BET bromodomain inhibition affects immune humoral responses both by direct inhibition of B-cell class switching and by modulating T cell help

Zeinab Dalloul[1], Marie Best[2], Pauline Chenuet[3,4], Sandrine Lenoir[1], Dieudonnée Togbé[4], Mylène Gador[2], Bernhard Ryffel[3], Valerie FJ Quesniaux[3], Yolla El Makhour[5], François Boyer[1], Jean-Claude Aldigier[1], Nicolas Fazilleau[2], Jeanne Cook-Moreau[1] & Michel Cogné[1]

(Article en préparation)

Les protéines incluant des « bromodomaines » sont des lecteurs spécifiques des lysines acétylées des histones. Elles contribuent à la force transcriptionnelle des éléments cis-régulateurs organisés en cluster dans les *superenhancers* (SE). La région régulatrice 3' du locus de la chaîne lourde (IgH) de l'immunoglobuline (3'RR) est un SE majeur, stimulant non seulement la transcription, mais aussi le recrutement de l'AID, l'apparition de cassures double brin et la synapse des DSB durant la commutation de classe (CSR).

L'objectif de ce travail est de déterminer si l'inhibition des BET par un inhibiteur classique de cette famille de protéines, la molécule JQ1, a un impact sur des fonctions de la lignée B que notre laboratoire a par ailleurs précédemment démontrées comme « superenhancer-dépendantes » (CSR, SHM et autres aspects des réponses immunitaires humorales), avec alors la possibilité d'une voie de modulation pharmacologique des réponses des anticorps spécifiques à l'antigène.

Nous avons donc évalué les réponses immunes en présence ou en abscence de JQ1, en monitorant un certain nomre de paramètres *in-vitro* et *in-vivo* (évalaution de la CSR par ELISA, ELISPOT, RT-qPCR, expériences de séquençage de nouvelle génération (NGS) et ChIP, altérations des cellules T par cytométrie de flux, inflammation pulmonaire *in-vivo* par recrutement de cellules inflammatoires, taux de cytokines dans BALF et évaluation de la fonction pulmonaire).

Une dose non toxique de JQ1 a réduit la CSR *in-vitro* et *in-vivo* chez des souris immunisées à l'OVA, tant au niveau de la sécrétion d'Ig que de l'expression à la surface des cellules B. Cela résulte d'une expression plus faible et du recrutement de l'AID au niveau du locus IgH, et d'une recombinaison plus faible des gènes constants IgH malgré des transcrits de la lignée germinale IgH pré-switchés préservés. Des niveaux plus faibles d'ARNs IgH 3'RR ont aussi indiqué une

activité réduite du *superenhancer* qui contrôle la CSR. Dans un modèle d'inflammation pulmonaire allergique, JQ1 a diminué le recrutement des lymphocytes, des macrophages et des éosinophiles dans le BALF, mais a augmenté les taux de certaines cytokines TH2 dans le BALF et la résistance des voies aériennes. La diminution drastique des nombres de TFH a également marqué des réponses d'Ag chez des souris immunisées par OVA, avec une polarisation accrue vers la production de cytokines Th2.

Au total, ces données suggèrent que l'inhibition de la bromodomaine peut avoir des effets immunomodulateurs majeurs sur les cellules B et T notamment en diminuant la CSR et pourrait donc aider à contrôler les troubles impliquant à la fois des anticorps transgéniques et des cytokines Th1, où les cytokines Th2 sont bénéfiques…). Avant d'aller plus loins, il importe cependant d'analyser complètement les répercussions sur la polarisation T car celles-ci sont à l'évidence complexe. Par rapport à l'effet simple d'inhibition du switch observé *in vitro*, l'effet global de la molécule JQ1 sur la globalité du système immunitaire *in vivo* pourrait donc être protéiforme voire paradoxal.

