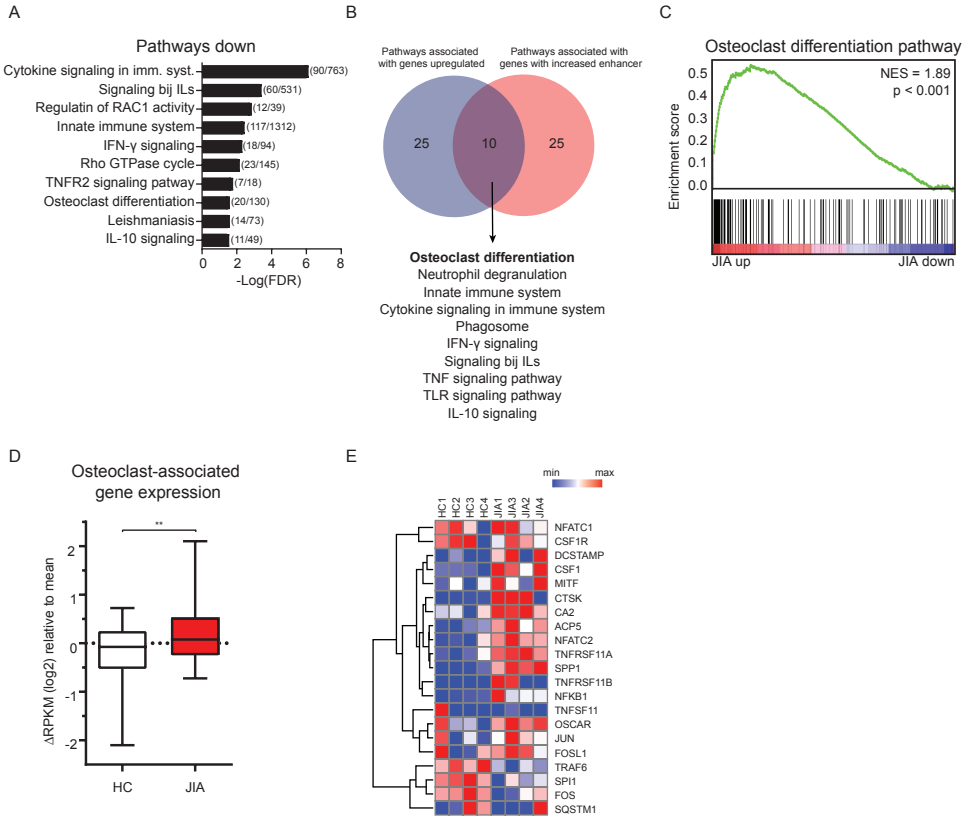
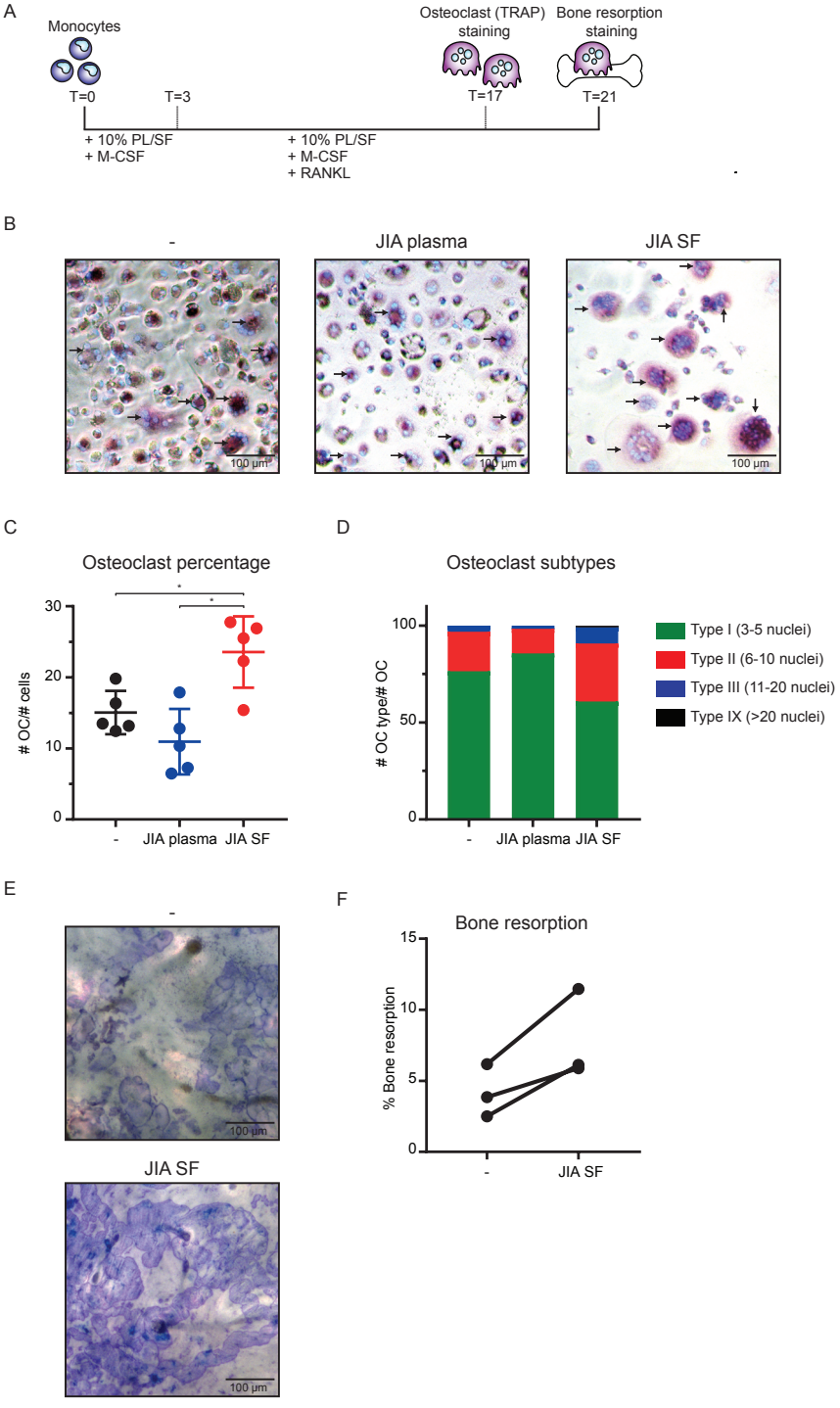


Figure 3. Epigenetic and transcriptional analyses of inflammatory site-derived monocytes indicate a role in osteoclast differentiation.
(**A**) Top 10 pathways related to genes associated with an increased enhancer in JIA and significantly downregulated by JQ1 in HC monocytes. (**B**) Overlap of pathways associated with genes upregulated in SF JIA monocytes and pathways associated with genes associated with an increased enhancer in JIA SF monocytes. (**C**) Gene set enrichment analysis of osteoclast differentiation-associated genes and genes differentially expressed in JIA SF monocytes. (**D**) Boxplot with 5%-95% whiskers displaying ΔFPKM (log2) values of osteoclast differentiation-associated genes relative to the mean of both HC and JIA. (**E**) Heatmap demonstrating the expression of selected osteoclast differentiation-associated genes in HC and JIA SF monocytes.

genes upregulated in JIA and genes associated with an increased enhancer also revealed the osteoclast differentiation pathway to be enriched in both groups (**Figure 3B**). Correspondingly, osteoclast differentiation-associated genes are enriched within the genes that are increased in JIA SF-derived monocytes and as a result expression of osteoclast differentiation-associated genes is increased within JIA SF monocytes (**Figure 3C-E**). Furthermore, these observations that osteoclast differentiation-associated genes are prone to inhibition by JQ1, validates that their expression likely depends on epigenetic regulation (**Figure 3F and Supplemental Figure 2, 3A and 3B**). Taken together, these data suggest that osteoclast differentiation is increased within the synovial environment and is (partially) regulated on the epigenetic level.


A
Monocytes
Osteoclast (TRAP) staining
Bone resorption staining
T=0
T=3
T=17
T=21
+ 10% PL/SF
+ M-CSF
+ 10% PL/SF
+ M-CSF
+ RANKL
B
-
JIA plasma
JIA SF
100 µm
C
Osteoclast percentage
OC/# cells
30
20
10
0
-
JIA plasma
JIA SF
D
Osteoclast subtypes
OC type/# OC
100
50
0
Type I (3-5 nuclei)
Type II (6-10 nuclei)
Type III (11-20 nuclei)
Type IX (>20 nuclei)
E
-
JIA SF
F
Bone resorption
% Bone resorption
15
10
5
0

◀ **Figure 4. JIA SF potentiates osteoclast differentiation.**
(**A**) Experimental setup of the differentiation of HC monocytes to osteoclasts in the presence of JIA plasma (PL) or JIA SF. (**B**) Representative pictures of TRAP (purple) and DAPI (blue) staining of monocytes after differentiation towards osteoclasts in the absence or presence of JIA plasma or SF. (**C**) Quantification of the percentage of osteoclasts after differentiation in the absence or presence of JIA plasma or SF (n=5). (**D**) Quantification of the types of osteoclasts after differentiation in the absence or presence of JIA plasma or SF (n=5). (**E**) Representative pictures of the amount of bone resorption in monocyte cultures after differentiation towards osteoclasts in the absence or presence of JIA SF. (**F**) Quantification of the percentage of bone resorption by osteoclasts cultured in the absence or presence of JIA plasma or SF (n=3).

JIA SF POTENTIATES THE DIFFERENTIATION OF MONOCYTES TOWARDS OSTEOCLASTS

To further explore the role of osteoclast differentiation within the synovial inflammatory environment, HC CD14$^+$ cells were differentiated towards osteoclasts using macrophage colony-stimulating factor 1 (M-CSF/CSF-1) and receptor activator of nuclear factor κB ligand (RANKL), in the absence or presence of JIA SF and JIA blood plasma (**Figure 4A**). JIA plasma did not affect osteoclast differentiation compared to HC plasma and is therefore used as a control situation in our experiments (**Supplemental Figure 4A**). Osteoclast formation was increased in monocyte cultures differentiated in the presence of JIA SF compared to JIA plasma and regular culture medium (**Figure 4B and 4C**). Next to the percentage of osteoclasts, the percentage of osteoclasts containing 6 or more nuclei is increased in the presence of JIA SF, indicating that SF enhances fusion during the differentiation period as well (**Figure 4D**). We also assessed whether JIA SF can enhance spontaneous osteoclast differentiation, *i.e.* osteoclast formation without addition of RANKL and M-CSF (**Supplemental Figure 4B**). Also in this setting, addition of JIA SF increased the percentage of osteoclasts being formed, as well as the percentage of osteoclasts containing 6 or more nuclei (**Supplemental Figure 4C-E**). To evaluate whether the osteoclasts formed in the presence of SF were functional, a bone resorption assay was performed in which cells were differentiation on bone and the percentage of bone resorption was determined. Osteoclasts formed in the presence of SF were functional and in line with the increased percentage of osteoclasts, the percentage of bone resorption was increased in the presence of JIA SF (**Figure 4E and Supplemental Figure 4D**). In summary, we demonstrated that JIA SF potentiates the differentiation of monocytes towards osteoclasts, indicating that the inflammatory synovial environment contributes to bone degradation within autoimmune arthritis.

DISCUSSION

Here, we characterized the active enhancer profile of JIA PB and SF-derived monocytes. We observed that the active enhancer profile of JIA PB monocytes resembles the enhancer landscape of HC monocytes, while JIA SF monocytes display a distinct active enhancer profile, reflecting the activated, inflammatory cellular phenotype. Furthermore, we demonstrated that arthritis-associated SNPs are enriched within enhancers increased in JIA SF monocytes, suggesting that these enhancer regions are involved in disease pathogenesis. For other

autoimmune diseases it has been demonstrated that SNPs located within enhancer regions can contribute to autoimmunity[31–33]. Therefore, it will be interesting to further investigate SNPs present within JIA enhancers and especially to study their effect on enhancer activity, as this could reveal whether SNPs actively contribute to defining the enhancer profile of autoimmune disease patient-derived cells. The altered enhancer landscape of JIA SF monocytes could also be a reflection of the highly inflammatory synovial environment. Since TNF-α has been described to shape the enhancer repertoire of endothelial cells, it seems plausible that pro-inflammatory mediators within the synovial compartment could affect the enhancer profile[34]. The enhancer profile of $CD4^+CD45RO^+$ T cells derived from the joint of JIA patients has also been demonstrated to be distinct compared to PB T cells, which argues for a role of the synovial environment within enhancer regulation[19]. However, arthritis-associated SNPs were also enriched within JIA T cell enhancer regions, indicating that probably intrinsic as well as extrinsic factors are involved in shaping the enhancer repertoire.

We observed that the active enhancer profile of JIA SF monocytes correlated with disease-associated gene expression. We further investigated epigenetic regulation of JIA-associated gene expression using the BET inhibitor JQ1[20]. JQ1 prevents binding of bromodomain proteins, especially BRD4, to acetylated chromatin regions, and thereby impairs recruitment of elongation factor complex (P-TEFb), which inhibits RNA polymerase II binding and activation, thus inhibiting transcription[35]. Since BRD4 has been demonstrated to preferentially localize to enhancer regions, BET inhibitors can be used to inhibit enhancer activity[36,37]. Analysis of the genes affected by JQ1 in HC monocytes revealed that JIA-associated genes were highly sensitive to downregulation by JQ1, indicating that expression of these genes is regulated on the epigenetic level. Furthermore, this indicates that BET inhibition of JIA SF monocytes could be effective in reducing disease-associated gene expression. It will therefore be interesting to treat JIA SF monocyte *in vitro* with JQ1 and analyze their activation status and gene expression. For JIA SF-derived T cells, JQ1 treatment has been demonstrated to reduce disease-associated gene expression and affected genes predominantly involved in cytokine – cytokine receptor interactions[19]. Monocytes produce pro-inflammatory cytokines that can stimulate T cells, for example TNF-α and IL-6, and vice versa, such as T cell-derived IFN-γ. This suggests that the effect of JQ1 on T cells will probably also indirectly affect monocytes and the other way around, indicating that BET inhibition within the synovial environment might have a synergistic effect. In addition, the BET inhibitor I-BET151 has been demonstrated to suppresses osteoclastogenesis *in vitro* and *in vivo* and decreased bone resorption in a serum-induced arthritis mouse model[38]. JIA and other forms of inflammatory arthritis, such as RA, psoriatic arthritis (PsA), and ankylosing spondylitis (AS), are characterized by bone erosion and our transcriptomic and epigenomic analyses indicated that osteoclast differentiation might be affected within the JIA synovial environment[39–42]. JQ1 and other BET inhibitors have been proven effective in suppressing inflammation in many preclinical models for autoimmune diseases and are currently being used in clinical trials against solid tumors and hematologic malignancies[43,44]. This indicates that BET inhibition might be a powerful, novel, therapeutic approach for the treatment of JIA and other autoimmune diseases.

We demonstrated that JIA SF potentiates the differentiation of HC monocytes towards osteoclasts *in vitro*. These findings suggest that (inflammatory) mediators present within the SF promote osteoclast differentiation. Indeed, two factors, M-CSF/CSF-1 and RANKL, have been described to be important for osteoclast differentiation and are increased in the SF of oligoarticular JIA patients compared to blood plasma levels, suggesting that these cytokines contribute to enhanced osteoclast formation (data not shown)[45]. However, also in the presence of recombinant RANKL and M-CSF, osteoclast formation is increased by the addition of SF, indicating that other mediators within the SF contribute to osteoclast differentiation. Indeed, several inflammatory cytokines have been linked to osteoclastogenesis. For example, TNF-α has been demonstrated to induce osteoclast formation *in vitro* and *in vivo* in a RANKL-dependent as well as a RANKL-independent manner[46–48]. IL-6 and IL-1 family members have been described to be involved in regulating the effects of TNF-α on osteoclastogenesis[49–52]. In addition, the Th17-associated cytokines IL-17 and IL-23 have been implicated in osteoclast differentiation[53–56]. Many of these cytokines have been demonstrated to be either elevated within the SF of JIA patients, such as IL-6 and TNF-α, or have been described to be involved in JIA pathogenesis, for example IL-17, indicating that these cytokines are likely candidates for the osteoclast-promoting effect of JIA SF[16,45,57]. The percentage of bone resorption was increased in osteoclast cultures in the presence of JIA SF. However, it remains to be determined whether this increase is related to the increased percentage of osteoclasts or whether the bone resorption capacity per osteoclast increased as well.

SF from RA and pyrophosphate arthropathy patients has been described to induce osteoclast formation, indicating that the potentiating effect of SF on osteoclast differentiation is not specific for JIA[58]. SF from non-inflammatory osteoarthritis (OA) patients does not increase osteoclast differentiation, suggesting that inflammatory mediators are responsible for promoting osteoclastogenesis. For PsA patients it has been demonstrated that the amount of osteoclast precursor cells within the blood is increased and PsA PBMCs are more prone to differentiate into osteoclasts *in vitro* compared to HC PBMCs[59]. This suggests that, next to extrinsic factors, also cell-intrinsic properties might promote bone degradation in autoimmune arthritis patients. To pursue this hypothesis we evaluated the osteoclast differentiation capacity of JIA PB SF-derived monocytes *in vitro*, but due to the high number of cells needed and impaired cell viability during the 17 day differentiation period, we were not able to reliably perform these assays. The osteoclast differentiation capacity of $CD14^+CD16^+$ cells is lower compared to $CD14^+CD16^-$ on bone[60]. The majority of $CD14^+$ cells within the synovial compartment of JIA patients are also $CD16^+$ (data not shown), indicating that careful evaluation of distinct monocyte subsets is necessary when comparing the osteoclast differentiation capacity of JIA monocytes with HC monocytes.

Altogether, we here demonstrated that enhancers in inflammatory site-derived monocytes contribute to disease-associated gene expression. In addition, transcriptomic and epigenomic analysis indicated that monocyte to osteoclast differentiation is increased within the synovial compartment. Indeed, we were able to demonstrate that JIA SF potentiates the differentiation

of HC monocytes towards osteoclasts *in vitro*. Inhibition of enhancer activity can reduce disease-associated gene expression and has been described to inhibit osteoclastogenesis, indicating that this might be a novel therapeutic strategy for the treatment of JIA and other forms of autoimmune arthritis.

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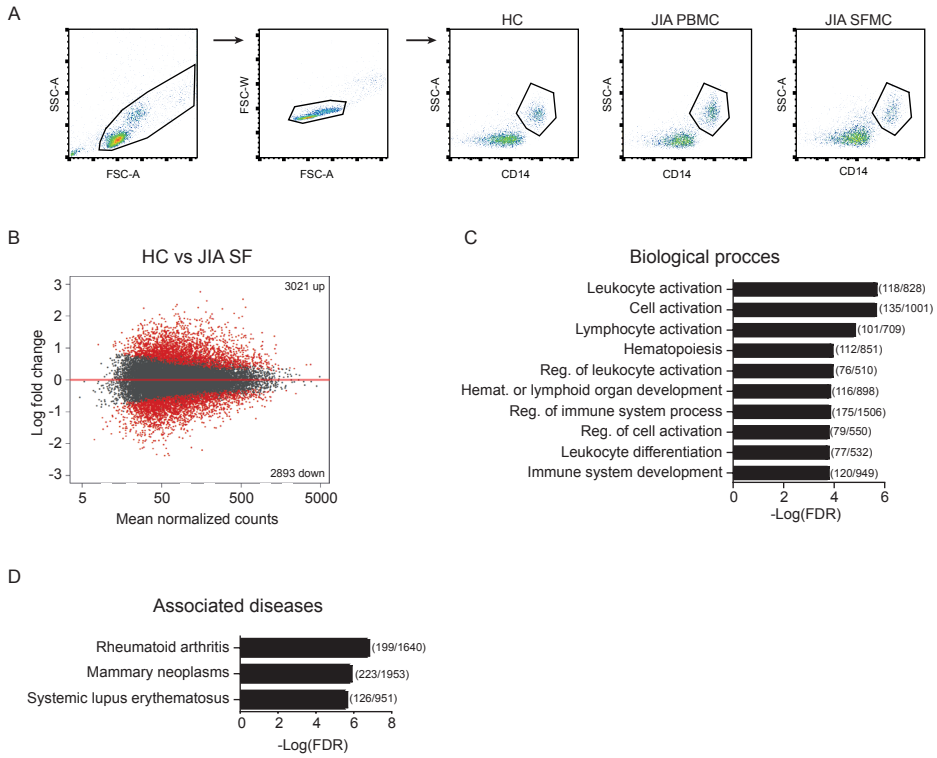
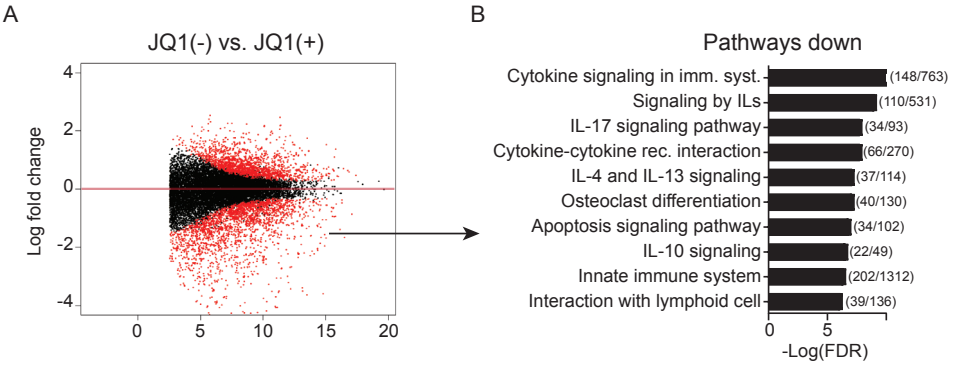
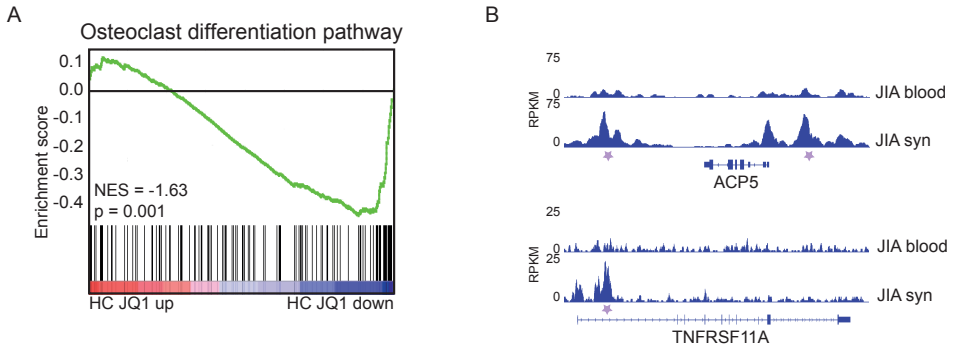
SUPPLEMENTAL INFORMATION



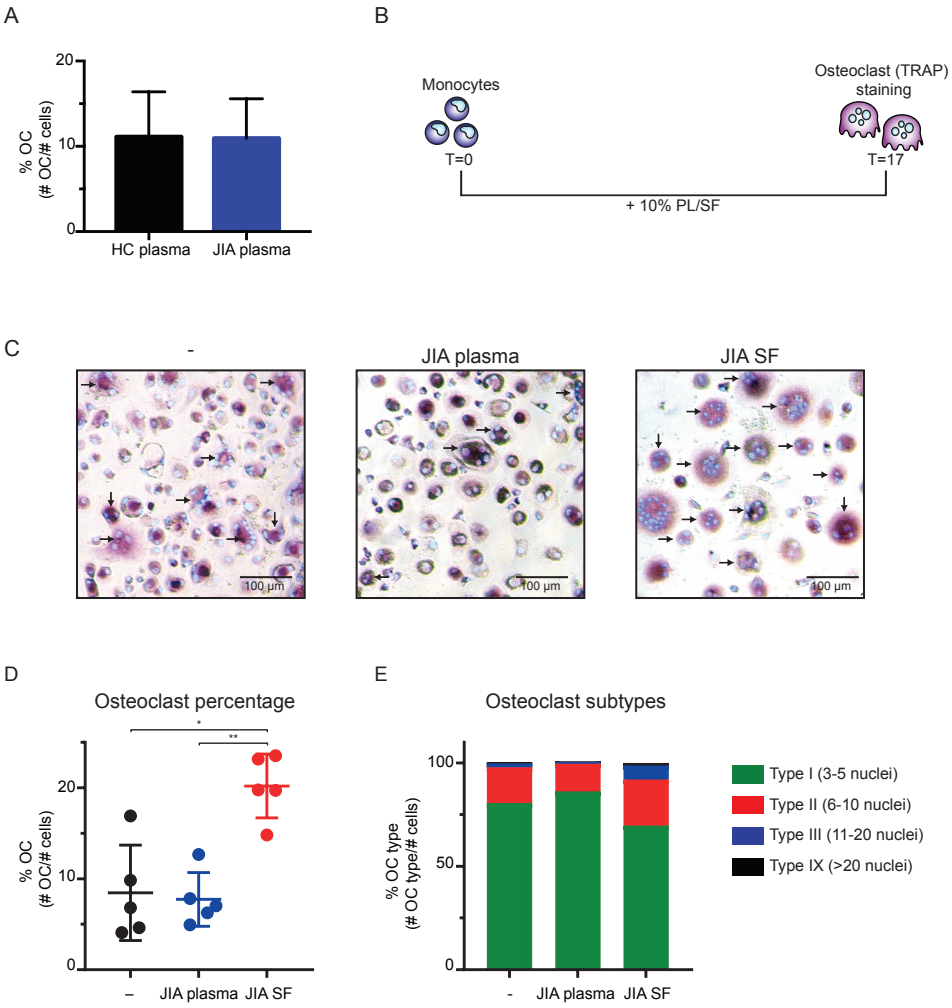
Supplemental Figure 1. Comparison of enhancers within HC and JIA SF monocytes.
(**A**) FACS strategy for the isolation of CD14$^+$ cells from HC PBMC, JIA PBMC, and JIA SFMC. (**B**) MA plot of differences in H3K27ac signal between HC and JIA SF-derived monocytes. Red dots indicate H3K27ac regions with a FDR<0.1. (**C**) Top 10 biological processes related to genes that are associated with a significantly increased enhancer in JIA SF-derived monocytes. (**D**) Top 3 diseases associated with genes that are associated with an increased enhancer in JIA SF-derived monocytes.



Supplemental Figure 2. Effect of JQ1 treatment on HC monocytes.
(**A**) MA plot of genes differentially expressed in HC monocytes upon JQ1(+) treatment. Red dots indicate genes with a FDR<0.05. (**B**) Top 10 pathways associated with genes significantly downregulated by JQ1(+).



