



# Imiquimod-induced psoriasis-like skin inflammation is suppressed by BET bromodomain inhibitor in mice through RORC/IL-17A pathway modulation



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## ABSTRACT

Psoriasis is one of the most common skin disorders characterized by erythematous plaques that result from hyperproliferative keratinocytes and infiltration of inflammatory leukocytes into dermis and epidermis. Recent studies suggest that IL-23/IL-17A/IL-22 cytokine axis plays an important role in the pathogenesis of psoriasis. The small molecule bromodomain and extraterminal domain (BET) inhibitors, that disrupt interaction of BET proteins with acetylated histones have recently demonstrated efficacy in various models of inflammation through suppression of several pathways, one of them being synthesis of IL-17A/IL-22 which primarily depends on transcription factor, retinoic acid receptor-related orphan receptor C (RORC). However, the efficacy and mechanistic aspect of a BET inhibitor in mouse model of skin inflammation has not been explored previously. Therefore, this study investigated the role of BET inhibitor, JQ-1 in mouse model of psoriasis-like inflammation. Mice were topically applied imiquimod (IMQ) to develop psoriasis-like inflammation on the shaved back and ear followed by assessment of skin inflammation (myeloperoxidase activity, ear thickness, and histopathology), RORC and its signature cytokines (IL-17A/IL-22). JQ-1 suppressed IMQ-induced skin inflammation as reflected by a decrease in ear thickness/myeloperoxidase activity, and RORC/IL-17A/IL-22 expression. Additionally, a ROR $\alpha$ / $\gamma$  agonist SR1078 was utilized to investigate the role of RORC in BET-mediated skin inflammation. SR1078 reversed the protective effect of JQ-1 on skin inflammation at both histological and molecular levels in the IMQ model. The current study suggests that BET bromodomains are involved in psoriasis-like inflammation through induction of RORC/IL-17A pathway. Therefore, inhibition of BET bromodomains may provide a new therapy against skin inflammation.

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## 1. Introduction

Psoriasis is one of the most common immune-mediated cutaneous inflammatory disorders that affects ~2% of the general population [1,2]. It is characterized by a thickened epidermis resulting from hyperproliferative keratinocytes, parakeratosis,

hyperkeratosis, and infiltration of inflammatory leukocytes into the dermis and epidermis. The resident/infiltrated immune cells in concert with keratinocytes produce inflammatory cytokines such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-23, IL-17A, and IL-22 which perpetuate and amplify the vicious cycle of inflammation. Among these cytokines, IL-23/IL-22/IL-17A axis has been shown to play a prominent role in the pathogenesis of psoriasis [3–6].

Recent studies suggest an important role of chromatin remodeling in the pathogenesis of various diseases through regulation of gene expression. For example, small molecule inhibitors that block interactions between chromatin protein domains such as histone deacetylases, DNA methyltransferases, and their activators have emerged as novel therapeutic approach for the treatment of diverse disorders such as cancer and inflammation [7–9]. In

**Abbreviations:** IMQ, imiquimod; MPO, myeloperoxidase; RORC, retinoic acid receptor-related orphan receptor C; BET, bromodomain and extraterminal domain.

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humans, there are at least 40 bromodomain proteins, which include histone acetyltransferases, helicases, and scaffolding proteins that control gene transcription. The bromodomain and extraterminal domain (BET) family is a distinct group of bromodomain proteins that in mammals includes BRD2, BRD3, BRD4, and BRDT [7,8,10]. BET proteins are readers of histone acetylation that regulate gene transcription and are involved in inflammatory response in various disorders. The tandem bromodomains on BET proteins form binding pockets that recognize acetylated lysine on histones. This feature of BET proteins confers them the ability to govern histone acetylation-dependent transcriptional regulation [11,12].

Recently, small molecule BET inhibitors through blocking the recruitment and function of BET proteins have demonstrated efficacy in various animal models of inflammation such as multiple sclerosis, colitis, rheumatoid arthritis and airways inflammation. BET inhibitors have shown suppression of inflammation via blockade of various transcription factors required for inflammatory response such as NF $\kappa$ B, STAT3, and RORC [13–15]. JQ1 was the first drug developed that specifically blocks interaction between BET proteins and acetylated histones. Following its efficacy in cancer models, several other BET inhibitors have been designed and tested in various disease models, all showing efficacy in inflammatory disease models [13–16]. However, the efficacy of a BET inhibitor in mouse model of psoriasis-like inflammation has not been explored previously.

Recent studies have demonstrated a fundamental role of IL-23/IL-17A/IL-22 axis in the pathogenesis of imiquimod (IMQ)-induced psoriasis-like inflammation in mice [17–19]. IMQ model resembles human psoriatic inflammation in many aspects, one of them being a major involvement of IL-23/IL-17A/IL-22 axis. This is confirmed by the efficacy of anti-IL23, -IL-17A and -IL-22 antibodies in mouse psoriasis-like skin inflammation models and anti-IL23/-IL-17A antibodies in human psoriatic patients [17,20–23]. The generation of IL-17A/IL-22 from both innate and adaptive immune cells mostly depends on RORC, as depicted by a lack of skin inflammation and the absence of IL-17A and IL-22 in RORC deficient mice treated with IMQ [18]. One of the mechanisms for the coordination of an inflammatory response by BET proteins is via the RORC/IL-17A; and IMQ mouse model depends on RORC and IL-17A/IL-22 pathway for skin inflammation [13,17,18,22]. Based on these observations, it was hypothesized that BET inhibitor JQ-1 could inhibit psoriasiform skin inflammation in this model through regulation of RORC. This study shows for the first time that BET-inhibitor JQ-1 suppresses IMQ-induced psoriasiform skin inflammation through suppression of the transcription factor, RORC and its signature inflammatory cytokines, IL-17A/IL-22.

## 2. Materials and methods

### 2.1. Animals

Male BALB/c (8–11 weeks; 20–25 g), free of specific pathogens, were used in the experiments. The animals were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were kept under standard laboratory conditions of 12-h light-dark cycle and 24–26°C ambient temperature. All experimental animals used in this study were under a protocol approved by Animal Care and Research Committee of College of Pharmacy, King Saud University.

### 2.2. IMQ-induced psoriasis-like skin inflammation in mice

Mice received a daily topical dose of 62.5 mg and 5 mg of commercially available IMQ cream (Aldara 5%; MEDA Pharma, Germany) on the shaved back and right ear respectively for 7

consecutive days, as previously described [17]. Control mice were topically applied a control vehicle cream (Vaseline; Fagron).

Mice were divided into the following groups in preventive treatment mode: Control group (Control): mice received only vehicle cream; inactive enantiomer and imiquimod treated group [(–)JQ-1+IMQ]: mice received inactive enantiomer (–)JQ-1 (Calbiochem, USA) in dimethyl sulfoxide (100  $\mu$ g/cm<sup>2</sup>) topically 1 h before topical application of commercially available IMQ cream (Aldara 5%; MEDA Pharma, Germany) on the shaved back and right ear for 7 consecutive days, once a day; active enantiomer and IMQ applied group [(+)JQ-1, 30  $\mu$ g+IMQ]: mice received active enantiomer (+)JQ-1 (Calbiochem, USA) in dimethyl sulfoxide (30  $\mu$ g/cm<sup>2</sup>) topically 1 h before topical application of IMQ on the shaved back and ear as described above; active enantiomer and IMQ applied group [(+)JQ-1, 100  $\mu$ g+IMQ]: mice received active enantiomer (+)JQ-1 in dimethyl sulfoxide (100  $\mu$ g/cm<sup>2</sup>) topically 1 h before topical application of IMQ on the shaved back and ear as described above.