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Keywords: B lymphocytes, T lymphocytes, class switch recombination, bromodomain inhibition, JQ1, allergic inflammation

Abstract

Inhibitors of bromodomain and extra terminal domain (BET) proteins show promising clinical activity in cancer most likely by targeting superenhancers and oncogene expression. By contrast, analysis of their impact on immune responses is still incomplete. We found that JQ1 treatment mechanically depresses class switch recombination (CSR) by decreasing AID loading onto IgH switch regions, in line with CSR dependency on the activity of the IgH locus 3'RR superenhancer. JQ1 also affected AID expression and had complex immunomodulatory effects on T-cells, altogether quantitatively and qualitatively affecting humoral immune responses.

Background: BET proteins are chromatin readers which accumulate at broken DNA ends before repair and bind superenhancers (SE) thus contributing to their transcriptional strength. The immunoglobulin heavy chain (IgH) locus 3'RR is a major SE, stimulating not only transcription but also recruitment of AID, occurrence of double-strand breaks (DSBs) and synapsis of DSBs during class-switch recombination (CSR).

Objective: To determine whether BET bromodomain inhibition by JQ1 impacts CSR upstream of the repair step, and globally impacts antigen specific responses in mice.

Methods: We measured *in-vitro* & *in-vivo* CSR by immunochemical and molecular biology assays, T-cell alterations by flow cytometry, lung inflammation by functional assays, inflammatory cell quantitation and cytokine levels in broncho-alveolar fluid (BALF).

Results: A non-toxic dose of JQ1 reduced CSR *in vitro* and *in-vivo* in OVA immunized mice, both at the level of Ig secretion and expression on B-cell surfaces. This resulted from lower expression and IgH recruitment of AID, and lower IgH recombination despite preserved abundant pre-switch IgH germline transcripts. Lower levels of IgH 3'RR eRNAs indicated decreased activity of the SE which controls CSR. In an allergic lung inflammation model, JQ1 decreased lymphocyte, macrophage, and eosinophil recruitment in BALF but increased cytokine levels in BALF and airway resistance. Drastic depression of T_{FH} numbers also marked Ag responses in OVA immunized mice, with increased polarization towards production of Th2 cytokines.

Conclusion: Altogether, these data suggest that bromodomain-inhibition has major immunomodulatory effects in B-cells, notably by decreasing multiple mechanistic aspects of CSR and antibody secretion, as well as in T-cells. It might thus help control disorders involving both class-switched antibodies and Th1 cytokines, such as auto-immune disorders affecting the central nervous system where Th2 cytokines are beneficial).

KEY MESSAGES:

Pharmacological targeting of humoral immune responses is possible via BET bromodomain inhibition and can alleviate specific antibody production and recruitment of inflammatory cells in models of antigen-induced inflammation.

Class-switch recombination is down-regulated *in vivo* and *in vitro* in a B-cell intrinsic manner by a drug targeting the binding of BET bromodomain factors to superenhancers.

During *in vivo* humoral immune responses, antigen-specific T-cells are quantitatively inhibited by BET treatment but also show major qualitative changes including increased production of TH2 cytokines.

Capsule summary: Targeting immunoglobulin class switching by BET-inhibition offers a novel immunomodulatory strategy which can reduce and modulate class-switched antibody production.

Keywords: Bromodomain inhibition, immunoglobulin class switch, B lymphocytes; immunopathology

Introduction

Bromodomain and extraterminal (BET) family proteins tightly bind acetylated lysine residues on the N-termini of histones and play important roles in gene expression, either alone or as part of protein complexes [1]. These factors are mostly enriched at positions of *cis*-regulatory elements such as active promoters, enhancers and to a higher extent superenhancers (SE), where they promote the recruitment of the Mediator complex and Polymerase II [2]. Hyperactivity of BET proteins contributes to the development of multiple diseases and notably cancer. Several small molecule BET inhibitors have thus entered into cancer therapy trials.

In malignant B-lineage cells, the BET protein BRD4 down-regulates translocated oncogenes associated with immunoglobulin heavy chain (IgH) locus regulatory elements [3]. In normal activated B-cells, BRD4 controls expression of Bcl-6, the master regulator of germinal center (GC) formation, and recruits non-homologous end joining (NHEJ) factors for repair of AID-initiated DNA breaks during class switch recombination (CSR) [4, 5]. It was however suggested that, before CSR, BET proteins do not contribute to the initiation of AID-mediated lesions in IgH genes [5].