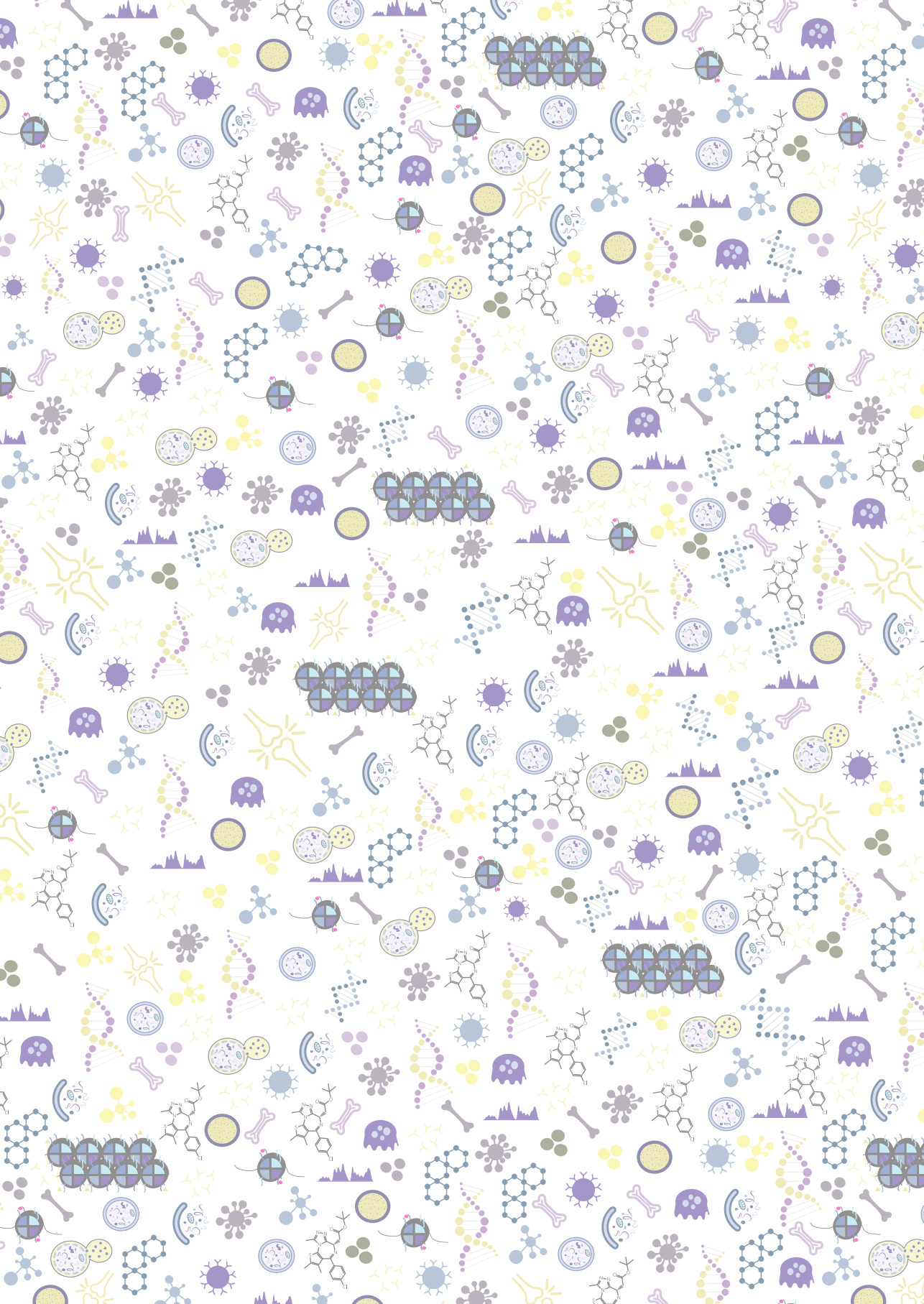
Supplemental Figure 3. Epigenetic regulation of osteoclast-associated genes.
(**A**) Gene set enrichment analysis of osteoclast differentiation-associated genes and genes affected by JQ1 in HC monocytes. (**B**) H3K27ac enrichment tracks of *ACP5* and *TNFRSF11A* for JIA peripheral blood-derived and SF-derived monocytes. Enhancer regions are indicated with a purple star.



Supplemental Figure 4. JIA SF induces osteoclast differentiation.
(**A**) Quantification of the percentage of osteoclasts after differentiation in the presence of HC or JIA plasma (HC plasma n=2; JIA plasma: n=5). (**B**) Outline of the experimental setup to induce monocyte to osteoclast differentiation with JIA plasma (PL) or JIA SF. (**C**) Representative pictures of TRAP (purple) and DAPI (blue) staining of osteoclast differentiation induced by JIA plasma or SF. (**D**) Quantification of the percentage of osteoclasts after induction of differentiation by JIA plasma or SF (n=5). (**E**) Quantification of the types of osteoclasts after induction of differentiation by JIA plasma or SF (n=5).

Supplemental Table 1. Genes belonging to the KEGG pathway "Osteoclast differentiation".

CYBB	TNFRSF11B	SQSTM1
CYLD	GAB2	PIK3R1
AKT3	CHUK	PIK3R2
LILRA1	FOSL1	LOC102725035
LILRB3	IFNAR1	NFKB1
TRAF2	IFNAR2	BTK
LILRA3	SIRPG	NFKB2
LILRA2	IFNB1	NFKBIA
TRAF6	TGFB1	MITF
CALCR	TYK2	TREM2
SPI1	IFNG	SYK
CAMK4	TGFB2	SOCS1
LILRB2	IFNGR1	AKT1
FOS	IFNGR2	AKT2
FOSB	JAK1	TNF
FOSL2	TGFBR1	TAB1
ACP5	TGFBR2	PLCG2
PIK3R3	TYROBP	MAPK1
NCF1	IRF9	MAPK3
MAP3K14	JUN	TNFRSF1A
SOCS3	JUNB	IKBKB
OSCAR	JUND	MAPK8
BLNK	FCGR2C	MAPK11
LILRA6	MAPK14	IL1A
GRB2	TNFSF11	IL1B
IKBKG	PPP3CA	MAPK9
NOX3	CSF1	FHL2
TAB2	NOX1	IL1R1
NCF2	PPP3CB	MAPK10
NCF4	MAPK12	MAPK13
RELA	CSF1R	MAP2K1
RELB	PPP3CC	MAP3K7
SIRPB1	PPP3R1	FYN
SIRPA	PPP3R2	MAP2K6
TNFRSF11A	FCGR1A	CTSK
LCK	NFATC1	MAP2K7
PPARG	FCGR2A	LILRB5
TEC	NFATC2	LILRA5
LCP2	FCGR2B	RAC1
CREB1	FCGR3A	LILRA4
ITGB3	FCGR3B	LILRB4
LILRB1	PIK3CA	CYBA
STAT1	PIK3CB	
STAT2	PIK3CD	



Increased autophagy contributes to the inflammatory phenotype of juvenile idiopathic arthritis synovial fluid T cells

5

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ABSTRACT

Juvenile Idiopathic Arthritis (JIA) is an autoimmune disease involving disturbed T cell homeostasis, marked by highly activated effector T cells. Autophagy, a lysosomal degradation pathway, is critical for maintaining cellular homeostasis by regulating survival, differentiation, and function of a large variety of cells, including T cells. We hypothesized that autophagy is affected in JIA T cells and investigated the effect of autophagy inhibition on the inflammatory phenotype of JIA T cells. Therefore, autophagy-related gene expression was analyzed in $CD4^+$ T cells from the synovial fluid (SF) of JIA patients and healthy controls (HC) using RNA-sequencing. Autophagy was measured by flow cytometry and Western Blot. The effect of autophagy inhibition, using hydroxychloroquine, on the cellular activation status was analyzed using flow cytometry and multiplex immunoassay. Autophagy was increased in T cells derived from the site of inflammation compared to cells from the peripheral blood of patients and healthy controls. This increase in autophagy was not induced by JIA SF, but is more likely the result of increased cellular activation. Inhibition of autophagy reduced proliferation, cytokine production, and activation marker expression of JIA SF-derived $CD4^+$ T cells. These data indicate that autophagy is increased in JIA SF-derived T cells and that targeting autophagy could be a promising therapeutic strategy to restore the disrupted T cell homeostasis in JIA.

INTRODUCTION

Juvenile Idiopathic Arthritis (JIA) describes a heterogeneous group of autoimmune conditions, characterized by chronic arthritis with an unknown cause and onset before the age of 16. Apart from several others, two main forms of JIA are recognized based on the number of joints affected: oligo- and polyarticular JIA[1]. Inflammation in the joint synovial fluid (SF) is characterized by an increase in autoreactive, highly activated effector T cells and impaired control by regulatory T cells, but the exact disease mechanism remains unclear[2].

Autophagy is a catabolic process essential for maintaining cellular homeostasis, including T cell homeostasis[3,4]. Conserved in evolution, it involves the sequestration of cytoplasmic components by formation of double-membrane vesicles and subsequent delivery to the lysosome for degradation. Autophagy has been linked to T cell activation, differentiation, and survival[5–7]. There are several indications that autophagy may play a role in autoimmunity, as single nucleotide polymorphisms (SNPs) in autophagy-related genes correlate with susceptibility to Crohn's disease and Systemic Lupus Erythematosus (SLE)[8,9]. In addition, RA serum, SLE serum, and SLE purified auto-antibodies can promote autophagy in healthy control (HC) T cells[10]. Furthermore, autophagy is increased in $CD4^+$ T cells obtained from the peripheral blood (PB) of Rheumatoid Arthritis (RA) patients[11].

So far, autophagy has not been studied in JIA and particularly not in primary cells derived from the site of inflammation. Since T cell homeostasis is disturbed in JIA and autophagy is important for T cell homeostasis and linked to autoimmunity, we hypothesized that autophagy is increased in JIA-patient derived T cells. Furthermore, as T cell homeostasis is predominantly disturbed at the site of inflammation, we tested whether inflammatory mediators in SF can induce autophagy. To examine whether autophagy contributes to the inflammatory phenotype of JIA T cells, we assessed the effect of autophagy inhibition on the activation status of JIA-derived T cells.

MATERIAL AND METHODS

SAMPLE COLLECTION AND CELL CULTURE

Samples were collected, isolated, and cultured as described previously[12]. Thirteen oligoarticular, eight extended oligoarticular, and three polyarticular JIA patients were included in this study. All patients had active disease and underwent therapeutic joint aspiration at the time of sampling. Patients were between 5 and 18 years of age and were either untreated or treated with methotrexate (MTX) and/or TNF blockers at the time of inclusion. Characteristics of patient samples used in this study are outlined in Supplemental Table 1. Thirty-two anonymous volunteers, between 18 and 65 years old, were included as HCs. The study procedures were approved by the Institutional Review Board of the University Medical Center Utrecht and performed according to the principles expressed in the Helsinki Declaration. Informed consent was obtained from all patients either directly or from parents/guardians when the patients were younger than age 12 years. To obtain cell-free plasma and SF, samples were centrifuged; supernatants were collected, and stored at -80 ºC. Where indicated, cells were stimulated with 1 µg/mL plate-bound α-CD3 (eBioscience, OKT3) or cultured with HCQ (Acros Organics), IL-6 (BD Biosciences) or TNF-α (Miltenyi).

ANALYSIS OF AUTOPHAGY-RELATED GENES

RNA-sequencing data from HC and JIA $CD4^+CD45RO^+$ T cells (GSE71595) were analyzed for autophagy-related genes, identified via the human autophagy database (available at http://autophagy.lu/)[12,13].

FLUORESCENCE-ACTIVATED CELL SORTING

Autophagy was analyzed using Cyto-ID autophagy detection kit (Enzo Life Sciences). Cells were cultured +/- HCQ, washed twice, and stained with Cyto-ID (1:500) for 25 minutes at 37 ºC. To measure autophagic flux, the relative MFI Cyto-ID +/- HCQ was used.

Apoptosis was analyzed using Annexin V Apoptosis Detection Kit (BD Biosciences) according to manufacturer's protocol. Apoptotic cells were defined as Annexin V^+.

To detect intracellular cytokine production, cultured cells were stimulated for 4 hours with PMA (20 ng/mL; Sigma) and ionomycin (1 µg/mL; Calbiochem), with Monensin (1/1500; BD Biosciences) for the last 3.5 hours. Cells were washed twice in FACS buffer (PBS with 2% FCS [Invitrogen] and 0.1% sodium azide [Sigma-Aldrich]) and subsequently stained with surface antibodies. Then, cells were washed twice in FACS buffer, fixed and permeabilized (eBioscience; according to manufacturer's instructions) and stained with cytokine antibodies.

WESTERN BLOT

$CD4^+$ T cells were isolated using Biotin Human $CD4^+$ T lymphocyte Enrichment Set-DM (BD IMag) according to manufacturer's protocol. Western blot was performed as described previously[11]. Antibodies: mouse anti-LC3 (Nanotools, 5F10) and goat anti-actin (Santa Cruz, sc-1616).

CELL PROLIFERATION

Prior to culture, cells were labeled with 2 μM Cell Tracer Violet (Invitrogen) for 7 minutes at 37 ºC. Labelling was blocked by adding 10 volumes cold serum.

MULTIPLEX IMMUNOASSAY

Luminex/xMAP was performed as described previously[12].

STATISTICS

Statistical analysis was performed using GraphPad Prism 6.0. Statistical tests used to test significance are specified in figure legends.

RESULTS

AUTOPHAGY IS INCREASED IN JIA SF-DERIVED T CELLS COMPARED TO JIA PB-DERIVED T CELLS.

To examine whether autophagy might be affected in JIA T cells, the expression of autophagy-related genes in HC and JIA SF-derived $CD4^+CD45RO^+$ T cells was analyzed utilizing RNA-seq (Figure 1A)[12]. Autophagy-related genes were significantly enriched within genes that are upregulated in JIA compared to HC, indicating that autophagy is disturbed in JIA T cells (**Figure 1A**, **1B**, and **Supplemental Table 1**). To validate that autophagy is increased in JIA T cells we determined autophagy in HC peripheral blood mononuclear cells (PBMC) and JIA PBMC and synovial fluid mononuclear cells (SFMC). To determine the rate of autophagy, *i.e.* autophagic flux, we cultured the cells with and without hydroxychloroquine (HCQ). HCQ affects lysosomal acidification, hence inhibiting degradation of autophagosomes, resulting in the accumulation of autophago(lyso)somes. Cyto-ID, a cationic amphiphilic tracer dye, specifically recognizes autophago(lyso)somes and can be quantified using flow cytometry[14]. The difference in MFI Cyto-ID of cells treated with and without HCQ was used to measure the autophagic flux (**Figure 1C**). This demonstrated that autophagy was significantly increased in SF-derived $CD4^+$ T cells compared to paired PB-derived $CD4^+$ T cells (**Figure 1D**). Similar observations were made for $CD8^+$ T cells (data not shown). The detected difference was not related to disease activity, disease subtype or treatment (**Supplemental Figure 1A-C**). Since autophagy was demonstrated to be increased in memory/effector T cells ($CD4^+CD45RO^+$) compared to naïve T cells ($CD4^+CD45RA^+$), and memory/effector T cells are present in large numbers in the inflamed synovium, autophagy was analyzed in each subset (**Figure 1E and Supplemental Figure 1D**)[15]. In both subsets a significant increase was detected in SF-derived T cells compared to PB-derived T cells, indicating that the increase in autophagy is not just a reflection of the increased amount of memory/effector T cells in the synovium.

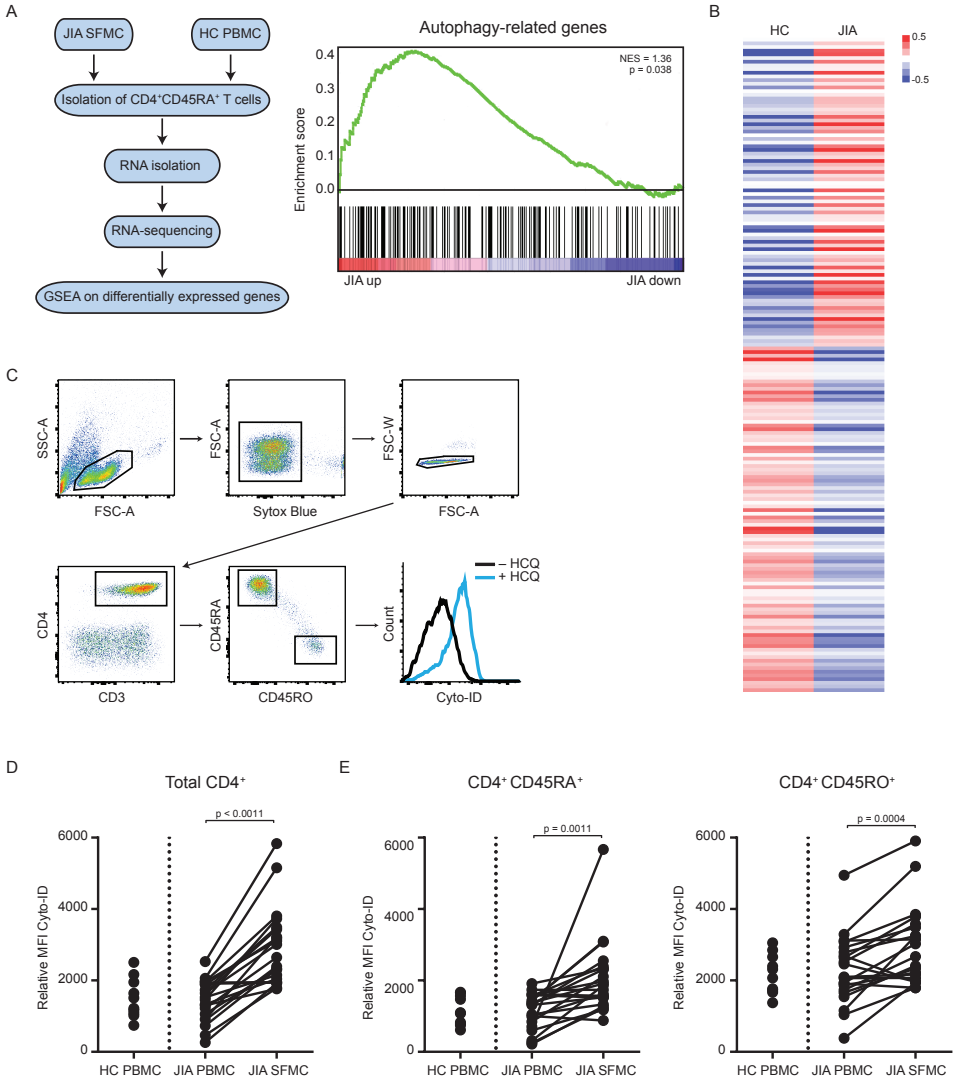

Figure 1. Autophagy is increased in JIA SF-derived CD4⁺ T cells compared to JIA and HC PB-derived CD4⁺ T cells. (A) Experimental setup to define genes differentially expressed between HC PB and JIA SF-derived CD4⁺CD45RO⁺ T cells using RNA-sequencing. **(B)** Gene Set Enrichment Analysis of autophagy-related genes. **(C)** Average log2 fold change of autophagy-related genes in HC and JIA CD4⁺CD45RO⁺ T cells. **(D)** Gating strategy used to analyse autophagy levels in CD4⁺, CD4⁺CD45RA⁺, and CD4⁺CD45RO⁺ T cells. Histogram depicts Cyto-ID signal of CD4⁺ T cells treated with or without HCQ. **(E)** Autophagy levels of CD4⁺ T cells in HC PBMC (n=11), JIA PBMC and SFMC (n=21, paired) cultured for 16 hours. Autophagy was determined by the relative MFI Cyto-ID, *i.e.* difference in MFI Cyto-ID between cells treated +/- 20 μM HCQ. **(F)** Relative MFI Cyto-ID of CD4⁺CD45RA⁺ and CD4⁺CD45RO⁺ T cells in HC PBMC, JIA PBMC and SFMC (cultured -/+ 20 μM HCQ). P-values were calculated using a paired Student's t-test.

AUTOPHAGY IS NOT INDUCED BY THE SF AND INHIBITION OF AUTOPHAGY IMPAIRS THE INFLAMMATORY PHENOTYPE OF JIA SF-DERIVED CD4$^+$ T CELLS.

As autophagy is increased in cells derived from the synovium, we aimed to determine whether inflammatory mediators in the SF can induce autophagy. HC PBMC were cultured with either AB serum, HC plasma or pooled oligoarticular JIA (oJIA) SF-samples and the autophagic flux was determined (**Figure 2A**). We did not detect a consistent effect of SF on autophagy under these conditions, neither with additional α-CD3 stimulation (data not shown), nor utilizing Western blot as technique to assess autophagy **(Figure 2B**). To validate these data, plasma and SF from different JIA subtypes, discriminated on the presence of antinuclear antibodies (ANA), were tested. Autophagy was not increased upon culture with these samples, neither in HC (**Figure 2C**) nor in JIA SF-derived T cells (**Figure 2D**). Furthermore, the effect of individual cytokines, present within the SF on autophagy, was analyzed. Both TNF-α and IL-6 did not affect autophagy (**Supplemental Figure 2A**). Next, we wondered what the effect is of increased autophagy on the cellular activation status of JIA SF-derived T cells. Since highly activated effector T cells is a key feature of JIA and autophagy is involved in T cell activation, we hypothesized that autophagy contributes to the inflammatory phenotype of SF-derived CD4$^+$ T cells[6]. To test this we cultured activated SFMC for 4 days with HCQ, to inhibit autophagy, and determined cellular activation status by flow cytometry. HCQ treatment resulted, as expected, in increased Cyto-ID levels (**Supplemental Figure 2B**) and did not affect apoptosis (**Figure 2E**). In contrast, proliferation and expression of the activation markers CD25 and CD279 was decreased upon HCQ treatment (**Figure 2F and 2G**). Furthermore, there was a significant decrease in cytokine production, measured both in the supernatant and intracellularly (**Figure 2H and 2I**). Altogether, these data demonstrate that culturing HC and JIA T cells in the presence of SF does not induce autophagy and that autophagy contributes to the inflammatory phenotype of SF-derived CD4$^+$ T cells.

DISCUSSION

Autophagy has recently been implicated to play a role in numerous autoimmune diseases[4]. Here, we studied autophagy in T cells of JIA patients and observed that autophagy is increased in T cells obtained from inflamed joints.

It remains to be determined whether the increase in autophagy at the site of inflammation is specific for JIA. Since T cell activation induces autophagy, the increase in autophagy in JIA T cells could be a consequence of their inflammatory phenotype[5,6,15]. Therefore, other diseases characterized by hyperactivated T cells will probably display a similar phenotype. For various rheumatic diseases, it has been demonstrated that autophagy is affected in disease-associated (immune) cells[16]. However, the alterations at the autophagy level differ from one disease and one cell type to another, making it difficult to pinpoint the exact role of autophagy in disease pathogenesis. This might also explain why, in contrast to what has been reported for SLE and RA serum, we did not observe that JIA SF induces autophagy in HC CD4$^+$ T cells[10]. For example,

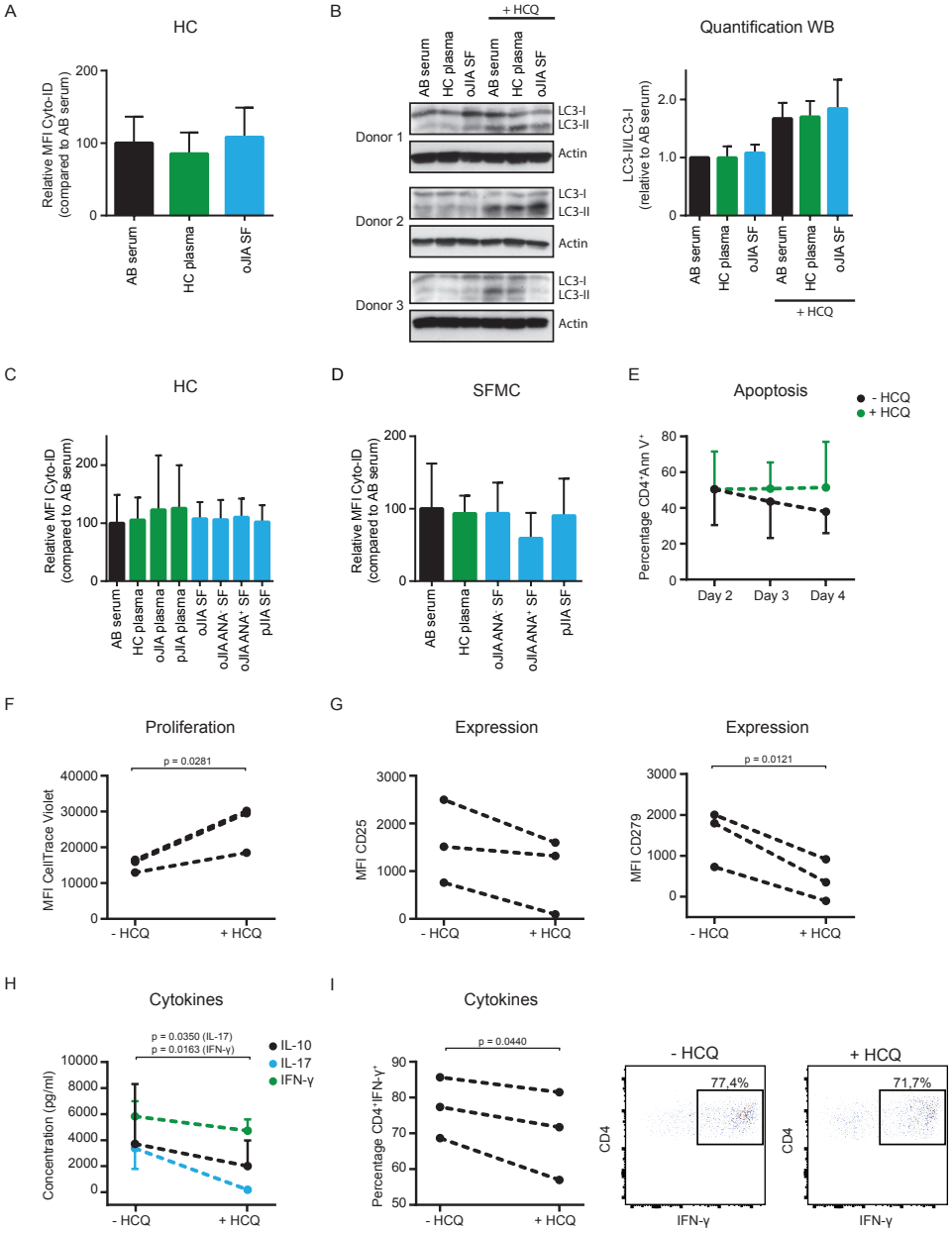

Figure 2. Autophagy is not induced by the SF and inhibition of autophagy impairs the inflammatory phenotype of JIA SF-derived CD4⁺ T cells.