In semi-therapeutic mode, mice were either treated with (–)JQ-1 or (+)JQ-1 two days after starting IMQ application. Mice were divided into the following groups in semi-therapeutic treatment mode: Imiquimod and inactive enantiomer treated group [IMQ+(–)JQ-1]: mice received topical application of commercially available IMQ cream (Aldara 5%; MEDA Pharma, Germany) on the shaved back and right ear for 7 consecutive days, once a day, however, treatment of inactive enantiomer (–)JQ-1 (Calbiochem, USA) in dimethyl sulfoxide (100  $\mu$ g/cm<sup>2</sup>) was carried out topically only for 5 days, once daily (from 3rd day onwards until day 7); Imiquimod and active enantiomer treated group [IMQ+(+)JQ-1, 30  $\mu$ g]: mice received topical application of IMQ cream as described above on the shaved back and right ear for 7 consecutive days, once daily and the treatment with active enantiomer (+)JQ-1 (30  $\mu$ g/cm<sup>2</sup> in dimethyl sulfoxide; Calbiochem, USA) was carried out topically only for 5 days, once daily (from 3rd day onwards until day 7); Imiquimod and active enantiomer treated group [IMQ+(+)JQ-1, 100  $\mu$ g]: mice received topical application of IMQ cream as described above on the shaved back and right ear for 7 consecutive days, once daily and the treatment with active enantiomer (+)JQ-1 (100  $\mu$ g/cm<sup>2</sup> in dimethyl sulfoxide; Calbiochem, USA) was carried out topically only for 5 days, once daily (from 3rd day onwards until day 7).

Mice were killed by cervical dislocation after 7 days followed by collection of blood and skin samples for different analyses. For acute experiments, (+)JQ-1 and IMQ were applied topically at the same concentrations as described above but animals were killed 12 h after the application of IMQ. The concentrations of (+)JQ-1 used in this study were 10, 30 and 100  $\mu$ g/cm<sup>2</sup>, but 10  $\mu$ g/cm<sup>2</sup> was excluded based on the lack of any significant effect in the skin inflammatory parameters.

To assess the role of RORC in BET mediated psoriasis-like inflammation in IMQ model; we used SR1078 (Tocris, UK), a selective ROR $\alpha$ / $\gamma$  agonist. For this purpose, SR1078 was applied topically at a concentration of 100  $\mu$ g/cm<sup>2</sup> in dimethyl sulfoxide (this concentration was chosen based on the lack of any significant effect by 30  $\mu$ g/cm<sup>2</sup> in the skin inflammatory parameters), once daily, 3 h after (+)JQ-1 (100  $\mu$ g/cm<sup>2</sup>) application in IMQ model for seven days.

### 2.3. Measurement of ear thickness

The ear thickness was measured in duplicate using a digital micrometer (Helios, China) on days 0, 2, 4, and 6 by an independent investigator blinded to the treatment groups. The increase in ear thickness was taken as a measure of skin inflammation.

### 2.4. Measurement of cytokines in skin and plasma by ELISA

Cytokines were measured in samples collected from the back skin/plasma on day 1/7. IL-17A, IL-22, IL-23, and TNF- $\alpha$  were

**Table 1**  
Primers sequence.

Targeted gene	Direction and sequence
IFN- $\gamma$	F: 5'-TCTGGGCTTCTCTCTGCGG-3'; R: 5'-GGCGCTGGACCTGTGGGTG-3'
IL-23 <sub>p19</sub>	F: 5'-GGAGCAACTTCACACCTCC-3'; R: 5'-GGCAGCTATGGCCAAAAGG-3'
STAT-3	F: 5'-CCCCCGTACCTGAAGACCAAG-3'; R: 5'-TCCTCACATGGGGAGGTAG-3'
TNF- $\alpha$	F: 5'-GCGGAGTCCGGGACAGTCTA-3'; R: 5'-GGGGGCTGGCTCTGTGAGGA-3'
GAPDH	F: 5'-CCAGCAAGGACACTGAGCAAG-3'; R: 5'-GGTCTGGGATGGAAATGTGAGGG-3'

IL, Interleukin; IFN- $\gamma$ , interferon gamma; STAT3, signal transducer and activator of transcription 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

estimated using ELISA plates as per manufacturer's protocol (R&D Systems and Biolegend, USA).

### 2.5. Flow cytometry

Single cell suspensions from the spleen were prepared and  $2.5 \times 10^6$  cells per staining were fluorescently labeled with monoclonal antibody against the CD4 T cell surface antigen (BioLegend, USA), followed by treatment with fixation and permeabilization solution (Miltenyi Biotec, Germany). Cells were then stained intracellularly with specific monoclonal antibodies against Foxp3, IFN- $\gamma$ , and IL-17A conjugated to PE or FITC (Miltenyi Biotec and BD Biosciences). The stained cells were acquired on a flow cytometer (Beckman Coulter, USA) and analyzed for the expression of phenotypic markers using Cytomics FC 500 software as described earlier by us [24].

### 2.6. Real-time PCR

Total RNA was isolated from the back skin of different groups using the TRIzol reagent from Life Technologies/Invitrogen followed by DNase treatment to eliminate potential genomic DNA contamination as described earlier [25]. This was followed by conversion of 1  $\mu$ g of total RNA into cDNA using High Capacity cDNA archive kit (Applied Biosystems, USA) according to the manufacturer's instructions.

IL-17A, TNF- $\alpha$ , IFN- $\gamma$ , IL-23<sub>p19</sub>, STAT-3, RORC, 18S rRNA and GAPDH mRNA levels were measured by real-time PCR analysis using the ABI PRISM 7500 sequence detection system (Applied Biosystems) as described by us recently [24,26]. The primers were purchased from Applied Biosystems (UK)/GenScript (USA) and sequences were selected from PubMed database (Table 1). For the real-time PCR of IL-17A, RORC and 18S rRNA, Taqman assays-on-demand gene expression kits were purchased from Applied Biosystems. The real-time PCR data were analyzed using the relative gene expression (i.e.,  $\Delta\Delta$ CT) method as described earlier [27].

### 2.7. Western immunoblotting

Aliquots of the supernatants isolated from back skin (30  $\mu$ g protein/well) of different experimental groups were separated on 10% SDS-PAGE as described previously by us [25,26]. Proteins were transferred to nitrocellulose membranes and then probed either with polyclonal rabbit RORC antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:500, or  $\beta$ -actin rabbit polyclonal antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:5000. This was followed by the incubation with the secondary horseradish peroxidase-conjugated antibodies (anti-rabbit IgG; Santa Cruz Biotechnology, USA) for 1 h at room temperature. Bands were visualized using the enhanced chemiluminescence method (GE Health Care, Mississauga, Canada) and quantified relative to  $\beta$ -actin bands

using the ImageJ® image processing program (National Institutes of Health, Bethesda, USA). Images were taken using a C-Digit chemiluminescent western blot scanner (LI-COR, Lincoln, USA).

