Original paper

Folia Neuropathologica

Inhibitor of bromodomain and extraterminal domain proteins decreases transcription of Cd33 in the brain of mice subjected to systemic inflammation; a promising strategy for neuroprotection

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Folia Neuropathol 2024; 62 (2): 127-135 *DOI: https://doi.org/10.5114/fn.2024.138140*

Abstract

The neuroinflammation is a crucial component of virtually all neurodegenerative disorders, including Alzheimer's disease (AD). The bacterial lipopolysaccharide (LPS), a potent activator of the innate immune system, was suggested to influence or even trigger the neuropathological alterations in AD. LPS-induced neuroinflammation involves changes in transcription of several genes, thus controlling these molecular processes may be a potentially efficient strategy to attenuate the progression of AD. Since genome-wide association studies showed that the majority of AD-related genetic risk factors (AD-GRF) are connected to the immune system, our aim was to identify AD-GRF affected in the hippocampus by LPS-induced systemic inflammatory response (SIR). Moreover, we analysed the role of bromodomain and extraterminal domain (BET) proteins, the readers of the acetylation code, in controlling the transcription of selected AD-GRF in the brain during neuroinflammation.

In our study, we used a mouse model of LPS-induced SIR and mouse microglial BV2 cells. JQ1 was used as an inhibitor of BET proteins. The level of mRNA was analysed using microarrays and qPCR.

Our data demonstrated that among the established AD-GRF, only the expression of Cd33 was significantly upregulated in the hippocampus during SIR. In parallel, we observed an increase in the expression of Brd4, a BET family member. JQ1 prevented an LPS-evoked increase in Cd33 expression in the hippocampus of mice. Moreover, JQ1 reduced Cd33 expression in BV2 microglial cells stimulated with blood serum from LPS-treated mice.

Our study suggests that LPS-evoked SIR may increase Cd33 gene expression in the brain, and inhibition of BET proteins through suppression of Cd33 expression could be a promising strategy in prevention or in slowing down the progression of neuroinflammation and may potentially affect the pathomechanism of AD.

Key words: *Alzheimer's disease, systemic inflammatory response, endotoxin, bromodomain and extraterminal domain proteins, hippocampus, microglia.*

Introduction

During the last decades, a growing body of epidemiological data has indicated a substantial role of the immune system and inflammation in the pathogenesis/pathomechanism of Alzheimer's disease (AD). The association between the frequency of viral and bacterial infections and the risk of AD was presented during the last decades [10,22,54]. Several studies have demonstrated the advantageous effect of long-term use

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Received: 04.10.2023, **Accepted:** 23.02.2024, **Online publication:** 28.05.2024

of non-steroidal anti-inflammatory drugs (NSAIDs) on the risk of developing AD [13,33,46]. Additionally, analysis of human brains demonstrated that neuroinflammation, changes in cytokine profiles, and markers of microglia and astrocyte activation are important components of Alzheimer's pathology [1].

Recent hypotheses have highlighted the role of Gram-negative bacteria and their lipopolysaccharides (LPS) in the pathomechanism of AD [9,21,36,60]. The genome-wide association studies (GWAS) established a set of 29 genes whose polymorphism significantly affects the risk of developing AD (AD-GRF; AD-related genetic risk factors) [4]. Interestingly, many of these genes are related to the innate mechanisms of the immune system and to the function of microglia, which suggests the crucial role of neuroinflammation and microglial phagocytosis in the pathomechanism of AD [51]. LPS is a potent bacterial endotoxin that is highly resistant to degradation by mammalian enzymes, resulting in a persistent inflammatory stimulus. Intraperitoneal injection of LPS is a well-established *in vivo* model of the systemic inflammatory response (SIR) worsening brain function, cognitive functions, and memory [19,23,26,34]. However, the impact of SIR on the transcription of AD-GRF in the brain has never been studied.