CSR crucially regulates the production of pro-inflammatory antibodies such as IgG and IgE, which are major actors of adverse immune reactions. In addition to AID, IgH accessibility to CSR requires T-cell derived cytokines promoting germline transcription (GT) of given constant (C) genes [6]. CSR is additionally governed by the IgH 3' regulatory region (3'RR), a large (> 20kb-long) SE flanking the 3' end of the locus, the strength of which is optimal after B-cell activation [7, 8, 9]. The 3'RR also promotes somatic hypermutation (SHM) and it is directly implicated and targeted by locus suicide recombination (LSR) [10]. We thus wished to evaluate whether, in addition to their role in NHEJ repair, BET proteins might impact early steps of CSR such as AID loading or synapsis between broken DNA ends. Such aspects of IgH locus physiology are indeed major aspects of its 3'RR/SE-dependent control. We thus checked whether a low, non-toxic, dose of the BET inhibitor JQ1 might affect 3'RR function and its impact on the mechanisms of CSR. We also tested whether JQ1, known as an anti-inflammatory and

anti-cancer agent [11, 12], might cumulate B-cell intrinsic effects as seen *in vitro*, with more global effects on humoral responses *in vivo* and then positioning it as a potential immunomodulatory agent capable of modifying or attenuating Ag-specific or allergic reactions.

Materials and Methods

Mice

WT mice were used in all experiments and maintained in our animal facilities, at 21–23°C with a 12-h light/dark cycle. Our research was approved by the local ethics committee review board. Three to five month old BALB/c or C57BL/6 mice were used for the *vitro* culture experiments. Six to eight week old BALB/c mice were used for *in vivo* treatment trials.

Cell cultures

Splenocytes were collected, red blood cells lysed and CD43+ cells depleted using CD43 microbeads (Miltenyi Biotec). B cells were cultured for 4 days (for ELISA, qPCR and flow cytometry assays) and for 2 days (for ChIP experiments), in RPMI containing 10% FCS with lipopolysaccharide (LPS) (1 µg/mL) + IL-4 (40 ng/mL) with or without JQ1 (TOCRIS). JQ1 was used at concentratio of 5, 10, 20, and 40 nM depending on the experiment.

Proliferation Assay (MTS)

B cells isolated from 6 mice were seeded in 96-well plates at a density of $1x10^5$ per well in 100 µL total volume , stimulated with LPS + IL-4 as aboveand treated with the following concentrations of JQ1: 5, 10, 20 and 40 nM. Control cells were stimulated with LPS+IL-4 but not treated. The experiment was stopped at day 4. For the MTS assay, the CellTiter 96H AQueous One Solution Cell Proliferation Assay kit (Promega) was used following the manufacturer's instructions. Briefly, at 3 h before the desired time point, 20 µl MTS reagent was added to each well and cells were incubated at 37 °C for 3 h. Absorbance was detected at 492 nm with a Morti microplate reader (ThermoScientific).

Class-specific ELISA

ELISA was performed on sera and supernatants from *in vitro* stimulated and JQ1 (10, 20 and 40 nM) treated and untreated primary B cells for the detection of IgM, IgG1 and IgE, secretion. Plates were coated with monoclonal antibodies specific for IgM , IgG1 or IgE (all from Southern Biotech) overnight. Sera or supernatants were added and incubated for 2h at 37°C. After washing, alkaline phosphatase (AP)-conjugates of goat anti mouse IgM, IgG1 and IgE (Southern Biotech) were incubated 1h at 37°C. After washing and addition of AP substrate (Sigma), absorbance was measured at 405 nm.

Anti-ovalbumin (OVA) specific antibodies produced *in vivo* after immunization were evaluated in sera from JQ1 treated or untreated mice (50 mg/kg). Briefly, plates were coated with 10 μg/mL ovalbumin overnight. Sera were then incubated for 2h at 37°C and plates were treated as above.