(A) Relative MFI Cyto-ID of CD4⁺ T cells in HC PBMC (n=13) cultured with 10% AB serum, HC plasma or oJIA SF for 16h -/+ 2.5 μM HCQ. **(B)** Western blot of CD4⁺ T cells from three independent healthy donors cultured with 10% AB serum, HC plasma or oJIA SF for 16h -/+ 2.5 μM HCQ, stimulated with α-CD3. Quantification of LC3-II/LC3-I normalized for actin is shown. **(C)** Relative MFI Cyto-ID of CD4⁺ T cells in HC PBMC (n=4) cultured with 10% AB serum, HC plasma pool, oJIA plasma, polyarticular JIA (pJIA) plasma, oJIA SF, oJIA ANA⁻ SF, oJIA ANA⁺ SF or pJIA SF. >>>

(D) Relative Cyto-ID MFI of $CD4^+$ T cells in JIA SFMC (n=5) cultured with 10% AB serum, HC plasma, oJIA ANA- SF, oJIA ANA+ SF or pJIA SF. **(E)** Percentage of $CD4^+$Annexin V^+ T cells after 2, 3 or 4 days of culture of JIA SFMC (n=3) with 0 or 20 μM HCQ, stimulated with α-CD3. **(F)** MFI of CellTrace Violet (CTV) of $CD4^+$ T cells after 4 days of culture of JIA SFMC (n=3) with 0 or 20 μM HCQ, stimulated with α-CD3. CTV MFI is inversely correlated to proliferation, *i.e.* higher CTV MFI means less proliferation. **(G)** MFI of activation markers on $CD4^+$T cells after 4 days of culture of JIA SFMC (n=3) with 0 or 20 HCQ, stimulated with α-CD3. **(H)** Supernatant cytokine concentrations of JIA SFMC (n=3) cultured for 4 days with 0 or 20 μM HCQ, stimulated with α-CD3. **(I)** Percentage of $CD4^+$IFN-γ^+ T cells (with representative FACS plots) after 4 days of culture of JIA SFMC (n=3) with 0 or 20 μM HCQ, stimulated with α-CD3. Mean (SD) is shown and significance was tested using repeated measures ANOVA followed by post-hoc testing with Sidak's procedure for multiple testing (A,B,C,D,E) or paired Student's t-test (F,G,H,I).

in contrast to JIA, in RA autophagy is increased in PB-derived T cells compared to HC cells and in SLE autophagy is increased in naïve $CD4^+$ T cells, but not in memory or $CD8^+$ T cells[10,11]. This suggests that autophagy may have distinct roles within different autoimmune diseases and that this might be related to differences in pathology and localization of the disease[10]. Furthermore, it is important to take into account that, next to the inflammatory mediators present in the SF, there are many more aspects present at the site of inflammation, such as non-soluble factors and cell-cell contact (for example with synovial fibroblasts), that can induce immune activation and thereby affect autophagy.

Additionally, it remains unclear whether increased autophagy is a cause or a consequence of disease pathology. Our data suggest that autophagy may be necessary to increase the intracellular nutrient supply to meet the metabolic demand of activated T cells, thus pointing to a secondary role for autophagy in inflammation. However, autophagy is also described to be important for self-tolerance by regulating MHC class II antigen presentation in the thymus, suggesting that deregulated autophagy might also play a role in the initiation of autoimmunity[17]. Here, we chose to measure autophagy using Western Blot for LC3-I/LC3-II and flow cytometry with Cyto-ID. The advantage of flow cytometry is the relatively little amount of cells needed and the medium- to high- throughput of this technique. Alternative methods to measure autophagy are for instance SQSTM1/p62 turnover and LC3-GFP fluorescent microscopy[18]. The latter also allows for visualization of the size of the autophagosomes, but requires cell transfection and is thus difficult to apply to primary, patient-derived cells. In this study we used HCQ to block autophagy. However, as HCQ affects lysosomal acidification, other lysosome-dependent processes could be affected as well[19]. To elucidate whether the effect of HCQ on the cellular activation status is primarily caused by autophagy inhibition, genetic manipulation of autophagy-related genes could be useful.

In conclusion, we demonstrated that autophagy is increased in synovial JIA T cells. This increased autophagy is not induced by the inflammatory environment, but rather seems to be related to increased immune activation. This can be decreased by inhibiting autophagy using HCQ, a compound that is already being used for the treatment of various diseases[16]. Therefore, autophagy inhibition might be a promising therapeutic approach to target activated, autoreactive T cells and restore the disrupted T cell homeostasis in JIA.

ACKNOWLEDGEMENTS

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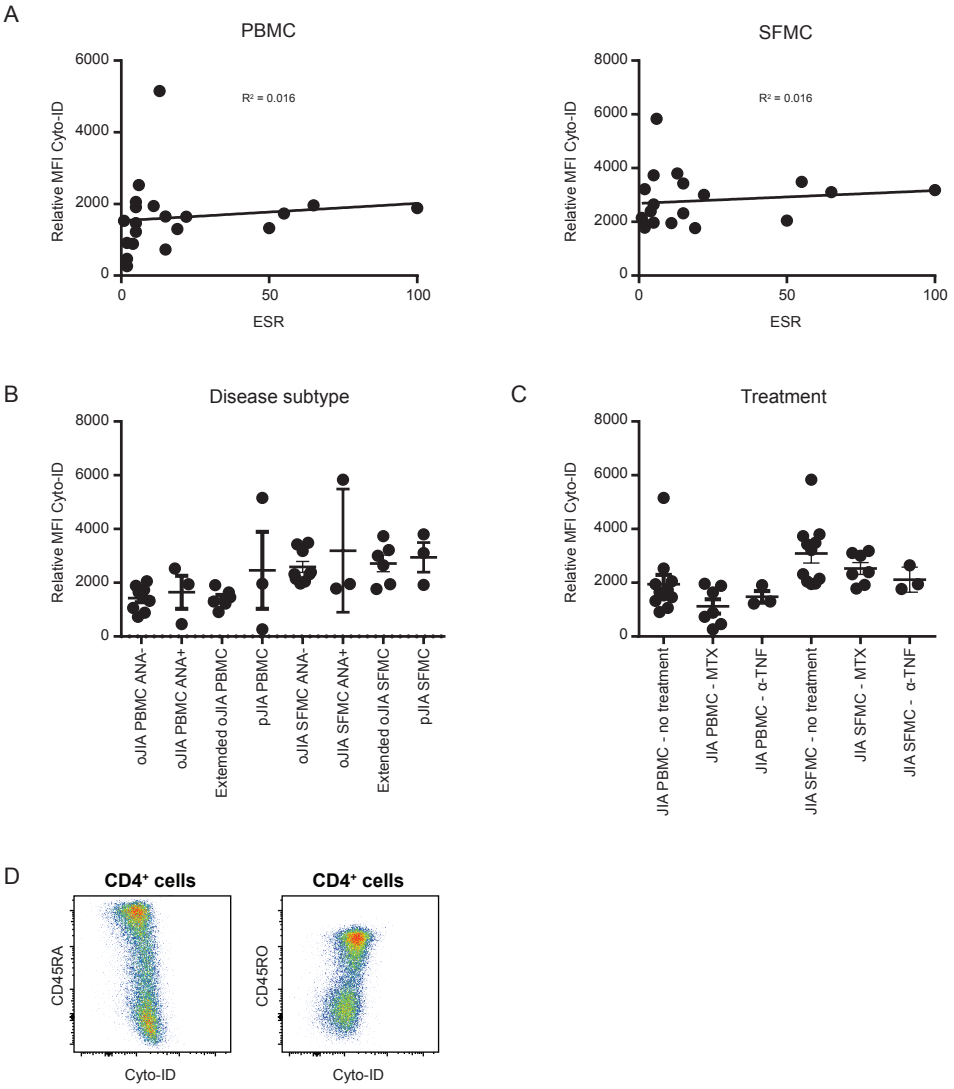
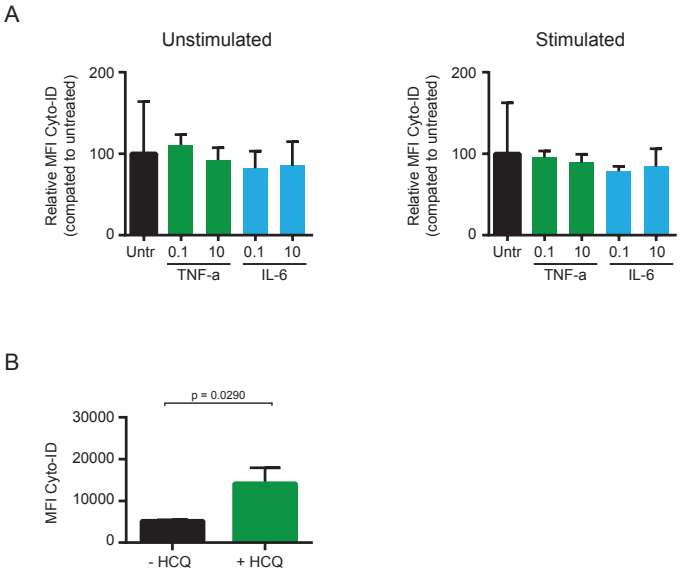
SUPPLEMENTAL INFORMATION



Supplemental Figure 1. No relation between autophagy levels and erythrocyte sedimentation rate (ESR), disease subtype, and treatment.

(**A**) Correlation between ESR (as a measure for disease activity) and relative MFI Cyto-ID of JIA PBMC and JIA SFMC-derived CD4⁺ T cells. (**B**) Relative MFI Cyto-ID of JIA PBMC and JIA SFM-derived CD4⁺ T cells classified based on disease subtype. (**C**) Relative MFI Cyto-ID of JIA PBMC and JIA SFM-derived CD4⁺ T cells classified based on treatment. Mean (SD) is shown and p-values were calculated using repeated measures ANOVA followed by post-hoc testing with Sidak's procedure for multiple testing. (**D**) FACS plots representing Cyto-ID signal of CD4⁺ cells stained for CD45RA (left) and CD45RO (right).



Supplemental Figure 2. HCQ blocks the autophagic flux.
(A) Relative MFI Cyto-ID of $CD4^+$ T cells in HC PBMC (n=4) cultured with 0.1 and 10 ng/ml TNF-α or IL-6 for 16h -/+ 2.5 μM HCQ, unstimulated (left) or stimulated with α-CD3 (right). **(B)** MFI Cyto-ID of $CD4^+$ T cells after 4 days of culture of JIA SFMC with 0 or 20 μM HCQ. Mean (SD) is shown and p-value was calculated using a paired Student's t-test.

Supplemental Table 1. Autophagy-related gene expression in HC and JIA CD4$^+$CD45RO$^+$ T cells.
Average log2 fold change of autophagy-related genes according to Figure 1B is shown.

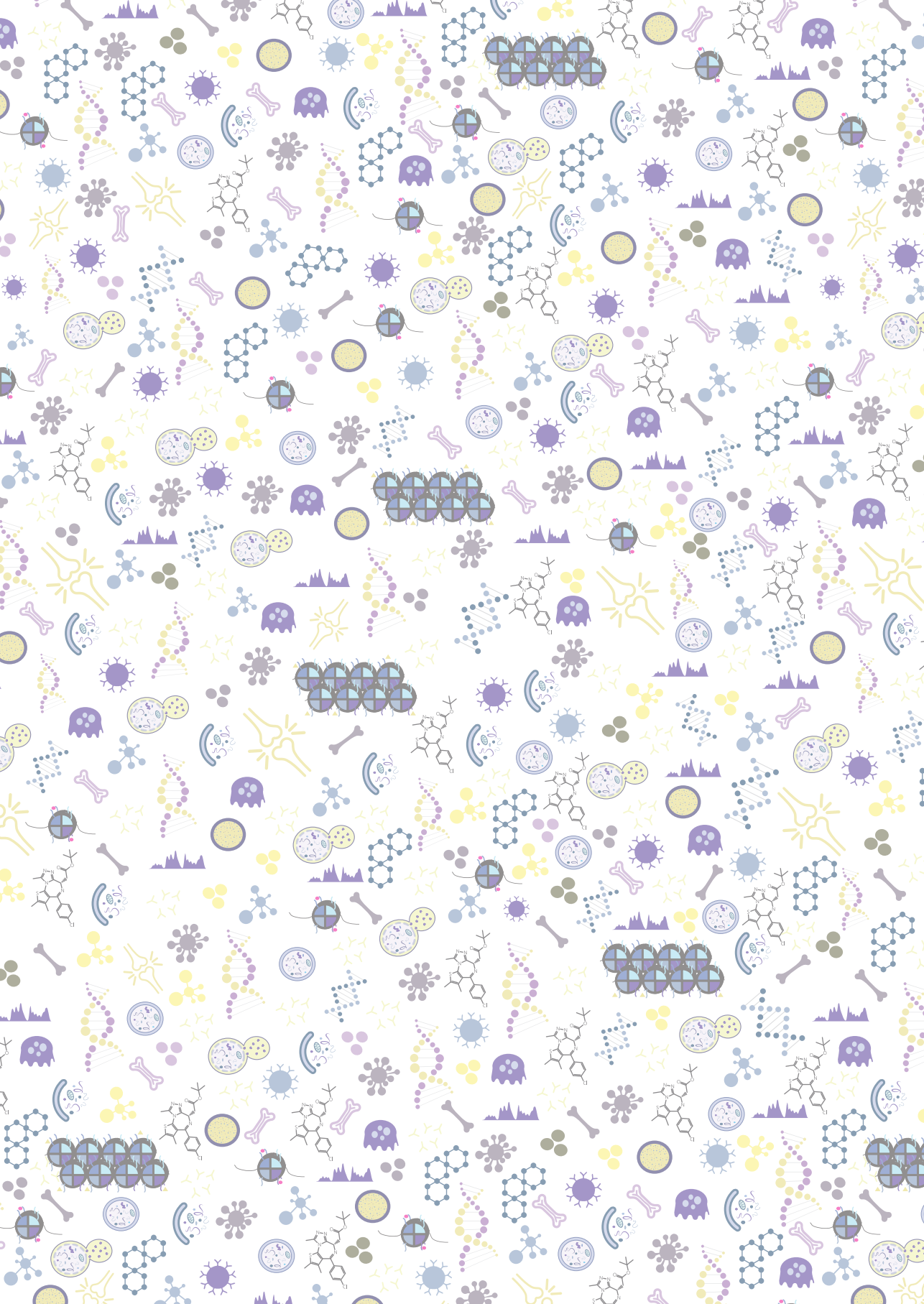
Gene name	HC	JIA	Gene name	HC	JIA
SH3GLB1	0,994226	1,005774	PIK3C3	0,973157	1,026843
PEA15	0,909977	1,090023	NPC1	0,729521	1,270479
ATF6	0,981253	1,018747	CAPNS1	0,947118	1,052882
IKBKE	0,682003	1,317997	PPP1R15A	0,81496	1,18504
CAPN2	0,65825	1,34175	SIRT2	0,957336	1,042664
ARNT	0,967378	1,032622	CFLAR	0,958253	1,041747
MAP1LC3C	0,742845	1,257155	CASP8	0,996046	1,003954
SIRT1	0,967623	1,032377	EIF2AK2	0,755281	1,244719
PTEN	0,972303	1,027697	CXCR4	0,485214	1,514786
FAS	0,620935	1,379065	ATG9A	0,986314	1,013686
BAG3	0,976445	1,023555	CHMP4B	0,906484	1,093516
PRKCQ	0,84203	1,15797	TP53INP2	0,67687	1,32313
ITGB1	0,958215	1,041785	BCL2L1	0,920665	1,079335
ATG13	0,795067	1,204933	APOL1	0,567337	1,432663
CAPN1	0,996933	1,003067	BID	0,982051	1,017949
CTSD	0,962108	1,037892	EDEM1	0,9084	1,0916
AMBRA1	0,87748	1,12252	MTMR14	0,861378	1,138622
RELA	0,879575	1,120425	PRKCD	0,944936	1,055064
CASP4	0,900468	1,099532	KLHL24	0,695137	1,304863
CASP1	0,944246	1,055754	TNFSF10	0,95354	1,04646
GABARAPL1	0,917212	1,082788	RUBCN	0,566467	1,433533
CDKN1B	0,704467	1,295533	CASP3	0,971635	1,028365
TBK1	0,895477	1,104523	SQSTM1	0,63073	1,36927
DRAM1	0,336862	1,663139	ARSB	0,809171	1,190829
PRKAB1	0,874943	1,125057	RAB24	0,721931	1,278069
IFNG	0,781691	1,218309	CDKN1A	0,196892	1,803108
LAMP1	0,998087	1,001913	FOXO3	0,794952	1,205048
FOXO1	0,87922	1,12078	VAMP3	0,918321	1,081679
TM9SF1	0,980463	1,019537	LAMP2	0,901397	1,098603
ZFYVE1	0,750953	1,249047	WDR45	0,767755	1,232245
SERPINA1	0,143042	1,856958	HDAC6	0,847342	1,152658
ATG2B	0,897666	1,102334	TSC1	0,917245	1,082755
GABARAPL2	0,93606	1,06394	DAPK1	0,096409	1,903591
CCL2	0,475494	1,524506	RB1CC1	0,842427	1,157573
NBR1	0,873098	1,126902	CTSB	0,753849	1,246151
CALCOCO2	0,923504	1,076496	IKBKB	0,831115	1,168885
ITGA3	0,726891	1,273109	BNIP3L	0,843293	1,156707
PRKAR1A	0,941316	1,058684	DNAJB9	0,947616	1,052384
SPHK1	0,892312	1,107688	RAC1	0,898582	1,101418
HGS	0,996113	1,003887	BAK1	0,925605	1,074395
GABARAP	0,988251	1,011749	HDAC1	1,144956	0,855044
ULK2	0,680143	1,319857	ATG4C	1,402463	0,597537
ERN1	0,999008	1,000992	GNAI3	1,125269	0,874731
WIPI1	0,785693	1,214307	IL24	1,463618	0,536382

Gene name	HC	JIA
CD46	1,048481	0,951519
MTOR	1,035148	0,964852
PARP1	1,037375	0,962625
MAPK8	1,018435	0,981565
KIF5B	1,028531	0,971469
SAR1A	1,111772	0,888228
BNIP3	1,185125	0,814875
FADD	1,109531	0,890469
ATG16L2	1,293309	0,706691
UVRAG	1,160584	0,839416
HSPA8	1,249746	0,750254
GAPDH	1,090497	0,909503
ATG101	1,051739	0,948261
DDIT3	1,079674	0,920326
CAMKK2	1,049725	0,950275
RB1	1,075964	0,924036
HIF1A	1,016888	0,983112
EIF2S1	1,214667	0,785333
FOS	1,284024	0,715976
ATG14	1,093238	0,906762
RAB11A	1,055578	0,944422
ULK3	1,067154	0,932846
TSC2	1,010381	0,989619
MLST8	1,232279	0,767721
EEF2K	1,197918	0,802082
SPNS1	1,016412	0,983588
USP10	1,143377	0,856623
MAP1LC3B	1,027109	0,972891
CLN3	1,091615	0,908385
MAPK3	1,068217	0,931783
VMP1	1,089736	0,910264
RPS6KB1	1,158924	0,841076
RPTOR	1,18483	0,81517
PELP1	1,137382	0,862618
TP53	1,060024	0,939976
BECN1	1,127498	0,872502
P4HB	1,050231	0,949769
BCL2	1,082387	0,917613
STK11	1,009558	0,990442
MAP2K7	1,052274	0,947726
ATG4D	1,281287	0,718713
BAX	1,015076	0,984924
EEF2	1,232948	0,767052
DNAJB1	1,129899	0,870101
BIRC6	1,034819	0,965181
ITGA6	1,389242	0,610758

Gene name	HC	JIA
ATIC	1,351496	0,648504
ATG16L1	1,064629	0,935371
ATG4B	1,026453	0,973547
RAB1A	1,108577	0,891423
EIF2AK3	1,055587	0,944413
NFE2L2	1,011905	0,988095
NCKAP1	1,120678	0,879322
FKBP1A	1,155881	0,844119
RGS19	1,211866	0,788134
ATF4	1,097837	0,902163
MAPK1	1,135659	0,864341
ST13	1,181504	0,818496
ITPR1	1,154577	0,845423
ATG7	1,099202	0,900798
RAB5A	1,018482	0,981518
CHMP2B	1,156205	0,843795
RAB7A	1,019718	0,980282
EIF4G1	1,02016	0,97984
RAF1	1,058792	0,941208
ATG3	1,034451	0,965549
PIK3R4	1,162689	0,837311
NFKB1	1,077142	0,922858
RAB33B	1,099069	0,900931
NAF1	1,222151	0,777849
PEX14	1,057881	0,942119
SESN2	1,049133	0,950867
RACK1	1,129908	0,870092
ATG4A	1,064356	0,935644
HSPA5	1,285458	0,714542
BAG1	1,206392	0,793608
MYC	1,212834	0,787166
EIF4EBP1	1,254222	0,745778
RHEB	1,081002	0,918998
NAMPT	1,03645	0,96355
WIPI2	1,076486	0,923514
GOPC	1,173078	0,826922
ATG5	1,110284	0,889716
PEX3	1,157943	0,842057
HSP90AB1	1,221237	0,778763
VEGFA	1,195729	0,804271
MAPK9	1,237846	0,762154
ATG12	1,100642	0,899358
CANX	1,117158	0,882842
BNIP1	1,190702	0,809298

Supplemental Table 2. Characteristics of patient samples.
Gender, age, diagnosis, and medication information of patient samples included in the study

Gender (M/F)		**8/16**
Age (Mean, St dev) Highest age – lowest age		10.6 (4.3) 4.7 – 18.3
Diagnosis	Oligo articular ANA-	9
	Oligo articular ANA+	4
	Extended oligoarticular	8
	Polyarticular RF-	3
Disease activity	#Inflamed joints (median, range)	2 (1-3)
	ESR (mean, St dev)	21.6 (27.1)
Systemic NSAID use	Methotrexate	8
	Biologicals (TNF blockers)	3
	Steroids	0



Transcriptional and epigenetic profiling of nutrient-deprived cells to identify novel regulators of autophagy

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ABSTRACT

Macroautophagy (hereafter autophagy) is a lysosomal degradation pathway critical for maintaining cellular homeostasis and viability, and is predominantly regarded as a rapid and dynamic cytoplasmic process. To increase our understanding of the transcriptional and epigenetic events associated with autophagy, we performed extensive genome-wide transcriptomic and epigenomic profiling after nutrient deprivation in human autophagy-proficient and autophagy-deficient cells. We observed that nutrient deprivation leads to the transcriptional induction of numerous autophagy-associated genes. These transcriptional changes are reflected at the epigenetic level (H3K4me3, H3K27ac, and H3K56ac) and are independent of autophagic flux. As a proof of principle that this resource can be used to identify novel autophagy regulators, we followed up on one identified target: EGR1 (early growth response 1), which indeed appears to be a central transcriptional regulator of autophagy by affecting autophagy-associated gene expression and autophagic flux. Taken together, these data stress the relevance of transcriptional and epigenetic regulation of autophagy and can be used as a resource to identify (novel) factors involved in autophagy regulation.

INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is a highly conserved catabolic mechanism, involving the sequestration of bulk cytoplasmic components by transient double-membrane compartments called phagophores; these mature into autophagosomes, which allow subsequent delivery of the cargo into lysosomes for degradation[1,2]. Autophagy is essential for maintaining cellular homeostasis by removal of damaged or unnecessary proteins and organelles, and is important for cell viability by maintaining the energy balance upon cellular stresses, such as nutrient starvation[3].

Because autophagy is a rapid, dynamic process that constantly requires adaptation to environmental changes, research has often focused on cytoplasmic post-translational modifications of autophagy-associated genes[4]. In fact, for a long time autophagy has been viewed as mainly a cytoplasmic process, especially because enucleated cells are still capable of undergoing autophagy[5]. Recently it is becoming apparent that transcriptional and epigenetic events are also involved in regulating autophagy[6]. One of the first transcription factors identified to be involved in autophagy regulation under amino acid and serum starvation is TFEB (transcription factor EB). Besides its role in regulating lysosomal biogenesis, TFEB is involved in autophagy initiation because its overexpression can induce autophagy[7]. This is in part established by direct binding to the promotor of a set of autophagy-associated genes and thereby increasing their gene expression[7]. Transcription factors that have been implicated in the regulation of specific autophagy-associated gene expression under various starvation conditions can have an enhancing effect, such as the FOXO (forkhead box O) family of transcription factors [reviewed in 8], or a suppressing effect, such as ZKSCAN3 (zinc finger protein with KRAB and SCAN domains 3)[9]. NFKB (nuclear factor kappa B) is a transcription factor with a dual effect on autophagy-associated gene expression, by inhibiting *BNIP3* (BCL2 interacting protein 3) transcription[10] and inducing *BECN1* (beclin 1)[11], *SQSTM1* (sequestosome 1)[12], and *BCL2*[13] expression. While these studies have shed light on the transcriptional regulation of autophagy, it is still incompletely understood which transcription factors are involved in autophagy modulation and whether autophagy itself has a feedback regulation on its transcriptional regulation.

In addition to transcriptional regulation, there is limited evidence demonstrating whether autophagy is epigenetically regulated. EHMT2/G9a (euchromatic histone lysine methyltransferase 2)[14] and EZH2 (enhancer of zeste 2 polycomb repressive complex 2 subunit)[15] have both been implicated in autophagy repression under serum starvation by increasing H3K9me2 and H3K27me3 histone mark levels, respectively, of certain autophagy-associated genes. Furthermore, autophagy induction has been demonstrated to affect total H3R17me2, H4K16ac, and H2BK120ub levels through CARM1 (coactivator associated arginine methyltransferase 1)[16], KAT8/hMOF (lysine acetyltransferase 8)[17], and the deubiquitinase USP44 (ubiquitin specific peptidase 44)[18], respectively. These alterations affect transcription of genes involved in (the regulation of) autophagy and therefore function as an epigenetic switch in autophagy regulation

under various starvation conditions and upon MTOR (mechanistic target of rapamycin kinase) inhibition. For example, autophagy induction downregulates KAT8, thereby decreasing H4K16 acetylation of autophagy-associated genes, which results in decreased gene expression. This reduces autophagy, thereby providing a feedback mechanism to control the amount of autophagy[17]. Furthermore, global changes in H4K20me3[19], H3K4me3[17], and H3K56ac[20] have been associated with autophagy induction, but whether and how this affects autophagy remains to be determined[17,19,20]. Importantly, extensive studies which assess and combine genome-wide transcriptomic and epigenomic events underlying autophagy are lacking. Taken together, further research is required to understand how, and which, epigenetic modifications contribute to the regulation of autophagy.

Here, we performed in-depth genome-wide transcriptional and epigenetic profiling to improve our understanding of the transcriptional and epigenetic events associated with amino acid and serum starvation-induced autophagy. RNA and chromatin immunoprecipitation (ChIP) sequencing of human cells revealed that nutrient deprivation leads to the transcriptional induction of many autophagy-associated genes. A similar induction was observed in autophagy-deficient cell lines, demonstrating that the induction of transcription of autophagy-associated genes is an autophagy-independent process in the cells used in this study. These transcriptional changes are reflected by POLR2/RNA polymerase 2 occupancy, and at the epigenetic level by H3K4me3, H3K27ac, and H3K56ac, indicating that the epigenome is involved in autophagy regulation. Our unbiased analyses identified EGR1 as a transcriptional regulator of many autophagy-associated genes, thereby affecting autophagy. This proof of principle demonstrates that these databases can function as a resource to further characterize the transcriptional and epigenetic events associated with autophagy, thereby facilitating the identification of (novel) mediators regulating autophagy in the future.

MATERIALS AND METHODS

CELL CULTURE

HAP1 WT (C631), *ATG7* KO (HZGHC000302c022), *RB1CC1* KO (HZGHC000567c007), and *EGR1* KO (HZGHC1958) cells were obtained from Horizon Genomics and cultured in Iscove's Modified Dulbecco's Medium (Gibco, 21980032; IMDM). U2OS (HTB-96) and HEK293 cells (CRL-1573) were obtained from ATCC and were cultured in Dulbecco's Modified Eagle Medium (Gibco, 31966021l; DMEM). Both IMDM and DMEM were supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco, 15070-063) and 10% heat-inactivated fetal calf serum (Sigma-Aldrich, F7524) and all cells were cultured at 37°C in 5% CO_2. For nutrient deprivation, cells were cultured with Earle's Balanced Salt Solution (Sigma-Aldrich, E288; EBSS). For overexpression, cells were transfected with 2 µg DNA using polyethylenimine (Polysciences, 23966-1). After 18 h, cells were washed with PBS (Sigma-Aldrich, D8537) and cultured for 24 h. For knockdown, cells were transfected with 25 pmol siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen, 13778150). After 18 h, cells were washed with PBS and cultured for 6 h.