The modulation of neuroinflammatory processes was demonstrated to protect the brain from LPS-induced dysfunction [17,59]. Among several anti-inflammatory strategies, inhibition of bromodomain and extraterminal domain (BET) proteins appears to be especially interesting. BET proteins are important epigenetic regulators of gene expression [12]. They are the readers of the acetylation code that in cooperation with transcription factors control the transcription [57]. Several BET inhibitors have shown anti-inflammatory properties in animal models of AD [39,43,49,50].

Therefore, in our study, we studied the impact of LPS-evoked systemic inflammation on the transcription of AD-GRF in the hippocampus of mice. Moreover, we analysed whether pharmacological inhibition of BET proteins may attenuate LPS-evoked alterations.

Material and methods

Animals and experimental design

The experiments were carried out on 3-month-old male C57BL/6J mice supplied by the Animal House of the Mossakowski Medical Research Institute, Polish Academy of Sciences (Warsaw, Poland). The animals were maintained under standard conditions, with controlled temperature (22°C ±10%) and humidity (55% ±10%). All of the experiments conducted on the animals were approved by the II Local Ethics Committee for Animal Experimentation in Warsaw (permission WAW2/060/2020) and carried out following EU Directive 2010/63/EU on the protection of animals used for scientific purposes, and complying with the ARRIVE guidelines. All efforts were made to minimize animal suffering and reduce the animals' number. All manipulations were performed quickly and gently to reduce the animal's stress.

(S)-(+)-tert-butyl 2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetate (JQ1), a highly specific and potent inhibitor of BET proteins (Sigma-Aldrich, St. Louis, MO, USA), solution was prepared as defined previously [35]. Shortly, it was dissolved in dimethyl sulfoxide and mixed 1 : 10 with 10% 2-hydroxypropyl-β-cyclodextrin. LPS (from *E. coli* serotype O55:B5; toxicity 1.5×10^7 EU/mg; Sigma-Aldrich) was dissolved in saline. All treatments were performed in the morning. Animals were intraperitoneally (i.p.) injected with JQ1 (50 mg/kg b.w. or respective volume of the vehicle) and 30 min later with LPS (i.p.; 1 mg/kg or respective volume of the vehicle). After 3 and 12 h, animals were anesthetized by isoflurane inhalation and decapitated. The blood was collected 12 hours after the administration of LPS. Directly after the formation of the clot, samples were centrifuged at 1000 × g for 5 minutes to separate the serum and immediately frozen and stored at –85°C.

Microarray analysis of gene expression

Twelve hours after administration of LPS (i.p.; 1 mg/kg b.w.), total RNA from the perfused mouse hippocampus was isolated and analysed by using the Affymetrix Gene Chip Mouse genome 430 2.0. (Affymetrix Inc., Santa Clara, CA, USA), as described previously [21]. The data were normalized with the GC-RMA method and log2 transformed. Full microarray data (CEL files) are available in a public repository at: https://osf.io/x3jub/.

Cell culture experiment

Murine microglial BV2 cells were obtained from Elabscience Biotechnology Inc. (Houston, TX, USA) [6]. The cells were cultured in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/ml streptomycin, and 50 units/ml penicillin in a 5% CO_2 atmosphere at 37°C. The passages below the twentieth were used. The cells were frequently tested to avoid mycoplasma contamination.

JQ1 was dissolved in DMSO at a 10 mM stock solution and then it was diluted with a culture medium and added to cells at a 50 nM concentration. Thirty minutes after JQ1 the murine blood serum from con-

trol animals or from LPS-treated animals was added to BV2 cells (final concentration: 2% v/v). After 12 h incubation, cells were washed twice with PBS, and RNA was isolated, as described below. In all experiments, the respective volume of vehicle was consequently added to corresponding groups.

PCR analysis of gene expression

Total RNA from the mouse hippocampus and from BV2 cells was extracted using TRI-reagent as described previously [44]. Reverse transcription and quantitative PCR were performed using pre-developed TaqMan Gene Expression Assays: *Cd33* (Mm00491152_m1), *Brd2* (Mm01271171_g1), *Brd3* (Mm01326697_m1), *Brd4* (Mm01350417_m1), *Tnf* (Mm00443258_m1), and *Gusb* (Mm01197698_m1) (Applied Biosystems, Foster City, CA, USA) [44]. The relative levels of mRNA were calculated using the ΔΔCt method. *Gusb* was used as a reference gene.