### 2.8. Measurement of myeloperoxidase activity

Right ears were collected from all groups on day 1/7 and homogenized in 50 mM potassium phosphate containing 0.5% hexadecyltrimethylammonium bromide (pH 5.4). Myeloperoxidase (MPO) activity in the supernatants was measured as an index of neutrophil infiltration into tissue [28]. In brief, the supernatants were mixed with MPO substrates buffer (0.167 mg/ml O-dianisidine, 0.0005% H<sub>2</sub>O<sub>2</sub>, 50 mM potassium phosphate), and incubated for 20 min at 25 °C. MPO activity was measured by determining the absorbance at 450 nm by a microplate reader.

### 2.9. Histopathological analysis

The mice were sacrificed 7 days after the induction of imiquimod-induced skin inflammation. Skin samples from the back were fixed in 10% buffered formalin. After paraffin embedding, 5  $\mu$ m-thick sections were cut and stained with H&E. Sections were examined by brightfield microscopy using Olympus IX51 microscope.

### 2.10. Chemicals

Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (USA).

### 2.11. Statistical analysis

The data were expressed as mean  $\pm$  SEM. Comparisons among different groups were analyzed by ANOVA (analysis of variance) followed by Tukey's multiple comparison tests. Comparison between two groups was carried out using Student's *t*-test. A '*P*' value of less than 0.05 was considered significant for all statistical tests. All the statistical analyses were performed using Graph Pad Prism statistical package.

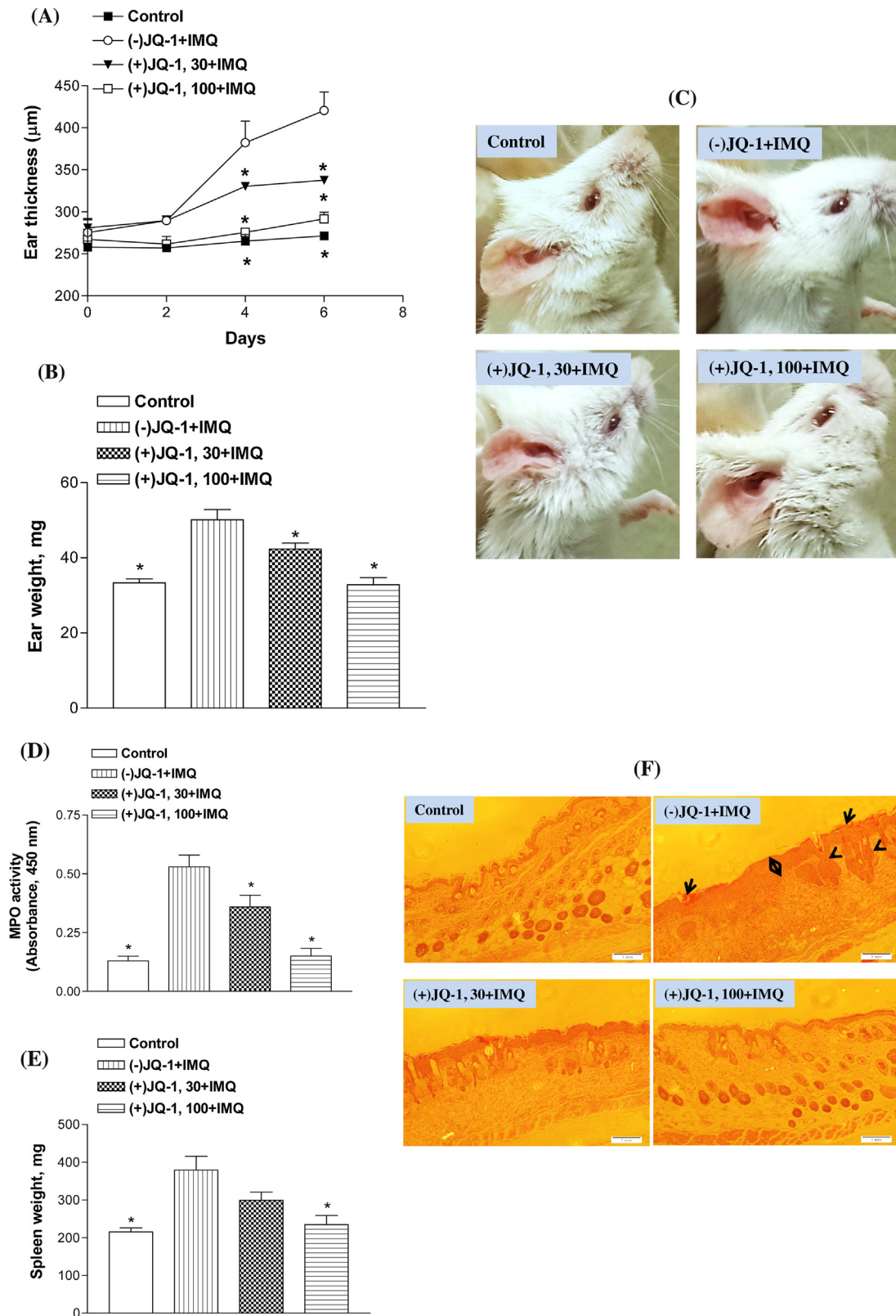
## 3. Results

### 3.1. IMQ induced skin inflammation is attenuated by BET inhibitor

IMQ application led to increased ear inflammation as evidenced by increased ear thickness, ear weight, erythema/swelling observed clinically (Fig. 1A–C) and MPO activity (Fig. 1D). Topical treatment with (+)JQ-1 dose dependently attenuated IMQ-induced increase in ear thickness/weight and MPO activity. Topical treatment with (+)JQ-1 dose dependently attenuated IMQ-induced increase in spleen weight also (Fig. 1E). Skin inflammation was also confirmed by histological examination which showed acanthosis, hyperkeratosis and elongation of rete-like ridge in IMQ treated group (Fig. 1F). All of these changes characteristic of psoriasis-like skin inflammation were markedly reduced by topical treatment with (+)JQ-1 as shown in Fig. 1F. These data show that BET inhibitor effectively suppresses IMQ-induced psoriasis-like inflammation in mice.