ANTIBODIES AND REAGENTS

The following antibodies were used: mouse anti-MAP1LC3B (Nanotools, 0231-100/LC3-5F10), rabbit anti-ATG7 (Cell Signaling Technology, 2631S), rabbit anti-RB1CC1/FIP200 (ITK diagnostics, A301-536A), rabbit anti-EGR1 (Cell Signaling Technology, 4154S), mouse anti-RPB1 (Euromedex; PB-7C2), rabbit anti-histone H3 acetyl K27 (Abcam, ab4729), rabbit anti-histone H3 acetyl K56 (Active Motif, 39281), rabbit anti-histone H3 trimethyl K4 (Active Motif, 39159), mouse anti-histone H3 (Active Motif, 39763), mouse anti-TUBA4A/tubulin (Sigma-Aldrich, T9026). pMXs-hs-EGR1 was a gift from Shinya Yamanaka (Addgene, 52724)[21]. For EGR1 knockdown, human SMARTpool *EGR1* siRNA (Dharmacon, M-006526-01-0005) was used. pBABE-puro-mCherry-EFGP-LC3B was a gift from Jayanta Debnath (Addgene, 22418)[22]. To increase the intensity of the fluorescence, the *EEF1A1/EF1α* promoter was cloned into the construct using NaeI restriction sites. bafilomycin A_1 was obtained from Sigma-Aldrich (B1793). Hydroxychloroquine (HCQ) was obtained from Acros Organics (263010250).

WESTERN BLOT

Western blot was performed as described previously[23]. In short, cells were lysed in Laemmli buffer (0.12 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 0.05 µg/µl bromophenol blue, 35 mM β-mercaptoethanol). Samples were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Merck, IPFL00010), probed with the indicated antibodies and analyzed using enhanced chemiluminescence (Thermo Fisher Scientific, 34075) or an Odyssey Sa Infrared Imaging System (LI-COR, Lincoln, NE, USA).

ELECTRON MICROSCOPY

Cells were fixed with 50% karnovsky fixative (2.5% glutaraldehyde [Merck, 104239], 2% paraformaldehyde [Sigma-Aldrich, 441244], 0.1 M phosphate buffer, pH 7.4 (0.019 M $NaH_2PO_4.1H_20$, 0.081 M $Na_2HPO_4.2H_2O$), 0.25 mM $CaCl_2$, 0.5 mM $MgCl_2$) by adding equal amount of fixative to the medium and incubating for 10 min. Then, fixative was replaced for fresh 50% karnovsky fixative and incubated for 2 h at room temperature. Cells were washed 3x 10 min with 0.1 M phosphate buffer, scraped and pelleted. Pellets were resuspended quickly in 2% low melting point agarose (Sigma-Aldrich, A9414-25G) at 63°C and immediately pelleted again. Pellets were incubated on ice for 30 min and cut into blocks, after which blocks were incubated in postfix solution (0.1 M phosphate buffer, pH 7.4, 1% OsO4 [Electron Microscopy Science, 19110], 1.5% $K_3[Fe\{CN\}_6]$ [Merck, 104984]) for 2 h, 4°C. Samples were washed 3x with distilled water and incubated in 0.5% uranyl acetate (Electron Microscopy Science, 041209AB) for 1 h, 4°C in the dark. Afterwards, samples were rinsed with distilled water and incubated in 70% acetone overnight. Dehydration was done with increasing amounts of pure acetone with a final step of 100% acetone (Merck, 1002991001). Epon infiltration (glycid ether 100 [Serva, 21045], 2-dodecenyl succinic anhydride [Serva 20755], methylnadic anhydride [Serva, 29452], N-benzyldimethylamine [Electron Microscopy Science, 11400-25] was done with acetone-epon mixtures with increasing amounts of epon and a final

step of 100% epon. After the last step of 100% epon, fresh epon was added and polymerized for 3 days at 60°C. Cutting of ultra-thin sections was done on a Leica UCT/FCS (Leica, Wetzlar, Germany). Staining was done with a Leica AC20 (Leica, Wetzlar, Germany) with 45 min 0.5% uranyl acetate (Laurylab, 705631095) at 20°C and 5 min Reynolds lead citrate (Leica, D151214) at 20°C. The samples were examined with a Jeol101 electron microscope (Jeol Europe, Nieuw-Vennep, The Netherlands).

CONFOCAL MICROSCOPY

Autophagy was analyzed using the pBABE-puro-Ef1alpha-mCherry-EFGP-LC3B construct (see section "antibodies and reagents" how construct was created). Cells were seeded in 8-well µ-slides (Ibidi, 80826) and cultured for 24 h, transfected with 0.2 µg DNA using polyethylenimine. After 18 h, cells were washed with PBS and cultured for 24 h. Next, cells were cultured for 6 h in either full IMDM or EBSS for 6 h, and 40 µM bafilomycin A_1 was added after 5.5 h. Cells were washed twice with PBS, fixed with 1% formaldehyde (Merck Millipore, 104003) and visualized with a Zeiss LSM 710 microscope (Carl Zeiss, Oberkochen, Germany) using the 63x objective.

APOPTOSIS MEASUREMENTS

Apoptosis was analyzed using the Annexin V Apoptosis Detection Kit (BD Biosciences, 556547) according to the manufacturer's protocol. Living cells were defined as $ANXA5^-$ $7\text{-}AAD^-$, early apoptotic cells as $ANXA5^+$ $7\text{-}AAD^-$, and late apoptotic cells as $ANXA5^+$ $7\text{-}AAD^+$.

QUANTITATIVE RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen, 74106) and cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad, 1708891). cDNA samples were amplified with SYBR Select mastermix (Life Technologies, 4472919) in a QuantStudio 12k flex (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. A list of primers used in this study can be found in Supplemental Table 2.

RNA-SEQUENCING AND ANALYSIS

Cells were cultured till 80% confluence in 6w plates (Thermo Fisher Scientific, 140675) and cultured for 6 h in full IMDM or EBSS. Total RNA was extracted from 3 independent biological replicates using the RNeasy kit. mRNA was isolated using NEXTflex®Poly(A) Beads (Bio Scientific, NOVA-512980), libraries were prepared using the NEXTflex®Rapid Directional RNA-Seq Kit (Bio Scientific, NOVA-513808) and samples were sequenced 75 base pair single-end on Illumina NextSeq500 (Illumina Inc., San Diego, CA, USA; Utrecht DNA Sequencing Facility). Reads were aligned to the human reference genome GRCh37 using STAR version 2.4.2a. Picard's AddOrReplaceReadGroups (v1.98) was used to add read groups to the BAM files, which were sorted with Sambamba v0.4.5 and transcript abundances were quantified with HTSeq-count version 0.6.1p1 using the union mode. Subsequently, reads per kilobase million sequenced

(RPKMs) were calculated with edgeR's RPKM function. Differentially expressed genes were identified using the DESeq2 package with standard settings. Genes with absolute padj<0.05 were considered as differentially expressed.

CHIP-SEQUENCING AND ANALYSIS

Cells were cultured until 80% confluence in 15-cm dishes (Corning, 430599) and cultured for 3 h in full IMDM or EBSS. Cells from 2 independent biological replicates were crosslinked in 1% formaldehyde, 5 mM HEPES-KOH, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 50 μM EGTA and after 10 min crosslinking was stopped by adding 0.1 M glycine. Nuclei were isolated in 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 (Sigma-Aldrich, 56741), 1% Triton X-100 (Sigma-Aldrich, T8787) and lysed in 20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.3% SDS. Lysates were resuspended in 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and sonicated using Covaris (Covaris, Woburn, MA, USA) for 8 min, maximum output. Sheared DNA was incubated overnight with the indicated antibodies pre-coupled to protein A/G magnetic beads (Pierce, 88802). Cells were washed and crosslinking was reversed by adding 1% SDS, 100 mM $NaHCO_3$, 200 mM NaCl, 300 μg/ml proteinase K (Invitrogen, 25530-015). DNA was purified using ChIP DNA Clean & Concentrator kit (Zymo Research, D5205), and end repair, A-tailing, and ligation of sequence adaptors was done using Truseq nano DNA sample preparation kit (Illumina, 20015965). Samples were PCR amplified, and barcoded libraries were sequenced 75 base pair single-end on Illumina NextSeq500. Peaks were called using Cisgenome 2.0[24] (–e 150 -maxgap 200 –minlen 200). Peak coordinates were stretched to at least 2000 base pairs and collapsed into a single list. Overlapping peaks were merged based on their outermost coordinates. Only peaks identified by at least 2 independent datasets were further analyzed. Peaks with differential H3K27ac, H3K56ac or H3K4me3 occupancy were identified using DESeq (padj<0.05)[25].

MOTIF ENRICHMENT ANALYSIS

H3K27ac, H3K56ac, and H3K4me3 peaks associated with autophagy-associated genes with log2FoldChange ≥0.5 upon starvation were overlapped with DNAse hypersensitivity sites, based on online DNAse-seq data in HAP1 cells (GEO GSE90371[26]). The overlapping peaks were analyzed for motif enrichment using the AME tool of MEME Suite[27].

GENE SET ENRICHMENT ANALYSIS (GSEA)

GSEA was performed using autophagy-associated genes that were identified via the human autophagy database (available at http://autophagy.lu/ and see Supplemental Table 1)[28]. Significance of the enrichment was calculated based on 1000 cycles of permutations.

STATISTICAL ANALYSIS

For ChIP-seq and RNA-seq analysis, p-values were adjusted with the Benjamini-Hochberg procedure and a false discovery rate (FDR) ≤0.05 was considered significant. Cell viability was analyzed using two-way ANOVA with Sidak correction for multiple testing. Correlation between

ChIP-seq and RNA-seq data and *EGR1* induction by serum and/or nutrient deprivation was determined using an ordinary one-way ANOVA with Dunnett's post-test. Starvation-induced changes of autophagy-associated genes, the POLR2 signal for key autophagy genes, and EGR1 knockdown, knockout, and overexpression were analyzed using Wilcoxon-matched pairs singed rank test (paired samples) or the Mann-Whitney U test (unpaired samples). Autophagic flux measurement using mCherry-EGFP-LC3B was analyzed using an unpaired t test with Welch's correction. All analyses were performed using GraphPad Prism (GraphPad Software).

DATA AVAILABILITY

The RNA-sequencing and ChIP-sequencing data from this publication have been deposited in the NCBI GEO database and together assigned the identifier GSE107603 (RNA-sequencing: GSE107600; ChIP-sequencing: GSE107599).

RESULTS

INCREASED EXPRESSION OF AUTOPHAGY-ASSOCIATED GENES UPON NUTRIENT DEPRIVATION

For a better understanding of the transcriptional changes initiated by starvation, cells were deprived of amino acids and serum for 6 h in EBSS (Earle's balanced salt solution; culture media without amino acids, serum and a low amount of glucose[29]), a common manner to starve cells and induce autophagy, and RNA-sequencing was performed. Nutrient deprivation of 6 h was chosen as this is long enough to allow for the detection of changes in the transcriptome and yet short enough to prevent interference of secondary modulators of transcriptional responses. We utilized the near-haploid human HAP1 cell line[30] in which autophagy genes can be readily manipulated, allowing us to study the effect of the autophagic flux on the transcriptome. Nutrient deprivation led to the induction of autophagy, as demonstrated by an increased autophagic flux as assessed by determining the levels of lipidated MAP1LC3B (microtubule associated protein 1 light chain 3 beta; hereafter referred to as LC3-II) in the presence or absence of bafilomycin A_1 (**Figure 1A**), an increase in autolysosomal structures (**Figure 1B**), and an increase in the number of $mCherry^+EGFP^+$ (yellow) and $mCherry^+$ (red) dots (**Figure 1C**). Cell viability was not significantly affected at this time point (**Supplemental Figure 1A**). Starvation had a profound effect on the transcriptome of these cells as many genes were significantly differentially expressed (**Figure 1D and 1E**). Analysis of genes affected by nutrient deprivation revealed that autophagy-associated genes were enriched within the genes upregulated upon nutrient deprivation (**Figure 1F; Supplemental Table 1**). The genes upregulated upon starvation also included the majority of the key genes regulating mammalian autophagosome formation, as defined by Mizushima *et al*.[31] (**Figure 1G**). The increased expression of genes associated with autophagy or involved in autophagosome formation, observed with RNA sequencing, was confirmed by qRT-PCR (**Supplemental Figure 1B**). Thus, nutrient deprivation induces autophagy, and in parallel induces the expression of autophagy-associated genes in HAP1 cells.

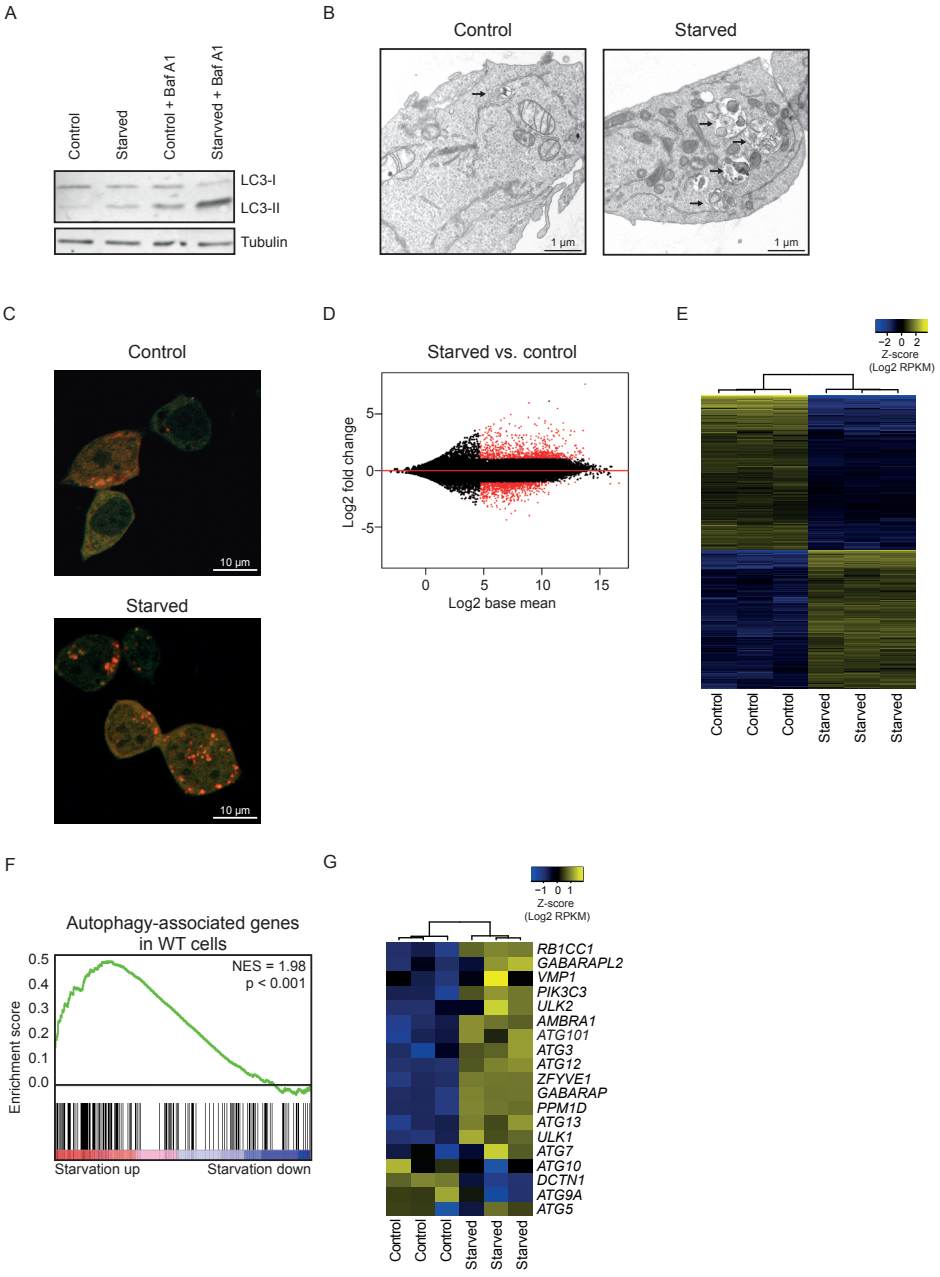

Figure 1. Increased expression of autophagy-associated genes after nutrient deprivation.
(**A**) Western Blot of HAP1 cells in control and starved (6 h EBSS) condition, with and without bafilomycin A_1 (40 nM). Representative blot is shown (n=4). (**B**) Representative EM images of HAP1 cells in control and starved (6 h EBSS) condition, treated with bafilomycin A_1. Autolysosomal structures are indicated by arrows. (**C**) Representative images of HAP1 cells transfected with a plasmid encoding mCherry-EGFP-LC3B in control and starved (6 h EBSS) condition.

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mCherry$^+$ EGFP$^+$ dots (yellow) are autophagosomes and mCherry$^+$ dots (red) are autolysosomes. (**D**) MA plot of HAP1 cells upon 6 h starvation with EBSS, displaying all expressed genes. Red dots indicate genes with a FDR <0.05. (**E**) Heatmap of genes differentially expressed in HAP1 cells after 6 h starvation with EBSS. (**F**) Gene set enrichment analysis for autophagy-associated genes in HAP1 cells upon starvation (6 h EBSS). (**G**) Heatmap depicting expression of key autophagy proteins upon starvation (6 h EBSS) of HAP1 cells.

EXPRESSION OF AUTOPHAGY-ASSOCIATED GENES IS INDEPENDENT OF AUTOPHAGIC FLUX

To determine whether autophagy is required for the increased expression of autophagy-associated genes upon starvation, CRISPR/Cas9-mediated ATG7 (autophagy related 7)- and RB1CC1/FIP200 (RB1 inducible coiled-coil 1)-deficient HAP1 cells were utilized (*ATG7* KO and *RB1CC1* KO), 2 genes belonging to 2 different ATG protein functional clusters[2] (**Supplemental Figure 2A and 2B**). These cells were unable to undergo normal autophagy, as demonstrated by the lack of LC3-II formation (**Figure 2A**) and the reduced formation of autophagosomal and autolysosomal structures (**Figure 2C and 2B**). Comparison of the transcriptomic changes of autophagy-deficient and wild-type (WT) cells upon nutrient deprivation demonstrated that both cell lines responded in a similar fashion (**Figure 2D and 2E**). This observation was supported by the analysis of differentially expressed genes upon starvation between WT and *ATG7* KO or *RB1CC1* KO cells, which revealed that only a few genes have a significantly different change in expression in *RB1CC1* KO cells upon nutrient deprivation compared to their change in WT cells (**Figure 2F**). Importantly, autophagy-associated genes were significantly enriched in genes increased in both autophagy-deficient cell lines (**Figure 2G**). Furthermore, expression of autophagy-associated genes was affected similarly in autophagy-deficient cells compared to WT cells upon starvation (**Figure 2D**, indicated by dark blue dots, **and Figure 2H**). These data demonstrate that the increased expression of autophagy-associated genes is not *per se* dependent on autophagic flux.

INCREASED TRANSCRIPTION OF AUTOPHAGY-ASSOCIATED GENES CONTRIBUTES TO INCREASED EXPRESSION OF AUTOPHAGY-ASSOCIATED GENES

To investigate whether the increased mRNA expression of autophagy-associated genes upon nutrient deprivation is the direct result of increased transcription, and to rule out that these differences are not only the result of increased mRNA stability, ChIP-sequencing for POLR2/Pol II (RNA polymerase II) was performed. mRNA expression as defined by RNA-sequencing directly correlated with POLR2 signal, indicating active transcription (**Figure 3A and 3B**). Moreover, genes identified based on RNA-sequencing as upregulated after nutrient deprivation showed indeed an increased POLR2 signal after starvation, and genes defined as downregulated displayed a decrease in POLR2 signal, demonstrating that transcription indeed contributed to the changes in gene expression (**Figure 3C**). Similarly, the POLR2 signal was increased for the majority of key genes involved in autophagosome formation upon starvation (**Figure 3D-F; Supplemental Figure 3**). Collectively these POLR2 ChIP-seq data demonstrate that increased transcription directly contributes to the increased expression of autophagy-associated genes in HAP1 cells.

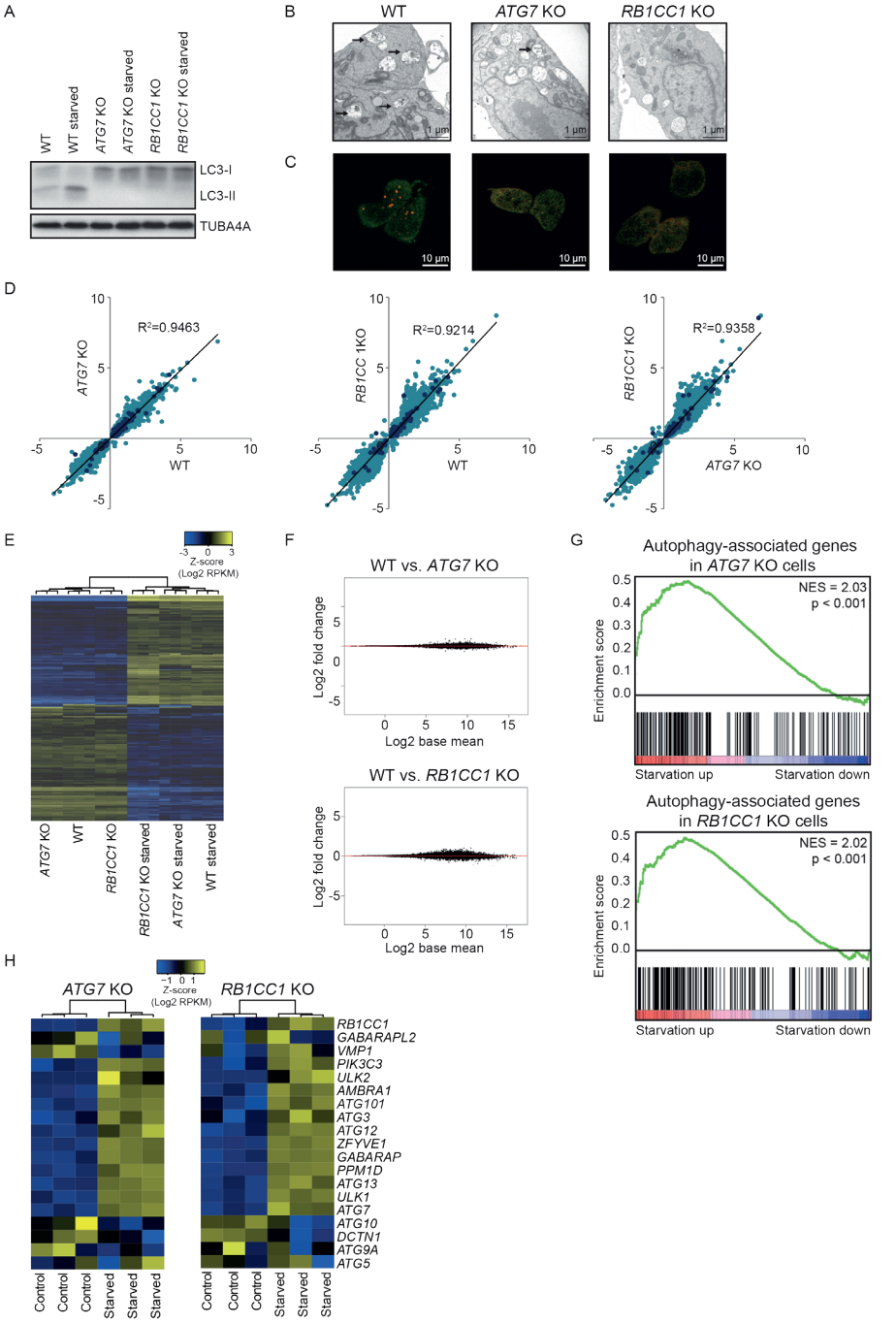

Figure 2. Increased expression of autophagy-associated genes upon nutrient deprivation in *ATG7* KO and *RB1CC1* KO cells.

(**A**) Western Blot of WT, *ATG7* KO, and *RB1CC1* KO HAP1 cells in control and starved (3 h EBSS) condition, treated

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with bafilomycin A_1 (40 nM). Representative blot is shown (n=4). (**B**) Representative EM images of WT, *ATG7* KO, and *RB1CC1* KO HAP1 cells in starved (6 h EBSS) condition, treated with bafilomycin A_1. Autolysosomes are indicated by arrows. (**C**) Representative images of HAP1 WT, *ATG7* KO, and *RB1CC1* KO cells transfected with a plasmid encoding mCherry-EGFP-LC3B in starved (6 h EBSS) condition. $mCherry^+$ $EGFP^+$ dots (yellow) are autophagosomes and $mCherry^+$ dots (red) are autolysosomes. (**D**) Fold change of significantly differentially expressed genes in either WT, *ATG7* KO and/or *RB1CC1* KO HAP1 cells. Blue dots represent autophagy-associated genes. (**E**) Heatmap of WT, *ATG7* KO, and *RB1CC1* KO HAP1 cells upon starvation (6 h EBSS) displaying genes significantly different in one of the cell lines. (**F**) MA plot of genes differentially expressed between WT and *ATG7* KO or *RB1CC1* KO HAP1 cells upon starvation (6 h EBSS). Red dots indicate genes with a FDR <0.05. (**G**) Gene set enrichment analysis of autophagy-associated genes in *ATG7* KO and *RB1CC1* KO HAP1 cells upon starvation (6 h EBSS). (**H**) Heatmap depicting expression of key autophagy proteins upon starvation (6 h EBSS) of *ATG7* KO and *RB1CC1* KO cells.

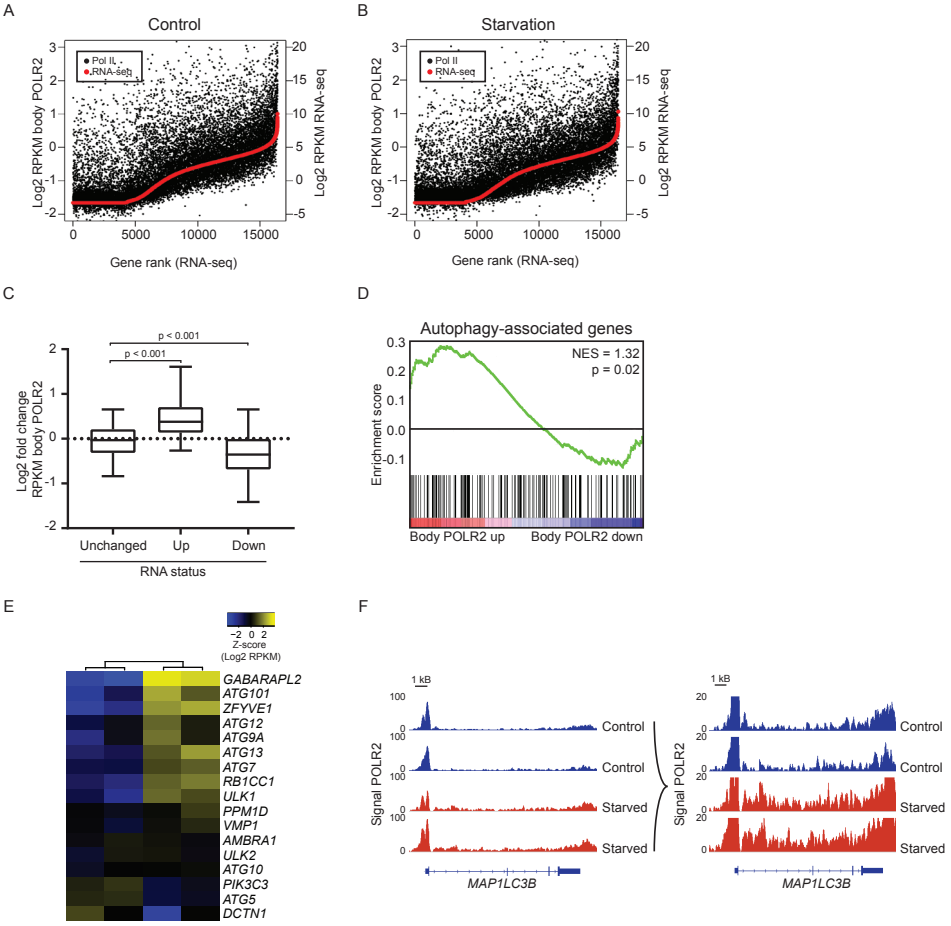

Figure 3. Increased transcription of autophagy-associated genes contributes to increased expression of autophagy-associated genes.