Statistics

Statistical analysis of data was performed with GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. The distribution of the data was analysed using the Shapiro-Wilk normality test. *N* refers to the number of animals in the experimental group or to the number of independent experiments *in vitro*.

Results

Systemic injection of LPS is a widely used model of the systemic inflammatory response (SIR). Our previous studies demonstrated that SIR evoked by a moderate dose of LPS (1 mg/kg b.w.) induced acute but transient neuroinflammatory processes in the brain, including alterations in gene expression patterns, oxidative stress, and changes in the activity of several enzymes, among them Gsk-3β, Cdk5 and other kinases [15-21,34]. These molecular alterations were accompanied by short-lasting sickness behaviour and cognitive impairment [34]. In the present study, we focused on genes that were identified by GWAS studies as AD-linked genetic risk factors [4,5]. In our analysis, we also included genes that cause a fully penetrant monogenic form of AD: *Psen1*, *Psen2,* and *App*. As shown in Figure 1, our microarray data revealed that twelve hours after peripheral injection of LPS into a mouse, the mRNA level of several AD-GRF in the hippocampus was altered. The most pronounced change was noticed

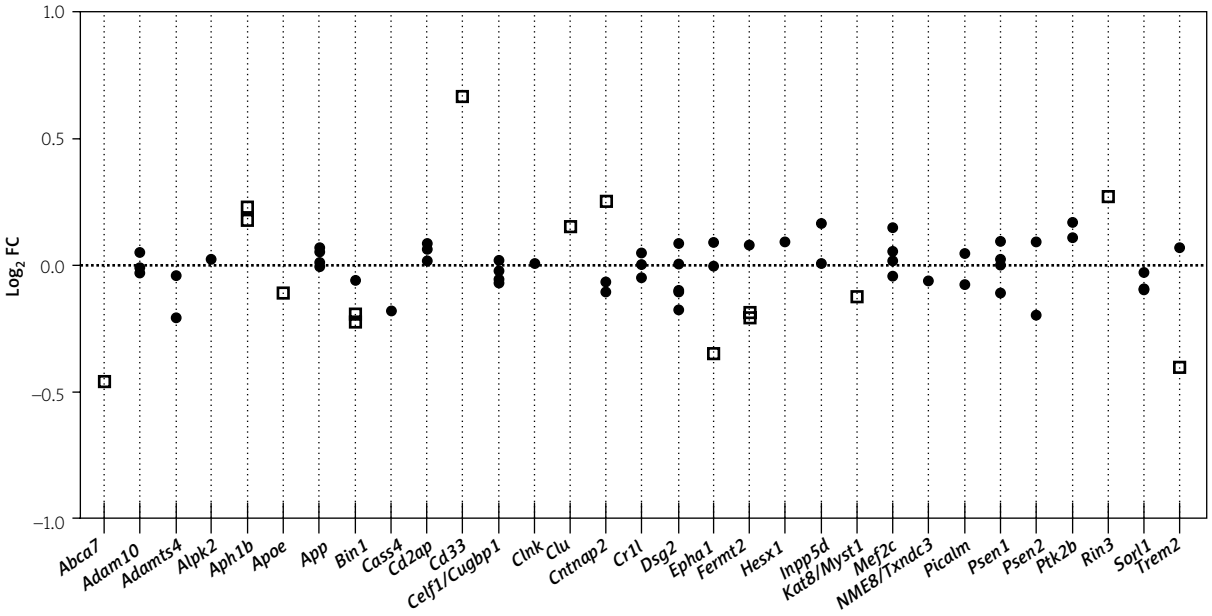

Fig. 1. Changes in mRNA levels of 31 genetic risk factors for Alzheimer's disease (AD) in the hippocampus of mice subjected to systemic inflammatory response (SIR) evoked by lipopolysaccharide (LPS). Microarray analysis was performed 12 hours after intraperitoneal administration of LPS. Each data point represents a mean value for a specific probe. The statistically significant change in expression ($p < 0.05$), compared to the control, was indicated by open squares. $N = 4$. FC – fold change.