ELISPOT

Specific IgG and IgM anti-OVA antibody secreting cells where quantified by ELISPOT using splenocytes from mice sacrificed 9 days after immunization and JQ1 treatment. Splenocytes were seeded in duplicate at a density starting at 5×10^5 /well, followed by 2-fold serial dilutions in culture medium in a 96-well MultiScreen HTS plate (Millipore) coated with 200 μg /well OVA. Cells were incubated overnight at 37 °C and then removed by washing with PBS/0.01 % Tween. Plates were incubated for 1 h at 37 °C with 1 μg/well alkaline phosphatase-coupled anti-IgG or anti-IgM. After washing, 100 μL BCIP/NBT alkaline phosphatase substrate (Millipore) were added. Plates were washed, dried, and images were taken with a NI-E microscope (Nikon) and analyzed for spot numbers using Nis-Ar software (Nikon).

Flow cytometry

Antibodies for staining were anti-mouse IgM-APC, CD19-APC-369 H7, IgG1-BV421, CD138-APC, B220-BV421 (all from BD Biosciences). Apoptosis was evaluated using AnnexinV-FITC (BD Biosciences) according to the manufacturer's instructions and 20 μL 7AAD (BD Pharmingen) was added 5 min prior to analysis.

Data were acquired on a Beckton Dickinson LSRII Fortessa cytometer and analyzed with the BD FACSDiva 6.1.3 and Infinity softwares.

Transcription analysis (RT-qPCR)

Iμ-Cμ, and Iγ1-Cγ1 germline transcripts and Iμ-Cγ1, Iμ-Cε post switch transcripts were quantified. Four day *in vitro* stimulated and JQ1 treated B cells were collected and RNA was extracted for evaluation of post switch and germline transcripts. RNA was prepared using standard techniques. cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Quantitative PCR was performed using power SYBR green (Applied Biosystems) and specific oligonucleotides (Iγ1 forward primer: 5' -GGCCCTTCCAGATCTTTGAG-3'; Cγ1 reverse primer: 5' -ATGGAGTTAGTTTGGGCAGCA-3'. Iμ forward primer: 5' -ACCTGGGAAT GTATGGTTGTGGCTT-3'; Cμ reverse primer 5' -TCTGAACCTTCAAGGATGC TCTTG-3'. Cε reverse primer: 5-AGCGATGAATGGAGTAGC-3').

All transcripts were normalized to Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) transcripts (reference Mm99999915-g1).

For Hs1.2 and Hs4 amplification the following primers were used: Hs1.2 forward: 5'-ATCAGTACCAGAAACAAGGC-3'; Hs1.2 reverse: 5'-TTGGGGTGAACCTGCAGC-3'; Hs4 forward: 5'-TTTAGTCTCAGCAAGACCC-3' and Hs4 reverse: 5'-AATGGGGCTTTCCACGCC-3'.

Amplification of Sμ/Sγ junctions and Ion torrent next generation sequencing NGS

DNA from LPS+IL-4 stimulated cells (with or without 40 nM JQ1) was extracted using a classical phenol/chloroform protocol. Sμ/Sγ junctions were amplified in triplicate by nested PCR with 100 ng DNA (Phusion HF polymerase; BioLabs) using the following primers: PCR1: Forward 5'- AGA GAC CTG CAG TTG AGG CC-3' and reverse 5'-TCA GGG AAR TAV CCY TTG ACC AGG CA-3', PCR 2: forward 5'-CCA GCC ACA GTA ATG ACC CAG-3' and reverse: 5'-CCA RKG GAT AGA CHG ATG GGG-3'. Each library was prepared using 200 ng PCR2 product. Barcoded libraries with 200-pb read lengths were prepared using Ion Xpress plus Fragment Library Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Each barcoded library was mixed

in equal amounts and diluted to 100 pM. Libraries were run on an Ion PI v3 chip on the Ion Proton sequencer (Life Technologies). Data analysis was performed using CSReport [13].