Rank analysis of gene body POLR2 occupancy and RNA-sequencing signal in control (**A**) condition and after starvation (**B**). Genes are ranked according to RNA-sequencing data. (**C**) Boxplots with 5%-95% whiskers displaying log2 fold change of body POLR2 signal for genes unchanged, increased, and decreased ≥ log1 based on RNA-sequencing. (**D**) Gene set enrichment analysis of autophagy-associated genes for genes associated with an alteration of body POLR2 signal upon starvation (3 h EBSS). (**E**) Heatmap depicting body POLR2 signal for key autophagy genes upon starvation (3 h EBSS). (**F**) Gene track for MAP1LC3B displaying ChIP-seq signals for POLR2.

INCREASED TRANSCRIPTION OF AUTOPHAGY-ASSOCIATED GENES IS REFLECTED AT THE EPIGENETIC LEVEL

To determine whether epigenetic modifications could contribute to the transcriptional changes upon nutrient deprivation, global levels of various histone marks (H4K16ac, H4K20me3, H3K9me2, H3K4me3, H3K27ac, and H3K56ac) were first assessed by western blotting 3 h after starvation. We did not observe an effect of nutrient-deprivation on global expression of these histone marks (**Supplemental Figure 4**). Next, to evaluate whether nutrient deprivation may result in a more specific redistribution of chromatin marks, ChIP-sequencing was performed for histone marks associated with active transcription (H3K4me3[32], H3K27ac[33], and H3K56ac[34]). Short-term nutrient deprivation resulted in alterations in all 3 histone marks (**Figure 4A**). As expected, the most pronounced effect was observed on the histone acetylation status, which has been demonstrated to be more dynamically regulated than methylation[35]. Increased H3K4me3, H3K27ac, and H3K56ac alterations directly correlated with increased mRNA expression (**Figure 4B**). Furthermore, autophagy-associated genes were enriched within the genes associated with an increase in H3K4me3, H3K27ac, or H3K56ac (**Figure 4C and 4D**). These data indicate that epigenetic alterations correlate with increased gene expression of autophagy-associated genes observed upon nutrient deprivation in HAP1 cells.

EPIGENETIC AND TRANSCRIPTOMIC ANALYSES IDENTIFY EGR1 AS A CANDIDATE TRANSCRIPTIONAL REGULATOR OF AUTOPHAGY

We next explored whether our epigenetic and transcriptomic datasets could be utilized to identify novel regulators of autophagy as a proof-of-principle exercise. To identify which transcription factor(s) could be involved in the increased transcription of autophagy-associated genes upon starvation, enrichment of transcription factor binding motifs in autophagy-associated genes was analyzed *in silico*. More specifically, open chromatin, indicated by H3K27ac, H3K56ac, or H3K4me3 peaks, associated with autophagy-associated genes with increased expression upon nutrient deprivation was combined with DNAse hypersensitivity data and analyzed for enrichment of transcription factor binding motifs (**Figure 5A**). For the 10 binding motifs with the highest enrichment, expression and induction of the corresponding transcription factors was assessed upon nutrient deprivation (**Figure 5B and 5C**). This analysis identified EGR1 as the transcription factor with the highest (increase in) expression under these conditions. Correspondingly, nutrient deprivation induced a strong increase in the POLR2 signal for *EGR1*, and EGR1 protein levels were demonstrated to increase upon starvation in both HAP1 and U2OS cell lines (**Figure 5D and 5E**). Serum or amino acid deprivation alone did not significantly affect *EGR1* expression (**Supplemental Figure 5**). To further validate the link between EGR1 and autophagy, we examined publically available EGR1 ChIP-sequencing data from 2 different lymphocytic cell lines, which indeed confirmed binding of EGR1 in the promotor region of many autophagy-related genes, including *MAP1LC3B* (**Figure 5F**). Furthermore, we identified the presence of 3 EGR1 motifs within the promoter region of *MAP1LC3B* corresponding to open chromatin regions in HAP1 cells. Altogether, these data identify EGR1 as a candidate transcriptional regulator of autophagy.

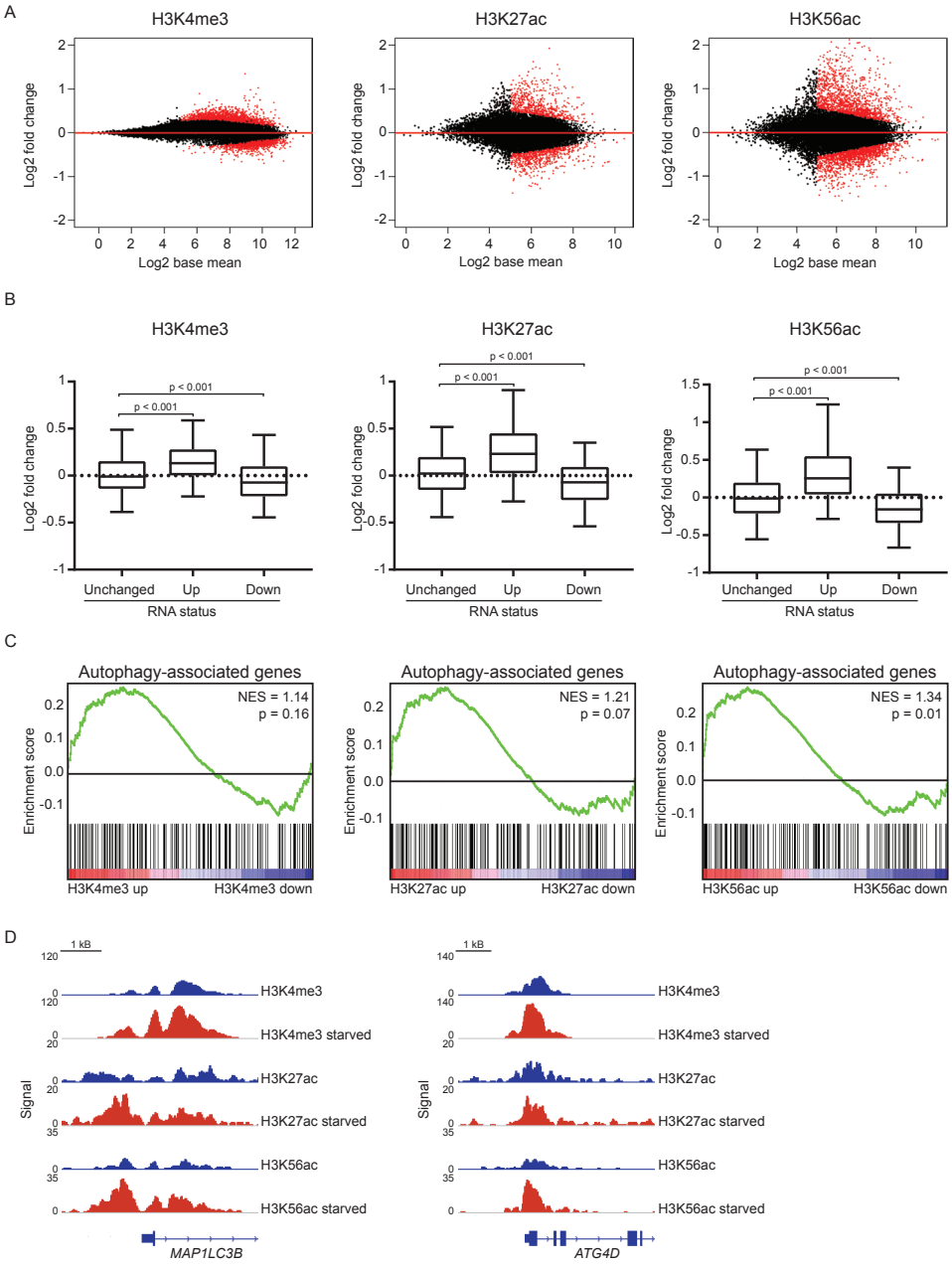

Figure 4. Increased transcription of autophagy-associated genes is reflected at the epigenetic level. (**A**) MA plots of H3K4me3, H3K27ac and H3K56ac signal upon starvation (3 h EBSS). Red dots indicate genes with a FDR <0.05. (**B**) Boxplots with 5%-95% whiskers displaying log2 fold change in H3K4me3, H3K27ac or H3K56ac signal for genes unchanged, increased, and decreased ≥ log1 based on RNA-sequencing. (**C**) Gene set enrichment analysis of autophagy-associated genes for genes associated with an alteration of H3K4me3, H3K27ac or H3K56ac signal upon starvation (3 h EBSS). (**D**) Gene tracks for *MAP1LC3B* and *ATG4D* displaying ChIP-seq signals for H3K4me3, H3K27ac, and H3K56ac with and without starvation (3 h EBSS).

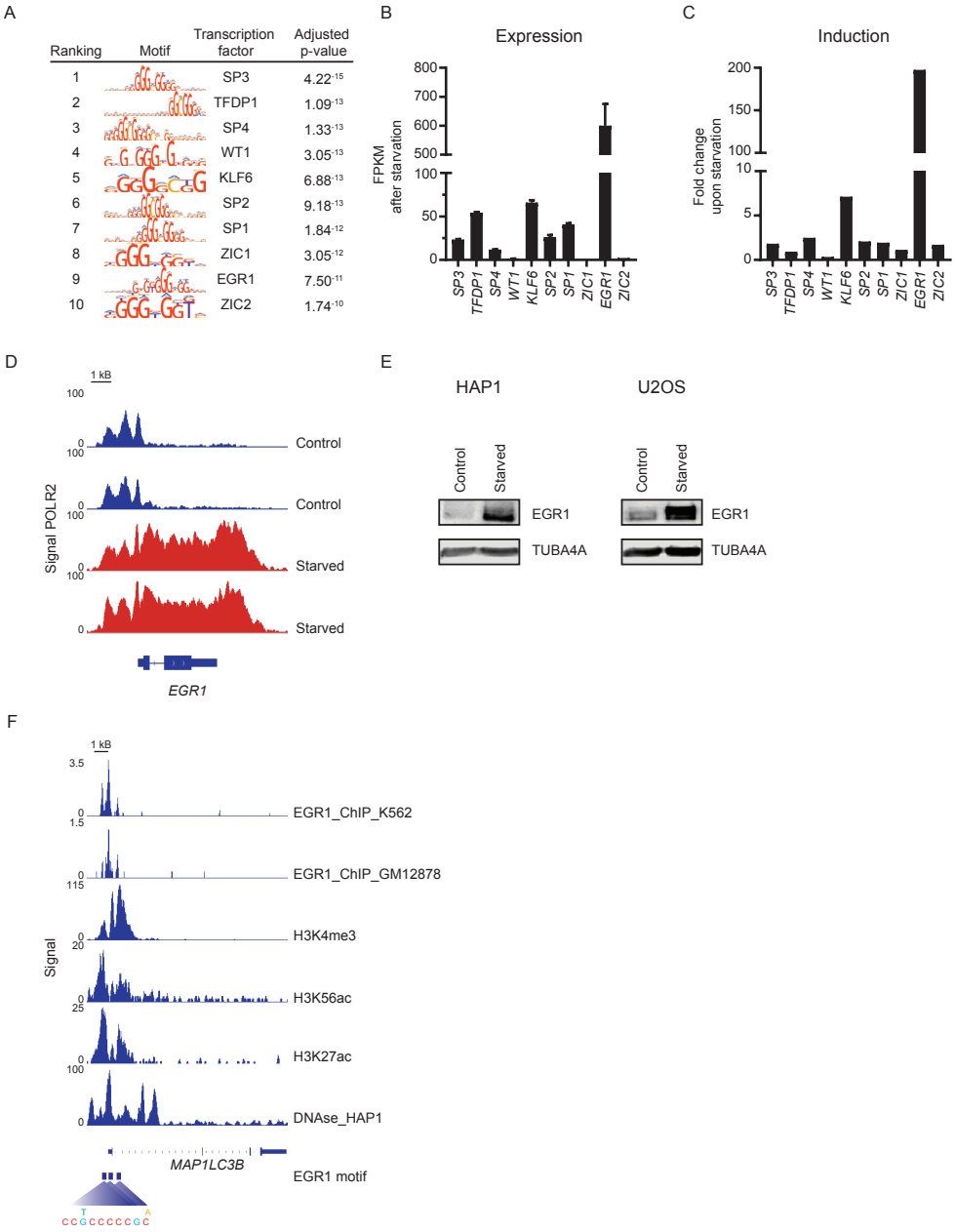

Figure 5. Epigenetic and transcriptomic analyses identify EGR1 as a candidate transcriptional regulator of autophagy.

(**A**) Top 10 transcription factor binding motifs enriched in autophagy-associated genes. Log2 FPKM (**B**) and fold change (**C**) after starvation (6 h EBSS) of transcription factors with binding motifs enriched in autophagy-associated genes. (**D**) Gene track for EGR1 displaying ChIP-seq signal for POLR2 with and without starvation (3 h EBSS). (**E**) EGR1 and TUBA4A/tubulin expression in HAP1 and U2OS cells upon starvation (6 h EBSS). Representative blots are shown (n=3). (**F**) Gene track for *MAP1LC3B* displaying ChIP-seq signals for EGR1 (obtained from GEO GSE32465, samples GSM803414 and GSM803434), H3K4me3, H3K27ac, H3K56ac, DNAse I hypersensitivity sites, and EGR1 motif.

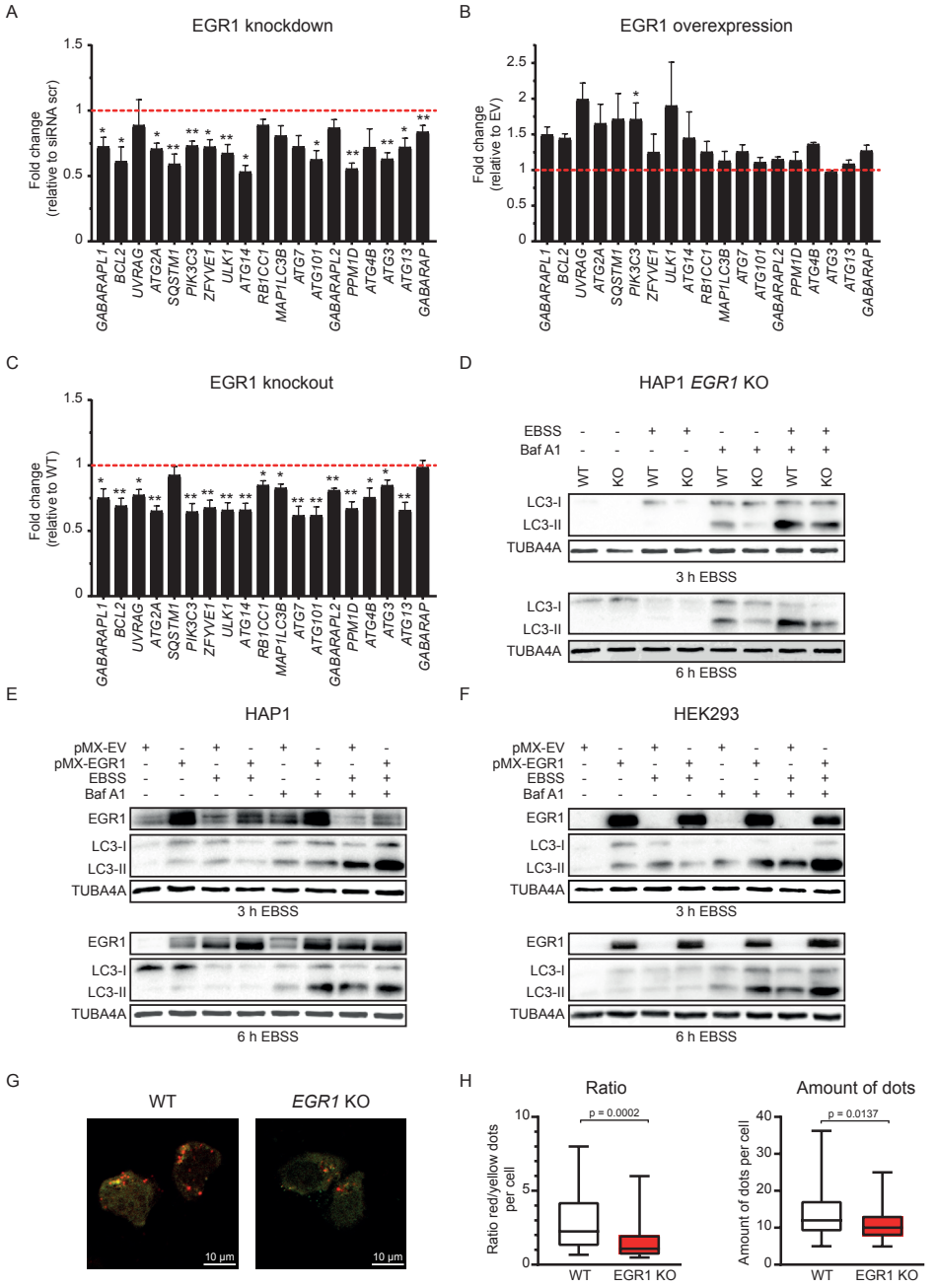

Figure 6. EGR1 acts as a transcriptional regulator of autophagy.

(**A**) Expression of autophagy-associated genes in HAP1 cells with and without EGR1 knockdown starved for 6 h with EBSS. Fold change relative to cells transfected with scrambled siRNA is shown. Data are represented as mean ± SEM (n=6-9). (**B**) Expression of autophagy-associated genes in HAP1 cells with and without EGR1 overexpression starved for 6 h with EBSS. Fold change relative to empty vector (EV)-transfected cells is shown. Data are represented

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as mean ± SEM (n=3-6). (**C**) Expression of autophagy-associated genes in HAP1 *EGR1* KO cells starved for 6 h with EBSS. Fold change relative to WT HAP1 starved for 6 h is shown. Data are represented as mean ± SEM (n=6). (**D**) LC3 and TUBA4A expression in WT and *EGR1* KO HAP1 cells in control and starved (3 h and 6 h EBSS) condition, treated with bafilomycin A_1 (40 nM). Representative blot is shown (3 h: n=4; 6 h: n=6). (**E**) EGR1, LC3, and TUBA4A expression in WT HAP1 cells with and without EGR1 overexpression in control and starved (3 h and 6 h EBSS) condition, treated with bafilomycin A_1 (40 nM). Representative blot is shown (3 h: n=3; 6 h: n=3). (**F**) LC3, EGR1, and TUBA4A expression in HEK293 cells with and without EGR1 overexpression in control and starved (3 h and 6 h EBSS) condition, treated with bafilomycin A_1 (40 nM). Representative blot is shown (3 h: n=3; 6 h: n=6). (**G**) Representative images of HAP1 WT and *EGR1* KO cells transfected with a plasmid encoding mCherry-EGFP-LC3B in starved (6 h EBSS) condition. $mCherry^+$ $EGFP^+$ dots (yellow) are autophagosomes and $mCherry^+$ dots (red) are autolysosomes. (**H**) Boxplots with 5%-95% whiskers displaying the ratio red vs. yellow dots per cell and the total amount of dots per cell (75 cells were counted within 2 independent experiments). * = p<0.05, ** = p<0.01, *** = p<0.001.

EGR1 ACTS AS A TRANSCRIPTIONAL REGULATOR OF AUTOPHAGY

To investigate whether EGR1 affects transcriptional regulation of autophagy, its expression levels were manipulated and autophagy-associated gene expression was analyzed. Upon nutrient deprivation, EGR1 knockdown resulted in a significant decrease in the transcription of the majority of autophagy-associated genes tested (**Figure 6A and Supplemental Figure 6A and 6B**). Overexpression of EGR1 had a modest effect on the transcription of autophagy-associated genes (**Figure 6B and Supplemental Figure 6C and 6D**). To substantiate these findings, we utilized CRISPR/Cas9-mediated EGR1-deficient HAP1 cells (*EGR1* KO) and assessed autophagy-associated gene expression upon nutrient deprivation. In agreement with knockdown of EGR1, the expression of the majority of autophagy-associated genes was decreased in *EGR1* KO cells compared to WT cells (**Figure 6C and Supplemental Figure 6E and 6F**). Altogether, this demonstrates that EGR1 transcriptionally regulates autophagy-associated genes. To determine whether the decreased expression of autophagy-associated genes affects the autophagic flux, the LC3-II:LC3-I ratio was analyzed in *EGR1* KO cells after 3 and 6 h of nutrient deprivation, in the presence or absence of bafilomycin A_1. Indeed, autophagy was reduced in the absence of *EGR1*, as indicated by the decreased LC3-II:LC3-I ratio compared to WT cells (**Figure 6D**). In contrast, overexpression of EGR1 in either HAP1 cells or HEK293 cells resulted in an increase in the autophagic flux, observed after 3 as well as 6 h of EBSS treatment (**Figure 6E and Figure 6F**). To validate the findings obtained with western blot, we transfected *EGR1* KO cells with an mCherry-EGFP-LC3B construct and quantified the amount of autophagosomes (yellow) and autolysosomes (red) after 6 h of nutrient deprivation (**Figure 6G**). In agreement with the decrease in LC3-II:LC3-I observed with western blot, the ratio of red:yellow dots and the total amount of dots was decreased in *EGR1* KO cells, suggesting a reduced autophagic flux compared to WT cells (**Figure 6H**). Altogether, nutrient deprivation induces EGR1 expression, which can subsequently induce autophagy through transcriptional control of numerous autophagy-associated genes, indicating that our datasets can indeed be utilized to identify (novel) regulators of autophagy.

DISCUSSION

Here, we generated an extensive transcriptomic and epigenomic database of human cells undergoing autophagy upon nutrient deprivation. We observed that nutrient deprivation induces an increase in expression of multiple autophagy-associated genes. This is in agreement with other studies, which analyzed the expression of a subset of proteins involved in autophagy under different starvation conditions, such as serum and amino acid deprivation[36] or glucose starvation[16]. Furthermore, we demonstrated that the expression of autophagy-associated genes was accompanied by an increase in POLR2 signal for these genes, validating that increased transcription contributes to the increased expression. We observed that nutrient deprivation had a similar effect on the transcriptome of *ATG7* and *RB1CC1* knockout cells compared to WT cells, including increased expression of autophagy-associated gene expression. This demonstrated that the transcriptional changes observed upon autophagy induction are not dependent on autophagic flux, but are rather the direct result of sensing nutrient deprivation[37]. This also indicates that within 6 h there is either limited feedback by autophagy itself on the transcriptional level, or that feedback still takes place in the autophagy-deficient cell lines, suggesting that autophagic flux is not itself necessary for feedback.

In *atg5*$^{-/-}$ mouse embryonic fibroblasts (MEFs), in contrast to WT MEFs, autophagy induction via rapamycin treatment does not lead to a decrease in H4K16ac, which is associated with downregulation of autophagy-associated genes and considered to be a feedback mechanisms[17]. This suggest that feedback at the transcriptional levels starts to become relevant after 6 h, or that these feedback loops are indirect, or that this is caused by differences between mice and men**.** Indeed, analysis of certain key autophagy genes in zebrafish has demonstrated that the increase in gene expression that is observed after 12 h of amino acid and serum starvation is absent after 24 h[36]. Additionally, our results indicate that the expression of autophagy-associated genes is not a measure of autophagic flux, because increased gene expression can still be observed in the absence of *ATG7* and *RB1CC1*, when no autophagic flux is present. In contrast to other reports, we did not observe an effect of autophagy induction on global H4K16ac, H3K4me3, and H3K56ac levels. This discrepancy could be due to different methods to induce autophagy, for example nutrient deprivation versus MTOR inhibition, differences in timing, and differences in the type or species of the employed cells. For example, rapamycin-induced downregulation of H3K4me3 in MEFs is not observed after glucose starvation[16,17].

As a proof of principle we used our transcriptional and epigenetic datasets and identified EGR1 as a potential transcriptional regulator of autophagy, because the EGR1 binding site is enriched within open chromatin regions of autophagy-associated genes, and EGR1 expression increases dramatically upon nutrient deprivation. *EGR1* is an immediate-early response gene, of which its expression can be induced within minutes after stimulation[38]. Mitogens[39,40], growth factors[41], and stress stimuli, such as cigarette smoke[42–44], hypoxia[45,46], and nutrient deprivation[47] regulate EGR1. For example, in agreement with our data, glucose restriction rapidly increases EGR1 protein levels in multiple cell lines[47]. The transcription factor EGR1 has been implicated in

numerous processes, for example apoptosis, angiogenesis, proliferation, cell differentiation, and migration[48,49]. EGR1 has been linked to cigarette smoke, hypoxia, and irradiation-induced autophagy, by induction of ATG4B [40] and LC3B protein or gene expression[43,46]. Additionally, *egr1*$^{-/-}$ mice are more resistant to the pro-autophagic effects of chronic cigarette smoke exposure[43]. However, there are also indications that EGR1 might act as a negative regulator of autophagy, either by affecting ATG12–ATG5 conjugation with ATG16L1[50] or by transcriptionally regulating the miRNA *MIR152*, which inhibits ATG14 and thereby decreases autophagy[51]. Our results are in line with a transcriptional activating role for EGR1 in autophagy and demonstrate that its transcriptional activity does not solely apply to *ATG4B* and *MAP1LC3B*, but to numerous autophagy-associated genes. *SQSTM1* and *GABARAP* (GABA type A receptor-associated protein) expression was not significantly affected in *EGR1* KO cells, whereas expression was decreased upon EGR1 knockdown. This discrepancy could be caused by adaptation of *EGR1* KO cells to the long-term absence of EGR1, as knockdown of EGR1 is transient. The modest effect of EGR1 overexpression on autophagy-associated gene expression compared to EGR1 knockdown or knockout could be caused by the starvation conditions under which these experiments were performed. Upon starvation, EGR1 expression is already high, therefore a knockdown/knockout approach is more likely to have a more pronounced effect on the expression of EGR1 and autophagy-associated genes. Overall, the fact that EGR1 was unbiasedly identified as a transcriptional regulator of autophagy in our transcriptomic and epigenetic analyses, indicates that our data can facilitate the identification of additional transcription factors involved in the regulation of autophagy.