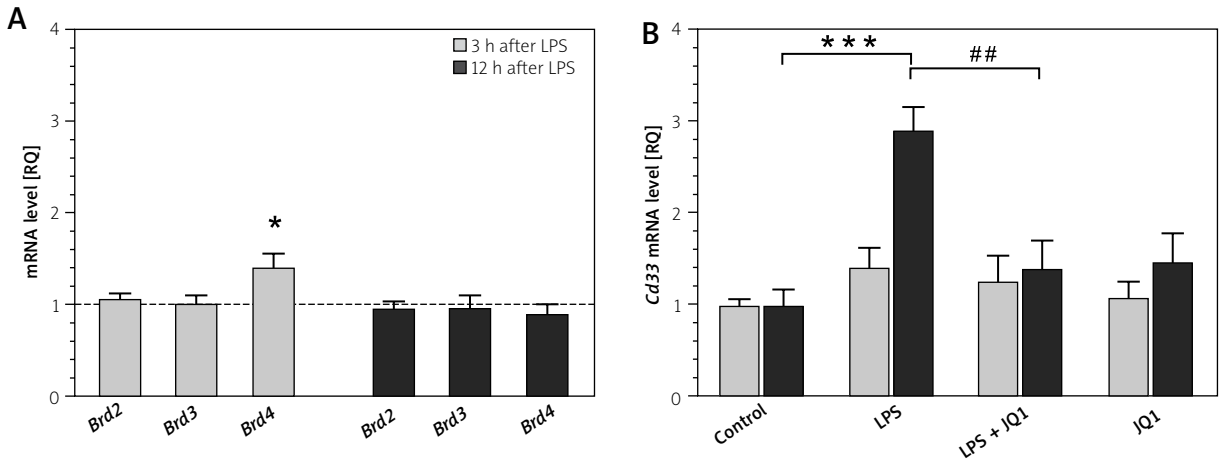

Fig. 2. Changes in mRNA levels of genes coding BET proteins and Cd33 in the mouse hippocampus 3 and 12 h after intraperitoneal injection of lipopolysaccharide (LPS). **A**) The impact of LPS on the transcription of *Brd2*, *Brd3*, and *Brd4* genes. The respective control level was presented as a dotted line. **B**) The effect of BET inhibitor, JQ1, on LPS-evoked alterations of *Cd33* expression in the hippocampus. The mRNA level was analysed by using the qPCR method. The data represent the mean values ± SEM from 6 animals. *,***$p < 0.05$, and $p < 0.001$, compared to the corresponding control, respectively; ##$p < 0.01$, compared to the LPS group.