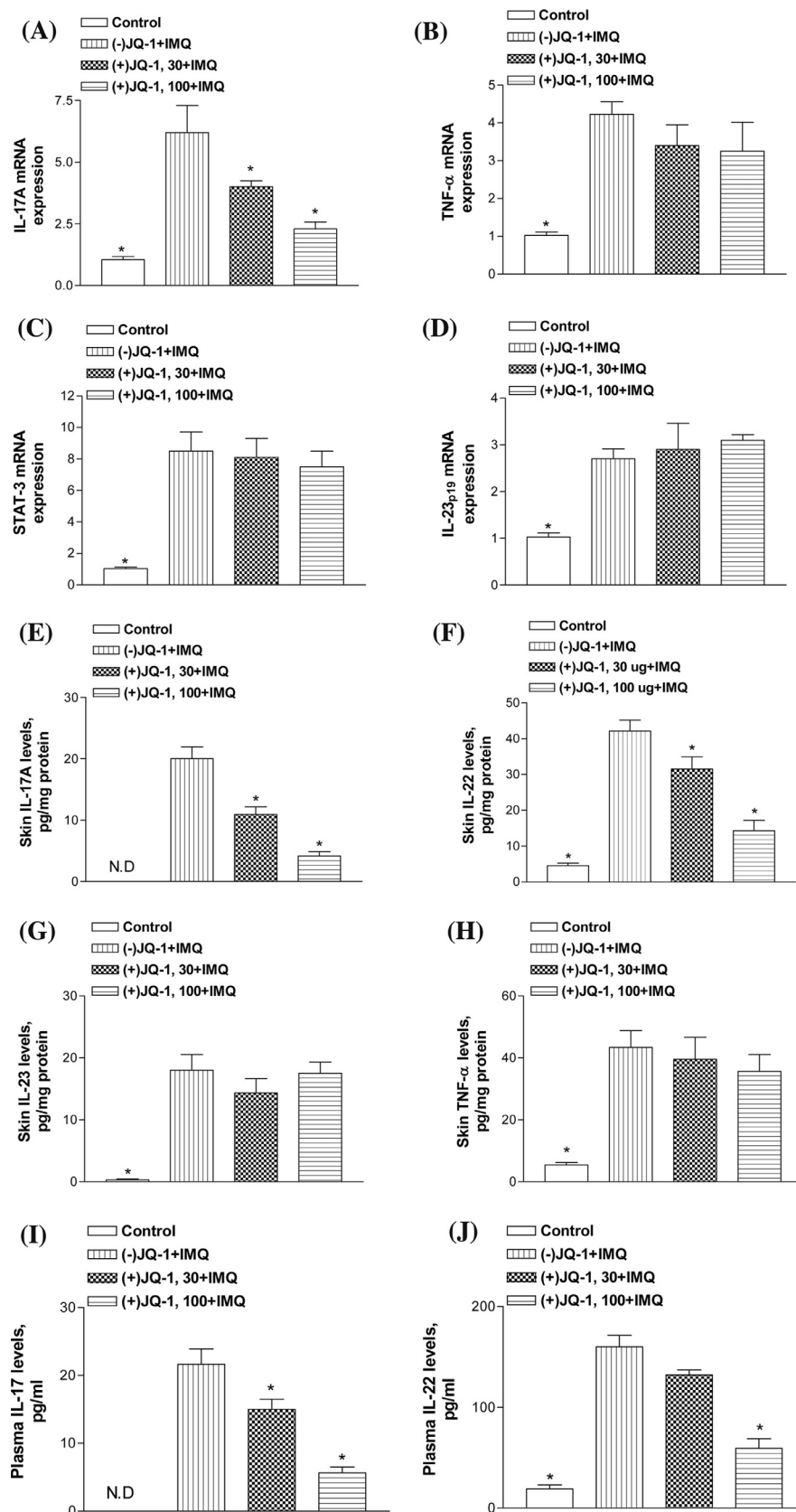
### 3.2. IMQ induced IL-17A, IL-22 and RORC are attenuated by BET inhibitor

Since IMQ-induced skin inflammation in mice has been shown to be dependent on IL-17A and IL-22, and BET inhibition has been shown to suppress them; therefore it was reasoned that BET inhibition by JQ-1 may also attenuate skin inflammation via reduction in

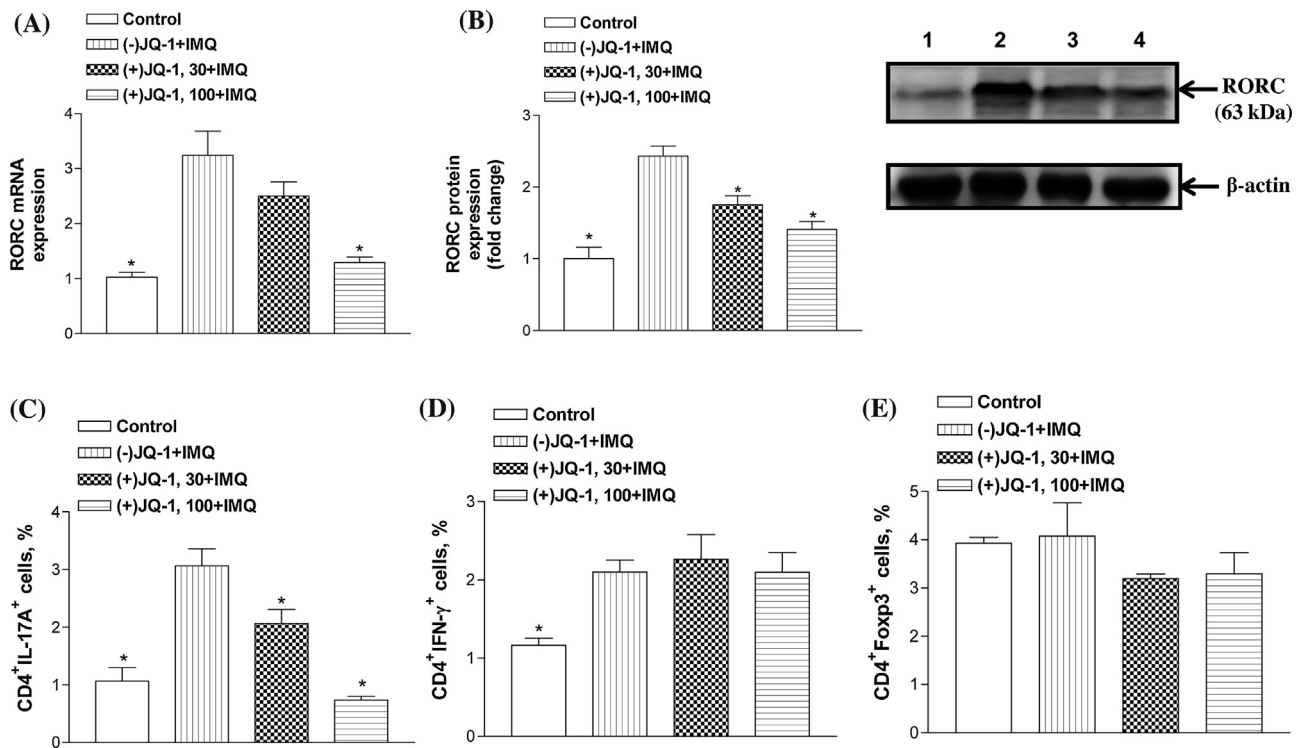


**Fig. 1.** Effect of BET inhibitor, (+)JQ-1 on IMQ-induced psoriasis-like inflammation. (A) Ear thickness, (B) ear weight, (C) clinical profile of right ear on 6th day, (D) ear myeloperoxidase (MPO) activity, (E) spleen weight, and (F) H&E staining of the shaved back (single arrows, double-headed arrow and arrow heads indicate hyperkeratosis, acanthosis, and elongation of rete-like ridges respectively). Ear thickness was measured using a digital micrometer. MPO activity was measured biochemically. Values are expressed as mean  $\pm$  SE,  $n = 6-8$ /group. \* $P < 0.05$ , vs. (-)JQ-1+IMQ group. Each photo/photomicrograph is a representative image from every group ( $n = 4-5$ /group; magnification,  $100\times$  for H&E staining).