Various signaling pathways have been reported to be involved in the transcriptional regulation of *EGR1*[52]. Reactive oxygen species (ROS), which can be induced by EBSS treatment, are a known inducer of autophagy[53]. Additionally, ROS production has been demonstrated to induce EGR1 expression in a MAPK/JNK- and MAPK/ERK-dependent manner[54]. Together, this suggests that nutrient deprivation may induce EGR1 expression through MAPK/JNKs and MAPK/ERKs. AMP-activated protein kinase (AMPK), a key energy sensor regulating cellular energy homeostasis, can induce autophagy through inactivation of MTOR complex 1[55] and phosphorylation of ULK1 (unc-51 like autophagy activating kinase 1), a rapid and cytoplasmic process[56]. Recently, also a nuclear role for AMPK has also been described; upon prolonged glucose starvation, nuclear AMPK expression and activation is increased, leading to initiation of the FOXO3-SKP2 (S-phase kinase associated protein 2)-CARM1 axis, which can transcriptionally regulate specific autophagy-associated genes[16]. AMPK activation has been demonstrated to induce EGR1 protein expression within 30 min[57,58]. These data indicate that the signaling pathway regulating the role of EGR1 in autophagy might involve AMPK. Because nuclear AMPK expression was only observed upon prolonged glucose starvation and the increase of EGR1 after starvation is rapid, it remains to be investigated whether nuclear AMPK is involved in the initial EGR1 induction or whether it is more important for maintaining EGR1 expression upon starvation.

In conclusion, our global transcriptomic and epigenomic profiling has demonstrated that nutrient deprivation regulates the transcriptional induction of autophagy-associated genes.

This increase in autophagy-associated gene expression is accompanied by changes in chromatin remodeling and is not regulated by the autophagic flux. Furthermore, as a proof of principle, our data identified EGR1 as a transcriptional regulator of serum and amino acid starvation-induced autophagy. Taken together, these data increase our understanding of the molecular pathways regulating autophagy and can be used as a resource to identify (novel) factors involved in autophagy regulation. Because autophagy has been implicated in numerous diseases, a better understanding of the molecular pathways and transcription factors regulating autophagy might lead to the development of novel strategies aimed at restoring autophagy levels in the context of disease, for example therapies targeting EGR1 expression[59].

ACKNOWLEDGEMENTS

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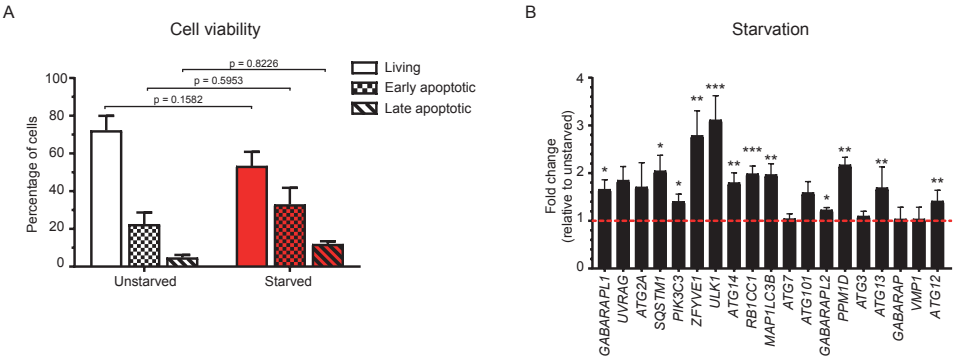
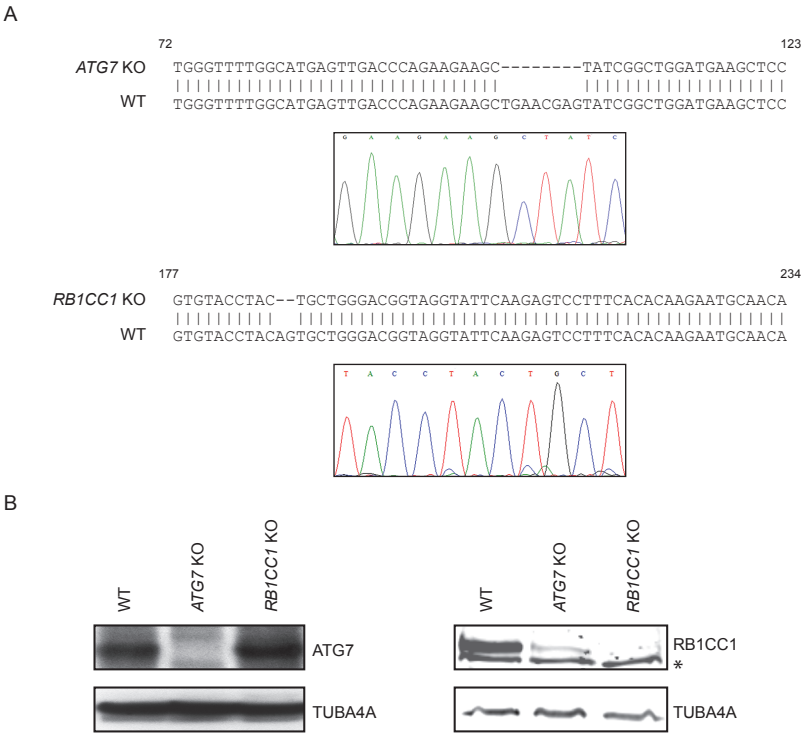
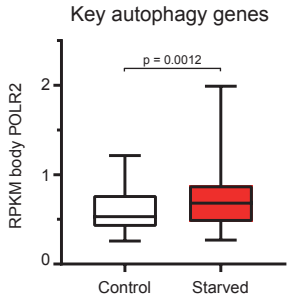
SUPPLEMENTAL INFORMATION



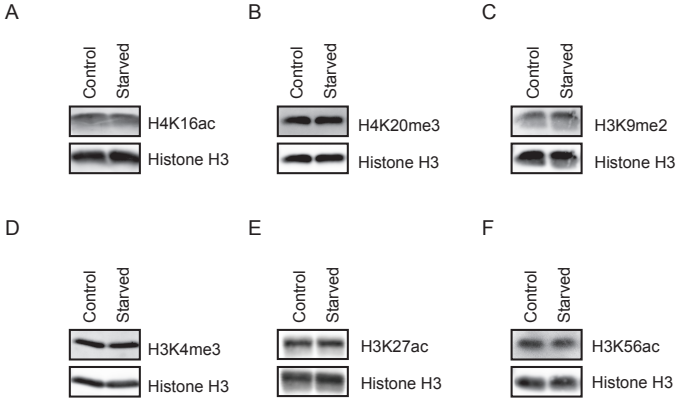
Supplemental Figure 1. Nutrient deprivation does not significantly affect cell viability and induces autophagy-associated gene expression.
(**A**) Percentage of living, early apoptotic, and late apoptotic cells in WT HAP1 cells in control and starved (6 h EBSS) conditions. Data are represented as mean ± SEM (n=3). (**B**) Expression of autophagy-associated genes in HAP1 cells upon starvation (6 h EBSS). Data are represented as mean ± SEM (n=9-12); * = p<0.05, ** = p<0.01, *** = p<0.001.



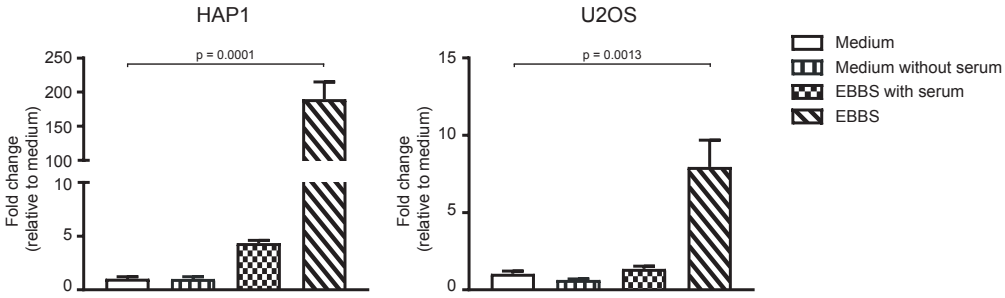
Supplemental Figure 2. Analysis of HAP1 *ATG7* KO and *RB1CC1* KO cells.
(**A**) Sequence alignment of exon 2 of *ATG7* for HAP1 WT and *ATG7* KO cells (top) and of exon 4 of *RB1CC1* for HAP1 WT and *RB1CC1* KO cells (bottom). (**B**) WB for ATG7, RB1CC1, and TUBA4A/tubulin in HAP1 WT, *ATG7* KO, and *RB1CC1* KO cells. Asterisk (*) indicates a nonspecific band observed with anti-RB1CC1/FIP200 antibody.



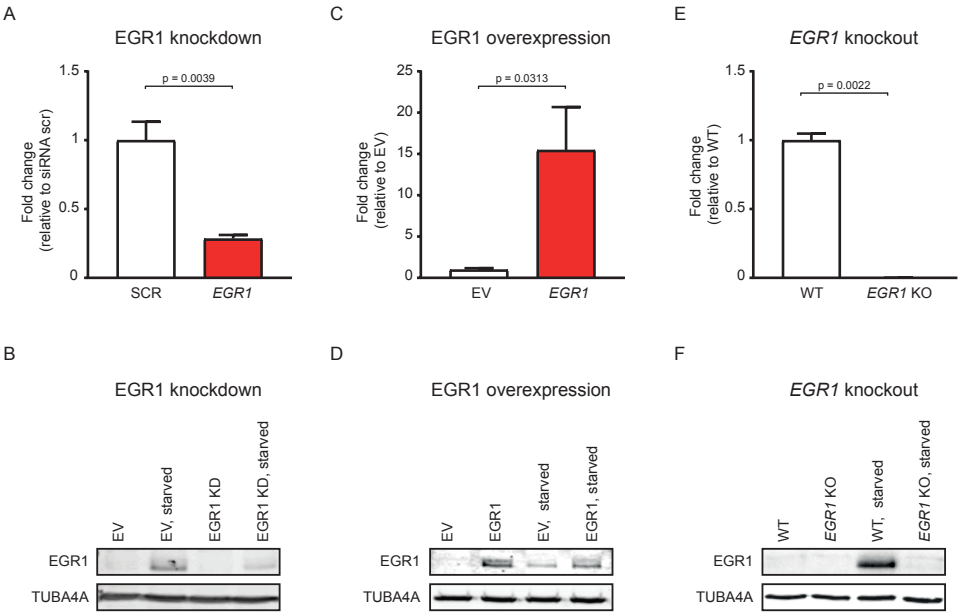
Supplemental Figure 3. Increased transcription of autophagy-related genes.
Boxplots with 5%-95% whiskers displaying log2 body POLR2 signal for key autophagy genes with and without starvation (3 h EBSS).



Supplemental Figure 4. Nutrient deprivation does not induce global changes of certain histone marks.
H4K16ac (**A**), H4K20me3 (**B**), H3K9me2 (**C**), H3K4me3 (**D**), H3K27ac (**E**) and H3K56ac (**F**) expression in HAP1 cells in control and starved (3 h EBSS) conditions. Representative blots are shown (n=3).



Supplemental Figure 5. *EGR1* induction by serum and/or amino acid deprivation.
EGR1 expression in HAP1 cells and U2OS cells cultured for 6 h in either medium supplemented with 10% serum (medium), medium without serum (= serum starvation), EBSS supplemented with 10% serum (= amino acid starvation), or EBSS (= serum and amino acid starvation). Fold change relative to cells cultured in medium is shown. Data are represented as mean ± SEM (n=3).



Supplemental Figure 6. Manipulation of EGR1 expression.
(**A**) *EGR1* mRNA expression in HAP1 cells with and without EGR1 knockdown starved for 6 h with EBSS. Fold change relative to cells transfected with siRNA scrambled cells is shown. Data are represented as mean ± SEM (n=9). (**B**) EGR1 and TUBA4A protein levels in HAP1 cells with and without EGR1 knockdown, with and without 6 h starvation (EBSS). Representative blot is shown (n=3). (**C**) *EGR1* mRNA expression in HAP1 cells with and without EGR1 overexpression starved for 6 h with EBSS. Fold change relative to empty vector (EV)-transfected cells is shown. Data are represented as mean ± SEM (n=6). (**D**) EGR1 and TUBA4A protein levels in HAP1 cells with and without EGR1 overexpression, with and without 6 h starvation (EBSS). Representative blot is shown (n=3). (**E**) *EGR1* mRNA expression in HAP1 WT and *EGR1* KO cells starved for 6 h with EBSS. Fold change relative to starved HAP1 WT cells is shown. Data are represented as mean ± SEM (n=6). (**F**) EGR1 and TUBA4A protein levels in HAP1 WT and *EGR1* KO cells with and without 6-h starvation (EBSS). Representative blot is shown (n=3).

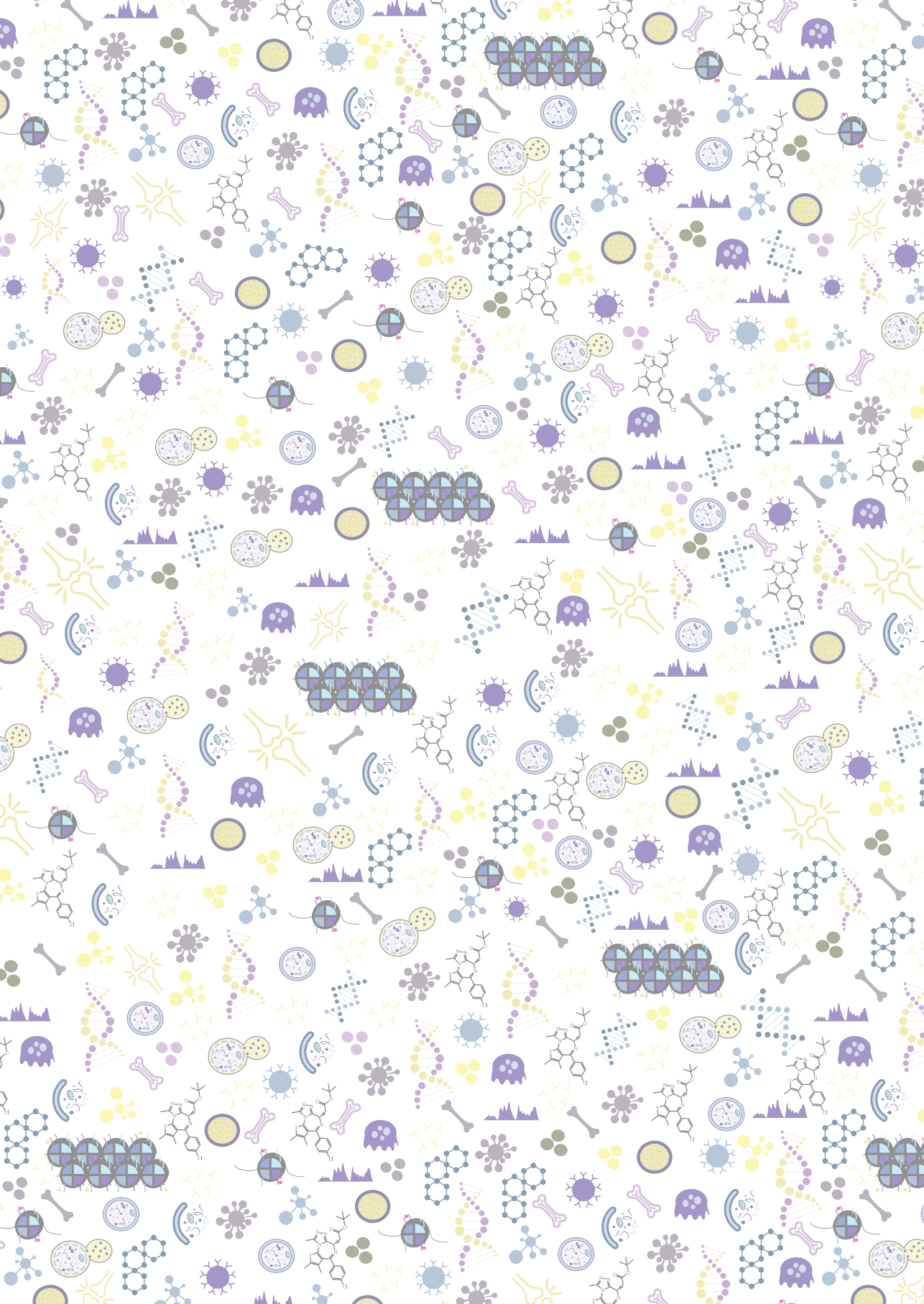
Supplemental Table 1. List of autophagy-associated genes.

AMBRA1	*CANX*	*EIF4G1*
APOL1	*CAPN1*	*ERBB2*
ARNT	*CAPN10*	*ERN1*
ARSA	*CAPN2*	*ERO1L*
ARSB	*CAPNS1*	*FADD*
ATF4	*CASP1*	*FAM48A*
ATF6	*CASP3*	*FAS*
ATG10	*CASP4*	*FKBP1A*
ATG12	*CASP8*	*FKBP1B*
ATG16L1	*CCL2*	*FOS*
ATG16L2	*CCR2*	*FOXO1*
ATG2A	*CD46*	*FOXO3*
ATG2B	*CDKN1A*	*GAA*
ATG3	*CDKN1B*	*GABARAP*
ATG4A	*CDKN2A*	*GABARAPL1*
ATG4B	*CFLAR*	*GABARAPL2*
ATG4C	*CHMP2B*	*GAPDH*
ATG4D	*CHMP4B*	*GNAI3*
ATG5	*CLN3*	*GNB2L1*
ATG7	*CTSB*	*GOPC*
ATG9A	*CTSD*	*GRID1*
ATG9B	*CTSL1*	*GRID2*
ATIC	*CX3CL1*	*HDAC1*
BAG1	*CXCR4*	*HDAC6*
BAG3	*DAPK1*	*HGS*
BAK1	*DAPK2*	*HIF1A*
BAX	*DDIT3*	*HSP90AB1*
BCL2	*DIRAS3*	*HSPA5*
BCL2L1	*DLC1*	*HSPA8*
BECN1	*DNAJB1*	*HSPB8*
BID	*DNAJB9*	*IFNG*
BIRC5	*DRAM1*	*IKBKB*
BIRC6	*EDEM1*	*IKBKE*
BNIP1	*EEF2*	*IL24*
BNIP3	*EEF2K*	*IRGM*
BNIP3L	*EGFR*	*ITGA3*
C12orf44	*EIF2AK2*	*ITGA6*
C17orf88	*EIF2AK3*	*ITGB1*
CALCOCO2	*EIF2S1*	*ITGB4*
CAMKK2	*EIF4EBP1*	*ITPR1*

GAA	*NRG1*	*SIRT2*
GABARAP	*NRG2*	*SPHK1*
GABARAPL1	*NRG3*	*SPNS1*
GABARAPL2	*P4HB*	*SQSTM1*
GAPDH	*PARK2*	*ST13*
GNAI3	*PARP1*	*STK11*
GNB2L1	*PEA15*	*TBK1*
GOPC	*PELP1*	*TM9SF1*
GRID1	*PEX14*	*TMEM49*
GRID2	*PEX3*	*TMEM74*
KIAA0226	*PIK3C3*	*TNFSF10*
KIAA0652	*PIK3R4*	*TP53*
KIAA0831	*PINK1*	*TP53INP2*
KIF5B	*PPP1R15A*	*TP63*
KLHL24	*PRKAB1*	*TP73*
LAMP1	*PRKAR1A*	*TSC1*
LAMP2	*PRKCD*	*TSC2*
MAP1LC3A	*PRKCQ*	*TUSC1*
MAP1LC3B	*PTEN*	*ULK1*
MAP1LC3C	*PTK6*	*ULK2*
MAP2K7	*RAB11A*	*ULK3*
MAPK1	*RAB1A*	*USP10*
MAPK3	*RAB24*	*UVRAG*
MAPK8	*RAB33B*	*VAMP3*
MAPK8IP1	*RAB5A*	*VAMP7*
MAPK9	*RAB7A*	*VEGFA*
MBTPS2	*RAC1*	*WDFY3*
MLST8	*RAF1*	*WDR45*
MTMR14	*RB1*	*WDR45L*
MTOR	*RB1CC1*	*WIPI1*
MYC	*RELA*	*WIPI2*
NAF1	*RGS19*	*ZFYVE1*
NAMPT	*RHEB*	
NBR1	*RPS6KB1*	
NCKAP1	*RPTOR*	
NFE2L2	*SAR1A*	
NFKB1	*SERPINA1*	
NKX2-3	*SESN2*	
NLRC4	*SH3GLB1*	
NPC1	*SIRT1*	

Supplemental Table 2. List of primers used in this study.

	qPCR primers
GABARAPL1	FW: ATGAAGTTCCAGTACAAGGAGGA RV: GCTTTTGGAGCCTTCTCTACAAT
BCL2	FW: GCCTTCTTTGAGTTCGGTGG RV: ATCTCCCGGTTGACGCTCT
UVRAG	FW: GATCGAGATGAGCGCCTCC RV: AAGACGCCGCTGCTGAG
ATG2A	FW: GCTCAGGGTACATGGAGCTG RV: CTCGTGGTCTGTAAGGCTCAC
SQSTM1	FW: ACCTTCTGGGCAAGGAGGACGC RV: CCCCGTCCTCATCGCGGTAGTG
PIK3C3	FW: GCTGTCCTGGAAGACCCAAT RV: TCAGCCATTCATTCCAGTTCCA
ZFYVE1	FW: CCTGGATGTCCCTTTATCCA RV: TCCTGGATGAGCTTCTCTGG
ULK1	FW: CAAGATCGCTGACTTCGGCT RV: CACTGGTAGACGATGGTGCC
ATG14	FW: CGGGACCTGGTGGACTCCGT RV: TCGATAAACCTCTCCCGGTCGC
RB1CC1/FIP200	FW: CTCAAACCAGGTGAGGGTGCTTCA RV: TGTTTTGTGCCTTTTTGGCTTGACA
MAP1LC3B	FW: AAGGCGCTTACAGCTCAATG RV: CTGGGAGGCATAGACCATGT
ATG7	FW: TAGCAGCCCACAGATGGAGTA RV: TCCCATGCCTCCTTTCTGGT
ATG101	FW: GTGGGAGCTGTTTTAACCGTG RV: GAGACCAGCTCCACAGTCCA
GABRAPL2	FW: TTGTTGTGCTCGGTGCGCTG RV: ACGCATCTGTGTTCCAGCGAGT
PPM1D	FW: GTTCCTCCGTGGCCTTTTTC RV: GGCCATTCCGCCAGTTTCTT
ATG4B	FW: TCGCTGTGGGGTTTTTCTGT RV: AGAATCTAGGGACAGGTTCAGGA
ATG3	FW: ACATGGCAATGGGCTACAGG RV: TTCCATCTGTTTGCACCGCT
ATG13	FW: TTTGTAGCCAGCATTAAGT RV: AAGGAAATCTGGATCAAGTGCAGTA
GABARAP	FW: GCTCCCAAAGCTCGGATAGG RV: TCTTCTTCATGGTGTTCCTGGT
EGR1	FW: CACCTGACCGCAGAGTCTTTT RV: GGCCAGTATAGGTGATGGGG
	Sequencing primers
ATG7	RV: CACCAGGTTTTGCATGGATATGTTA
RB1CC1/FIP200	FW: TGTTTTTGGGGAAGGTTTTAGAGTG



General discussion 7

Numerous studies focusing on the genetic basis of autoimmunity have been performed to unravel the molecular mechanisms underlying autoimmune diseases[1]. Although multiple risk variants associated with autoimmunity have been described, it has been proven difficult to translate these findings into novel insights of disease pathogenesis, and to demonstrate the molecular consequence of these variants. One of the reasons for this is that it has become increasingly clear that autoimmune diseases are characterized by a complex interplay of genetic as well as epigenetic mechanisms[2]. In this thesis we sought to characterize epigenetic mechanisms, focusing on enhancers, which are involved in the pathogenesis of autoimmune diseases (**Chapter 3 and 4**). In addition, we studied autophagy in the context of autoimmune disease and its epigenetic and transcriptional regulation (**Chapter 5 and 6**). To this end, we used primary cells obtained from the site of inflammation of Juvenile Idiopathic Arthritis (JIA) patients, as they provide a reflection of the chronic inflammation characteristic of autoimmune diseases. In this thesis, we demonstrate that altered enhancer regulation and autophagy is associated with autoimmunity. The implications of these observations regarding autoimmune disease pathogenesis are discussed below. The current treatment strategies for autoimmune diseases are effective in the majority of patients, but since some patients become resistant to therapy, only show partial remission or do not respond at all, there is still a medical need for the development of novel therapeutic strategies. The work described in this thesis aids in the identification of novel therapeutic targets. Here, we will discuss these findings in view of their potential use as therapeutic strategy for the treatment of autoimmune disease.

ALTERED ENHANCER PROFILE IN JIA PATIENT CELLS: A CAUSE OR CONSEQUENCE OF THE DISEASE?

In **Chapter 3** and **4** we demonstrate that T cells and monocytes obtained from the synovial fluid (SF) of JIA patients display an altered enhancer profile compared to healthy immune cells, which correlates with disease-associated gene expression. We can only speculate whether this altered enhancer landscape and the associated gene expression is a cause or a consequence of the disease. If it is a consequence of the disease, this is probably the result of the highly inflammatory environment where the cells are located. Stimuli within the environment can affect expression levels of histone modifying enzymes, such as acetyltransferases and demethylases, which can shape the enhancer repertoire. For JIA T cells we observed increased mRNA expression of the *K (lysine) acetyltransferase 2B* (*KAT2B*), which could contribute to the increased H3K27ac signal on enhancers. In JIA monocytes we observed increased mRNA expression of the *lysine demethylase 6B* (*KDM6B*/*JMJD3*), which is also associated with an increased enhancer. KDM6B is a H3K27me3-specific demethylases and its expression in macrophages is induced by LPS and inflammatory cytokines[3]. A decrease in tri-methylation of H3K27, due to increased KDM6B expression, might be associated with an increase in acetylation of H3K27. For monocytes obtained from systemic lupus erythematosus (SLE) patients, *KDM6B* mRNA levels haven been demonstrated to be increased as well[4,5]. In addition, increased mRNA and protein expression of KDM6B has been described in $CD4^+$ T cells from SLE patients, were it contributes to increased

CD11a expression, which has been implicated to be involved in SLE pathogenesis[6]. This suggests that increased *KDM6B* expression might be a molecular mechanism that contributes to autoimmune disease pathogenesis. A confounding factor in our studies is the comparison of healthy peripheral blood derived cells with cells derived from the synovial compartment of patients. It is difficult to discriminate whether the differences in enhancer landscape and gene expression are solely due to the disease, or whether they also reflect the differential location within the body. As it has been demonstrated for tissue-resident macrophages that the epigenetic landscape is highly dependent on the (local) microenvironment, it seems likely that the observed differences are a combination of the disease and the location[7,8]. Culturing patient-derived immune cells *in vitro* instead of analyzing them *ex vivo* can give insight into environment-dependent and environment-independent epigenetic modifications associated with the disease. This is especially relevant since inflammatory mediators present within the inflamed synovial environment can affect the enhancer landscape via activation of transcription factors, such as tumor necrosis factor α (TNF-α) via activation of nuclear factor κB (NF- κB), suggesting that the altered enhancer repertoire of JIA patient-derived immune cells might be a consequence of the disease[9]. This can be tested *in vitro* by culturing healthy control cells in the presence of SF or individual pro-inflammatory cytokines and perform ChIP-sequencing for H3K27ac to determine the active enhancer profile. The expression of histone modifying enzymes could also be affected by single nucleotide polymorphisms (SNPs), indicating that the resulting differences in the enhancer landscape could also be viewed as a cause of the disease, instead of a consequence. Moreover, SNPs can immediately affect enhancer formation by impairing transcription factor binding sites, which will be discussed in more detail below. Altogether, it seems plausible that the enhancer landscape of autoimmune disease patient-derived cells is affected by intrinsic factors, such as SNPs, and environmental factors, for example pro-inflammatory cytokines within the SF, and thus is a cause as well as a consequence of the disease. Furthermore, this suggests that the enhancer landscape is very dynamic and that feedback loop exists, namely increased pro-inflammatory cytokine expression due to increased enhancer activity can shape the enhancer landscape and contribute to altered gene expression.