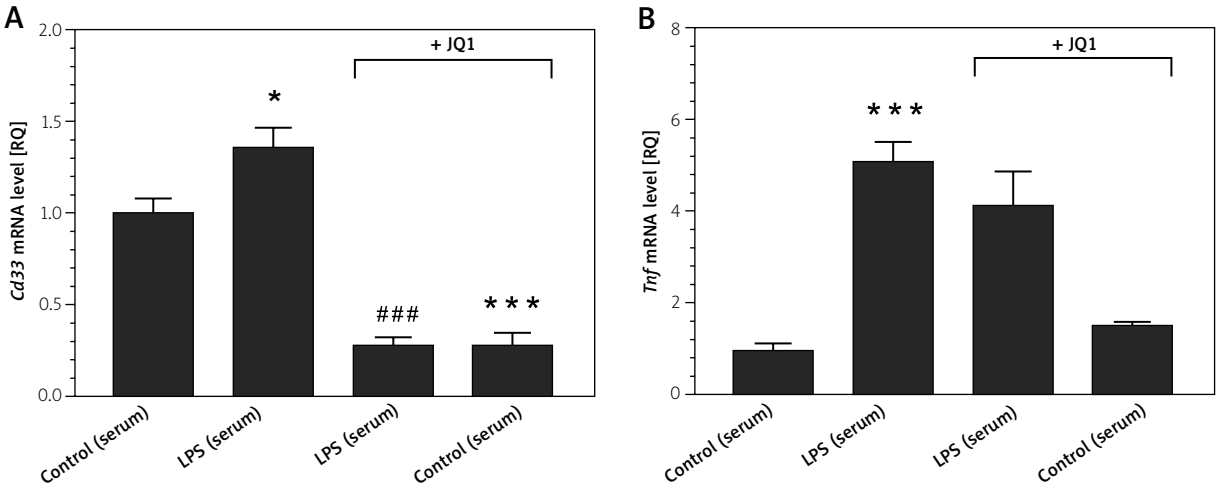

Fig. 3. The effect of BET proteins inhibitor, JQ1, on the mRNA level of *Cd33* and *Tnf* in the mouse microglial BV2 cells after 12 h incubation in the presence of blood serum from control and lipopolysaccharide (LPS)-treated mice. Mice received intraperitoneal injections of saline or LPS, then after 12 h blood was collected, and serum was prepared. BV2 cells were incubated in the presence of JQ1 (50 nM) for 30 min, then mice blood serum was added and incubation was continued for 12 h. The mRNA levels of *Cd33* (**A**) and the mRNA level of *Tnf* (**B**) were analysed by using the qPCR method. The data represent the mean values ± SEM from 5 independent experiments. *,***$p < 0.05$, and $p < 0.001$, compared to the corresponding control, respectively; ###$p < 0.001$, compared to the respective LPS group.

for the *Cd33* gene, whose expression was upregulated by 58% ($p < 0.001$).

The alterations in *Cd33* expression were verified on a separate set of animals by using the quantitative PCR method. Additionally, the effect of JQ1, a very selective inhibitor of BET proteins, was analysed. Our data demonstrated (Fig. 2A) that among the three brain-expressed isoforms of BET proteins (Brd2, Brd3, and Brd4), the expression of the *Brd4* gene increased three hours after injection of LPS. As shown in Figure 2B, three hours

after administration of LPS, neither LPS nor JQ1 affected the mRNA level for *Cd33*. However, twelve hours after peripheral administration of LPS, the mRNA level for the *Cd33* gene was significantly increased in the hippocampus, probably in microglia cells. Moreover, JQ1 efficiently prevented LPS-evoked upregulation of *Cd33*.

To confirm that inflammatory conditions increase the expression of *Cd33* in microglial cells, in a separate experiment *in vitro*, we analysed the impact of JQ1 on the level of *Cd33* mRNA in stimulated mouse microglial BV2 cells. As demonstrated in Figure 3, stimulation of BV2 cells for twelve hours with blood serum from LPS-treated mice induced a substantial increase in the expression of *Cd33* and *Tnf* genes, compared to cells incubated with serum from control mice. At this timepoint, JQ1 significantly reduced the mRNA level of *Cd33*, in both stimulated (79% decrease) and non-stimulated cells (72% decrease), but did not affect the expression of the *Tnf* gene.

Discussion

Our data demonstrated hippocampal expression of 29 genes that were identified as bearing single nucleotide polymorphisms affecting the risk of developing AD [4]. In addition, we examined genes that are responsible for a genetic form of AD: *Psen1*, *Psen2*, and *App*. We hypothesized that inflammation may evoke changes in the expression of these genes in the brain, which, in consequence, might contribute to the pathomechanism of AD. By using a mouse model of the systemic inflammatory response (SIR) evoked by peripheral administration of LPS we identified a significant increase in the mRNA level for *Cd33* in the hippocampus. To our knowledge, the change in CD33 expression in the brain due to SIR has not been previously reported, but it seems that it could promote molecular alterations leading to neurodegeneration.