**Fig. 2.** Effect of BET inhibitor, (+)JQ-1 on IMQ-induced changes in mRNA/protein levels of inflammatory genes in the back skin and plasma. (A) IL-17A mRNA expression, (B) TNF-α mRNA expression, (C) STAT-3 mRNA expression, (D) IL-23<sub>p19</sub> mRNA expression, (E) IL-17A protein levels, (F) IL-22 protein levels, (G) IL-23 protein levels, (H) TNF-α protein levels, (I) IL-17A protein levels in plasma, and (J) IL-22 protein levels in plasma. Expression of the genes in different groups was assessed by real time PCR. For mRNA expression by comparative  $C_T$  method using real time PCR, the first column was made as the calibrator against which the other groups were compared. Cytokines were measured using standard ELISA kits. N.D. on the graph stands for 'Not Detected'. Values are expressed as mean  $\pm$  SE,  $n = 6-8$ /group. \* $P < 0.05$ , vs. (-)JQ-1+IMQ group.



**Fig. 3.** Effect of BET inhibitor, (+)JQ-1 on IMQ-induced changes in expression of inflammatory markers in the back skin and splenic CD4<sup>+</sup> T cells. (A) Skin RORC mRNA expression, (B) skin RORC protein expression, (C) CD4<sup>+</sup>IL-17A<sup>+</sup> T cells % in splenocytes, (D) CD4<sup>+</sup>IFN-γ<sup>+</sup> T cells % in splenocytes, and (E) CD4<sup>+</sup>FOXP3<sup>+</sup> T cells % in splenocytes. Expression of the genes in different groups was assessed by real time PCR. For mRNA expression by comparative C<sub>t</sub> method using real time PCR, the first column was made as the calibrator against which the other groups were compared. Expression markers in splenic CD4<sup>+</sup> T cells were analyzed by flow cytometry. RORC protein expression was assessed by western blot. Bands 1, 2, 3, and 4 in the western blot B represent control, (-)JQ-1+IMQ, (+)JQ-1,30+IMQ, and (+)JQ-1,100+IMQ groups respectively. Values are expressed as mean ± SE, n = 4–8/group. \*P < 0.05, vs. (-)JQ-1+IMQ group.

these cytokines. Application of IMQ led to up regulation of IL-17A, IL-23<sub>p19</sub>, STAT-3, and TNF-α mRNA expression as compared to control mice (Fig. 2A–D), whereas there was no significant increase in IFN-γ expression (data not shown). Application of IMQ also led to increased protein release of IL-17A, IL-22, TNF-α, and IL-23 in skin as compared to control mice (Fig. 2E–H). Topical treatment with (+)JQ-1 dose dependently attenuated IMQ-induced increase in protein/mRNA levels of only Th-17 related cytokines (IL-17A/IL-22) but not other inflammatory markers/cytokines, i.e. IL-23, STAT-3 and TNF-α (Fig. 2A–H). This suggests that BET inhibitor specifically attenuates Th17 signature cytokines because there is no effect on other cytokines. Therefore, efficacy of BET inhibitor may be due to its suppression on IL-17A and IL-22 in IMQ skin inflammation model.

Until now we showed that IMQ-induced skin inflammation and IL-17A/IL-22 were suppressed by BET inhibitor, next we wanted to investigate if RORC was also affected by JQ-1. We hypothesized that BET proteins may be linked to synthesis of IL-17A/IL-22 via regulation of RORC. Our data shows that IMQ leads to activation and up regulation of RORC in skin at both mRNA and protein levels (Fig. 3A and B), which was associated with an increase in splenic CD4<sup>+</sup> IL17A<sup>+</sup> T lymphocytes (Fig. 3C). JQ-1 treatment led to down regulation of RORC mRNA and protein expression in skin with a concomitant decrease in splenic CD4<sup>+</sup> IL-17A<sup>+</sup> T cells (Fig. 3A–C), but not CD4<sup>+</sup> FOXP3<sup>+</sup> and CD4<sup>+</sup> IFN-γ<sup>+</sup> T cells (Fig. 3D and E) which are markers of Treg and Th1 cells respectively. These data show that BET inhibitor suppresses IL-17A expression via regulation of RORC.

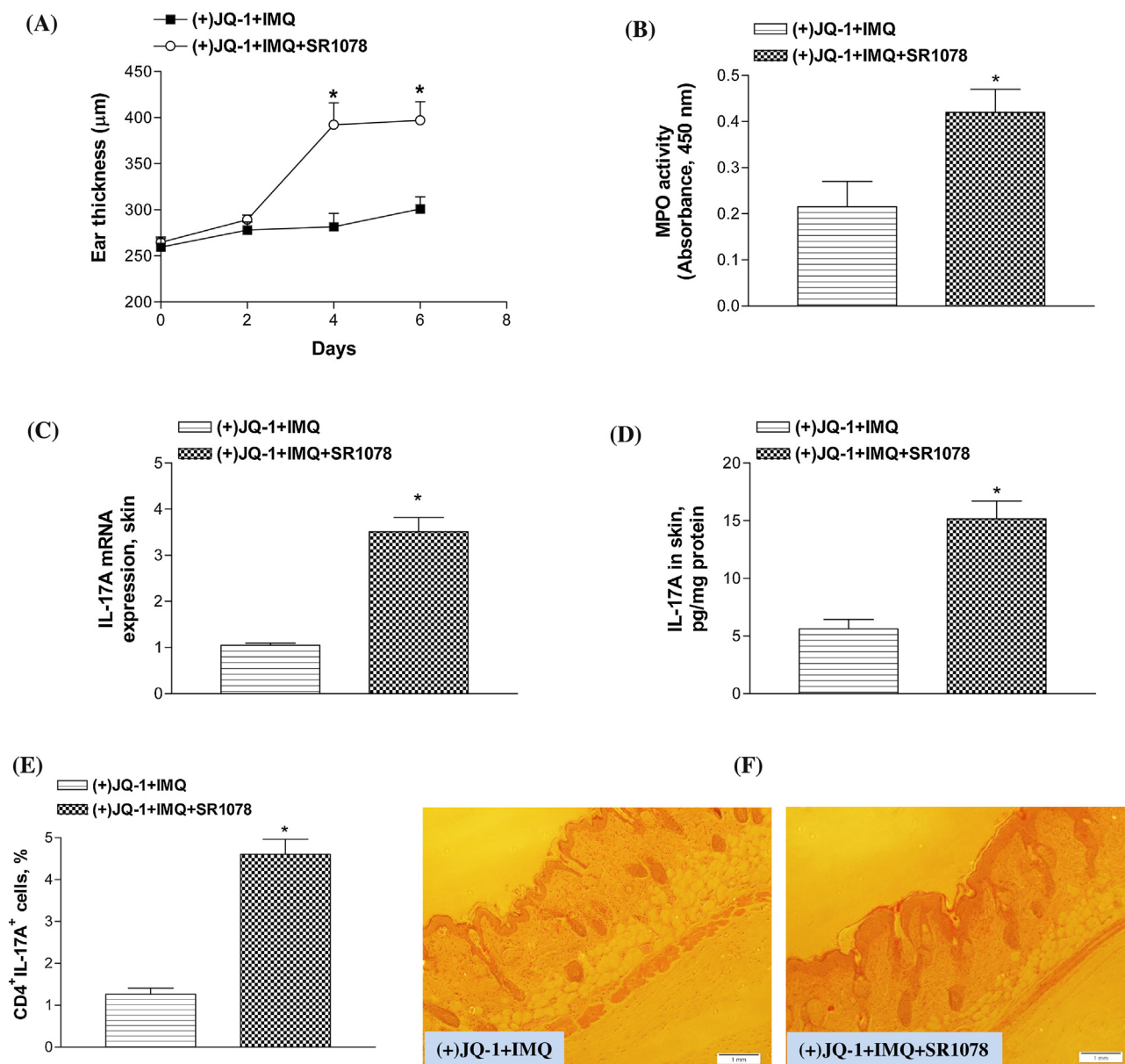
### 3.3. BET inhibitor induced protective effect is reversed by selective RORα/γ agonist in IMQ model