ChIP experiments

ChIP experiments were done on LPS+IL-4 stimulated CD43neg spleen cells incubated with or without 40 nM JQ1. Briefly, 15×10^6 B-cells were cross-linked at room temperature for 15 min in 15 mL PBS with 1% formaldehyde. The reaction was quenched with 2.125M glycine. After lysis, chromatin was sonicated to 0.5–1 kb using a Vibracell 75043 (Thermo Fisher Scientific). Following dilution in ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl), chromatin was precleared by rotating for 2h at 4 °C with 50 mL of 50% protein A/G slurry (0.2 mg / mL sheared salmon sperm DNA, 0.5 mg/mL BSA, and 50% protein A/G; Sigma). Cell equivalents (1×10^6) were saved as input, and the remaining cell equivalents were incubated overnight with anti-AID rabbit polyclonal antibodies (kindly provided by Dr. P. Gearhart) or control antibodies. Immune complexes were precipitated by the addition of protein A/G. Cross-linking was reversed by overnight incubation (70 °C) in TE buffer with 0.02% SDS and chromatin was phenol/chloroform extracted. QPCR assays used for evaluating precipitated DNA from Sμ, Sγ1 and Sε used the following primers:

- Sμ Fw: 5'-TCTAAAATGCGCTAAACTGAGG-3'
- Sμ Rev: 5'-AGCGTAGCATAGCTGAGCTC -3'
- Sγ1 Fw: 5'- GGGGGAGGAGATATCCAAGA -3'
- Sγ1 Rev: 5'- CAGCTCTTTTGCAGGTCTTG-3'
- Sε Fw: 5'- CTTGACCACCGAATGTCCTT -3'
- Sε Rev: 5'- GATTCCTCTCCAGCCTCTCC-3'

Ovalbumin (OVA) induced allergic asthma

BALB/c female mice at 8 weeks of age were immunized intraperitonealy (i.p.) on days 0, 7 and 14 with 20 μg ovalbumin grade V (Sigma France) emulsified in 2 mg aluminium hydroxide gel (total volume 200 μL). For control mice, 200 μL saline was injected. Mice were challenged at days 21-24 with 10μg OVA by intra-tracheal administration to

provoke allergic asthma with analysis of the allergic response at day 25. Control mice received saline.

Bronchoalveolar Lavage (BAL) and differential cell counts

BAL was performed by washing the lungs four times with 0.5 mL saline solution at room temperature. After centrifugation at 400 x g for 10 min at 4°C, supernatant from the first lavage (cell-free BAL fluid) was stored at -80°C for cytokine analysis. Cells were diluted with Turk's solution, counted and 200,000 cells were centrifuged onto microscopic slides (cytospin at 1,000 rpm for 10 min, at RT). Air-dried preparations were fixed and stained with Diff-Quik (#130832, Medion Diagnostics AG, Merz & Dade, Germany). Differential counts were made by oil immersion light microscopy. Two hundred cells were observed to determine the relative percentage of each cell type and absolute number of the differential cell count.

Lung homogenization and EPO activity

After bronchoalveolar lavage, the entire lung was perfused with isotonic solution through the right heart ventricle to flush the vascular content and lungs were frozen at -20°C until use. Lungs were homogenized in 0.05M Tris-HCl, pH 8 with an Ultra Turrax T18 apparatus (UltraTurrax, IKA), centrifuged and the supernatant was aliquoted. Following centrifugation, EPO activity was determined in supernatants from lungs by colorimetric assay. Following centrifugation, 100 µL supernatants were placed in a plate with 50 µL substrate solution, corresponding to 11 mL Tris HCl + 200mM O-phenylenediamine dihydrochloride pellets + 100 µL 30% H_2O_2). After 1 hour incubation at 37°C in a shaker, EPO activity was determined as absorbance at 490 nm against medium.

Lung histology

After BAL and lung perfusion, the left lobe was fixed in 4% buffered formaldehyde (#15225582, Fisher Scientific) for a minimum 24h for standard microscopic analysis (Leica microscope). Sections (3-µm) were stained with haematoxylin & eosin (H&E) and Periodic Acid Schiff (PAS) using standard techniques. Inflammatory cell infiltrate including eosinophils was assessed by a semi-quantitative score on H&E sections (0-5), while mucus production was assessed on PAS stained sections with mucopolysaccharide

at the apical pole of goblet cells from bronchioles by comparable semi-quantitative score (0-5) by two independent observers.

Cytokine quantification

Th1/Th2 (IL-4, IL-5, IL-13 and IFN-γ) cytokines concentrations in BALF and lung homogenate were determined by Luminex immunoassay (Millipore) by using MagPix system (BioRad) according to manufacturer's instructions.