It was recently suggested that bacterial endotoxins (LPS) derived from the gut microbiota or originating from invading bacteria may significantly contribute to stimulation or the overactivation of neuroinflammation in AD [26,36,61], however, the mechanism remains unclear. Therefore, the identification of specific molecules that mediate LPS-related neuroinflammation may potentially facilitate the development of novel targets for therapy in AD. This disease is the major cause of dementia in the elderly, but despite decades of extensive research, the primary trigger is still unknown, and we still do not have efficient curation. Epidemiological data, GWAS, and analysis of human AD brains suggested neuroinflammation as a driving force in the pathomechanism or even pathogenesis of AD [1,4,47].

CD33 (Siglec-3) is a member of the Siglec family (sialic acid-binding immunoglobulin-like lectins), which in the brain is expressed mainly in microglial cells [14,25]. In humans, due to alternative splicing, two isoforms of CD33 are synthesised: a full-length protein hCD33M (M = 'Major'; 90%), and devoid of exon 2 short protein hCD33m (m = 'minor'; 10%) [25]. There is compelling evidence that CD33 affects the pathology associated with Aβ accumulation in AD by impairing Aβ clearance by microglia active during neuroinflammation [30]. A level of sialoglycan ligand for CD33, $(RPTP\zeta)^{S3L}$, is about twofold higher in the brains of AD patients than in the age-matched healthy controls [28]. Also, a large family-based GWAS indicated *CD33* as one of the top-level AD risk-related genes [3,58]. Two single nucleotide polymorphisms (SNP) in the *CD33* gene are considered crucial: rs3865444, which is located upstream of *CD33*, and rs12459419, which is located within the second exon [3]. The risk allele of rs3865444 evokes higher cell surface expression of CD33 and is related to the decline of cognitive functions [7,52]. The occurrence of the minor (protective) rs3865444 polymorphism was related with a decreased level of functional CD33 protein and with decreased levels of insoluble Aβ in the brains of AD patients [30]. The common allele rs12459419 favours the synthesis of a full-length CD33M, whereas the protective allele favours the synthesis of a short CD33m isoform. Interestingly, that 'protective' allele was suggested to be derived, human-specific, and reflect evolutionary pressure for longevity in humans [53]. Also, rs3826656 and rs2455069 have been associated with a higher risk of AD [3,55]. In AD brains, the level of CD33 is increased and correlates with a higher Aβ level and cognitive decline. Moreover, experimental studies demonstrated that CD33 reduces the clearance of $A\beta_{42}$ by microglia; therefore, it was suggested that CD33 protein may play a crucial role in the pathomechanism of AD, and its attenuation may be beneficial [31]. Indeed, the inactivation of CD33 in genetic mouse models of AD decreased levels of insoluble $A\beta_{1\text{-}42}$ in the brain and alleviated Aβ plaque pathology [29,30]. Moreover, expression of the human full-length variant of CD33 in 5×FAD mice increased Aβ pathology in the brain, but the protective variant had the opposite effect [24].

An important question arises about the impact of aging on the brain expression of CD33, which could potentially play a key role in the pathomechanism of AD. Unfortunately, the available data are inconsistent. In BALB/c mice, aging had no impact on *Cd33* expression level (GEO Profiles:12080108) [27]. Also, studies on human post-mortem brain tissues gave varying results (GEO profiles 5718268 and 117386467) [41,42], therefore, additional research is necessary to answer this question.