Further, to confirm the role of RORC in BET mediated psoriasis-like inflammation, we treated mice with SR1078, a RORα/γ

agonist after JQ-1 (100 μg/cm<sup>2</sup>) and IMQ application to investigate if changes caused by JQ-1 get reversed. Our data shows that SR1078 reverses the protective effect of JQ-1 in the IMQ model (Fig. 4). This was reflected by an increase in ear thickness (Fig. 4A), MPO levels (Fig. 4B), skin IL-17A mRNA expression (Fig. 4C) and skin IL-17A protein levels (Fig. 4D) after 7 days in JQ-1+IMQ+SR1078 group as compared to JQ-1+IMQ group. Further, flow cytometry showed increased CD4<sup>+</sup>IL17A<sup>+</sup> T cells in JQ-1+IMQ+SR1078 group as compared to JQ-1+IMQ group (Fig. 4E). CD4<sup>+</sup>IFN-γ<sup>+</sup> T cells and CD4<sup>+</sup>Foxp3<sup>+</sup> T cells were unaffected by treatment of SR1078 (data not shown). Histopathology of the skin confirmed the findings observed at the biochemical and molecular levels, and showed increased acanthosis/hyperkeratosis in JQ-1+IMQ+SR1078 group as compared to JQ-1+IMQ group (Fig. 4F). These data show that SR1078 specifically reverses BET-mediated skin inflammation via inhibition of RORC and its signature cytokines.

### 3.4. BET inhibitor shows protective effect both before and after development of skin inflammation in IMQ model

Next we turned our attention to investigate the role of BET bromodomain inhibition at early stages of the disease development in IMQ model. Therefore, a short protocol was utilized for this purpose in which IMQ was applied only once followed by measurement of various parameters after 12 h. Application of IMQ led to increased ear MPO activity (Fig. 5A), and IL-17A/IL-22 levels in skin (Fig. 5A–C) and plasma (Fig. 5D and E) without having much effect on IL-17A expression in splenic CD4<sup>+</sup> T cells (data not shown). BET inhibitor suppressed IMQ-induced increase in ear MPO activity, and IL-17A/IL-22 protein levels in both skin and plasma dose dependently (Fig. 5A–E). These data suggest that immediate



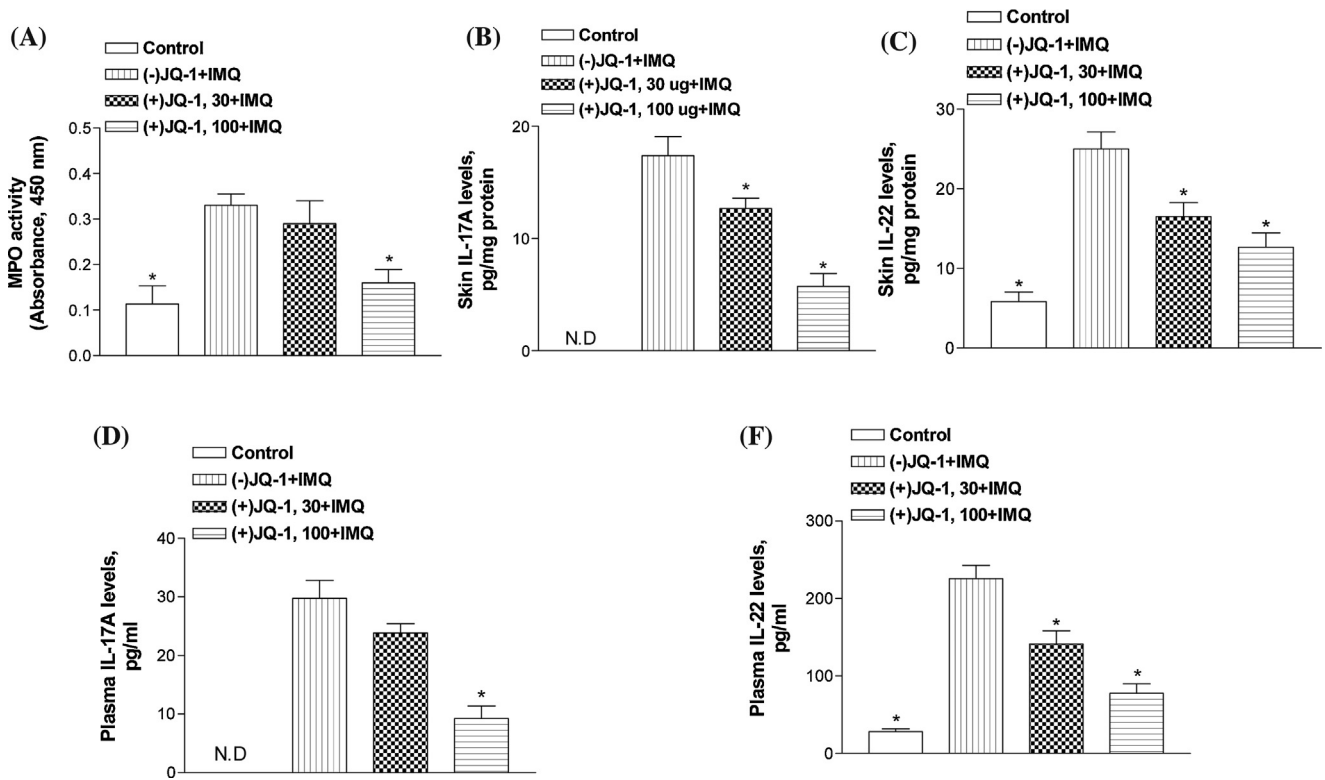
**Fig. 4.** Reversal of protective effect of BET inhibitor, (+)JQ-1 in the IMQ skin inflammation model by SR1078, ROR $\alpha/\gamma$  agonist at the end of the study. (A) Ear thickness, (B) ear myeloperoxidase (MPO) activity, (C) skin IL-17A mRNA expression, (D) skin IL-17A protein levels, (E) CD4<sup>+</sup>IFN- $\gamma$  T cells % in splenocytes, and (F) H&E staining of the shaved back. Ear thickness was measured using a digital micrometer. MPO activity was measured biochemically. Expression of the genes in two groups was assessed by real time PCR. For mRNA expression by comparative C<sub>T</sub> method using real time PCR, the first column was made as the calibrator against which the other group was compared. Cytokines were measured using standard ELISA kits. Expression markers in splenic CD4<sup>+</sup> T cells were analyzed by flow cytometry. Values are expressed as mean  $\pm$  SE,  $n = 4-6$ /group. \* $P < 0.05$ , vs. (+)JQ-1+IMQ group. Each photomicrograph is a representative image from every group ( $n = 4-5$ /group; magnification, 100 $\times$  for H&E staining).

synthesis and release of these cytokines from innate immune cells is also inhibited by BET inhibition.