T cell assessment

Immunization and reagents: C57BL/6 ($CD45.2^+$) mice were purchased from Centre d'Elevage Janvier (Genest Saint Isle, France). All mice were bred in UMS006 and only 8-12 week old females of were used for experimental procedures. Alum was from Thermo Scientific. 1W1K (EAWGALANKAVDKA)-OVA was from Genecust. Mice were either immunized sc (subcutaneous) at the tail base or i.p. with 40 µg 1W1K-OVA in the indicated adjuvant. 50 mg/kg JQ1 was injected i.p daily.

ELISA: ELISA plates (Thermo scientific) were coated with 10 µg/ml OVA. IgG, IgG1 and IgM were detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Southern Biotech). The HRP substrate o-Phenylenediamine dihydrochloride (OPD) was purchased from Sigma-Aldrich.

Flow cytometry analysis: Cell suspensions were prepared in PBS/2% FCS, 5mM EDTA. For Ag-specific Th and B cell analyses, organs were dissociated, filtered and treated with 2.4G2 for 10 minutes. To track antigen-specific $CD4^+$ T cells, cells were incubated with PE-1W1K-IAb tetramer (7µg ml^{-1}) and APC anti-CXCR5 (REA 215, Miltenyi Biotec, 1:10) or BV421 anti-CXCR5 (2G8, BD, 1:50) for 2 hours at room temperature. The tetramer 1W1K-IAb was obtained from NIH Tetramer core facility. To track antigen-specific B cells, cells were stained for 60 minutes with OVA-Alexa488 (Invitrogen) at a final concentration of 1µg /mL. Cells were then incubated on ice for 45 min with fluorophore labeled mAbs. The following mAbs purchased from BD Biosciences were used: anti-CD4 (RM4-5, 1:200), anti-CD138 (281-2, 1:500), anti-CD95 (Jo2, 1:500), anti-GL7 (GL-7, 1:500), anti-B220 (RA3-6B2, 1:500), anti-GATA3 (L50-823, 1:200), anti-IL-4 (11B11, 1:100), anti-IL-10 (JES5-16E3, 1:200). The following mAbs purchased

from eBioscience were used: anti-PD1 (J43, 1:500), anti-Foxp3 (JFK-16s, 1:200), anti-IFNγ (XMG1.2, 1:100), anti-CD4 (RM4-5, 1:1000), anti-Tbet (4B10, 1:200), anti-IL-13 and anti-CD44 (IM7, 1:800), anti-IgD (11-26c, 1:1000) from Biolegend was used. For IFNγ, IL4, IL13 and IL10 intracellular staining cell suspensions were incubated either for 4 hr at 37°C in the presence of Golgi Plug (BD Bioscience), 4µM Monensin, 50 ng/ml PMA and 2µM Ionomycin, or with 1W1K (10µg/ml) overnight and with Golgi Plug for 1 hr at 37°C. Then, cell suspensions were fixed and permeabilized using Invitrogen Fixation/Permeabilisation kit. Fixable Viability Dye eFluor506 (eBioscience) was used for dead cell exclusion. Data were collected on a BD LSRII-Fortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Statistical analysis

Statistical tests were performed using GraphPad Prism (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Results

Evaluation of JQ1 toxicity

JQ1 has been widely tested as an anti-cancer agent. It proved effectvet *in vivo* against mouse tumors, with a maximum tolerated dose of 50 mg/kg/day, and showed anti-proliferative effects *in vitro*. To assess its role on B-cells while minimizing non-specific effects, we used low doses in the 5 to 40 nM range for *in vitro* assays, and chose the non-toxic dose of 50 mg/kg/day for *in vivo* assays. We validated *in vitro* that the latter low doses did not significantly affect B-cell growth in cultures stimulated by LPS+IL-4 for 4 days (Fig.1A). In these cultures, the percentage of apoptotic cells did not vary when cells were cultured alone or with 40 nM JQ1 (Fig.1B).

Evaluation of *in vitro* CSR by cell cytometry and ELISA

To evaluate whether JQ1 modulated class-switching, mouse splenocytes were stimulated for four days *in vitro* by LPS + IL4 known to boost CSR and further expression of class-switched IgG1 and IgE.