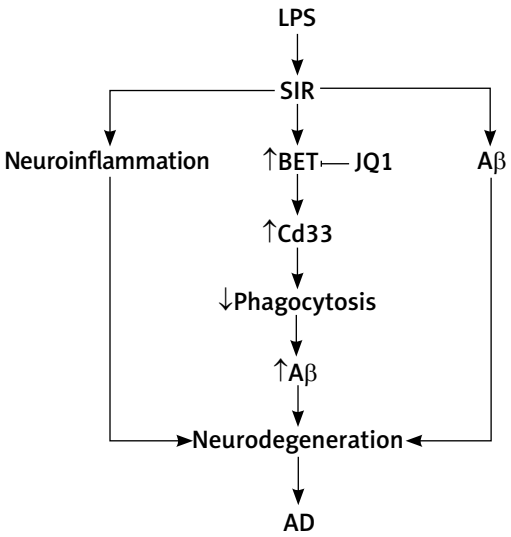

Fig. 4. Systemic inflammatory response (SIR), a trigger of CD33, could lead to neurodegeneration and Alzheimer's disease (AD). Lipopolysaccharide (LPS)-evoked SIR induces neuroinflammatory processes [16,20], accumulation of Aβ [37,56], and a BET-dependent increase in the expression of the *Cd33* – AD risk factor in the hippocampus. These processes may contribute to neurodegeneration and could be associated with AD. Enhanced expression of CD33 leads to inhibition of microglial phagocytosis and, in consequence, an increase in Aβ load in the brain. JQ1, an inhibitor of BET proteins, prevents SIR-related upregulation of *Cd33* expression.

Another important issue is the similarity between human and murine CD33. The fundamental physiological differences between humans and mice are evident, but still, a relatively large part of the human genome (about 40%) has a homologous locus in the mouse genome [38]. The human *CD33* and mouse *Cd33* genes have a similar structure and chromosomal position, and their protein sequence identity is 62% within extracellular domains [25]. The murine Cd33 lacks the characteristic ITIM (immunoreceptor tyrosine-based inhibitory motif) domain, which suggests that murine Cd33 may not precisely replicate the action of human CD33. Even though gene control systems are similar in mice and humans, some RNA expression diversity exists [11,40]. The previous reports demonstrated some variances in expression patterns and ligand recognition between human and murine CD33 [8]. However, both human and mouse CD33 inhibited phagocytosis of Aβ [30]. Therefore, future research should be performed to confirm our observations in human cells.

Because inhibitors of BET proteins were demonstrated to efficiently attenuate several LPS-evoked changes [2,32], the second goal of this study was to analyse the impact of JQ1, an inhibitor of BET proteins, on LPS-changed expression of AD-GRF genes in the hippocampus during systemic inflammation. In mice, JQ1 is well tolerated even after chronic treatment, and it efficiently enters the brain (AUC_{brain}/AUC_{plasma} = 98%) [43,45,48]. Our previous data indicated that in mouse microglia *in vitro* JQ1 reduced the expression of the *Cd33* gene by 83% [44]. In the current study, it was observed that JQ1 reduced the level of *Cd33* mRNA in the mouse hippocampus during SIR, but had no effect in the corresponding control. We can assume the cerebral action of JQ1, therefore, its inhibitory effect on microglial phagocytosis [44] cannot be completely excluded. Based on these data, we propose that inhibitors of BET proteins may prevent inflammation-evoked changes in the expression of the *Cd33* gene and, therefore, may be used to attenuate CD33-dependent signalling.

In summary, our study demonstrated the upregulation of the *Cd33*, a well-established genetic risk factor for AD, in the mouse hippocampus during LPS-evoked systemic inflammation. Moreover, our data indicated that an inhibitor of BET proteins prevented the activation of the *Cd33* gene in hippocampal cells during SIR. These results suggest that inhibitors of BET proteins may be suitable for prevention or for slowing down the progression of neuroinflammatory processes, which may be critical events in brain function and in the pathogenesis/pathomechanism of AD (Fig. 4).

Acknowledgments

The authors thank Elżbieta Gawinek for technical support.

Datasets/data availability statement

The data supporting the findings of this study are available on request from the corresponding author. Full microarray data (CEL files) are available in a public repository at: https://osf.io/x3jub/.

Funding

This research was supported by the National Science Centre, grant number 2018/31/B/NZ4/01379.

Disclosures

The study was approved by the II Local Ethics Committee for Animal Experimentation in Warsaw (permission WAW2/060/2020).

The authors report no conflict of interest.

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