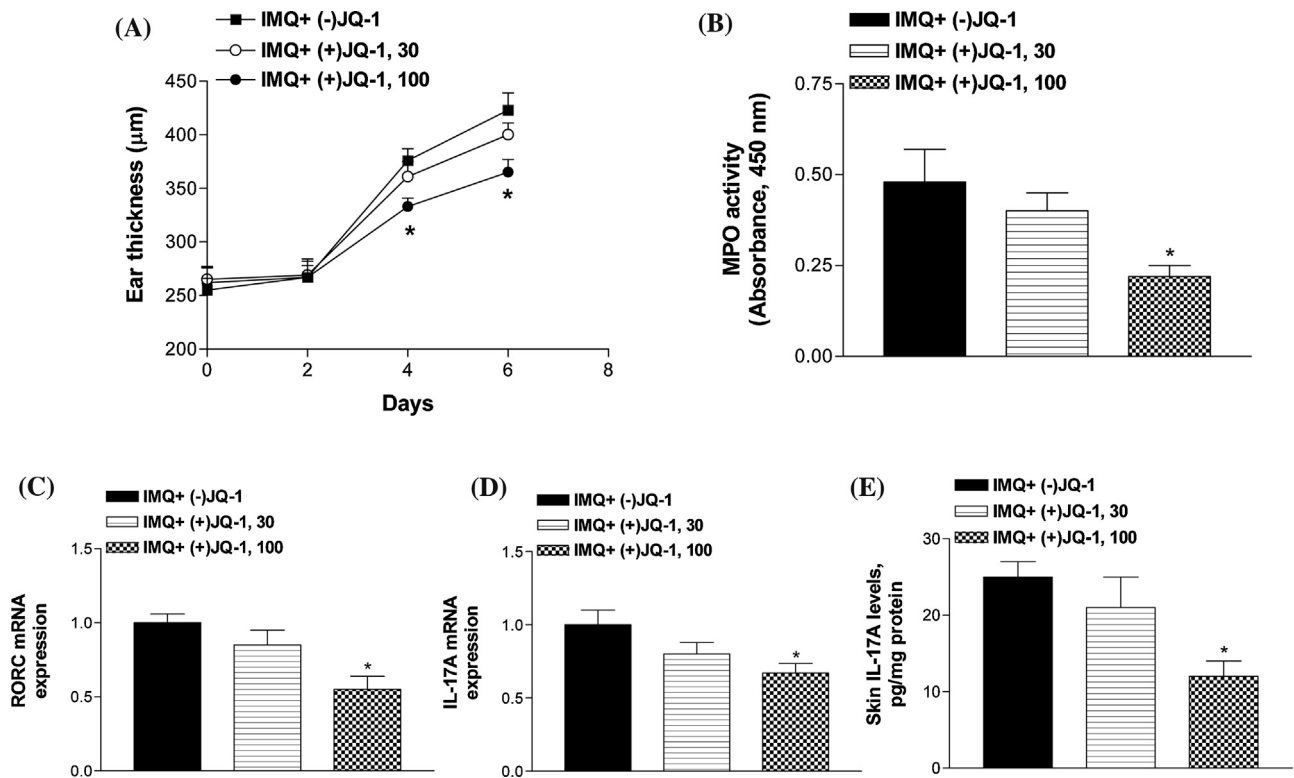
Finally, efficacy of (+)JQ-1 was tested in semi-therapeutic mode in IMQ model after initiation of skin inflammation where mice were treated with (+)JQ-1 from 3rd day onwards until the end of the study (7th day). Our data show that (+)JQ-1 (100  $\mu\text{g}/\text{cm}^2$ ) attenuates IMQ-induced skin inflammation at the end of the study. This was reflected by a decrease in ear thickness (Fig. 6A), MPO activity (Fig. 6B), skin RORC mRNA expression (Fig. 6C), skin IL-17A mRNA expression (Fig. 6D) and skin IL-17A protein levels (Fig. 6E). Overall, our data show that BET bromodomains regulate both immediate and late events in innate and adaptive immune cells in the IMQ model of skin inflammation via RORC and its signature cytokines, i.e. IL-17A and IL-22.

#### 4. Discussion

The IL-23/IL17A/IL-22 pathway plays a major role in the pathogenesis of IMQ-induced psoriasis-like skin inflammation. This was demonstrated by increased expression and release of IL-23/IL17A/IL-22 in the skin/blood of IMQ-treated mice in our study. JQ-1 which inhibits the recruitment of BET bromodomains to the transcriptional sites attenuated skin inflammation in IMQ model. JQ-1 suppressed skin inflammation in IMQ model by suppression of transcription factor, RORC and its effector cytokines, i.e. IL-17A and IL-22. These effects were specific as Th1/Treg markers were not affected by JQ-1. These data suggest that BET bromodomains are involved in the pathogenesis of psoriasis like skin inflammation via regulation of RORC/IL-17A/IL-22 pathway.



**Fig. 5.** Protective effect of BET inhibitor, (+)JQ-1 in the IMQ skin inflammation model using a short protocol. (A) Ear myeloperoxidase (MPO) activity, (B) skin IL-17A protein levels, (C) skin IL-22 protein levels, (D) plasma IL-17A protein levels, (E) plasma IL-22 protein levels. MPO activity was measured biochemically. Cytokines were measured using standard ELISA kits. N.D. on the graph stands for 'Not Detected'. Values are expressed as mean  $\pm$  SE,  $n = 5-6$ /group. \* $P < 0.05$ , vs. (-)JQ-1+IMQ group.



**Fig. 6.** Protective effect of BET inhibitor, (+)JQ-1 in the IMQ skin inflammation model using semi-therapeutic treatment mode. (A) Ear thickness, (B) ear myeloperoxidase (MPO) activity, (C) skin RORC mRNA levels, (D) skin IL-17A mRNA levels, (E) skin IL-17A protein levels. Ear thickness was measured using a digital micrometer. MPO activity was measured biochemically. Expression of the genes in different groups was assessed by real time PCR. For mRNA expression by comparative  $C_T$  method using real time PCR, the first column was made as the calibrator against which other groups were compared. MPO activity was measured biochemically. Cytokines were measured using standard ELISA kits. N.D. on the graph stands for 'Not Detected'. Values are expressed as mean  $\pm$  SE,  $n = 5-6$ /group. \* $P < 0.05$ , vs. IMQ+ (-)JQ-1 group.