SNPS AND ENHANCERS: A DYNAMIC INTERPLAY?

We observed that arthritis-associated SNPs are enriched within enhancer and super-enhancer regions in healthy control cells but even more within these regions in JIA SF-derived cells (**Chapter 3 and 4**). An example is a *CXCR4* variant present within a super-enhancer associated with *CXCR4* in JIA T cells. *CXCR4* risk variants have been correlated with decreased *CXCR4* mRNA expression in umbilical cord T cells, but whether and how *CXCR4* variants are involved in JIA pathogenesis remains to be determined[10]. To study the effect of *CXCR4* and other variants in more detail, for example to investigate the effect on (super-)enhancer formation, it will be informative to analyze the enhancer landscape in cells with and without the specific variant. Especially the circularized chromosome conformation capture (4C) sequencing technology, which analyzes all genomic regions that interact with a particular region of interest, can add

to the understanding how a non-coding genetic variant can contribute to disease on a molecular level[11,12]. Promoter-enhancer interaction can for instance be impaired when a certain variant disrupts a transcription factor binding site. This has for example been demonstrated for two SLE-associated SNPs, which disrupt NF-κB binding to the enhancer of *TNF-α-induced protein 3* (*TNFAIP3*), which encodes for A20[13]. As a result, *A20* expression is reduced, and since A20 is a negative regulator of NF-κB, this eventually leads to increased NF-κB activity, which might contribute to disease pathogenesis. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology can also be used to study the effect of genetic variation on enhancer formation and gene expression, by genetically introducing a known variant in the genome of a desired cell type[14]. Furthermore, the effect of *de novo* enhancer formation can be studied *in vitro* using a catalytic death Cas9 (dCas9) protein fused to the catalytic core domain of the histone acetyltransferase p300[15]. In combination with a guide RNA which localizes the dCas9/p300 fusion protein to a desired place within the genome, this will result in H3K27 acetylation and therefore eventually to enhancer formation. The CRISPR/Cas9 technology has nowadays been successfully applied in human primary cells, making it possible to dissect transcriptional enhancer activity within the relevant cell types and eventually also in patient-derived cells.

AUTOIMMUNE DISEASES: SHARED PATHOGENESIS?

A recent study investigating DNA methylation in peripheral blood $CD4^+$ T cells from oligoarticular JIA patients did not reveal substantial differences compared to age and sex matched controls[16]. Since altered DNA methylation has been reported extensively for rheumatoid arthritis (RA) $CD4^+$ T cells, this suggests that differential molecular mechanisms contribute to these two diseases[17]. Epigenetic studies assessing the enhancer profile of autoimmune disease patient-derived cells, such as RA and SLE patients, are now starting to emerge and it will be highly interesting to compare these profiles with the epigenetic landscape of JIA patients. We observed a significant overlap of autoimmune disease-associated SNPs with enhancers and super-enhancers in JIA, but not for non-autoimmune disease-associated SNPs, suggesting a certain degree of shared pathogenesis (**Chapter 3**). This is in line with observations that many genomic loci harboring risk variants associated with autoimmunity are shared between different autoimmune diseases[18–20]. In addition, comparison of genes differentially expressed in RA and JIA monocytes, both compared to healthy control monocytes, reveals that genes upregulated in JIA SF monocytes are enriched within the genes upregulated in RA SF monocytes and vice versa (**Figure 1**)[21]. To improve our understanding of the shared pathogenesis between autoimmune diseases, it will be very informative to characterize multiple autoimmune disease patient-derived cells on the genetic, epigenomic, and transcriptomic level in one single study, as this allows for extensive comparison.

What is currently lacking in the majority of studies examining enhancer biology within primary (patient-derived) immune cells is information about the 3D conformation of the genome, since this information is lost when ChIP-sequencing for histone marks characteristic of (active)

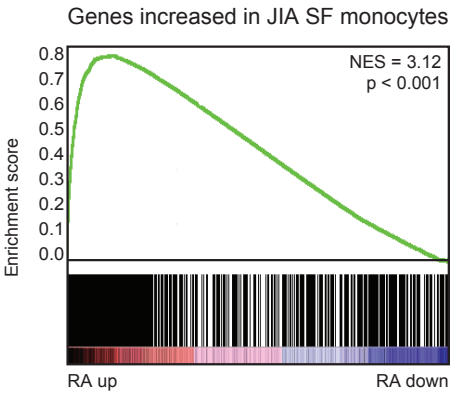


Figure 1. Comparison of genes upregulated in RA SF and JIA SF monocytes.
Gene set enrichment analysis demonstrating that genes significantly increased in JIA SF-derived monocytes are enriched within the genes upregulated in RA SF-derived monocytes.

enhancers is performed. A common assumption, which we also used for our studies, is that enhancers regulate the gene of which the transcriptional start site is closest to the enhancer. However, chromosome conformation capture-techniques (3C-based technologies), which are able to capture the physical interactions between enhancers and promoters, indicate that only 27-40% of the active enhancers indeed interact with their nearest promoter[22,23]. Instead, these techniques have demonstrated that chromosomes are spatially subdivided and that physical chromosome interactions occur more frequently between regions belonging to the same spatial domain than interactions between neighboring domains[24,25]. So far, it has been challenging to perform techniques that take into account the 3D genome conformation on patient samples given the high amount of cells that is needed. For the future, it will therefore be of great interest to further develop and improve these techniques so they can be used widely using small cell numbers.

MECHANISMS UNDERLYING THE SPECIFICITY OF BET-INHIBITORS

In **Chapter 3 and 4** we demonstrate that treatment of JIA patient cells with JQ1, a small molecule inhibitor of the bromodomain extra-terminal (BET) family of proteins, reduces disease-associated gene expression. The BET protein family consists of bromodomain-containing protein 2 (BRD2), BRD3, BRD4, and bromodomain testis-specific protein, which are all chromatin reader proteins that bind specific acetylated residues, such as histone proteins[26]. Competitive binding of JQ1, and other BET inhibitors, to the bromodomain of BRD proteins prevents binding of BRD proteins to acetylated chromatin and thereby impairs recruitment of positive transcription elongation factor complex (P-TEFb), which impairs RNA polymerase II binding and activation, thus inhibiting transcription[27]. Since BRD4 has been demonstrated to be essential for P-TEFb recruitment, transcriptional effects of BET inhibitors are predominantly linked to BRD4 inhibition[28]. Given their non-selective mechanisms of action, which is inhibition

of proteins involved in transcriptional initiation, it is rather surprising that the actual effects of several BET-inhibitors in *in vitro* and *in vivo* preclinical models are quite specific. For example, treatment of LPS-stimulated macrophages affects a specific subset of LPS-induced genes, while housekeeping genes are not affected[29]. In addition, in tumor cells there is selective inhibition of tumor oncogenes and for several animal cancer models it has been demonstrated that BET-inhibitors preferentially target tumor cells and hardly affect normal tissue[30–34]. The mechanisms underlying the gene- and context-specific selectivity of BET-inhibitors are incompletely understood, but it seems that BET inhibitors preferentially target highly or dynamically regulated genes, that is genes that are regulated by multiple large enhancers or super-enhancers, genes that are expressed in a lineage-specific manner, and genes that are dynamically expressed upon certain external stimuli[35]. This preferential inhibition is amongst others related to the BRD4 protein levels present at the regulatory elements involved in transcription of these genes. BRD4 is abundantly present at enhancers and increased BRD4 levels have been observed at super-enhancers, which is in line with the preferential inhibition of super-enhancer driven gene expression by BET inhibitors[30,32,36]. Furthermore, gene selectivity could depend on the transcription factors associated with BRD4, since BRD4 can bind to specific acetylated regions of transcription factors. In acute myeloid leukemia cells, several hematopoietic transcription factors recruit p300 which acetylates the surrounding histone proteins as well as the transcription factors itself, which both promote BRD4 recruitment[37]. In addition, JQ1 treatment strongly suppresses the downstream target genes of these hematopoietic transcription factors, while transcription factor DNA binding is not affected, suggesting that lineage-specific transcription factors can affect BRD4 occupancy and thereby contribute to the specificity of BET inhibitors[37]. That this concept does not only apply to leukemia cells is underscored by similar observations in prostate cancer, breast cancer and lung cancer, where the anti-cancer effects of BET inhibitors are related to functional suppression of the androgen receptor, estrogen receptor, and NF-κB, respectively[38–40]. External stimuli, such as pro-inflammatory cytokines, can induce the formation of *de novo* or latent enhancers, which are defined as genomic regions that are unbound by transcription factors and do not contain enhancer-associated histone marks, but that acquire these features upon stimulation[41,42]. BRD4 has been demonstrated to rapidly redistribute towards *de novo* (super-)enhancer regions upon stimulation[9,43]. In agreement with this observation, *de novo* (super-)enhancers, for example induced by TNF-α and LPS, are highly susceptible to BET inhibitors[9,29,41,43]. *De novo* or latent enhancers are highly dynamic and very cell type- and stimuli-specific, as they are defined by stimuli-dependent and lineage-restricted transcription factors. The increased localization of BRD4 at latent or *de novo* context-specific genomic regions therefore also contributes to the gene- and context-specific effect of BET inhibitors. This, together with the specificity that is provided by association with acetylated lineage-specific transcription factors, provides an understanding of the broad effectivity of BET inhibitors in multiple cancer models, which are each characterized by diverse sets of (onco)genes[27].

NEED FOR THE DEVELOPMENT OF "BETTER" INHIBITORS

Given the promising preclinical results of BET inhibition, there are currently 14 BET inhibitors in early phase clinical trials against solid tumors and hematologic malignancies[44]. The first results are promising with a subset of patients demonstrating a complete or partial response and with side-effects being limited to thrombocytopenia, gastrointestinal adverse effects, and fatigue[45]. The majority of BET inhibitors will therefore probably be further tested in phase II clinical trials. However, compared to preclinical studies, the overall clinical results are modest and relapses have been described several months after start of the treatment[45]. This suggests that there is need for the development of "BETter" inhibitors. This could for example be achieved by generating small molecule inhibitors with a prolonged half-life, by developing inhibitors that can inhibit individual BRD proteins, or by developing inhibitors that target one of the two bromodomains of BRD proteins as this might increase their specificity. Regarding the latter, several examples have already been developed and are now being tested in clinical trials[46–50]. Furthermore, an alternative approach has been described that provides the opportunity to specifically target individual BRD proteins for degradation by the proteasome[51–53]. This approach, referred to as proteolysis targeting chimera (PROTAC), conjugates a BRD binding moiety to a chemical structure that promotes recruitment of E3 ubiquitin ligases. The resulting poly-ubiquitinated BRD protein is subsequently recognized and degraded by the proteasome. Initial preclinical results indicate that BRD degradation might be more effective than BRD inhibition, suggesting that small molecule BET degraders, such as dBET1/6, ARV-825, and MZ1, might have clinical potential[51–53]. In addition, more clinical research is needed to get a better estimation of the correct dosage of BET inhibitors and degraders, since off-target effects might be related to the amount of BET inhibitor or degrader that is administered.

In view of their potential use for the treatment of autoimmune diseases, the need for the development of more specific BET inhibitors or BET degraders is especially relevant, since the accepted side effects of therapeutics used for the treatment of autoimmune diseases differ from those used for cancer treatment. Compared to the current therapeutic strategies used for autoimmune diseases, such as biologicals, BET inhibitors provide the advantage that they inhibit production of multiple pro-inflammatory cytokines instead of inhibiting or blocking the function of a single pro-inflammatory mediator. Since local or systemic inflammation is characterized by various (self)regulatory feedback loops, BET inhibitors might be a powerful therapeutic approach to perturb these feedback loops from an upstream point (**Chapter 2**). Alternatively, combination therapy with biologicals might be even a more potent therapeutic strategy, since biologicals will prevent the induction of *de novo* (super-)enhancers by pro-inflammatory cytokines present within the local inflammatory environment.

HDAC INHIBITION AND BET INHIBITION IN AUTOIMMUNE DISEASES: A CONFLICTING PARADIGM?

In several preclinical models for immunological diseases, histone deacetylase (HDAC) inhibitors have been demonstrated to be highly effective in suppression of inflammation, suggesting that

they might be novel therapeutic candidates for the treatment of autoimmune diseases. Since HDAC inhibitors increase the acetylation status of histones, it seems counterintuitive that HDAC inhibitors and BET-inhibitors can both be effective in the treatment of autoimmune diseases. One explanation for the overlapping effects of HDAC and BET inhibitors could be that the immune suppressive effect of HDAC inhibitors is also mediated by the affecting the acetylation status of non-histone proteins, such as transcription factors and other pivotal mediators of inflammatory signaling pathways[54]. However, since multiple HDAC inhibitors, targeting distinct HDACs, have been demonstrated to be immune suppressive in autoimmune disease models, this is probably not the sole reason for the overlapping effects of HDAC inhibitors and BET-inhibitors on gene expression[55]. An alternative explanation is given by the observation that HDAC inhibition alters BRD4 localization[56,57]. Due to the increased chromatin acetylation status upon HDAC inhibition, BRD4 cannot localize to its preferred genomic regions anymore, a process referred to as 'epigenetic confusion'. More specifically, BRD4 binding close to the transcription start site is lost while BRD4 binding at newly acetylated sites within intergenic regions is increased. Disturbing BRD4 genomic binding in this sense is similar to BRD4 inhibition by BET-inhibitors, therefore resulting in a comparable effect of HDAC inhibitors and BET inhibitors[58,59]. Furthermore, this suggests that HDAC inhibitors and BET-inhibitors might act in concert and that a combination might synergistically reduce hyperactive immune responses[58,60]. Indeed, combination therapy of HDAC inhibitors and BET-inhibitors has been proven effective in preclinical cancer studies. Moreover, a dual active HDAC/BET small molecule inhibitor, DUAL946, has been developed which is capable of inhibiting HDAC class I and IIb as well as bromodomain proteins at sub-micromolar concentrations[61]. However, since the exact mechanisms underlying the synergistic effects of HDAC and BET inhibitors are not fully understood and so far predominantly linked to the induction of apoptosis, it remains to be investigated whether combination therapy might also be effective in the treatment of autoimmune diseases.

EPIGENETIC INHIBITION OF OSTEOCLAST DIFFERENTIATION

The observation that increased enhancers in JIA SF-derived monocytes are associated with osteoclast-associated genes, suggests that epigenetic mechanisms contribute to osteoclast-associated gene expression (**Chapter 4**). Furthermore, this indicates that inhibiting enhancer activity might impair osteoclast differentiation. Indeed, JQ1 reduces bone destruction in experimental periodontitis and I-BET151 suppresses osteoclastogenesis *in vitro* and *in vivo* and decreases bone resorption in a serum-induced arthritis mouse model[62,63]. This inhibitory effect is mediated by inhibition of RANKL-induced *MYC* expression, which is necessary for the transcription of *NFATC1*, a master regulator of osteoclastogenesis. In addition, I-BET151 indirectly inhibits osteoclast differentiation by suppressing the induction of IL-1 family members, which can stimulate osteoclastogenesis[62,64]. Rheumatic diseases are characterized by bone degradation and biologicals inhibiting TNF-α or TNF-α signaling have been demonstrated to be effective in the majority of patients[65]. This effectivity has been attributed to their immune suppressive effect as well as their inhibitory effect on osteoclastogenesis. This indicates that BET inhibitors, which

have a similar dual mechanism of action, might be effective in the treatment of rheumatic diseases as well. KDM6B is induced by RANKL stimulation and is involved in H3K27me3 demethylation of *Nfatc1*, which is essential for NFATc1 induction[66]. In addition, KDM6B knockdown inhibits RANKL-induced osteoclastogenesis *in vitro*. *KDM6B* expression is increased in JIA SF-derived monocytes, indicating that increased *KDM6B* might promote the differentiation of JIA monocytes towards osteoclasts. KDM6B can be inhibited with the small molecule GSK-J4 and GSK-J4 treatment of NK cells co-cultured with monocytes has been demonstrated to inhibit NK cell-mediated osteoclastogenesis, by reduction of RANKL expression[67,68]. Together with the potential role of KDM6B in shaping the enhancer repertoire of JIA SF monocytes, this indicates that KDM6B inhibition might be effective in the treatment of rheumatic diseases.

CELLS AND SOLUBLE FACTORS POTENTIALLY CONTRIBUTING TO OSTEOCLAST DIFFERENTIATION WITHIN THE JOINT

Increased enhancer activity and increased expression of osteoclast-associated genes in JIA SF-derived monocytes, suggests that JIA SF-derived monocytes are maybe "primed" to differentiate into osteoclasts. We evaluated the osteoclast differentiation capacity of JIA SF-derived monocytes *in vitro*, but due to the high number of cells needed and impaired cell viability during the 17 day differentiation period, we were not able to test this properly. However, we were able to demonstrate that SF from JIA patients induces osteoclast differentiation of healthy control monocytes, which also correlates with increased bone resorption (**Chapter 4**). The increased osteoclastogenic potential of SF is not specific for JIA, since SF from RA and pyrophosphate arthropathy patients has also been described to induce osteoclast formation[69]. *In vitro* osteoclast differentiation of $CD14^+$ cells obtained from the synovial compartment of RA, inflammatory OA, and non-inflammatory OA patients is comparable, but in the presence of $CD14^-$ cells, obtained from the same compartment, osteoclast differentiation is significantly enhanced within RA and inflammatory OA-derived $CD14^+/CD14^-$ co-cultures[70]. In addition, SF from non-inflammatory osteoarthritis (OA) patients does not induce osteoclast differentiation[69]. Taken together, this indicates that $CD14^-$ cells within the inflamed synovial compartment produce inflammatory soluble factor(s) that can induce osteoclast differentiation. Osteoclast differentiation is dependent on macrophage colony-stimulating factor 1 (M-CSF/CSF-1) and receptor activator of nuclear factor κB ligand (RANKL)[71]. RANKL levels are elevated in the SF of oligoarticular JIA patients compared to blood plasma levels and M-CSF and RANKL levels are increased in the SF of RA patients compared to OA patients, indicating that these cytokines might indeed contribute to the induction of osteoclast formation by SF[72–74]. Also in the presence of recombinant M-CSF and RANKL osteoclast formation is enhanced by JIA SF, suggesting that other pro-inflammatory mediators present within the SF potentiate osteoclast differentiation. In line with this, several pro-inflammatory cytokines, such as TNF-α, IL-17, and IL-6, have been demonstrated to promote osteoclast differentiation and these cytokines have also been implicated in the pathogenesis of JIA[75–84]. Besides, IL-6 levels are significantly elevated within the SF of JIA patients compared to plasma[74]. RA synovial fibroblasts produce M-CSF and RANKL

and are $CD14^-$, implying that these cells might promote osteoclast differentiation within the joint[85,86]. Furthermore, T cells are likely candidates to contribute to synovial osteoclast differentiation as they are abundantly present within the joint, are capable of producing RANKL, TNF-α, and IL-17, and have been demonstrated to stimulate monocytes to differentiate towards osteoclasts[73,85,87].

THE ROLE OF AUTOPHAGY WITHIN THE SYNOVIAL COMPARTMENT

Autophagy is associated with osteoclast differentiation, as genetic and pharmacologic inhibition of autophagy reduces osteoclast differentiation *in vitro* and bone destruction is reduced in an autophagy-deficient animal model[88,89]. Whether autophagy is increased in monocytes obtained from the SF of JIA and RA patients has not been investigated, but expression of *EGR1*, a transcriptional regulator of autophagy, is increased in JIA SF-derived monocytes (data not shown). It could be speculated that increased autophagy levels within JIA SF monocytes might promote their differentiation towards osteoclasts. Furthermore, since SF induces osteoclast differentiation of monocytes, this raises the question whether SF might be capable of inducing autophagy in monocytes, and if so, whether this contributes to the osteoclast-inducing effect of SF. Monitoring autophagy levels and eventually manipulating autophagy levels during SF-induced osteoclast differentiation of monocytes will give more insight into the role of autophagy in osteoclast differentiation within the synovial compartment.

We did analyze autophagy in T cells obtained from the synovial compartment of JIA patients, and observed that autophagy is increased in SF-derived T cells (**Chapter 5**). Since autophagy is not increased in T cells within the peripheral blood of JIA patients, this increase in autophagy could be caused by the synovial environment. We tested this hypothesis by culturing healthy control T cells in the presence of SF *in vitro*, but we could not observe an autophagy-inducing effect of the SF. However, due to limitations of our experimental set-up, meaning that cells were cultured in only 10% SF since higher concentrations of SF affect cell viability, we cannot completely rule out that SF does not affect autophagy. Furthermore, the inflamed synovial compartment is a combination of soluble pro-inflammatory mediators and infiltrated cells and its composition is probably dynamic, while our experimental set-up does not take into account the interplay between T cells and other cells present within the synovial compartment and provides a static reflection of the SF. Due to the rapid influx of a high number of cells within the synovial compartment, one can imagine that nutrient availability might be limited. Nutrient deprivation is a known trigger of autophagy and might therefore contribute to the increased autophagy in SF-derived T cells[90]. In addition, the increase in autophagy might be a reflection of the highly activated phenotype of JIA SF T cells, as T cell activation is known to induce autophagy and T cell-derived cytokines signaling via the common cytokine receptor γ-chain, such as IL-2 and IL-4, can stimulate autophagy[91,92]. Alternatively, since autophagy has been described to act as a tolerance-avoidance mechanism in $CD4^+$ T cells by degrading negative regulators of T cell activation, increased autophagy can also contribute to the hyper-responsive state of JIA SF T cells and thereby contribute to disease pathogenesis (**Figure 2**)[93].

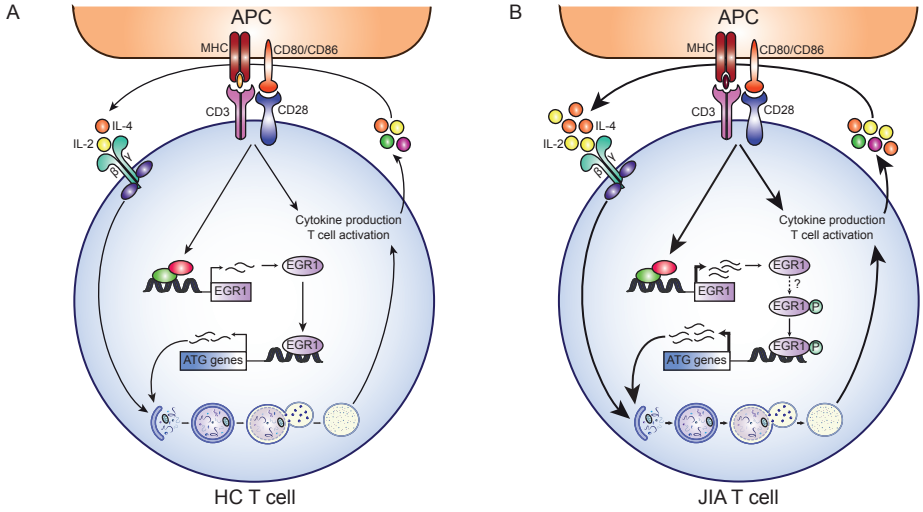


Figure 2. Hypothetical model of the molecular mechanisms underlying autophagy in HC and JIA T cells. (**A**) In a healthy control (HC) T cell, T cell receptor (CD3 and CD28) stimulation by an antigen-derived peptide presented by an antigen presenting cell (APC) leads, next to T cell activation and cytokine secretion, to the induction of *EGR1* expression. EGR1 transcriptionally regulates autophagy-associated gene expression and is therefore involved in autophagy induction upon T cell stimulation. Autophagy can contribute to T cell activation by the sequestration of negative regulators of T cell signaling. Cytokines, produced by activated T cells, that signal via the common cytokine receptor γ-chain can stimulate autophagy in an autocrine and paracrine fashion. (**B)** In a JIA T cell, antigen presentation, for example an auto-antigen, leads to enhanced T cell activation, cytokine production, and EGR1 expression. In addition, EGR1 phosphorylation might be increased, which increases the DNA binding activtity and thus results in increased expression of autophagy-associated genes. Increased autophagy enhances T cell activation and thus contributes to increased secretion of common γ-chain cytokines, which stimulates autophagy within JIA T cells and thereby creates a pro-inflammatory feedback loop.

Compared to healthy control T cells, we observed that autophagy-associated genes are enriched within the genes increased in JIA SF-derived T cells, which might contribute to the increased autophagy levels within these cells. It will be interesting to investigate whether this enrichment is also observed in peripheral blood-derived JIA T cells, as this might give some indications whether increased autophagy is a contributing factor to JIA development or rather occurs as a consequence of the disease. If autophagy-associated gene expression is increased in JIA peripheral blood-derived T cells, this also suggests that another, unknown, trigger is necessary to actually increase autophagy levels. Therefore, it might be interesting to analyze whether culturing JIA peripheral blood-derived T cells in the presence of SF will affect autophagy levels.

AUTOPHAGY MODULATION FOR THE TREATMENT OF AUTOIMMUNE DISEASES

Autophagy inhibition using hydroxychloroquine (HCQ) decreased the inflammatory phenotype of JIA SF-derived T cells, suggesting that inhibition of autophagy might be a novel therapeutic approach to suppress T-cell mediated immune responses (**Chapter 5**). In line with this, autophagy inhibition of JIA SF-derived T cells using 3-methyladenine induces a hypo-responsive,

anergic state, indicating that autophagy inhibition might be a manner to restore T cell tolerance in JIA patients[93]. Remarkably, HCQ has been described as a disease-modifying anti-rheumatic drugs (DMARD) and is being used for the treatment of RA and SLE[94,95]. HCQ is a lipophilic drug that accumulates in endosomes and lysosomes, induces disorganization of the Golgi complex and the endo-lysosomal system, and inhibits autophagy by impairing fusion of autophagosomes with lysosomes[96]. The exact mechanisms underlying the anti-inflammatory effects of HCQ are not clear, but it is thought that it is related to interference with MHC class II antigen presentation and inhibition of endosomal TLR signaling[97,98]. Autophagy has been described to be involved in these processes and autophagy is increased in peripheral blood-derived $CD4^+$ T cells from RA and SLE patients[99–101]. Together, these data suggest that the immunosuppressive effects of HCQ might be related to its effect on autophagy.

Counterintuitively, rapamycin, which is a known inducer of autophagy, has been demonstrated to be effective in the treatment of lupus animals models, where it reduces autoantibody production[102,103]. Furthermore, rapamycin administration to SLE patients improves disease activity in a rapid and long-lasting manner[104,105]. Rapamycin is an inhibitor of mammalian target of rapamycin (mTOR), which is involved in a plethora of cellular signaling processes, and mTOR activation has been implicated in SLE pathogenesis[106]. In addition, there are two case reports that link mutations in tuberous sclerosis 1 (TSC1) or TSC2, which are both negative regulators of mTOR, to a lupus-like phenotype[94,107]. How induction of autophagy, via mTOR inhibition by rapamycin, as well as inhibition of autophagy by HCQ, can both be effective in reducing an autoimmune response remains to be investigated. An mTOR-independent pathway that induces autophagy has been described, suggesting that rapamycin treatment of SLE patients might selectively affect autophagy[108]. Furthermore, HCQ and rapamycin are both non-specific modulators of autophagy since besides autophagy many other cellular processes are affected, indicating that it is difficult to assign their immunosuppressive effects solely to their effect on autophagy. In line with this, we and others have observed that rapamycin does not modulate autophagy in $CD4^+$ T cells *in vitro*[92]. In addition, the effect of targeting autophagy might be highly dependent on the cell type and the context in which this takes place, as autophagy can either promote cell survival as well as induce cell death[109]. Therefore, more research is needed to better understand the exact role of autophagy within autoimmune diseases and there is a need for the development for specific inhibitors of autophagy.