Psoriatic inflammation is characterized by acanthosis, hyperkeratosis and infiltration of inflammatory cell into dermis/epidermis. All of these events ultimately lead to erythematous plaque formation, and eventually loss of the protective skin barrier [3,6]. IMQ model recapitulates some of these features very well as also shown in our study (increased thickening of epidermal layer, elongation of rete-like ridges and hyperkeratosis after IMQ treatment). There is growing evidence that at the molecular level, IMQ induced skin inflammation in mice involves IL-22/IL-17A axis, which is controlled by the transcription factor, RORC [17,18,22,29]. This is also confirmed by human psoriatic patients that show increased IL-17A/IL-22 levels in their blood and skin [30,31]. The precipitating event for the synthesis of IL-17A/IL-22 in immune cells is thought to be the release of IL-23 from activated dendritic cells after toll-like receptor ligation. These cytokines cooperatively stimulate keratinocytes to produce a variety of growth factors and inflammatory mediators, ultimately fuelling the vicious cycle of psoriatic inflammation [6,29]. Keeping these studies in mind, it was hypothesized that BET bromodomains may play an important role in psoriasis-like skin inflammation.

The BET protein family consisting of BRD2, BRD3, BRD4 and BRDT modulate gene expression by acting as readers of protein acetylation on histones and other proteins through their highly conserved bromodomains [8,10]. BET proteins recruit transcription factors and chromatin remodeling complexes to the gene promoter by providing a scaffold for transcriptional activation or repression. This process gives BET bromodomains a control over a wide range of cellular processes ranging from inflammation to cellular differentiation. BET inhibitors such as JQ-1 and IBET-762 block interactions between acetylated histones and BET bromodomain's acetyllysine-binding pocket, thus conferring them high specificity [10,11,13]. Since these interactions were initially reported to play a significant role in tumorigenesis, BET inhibitors were thus, first tested in cancer models in which they showed good efficacy both in vitro and in vivo [16]. However, recent research has unraveled the role of BET bromodomains in a variety of inflammatory and autoimmune diseases. This is well supported by studies that show physical association of BET bromodomains such as BRD2 and BRD4 with promoters of inflammatory genes such as NF- $\kappa$ B, IL-6, and TNF- $\alpha$ , and BET inhibitors block this association [11,13]. Based on these observations, BET inhibitors have shown good therapeutic potential in models of airway inflammation, multiple sclerosis, rheumatoid arthritis, and colitis. For example, BET inhibitors, JQ-1 and IBET-762 have shown protection against joint and neuronal inflammation in mouse models of rheumatoid arthritis and multiple sclerosis respectively [13,14]. However, no study so far has explored the role of BET inhibitor in a mouse model of psoriasis-like inflammation.

It is currently unknown whether BET proteins are directly involved in the pathogenesis of psoriasis-like inflammation in a mouse model. We hypothesized that BET proteins are involved in the regulation of IL-17A/IL-22 signaling through modulation of RORC. Our study shows that BET inhibition has the capability to dampen psoriasis-like inflammation. It was reflected by a decrease in skin inflammation at histological level along with inflammatory markers, MPO, IL-17A and IL-22 by JQ-1 treatment in IMQ model. This suggests that BET inhibition effectively suppresses skin inflammation through inhibition of IL-17A/IL-22. Suppression/neutralization of IL-17A/IL-22/IL-23 by using antibodies have been shown to reduce psoriasis-like inflammation in animals and humans in earlier studies [17,19,21–23,32]. Our study confirms these earlier observations.

It is also now well established that synthesis of Th17 signature cytokines, IL-17A and IL-22 is under the control of transcription factor, RORC [33,34]. Our study also shows involvement of RORC

in production of IL-17A/IL-22 in skin and splenic CD4+ T cells, and subsequent psoriasis-like inflammation in IMQ model both at early and later stages of the disease. Our data suggests that BET bromodomains are critically involved in IMQ induced initiation and progression of psoriasis-like inflammation in mice through RORC and its signature cytokines. This is supported by data that show simultaneous attenuation of skin inflammation along with inhibition of RORC and its signature cytokines (IL-17A/IL-22) by JQ-1. It is further corroborated by lack of any effect on Th1/Treg markers by JQ-1 treatment in IMQ model. A recent study by Pantelyushin et al. [18] showed that RORC deficient mice were fully protected from IMQ-induced psoriatic inflammation which was due to absence of IL-17A and IL-22 release from skin innate lymphocytes and  $\gamma\delta$  T cells. Another study showed that TLR4 agonist, lipopolysaccharide led to induction of RORC in a murine macrophage cell line and RORC inhibition led to suppression of joint inflammation in a mouse model of collagen induced arthritis [35]. On the contrary, RORC overexpression led to increased airway inflammation via increased IL-17A/IL-22 pathway [36]. Our study supports these observations and adds new information in that BET inhibitor leads to suppression of skin inflammation via regulation of RORC and its signature cytokines.

To further confirm the role of RORC in BET-mediated skin inflammation in IMQ model, we utilized a strategy wherein IMQ+JQ-1 treated mice were administered a ROR $\alpha/\gamma$  agonist, SR1078. Administration of agonist led to worsening of skin inflammatory parameters at histological, biochemical and molecular levels at the end of the study. This shows that RORC activation is important in BET-mediated skin inflammation in the IMQ model. The findings from the present study also show that IL-23, TNF- $\alpha$ , IFN- $\gamma$  and STAT-3 expression levels remain unaffected by BET inhibitor, which further suggest that RORC is specifically regulated by BET bromodomains. This is also supported by a recent study that shows that JQ-1 treatment and BRD2/BRD4 knockdown specifically causes downregulation of RORC and its signature cytokines in human and murine CD4+ T cells, whereas RORC unrelated gene products remain unaffected. JQ-1 also reduced joint and neuronal inflammation in mice models of collagen induced arthritis and experimental autoimmune encephalomyelitis models respectively in the same study [13].

Our data also showed immediate release of RORC signature cytokines such as IL-17A and IL-22 just after 12 hr of IMQ application and this was also suppressed by BET inhibitor JQ-1. Immediate sources of these cytokines could be innate lymphoid cells and  $\gamma\delta$  T cells, and late sources could be CD4+IL17A+ T cells in addition to innate immune cells. Moreover, it is now well established that all of these immune cells require expression of RORC for synthesis of IL-17A and related cytokines [18,19,29,37–39]. Our data also showed suppression of RORC/IL-17A and other inflammatory parameters by JQ-1 after IMQ-induced skin inflammation (Fig. 6). Therefore, our study suggests that BET inhibition suppresses not only expression of RORC and its signature cytokines (IL-17A/IL-22) in preventive mode but also after initiation of skin inflammation in IMQ model. However, efficacy of BET inhibitors in a long-term disease model of skin inflammation needs to be investigated.

In conclusion, our study shows that IMQ application leads to enhancement of IL-17A and IL-22 via RORC activation. BET inhibitor JQ-1 is able to suppress IMQ-induced psoriasis-like inflammation via inhibition of RORC and its signature cytokines. These data suggest that BET bromodomain inhibition could be developed into a new therapeutic strategy in combating psoriatic inflammation.

#### Conflict of interest

The authors declare no conflict of interest.

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