A ROLE FOR EGR1 IN AUTOPHAGY REGULATION IN JIA?

In **Chapter 6** we analyzed the transcriptional and epigenetic events associated with autophagy induction by nutrient deprivation. We identified early growth response protein 1 (EGR1) as a novel transcriptional regulator of autophagy. EGR1 is a stress response protein that is induced upon several stimuli, for example hypoxia, mitogens, and growth factors, and has been implicated in numerous processes, such as apoptosis and cellular proliferation and differentiation[110–116]. In line with our data obtained in adherent cell lines, a recent study investigating transcriptomic differences within Jurkat T cells after a specific starvation regime

also identified EGR1 as one of the key regulators of autophagy[117]. In addition, T cell activation via CD8/CD28 stimulation or PMA/ionomycin, both induce autophagy and EGR1 protein expression[118,119]. This supports the idea that EGR1 is a general transcription factor involved in regulation of the autophagy response in a wide variety of cells. It is therefore tempting to speculate that targeting EGR1 might an alternative manner to modulate autophagy. Additionally, this also indicates that dysregulation of autophagy might be related to impaired EGR1 expression or activity. Though, *EGR1* mRNA expression is not increased in JIA SF-derived T cells compared to healthy control T cells (data not shown). Since *EGR1* mRNA expression has only been investigated in healthy control and JIA T cells upon *in vitro* stimulation, which enhances EGR1 expression, it will still be interesting to investigate *ex vivo EGR1* mRNA levels. Furthermore, EGR1 activity and stability has been demonstrated to be regulated by post-translational modifications, namely EGR1 phosphorylation increases its DNA binding activity, acetylation decreases EGR1 transcriptional activity, and EGR1 sumoylation and ubiquitination are linked to proteasome-mediated degradation[120–122]. In addition, in unstimulated cells, EGR1 is not or weakly phosphorylated, while in stimulated cells (hyper)phosphorylated forms of EGR1 have been detected[123,124]. Therefore, it will be informative to study EGR1 transcriptional activity as well EGR1 protein levels in JIA SF-derived T cells (**Figure 2**). It is also plausible that EGR1 is not involved in autophagy regulation in JIA SF-derived T cells, as EGR1 is preferentially expressed in Th2 cells and the majority of T cells infiltrating the synovial joint in JIA are Th1 cells[119,125].

"BETTING" ON AUTOPHAGY

BRD4 has been demonstrated to act as a transcriptional repressor of autophagy and lysosomal genes, by the recruitment of methyltransferase G9a[126]. This suggests that BET inhibitors and BET degraders will induce autophagy. Indeed, JQ1 treatment increases the autophagic flux and enhances autophagy-related gene expression[126]. There seems to be some specificity regarding the types of autophagy regulated by BRD4, since xenophagy and mitophagy are not affected upon BRD4 inhibition. It remains to be investigated what the effect of autophagy induction by BET inhibitors is in view of their potential use for the treatment of autoimmune diseases. Especially since autophagy has been implicated to promote osteoclast differentiation, while BET inhibitors suppress osteoclast differentiation[62,88,89]. Because both autophagy-inducing as well as autophagy-inhibiting drugs are capable of immune suppression, the actual effect of autophagy induction by BET-inhibitors probably heavily depends on the disease-specific context. Furthermore, if BET inhibitors will be administered in combination with other therapeutics, these will likely affect autophagy as well. Indeed, TNF-a induces autophagy in RA synovial fibroblasts and preliminary data suggests that etanercept inhibits autophagy in RA synovial fibroblasts[127,128]. The proto-oncogene *MYC* is highly sensitive to BET inhibition and has been implicated to be an important regulator of autophagy[117]. Indeed, MYC inhibition increases autophagy in Jurkat T cells suggesting that the autophagy-promoting effect of BRD4 inhibition is partially mediated by inhibition of MYC[117]. In addition, a study investigating the effects of the novel BET inhibitor RVX2135 reported increased *Egr1* expression upon RVX2135 treatment. This indicates that EGR1 might also play a role in the autophagy-inducing effect of BET inhibitors.

CONCLUDING REMARKS

Altogether, the studies described in this thesis provide novel insight into epigenetic processes and autophagy regulation in an autoimmune disease setting and demonstrate that altered enhancer regulation and autophagy are associated with autoimmunity (**Figure 3**). Small molecule inhibitors targeting the molecular mechanisms described in this thesis have recently been developed or are currently under development, indicating that it will be of interest to test the potential of these inhibitors for the treatment of autoimmune diseases. However, since many molecular processes are interconnected and the specificity of several small molecule inhibitors remains limited, more research is needed to fully understand the interplay between these molecular mechanisms in relation to autoimmunity and it is necessary to develop novel, selective, therapeutic approaches.

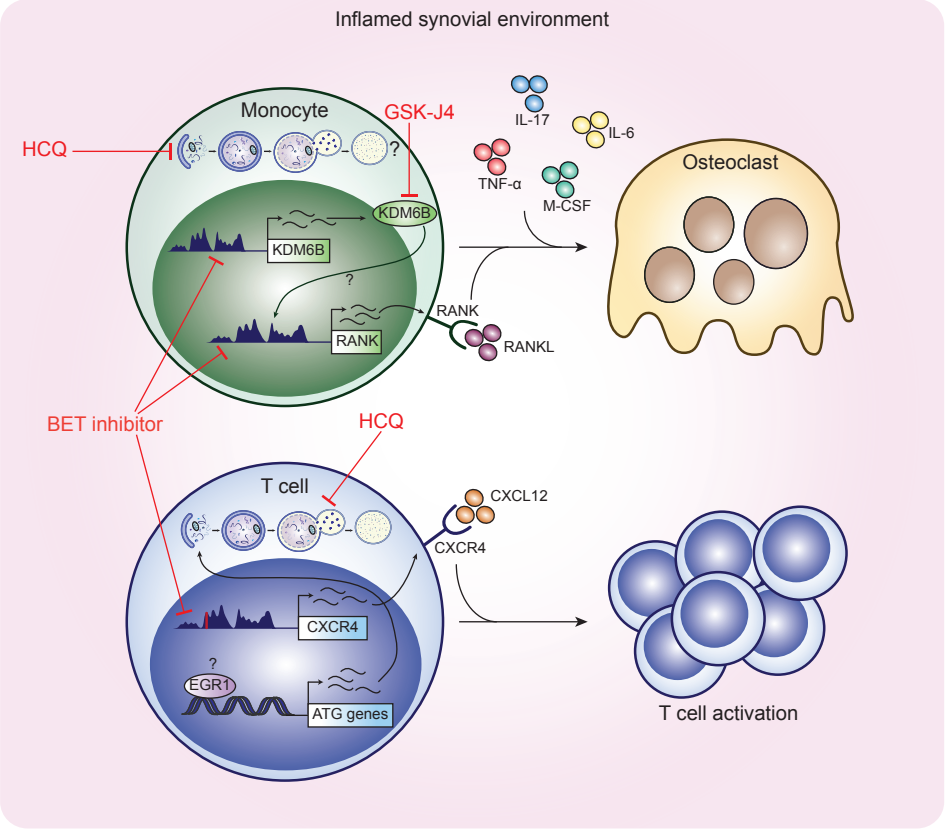


Figure 3. Hypothetical model of the molecular mechanisms underlying synovial inflammation and therapeutic strategies targeting these mechanisms.
The inflamed synovial environment contains many proinflammatory mediators that can stimulate the differentiation of monocytes towards osteoclasts. Increased enhancer activity contributes to increased expression of *RANK* within JIA monocytes which upon stimulation by RANKL, present within the synovial fluid (SF), promotes osteoclast differentiation. *KDM6B*, which expression is increased in JIA monocytes, can shape the JIA monocyte enhancer landscape and has been implicated in RANKL-induced osteoclastogenesis. GSK-J4 inhibits KDM6B and therefore inhibits osteoclastogenesis within the synovial joint. Autophagy is increased in JIA T cells and contributes to their inflammatory phenotype. EGR1 is a transcriptional regulator of autophagy and can contribute the increased expression of autophagy-associated gene expression in JIA SF T cells, and thus promote autophagy. HCQ inhibits autophagy and reduces the activated phenotype of JIA T cells. Autophagy is associated with osteoclast differentiation and might therefore also be affected in JIA monocytes. Increased enhancer activity within JIA T cells contributes to the increased expression of cytokine and chemokine receptors, for example *CXCR4*, and stimulation contributes to T cell activation. In addition, an arthritis-associated SNP (indicated in red) is present within the enhancer of *CXCR4* in JIA T cells, which could contribute to increased *CXCR4* expression. BET inhibitors reduce enhancer-mediated transcription and since enhancers contribute to disease-associated gene expression in JIA T cells and monocytes, this could be a novel therapeutic strategy for the treatment of JIA.

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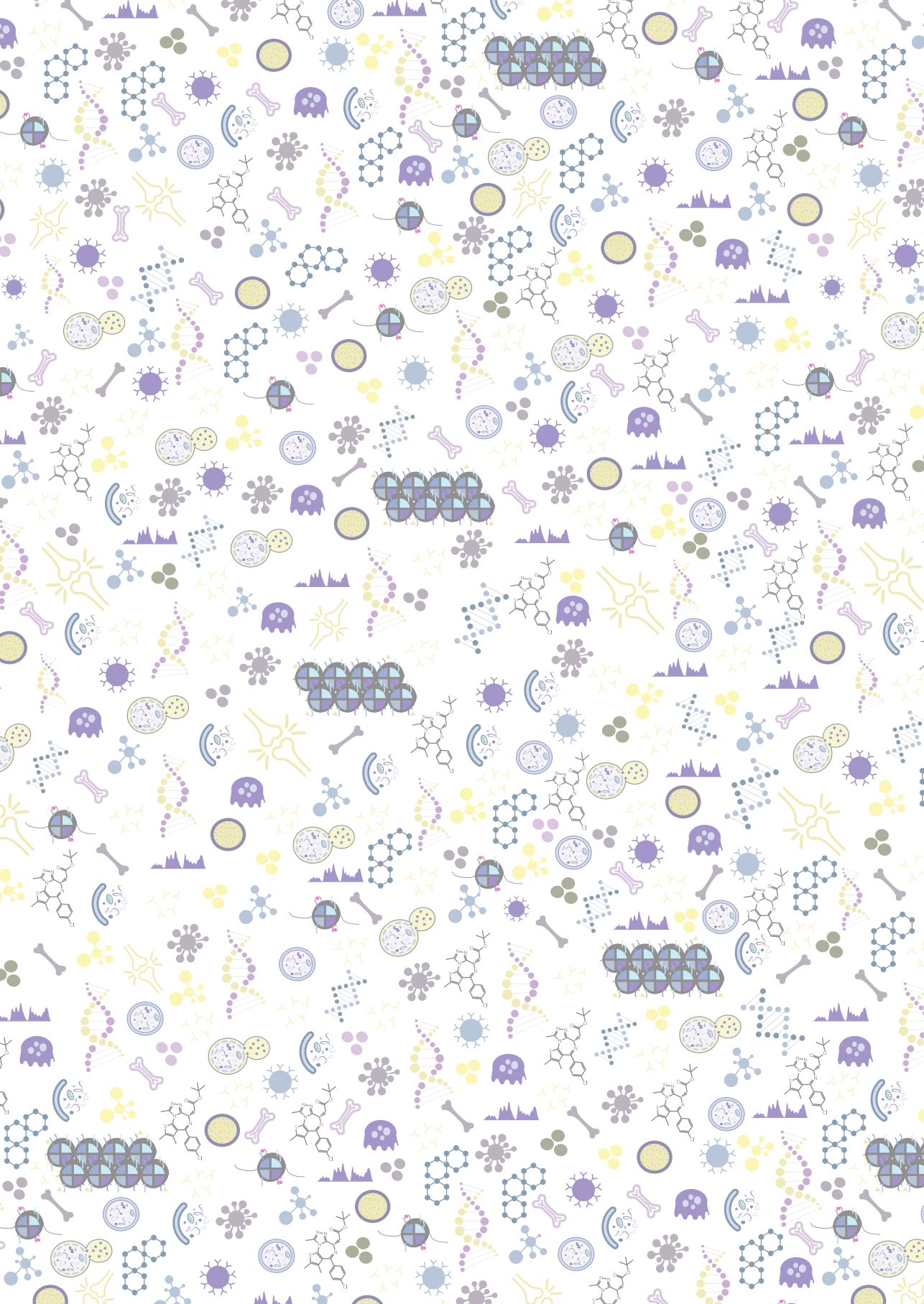

Nederlandse samenvatting
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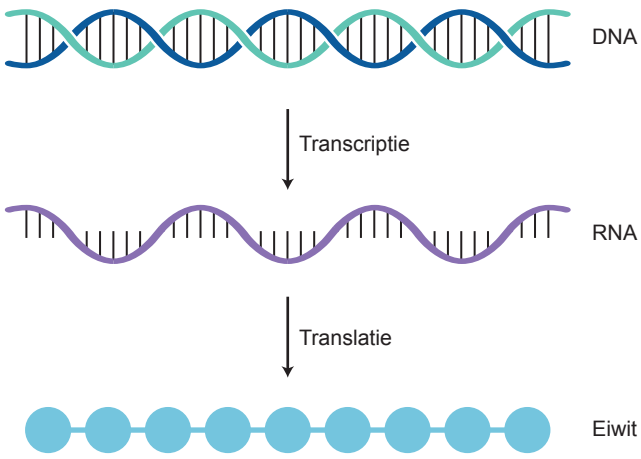
NEDERLANDSE SAMENVATTING

Het afweersysteem, ook wel immuunsysteem genoemd, is erg belangrijk om ons lichaam te beschermen tegen ziekteverwekkers zoals virussen en bacteriën. Een goed werkend immuunsysteem is in staat om ziekteverwekkers te onderscheiden van lichaamseigen moleculen en komt daardoor alleen in actie als er een ziekteverwekker het lichaam binnendringt. Er zijn helaas ook gevallen waarbij het immuunsysteem dit onderscheid minder goed kan maken en dus in actie komt tegen lichaamseigen moleculen. In zo'n geval spreken we van een auto-immuunziekte. Het is nog niet geheel duidelijk hoe auto-immuunziekten precies ontstaan. Dat komt onder andere doordat auto-immuunziekten multifactoriële ziekten zijn, dat wil zeggen dat er meerdere factoren bijdragen aan het ontstaan van de ziekte. Zo zijn er bepaalde genetische veranderingen die in verband worden gebracht met auto-immuunziekten, maar ook de omgeving en zogenaamde epigenetische factoren spelen een rol. Epigenetische factoren bepalen of genen 'aan' of 'uit' staan. Als genen 'aan' staan houdt dat in dat het DNA afgelezen wordt en het gen tot uiting (expressie) kan komen. Van het DNA wordt dan een kopie gemaakt in de vorm van een RNA-molecuul, dit proces wordt transcriptie genoemd. Het RNA kan vervolgens afgelezen worden en vertaald worden naar eiwit, via een proces genaamd translatie (**Figuur 1**). Bepaalde eiwitten zijn belangrijke signaalmoleculen die een bepalende rol hebben in de functie van een cel. Een darmcel bijvoorbeeld bevat exact hetzelfde DNA als een spiercel, maar beide cellen bevatten verschillende eiwitten en hebben daarom een totaal verschillende functie. Epigenetische factoren bepalen welke eiwitten geproduceerd worden in een cel en zijn daarom zeer bepalend voor de functie en activiteit van een cel.

VOLUMEKNOPPEN

In ons onderzoek hebben we specifiek gekeken naar epigenetische regulatie van *enhancers*, dit zijn een soort volumeknoppen die bepalen hoe hard een gen aanstaat. In gezonde cellen zijn deze volumeknoppen goed bestudeerd, maar het is niet duidelijk hoe deze volumeknoppen ingesteld staan in het geval van een auto-immuunziekte. In **hoofdstuk 2** bespreken we wat er bekend is over enhancers in relatie tot auto-immuunziekten en hoe nieuwe inzichten in enhancer-regulatie kunnen bijdragen aan de ontwikkeling van nieuwe therapeutische strategieën voor auto-immuunziekten. Om meer inzicht te krijgen hoe enhancers kunnen bijdragen aan ziekte, hebben we in **hoofdstuk 3 en 4** enhancers onderzocht in twee typen immuuncellen, T-cellen en monocyten, afkomstig van patiënten die lijden aan de auto-immuunziekte jeugdreuma. Jeugdreuma wordt gekenmerkt door ontstoken gewrichten en uit deze ontstoken gewrichten kunnen immuuncellen en gewrichtsvloeistof (synoviale vloeistof) gehaald worden, die gebruikt kunnen worden voor onderzoek. Ons onderzoek toont aan dat een aantal van de volumeknoppen in immuuncellen van jeugdreumapatiënten anders ingesteld staan vergeleken met de volumeknoppen in gezonde cellen. Hierdoor komen de genen waarbij de volumeknoppen verkeerd staan afgesteld te veel of te weinig tot expressie. De genen die te hard aan staan zijn geassocieerd met de ziekte en op deze wijze dragen enhancers bij aan de sterke expressie van

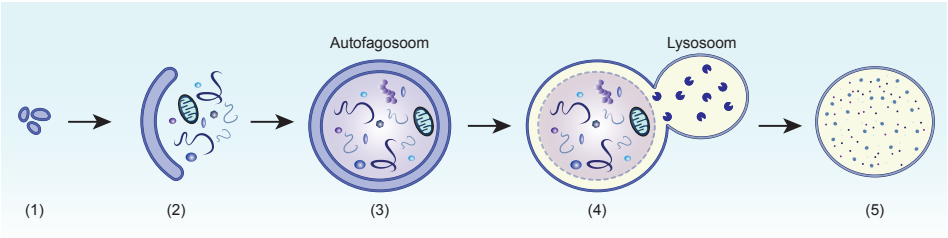


Figuur 1. De weg van DNA naar eiwit.
Als een gen op het DNA wordt afgelezen, wordt er een kopie gemaakt in de vorm van een RNA-molecuul (transcriptie). Het RNA kan vervolgens afgelezen worden en vertaald worden naar een eiwit (translatie).

ziekte-geassocieerde genen. Hieruit kunnen we concluderen dat epigenetische regulatie van enhancers een belangrijke rol speelt bij auto-immuunziekten. Het remmen van enhancers, dat wil zeggen het lager zetten van de volumeknoppen, in T-cellen afkomstig van jeugdreumapatiënten zorgt specifiek voor vermindering van de expressie van ziekte-geassocieerde genen. Dit suggereert dat remming van enhancers mogelijk een nieuwe therapie kan zijn in de behandeling van auto-immuunziekten. De volumeknoppen in monocyten van jeugdreumapatiënten zorgen opvallend vaak voor verhoogde expressie van genen betrokken bij osteoclastdifferentiatie. Osteoclasten zijn cellen die bot af kunnen breken en monocyten kunnen zich dusdanig ontwikkelen dat ze een osteoclast worden, dit proces wordt differentiatie genoemd. Aangezien we deze link met osteoclastdifferentiatie specifiek zagen in monocyten afkomstig uit het gewricht, hebben we in **hoofdstuk 4** onderzocht of gewrichtsvloeistof hier een effect op kan hebben. Inderdaad bleek dat als we gewrichtsvloeistof toevoegen aan gezonde monocyten afkomstig uit het bloed, er na enige tijd osteoclasten gevormd worden die in staat zijn bot af te breken. Aangezien botafbraak in de gewrichten een kenmerk is van (jeugd)reuma laat dit nogmaals zien dat enhancers kunnen bijdragen aan auto-immuunziekten. Dit betekent dat remming van enhancers waarschijnlijk ook een remmend effect zal hebben op de vorming van osteoclasten en dus botafbraak.

RECYCLING

In **hoofdstuk 5** hebben we een ander proces bestudeerd in T-cellen van jeugdreumapatiënten, namelijk autofagie. Autofagie betekent letterlijk "jezelf eten" en beschrijft een recycleproces waarbij cellen componenten van zichzelf afbreken en hergebruiken voor een ander doel (**Figuur 2**). Een cel doet dit als er bijvoorbeeld niet genoeg voedingsstoffen in de omgeving aanwezig



Figuur 2. Schematische weergave van het proces autofagie.
Autofagie begint met de formatie van blaasachtige structuren (1). Deze structuren kunnen samenvoegen waardoor een soort schepnet wordt gevormd dat bepaalde onderdelen binnenin de cel kan wegvangen (2). Het schepnet met gevangen onderdelen vormt uiteindelijke een soort blaasje, dit wordt een autofagosoom genoemd (3). Een autofagosoom kan samenvoegen met een ander blaasje, het lysosoom (4). Het lysosoom bevat allerlei eiwitten die de inhoud van het autofagosoom kunnen afbreken (5). Deze afbraakproducten worden vervolgens gerecycled en kunnen uiteindelijk gebruikt worden als energiebron of dienen als bouwstenen voor de cel.

zijn om in leven te kunnen blijven; de afbraakproducten kunnen namelijk gebruikt worden als energiebron voor de cel. Daarnaast is autofagie een manier voor cellen om cellulaire componenten op te ruimen die niet meer nodig zijn. Ons onderzoek heeft aangetoond dat in T-cellen van jeugdreumapatiënten meer autofagie plaatsvindt dan in T-cellen van gezonde personen. T-cellen van jeugdreumapatiënten staan erom bekend dat ze erg actief zijn, dat betekent dat ze erg snel delen en veel ontstekingsstoffen produceren. Het remmen van autofagie in deze cellen zorgt ervoor dat de cellen minder delen en minder ontstekingsstoffen produceren. Dit suggereert dat het remmen van autofagie mogelijk een manier is om de T-cellen van jeugdreumapatiënten, en van andere patiënten die lijden aan een auto-immuunziekte, weer tot rust te brengen. In het geval van jeugdreuma kan dit leiden tot vermindering van de ontstekingen in de gewrichten.
Om autofagie nog beter te kunnen begrijpen, hebben we dit proces in **hoofdstuk 6** meer in detail bestudeerd. Hiervoor hebben we normale cellen en cellen die geen autofagie kunnen ondergaan (zogeheten 'autofagie-deficiënte cellen'), gedurende een bepaalde periode geen voedingsstoffen gegeven, waardoor het proces van autofagie aangezet wordt. Als we kijken naar de transcriptie van genen die belangrijk zijn voor autofagie, zien we dat die verhoogd is in zowel de normale cellen als in de 'autofagie deficiënte cellen' na het aanzetten van autofagie. Dit betekent dat transcriptie van deze genen niet afhankelijk is van het autofagie proces zelf. Om te achterhalen welk moleculen, zogeheten transcriptiefactoren, verantwoordelijk zijn voor de verhoogde transcriptie van deze genen, hebben we gemeten welke transcriptiefactoren worden geproduceerd na het aanzetten van autofagie. Eén van deze factoren is EGR1, en EGR1 blijkt inderdaad bij te dragen aan de transcriptie van genen betrokken bij autofagie. Als we de hoeveelheid EGR1 in een cel verminderen of verhogen zien we ook een vermindering of verhoging van autofagie. Dit geeft aan dat EGR1 betrokken is bij de regulatie van autofagie en dat medicijnen die EGR1 remmen de hoeveelheid autofagie in een cel kunnen verminderen.

Aangezien voor meerdere (auto-immuun)ziekten, zoals jeugdreuma, verhoogde autofagie is beschreven, zou vermindering van autofagie in de toekomst een manier kunnen zijn om deze ziekten te behandelen.
Concluderend, geven de onderzoeken beschreven in dit proefschrift inzicht in epigenetische veranderingen en (de regulatie van) autofagie in auto-immuunziekten. Medicijnen die de in dit proefschrift beschreven processen beïnvloeden zijn recent op de markt gebracht, of worden momenteel ontwikkeld. Het zal in de toekomst erg interessant zijn om te testen of deze medicijnen effectief zijn in de behandeling van auto-immuunziekten.

DANKWOORD

Zoals Johan Cruijff het ooit verwoorde "*Alleen kan je niks, je moet het samen doen*", zonder heel veel hulp zowel binnen als buiten het lab was dit proefschrift er niet geweest. Daarom bij deze een woord van dank aan alle mensen die hebben bijgedragen aan het tot stand komen van dit 'boekje'.

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Paul, ik ben heel blij dat ik onderdeel van het 'Cofferlab' heb mogen zijn. Hoewel jouw begeleidende rol gedurende de jaren (letterlijk en figuurlijk) steeds meer op afstand kwam te staan was je er altijd op de momenten en in de perioden dat het nodig was. Na een meeting met jou lijken dingen altijd veel simpeler en vraag ik me vaak af waarom ik niet eerder bij je langs ben gegaan. Hoewel er geen traditionele co-IP te vinden is in dit proefschrift, hoop ik dat het toch een 'Cofferlab'-waardig boekje is geworden!

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Josca: huisgenootje, vriendin, 'moeder', dilemma-sparringpartner, ceremoniemeester, paranimf,

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CURRICULUM VITAE

Janneke Peeters was born on November 26th, 1988 in Lelystad, the Netherlands. In 2007 she completed secondary education at Scholengemeenschap Lelystad and in the same year she started the bachelor's programme Biomedical Sciences at Utrecht University, The Netherlands. During her bachelor study she participated in the *extracurricular* honors program of Biomedical Sciences and performed a literature study on diet-induced mechanism affecting PPAR-γ activity under the supervision of Dr. Eric Kalkhoven at the University Medical Center Utrecht (UMCU). After receiving her bachelor's degree *cum laude* in 2010, she started with the master's programme Biology of Disease at Utrecht University. As part of this master study she worked on a 9-month research project investigating interference of the Epstein-Barr virus with Toll-Like Receptor signaling, under the supervision of Michiel van Gent and Dr. Maaike Ressing at the UMCU. In addition, she wrote a thesis on the causes and contribution of genetic instability in cancer under the supervision of Prof. Geert Kops at the UMCU. In 2012, Janneke worked on an 8-month research project focusing on the development of aerolysin as a biological tool under the supervision of Dr. Carla Guimaraes in the laboratory of Prof. Hidde Ploegh at the Whitehead Institute, Cambridge, USA. She completed her master's degree *cum laude* in 2012. In 2013, she obtained a grant from the Netherlands Organization for Scientific Research (NWO) to start her PhD training in the laboratory of Prof. Paul Coffer at the UMCU under the supervision of Dr. Jorg van Loosdregt. During her PhD training she studied transcriptional and epigenetic mechanisms underlying autoimmune diseases, with a special focus on autophagy and enhancer regulation in juvenile idiopathic arthritis. The findings obtained during these studies are described in this thesis. In Spring 2019, Janneke will start as a post-doctoral research associate in the laboratory of Dr. Michel Dupage at the University of California, Berkeley, USA were she will study epigenetic regulation of regulatory T cells in cancer.

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