Direct evaluation of class switching in B-lymphocytes, by following cell-surface BCR expression after LPS+IL-4 stimulation, showed a strong reduction in the amount of class-switched cells observed, with a 1-fold reduction at 20 nM JQ1, a 3-fold reduction at 40 nM JQ1, and a reciprocal increase in IgM+ CD19+ un-switched cells (Fig. 2A).

Parallel ELISA evaluation of Ig secretion in cell supernatants showed no reduction in IgM levels. By contrast and to a much stronger extent than for BCR expression, secretion of class-switched Ig produced in such conditions (*i.e.* IgG1 and IgE with LPS+ IL-4), decreased at all concentrations of JQ1 (Fig. 2B).

Beside these variations in BCR class-switching and Ig secretion, we confirmed that B-cell counts, *i.e.* absolute numbers of CD19+ cells obtained after *in vitro* stimulation, were not significantly changed in 4 day- *in vitro* cultures stimulated with or without JQ1 (as expected from the preliminary evaluation of cell viability in similar conditions) (Fig. 3A). In contrast, differentiation into CD138+ plasmablasts was decreased by 50%, thus likely contributing to a global reduction in Ig secretion (Fig. 3B).

CSR blockade occurs downstream from IgH germline transcription

We measured AID loading of on target S-regions by ChIPexperiments in chromatin prepared from cells stimulated for CSR, and observed a drastic reduction in AID-recruitment to Sμ, Sγ1 as well as Sε regions in LPS+IL-4 stimulated B-cells (Fig 3C). Compared to this drastic reduction, transcription of the AICDA gene encoding AID was also affected by JQ1 in LPS+IL4-stimulated cells (Fig 3D).

Thus, we quantified, two types of IgH constant gene transcripts in LPS/IL-4 stimulated B cells, respectively specific for the "pre-CSR" B-cell stage (Iγ1-Cγ1 and Iμ-Cμ germline transcripts originating from un-switched B-cells) and the "post-CSR" stage (*i.e.* Iμ-Cγ1 and Iμ-Cε switched transcripts). Upon JQ1 treatment, we observed an increase in the "pre-CSR" transcripts" which are the usual hallmarks of accessibility of the IgH locus to CSR. This also indicated that IgH gene transcription was not globally decreased in cells treated with JQ1. By contrast, a specific decrease in IgG1 and IgE class-switched transcripts was observed (Fig. 3E). This precisely situated the blockade in-between the stages of "pre-

CSR" transcription (increased) and "post-CSR transcription" (decreased) and pointed to a direct alteration of the recombination step itself.

Positions and structure of CSR junctions

CSR junctions from Sμ to Sγ1 were amplified by PCR with specific primers, sequenced by NGS and analyzed as previously described according to their positions in the S region and their nature. The most striking change in junctions sequenced after JQ1 treatment of B cells was a dramatic ~ 4-fold decrease in number (Fig 4A). The repair process after the occurrence of DSBs was by contrast unchanged with regard to the use of non-homologous-end joining (NHEJ) pathway *versus* the alternate- microhomology-mediated-end joining (MMEJ): the strong predominance of NHEJ-mediated blunt junctions which is usual in CSR was thus maintained (Fig 4B). Regarding the location of breakage, a modest difference induced by JQ1 treatment was increased proximity of breaks to AID-targeted cytosines of RGYW/WRCY motifs, indicating that while AID loading was decreased in the presence of JQ1, breaks were then somehow more focused on canonical AID target sites (Fig 4C).

3'RR transcription

As for other enhancers, accessibility and activity of the IgH 3'RR can be assessed by evaluating transcription of core enhancer elements into eRNA. Comparison of untreated and treated cells after 4-days stimulation by LPS+IL4 revealed a major dose-dependent reduction in eRNA transcripts from the 3'RR Hs1,2 and, to a lesser extent, Hs4 core enhancer elements (Fig 5).

Specific antibody secretion and *in vivo* CSR after mouse immunization

Mice were immunized by ovalbumin in order to follow primary and secondary responses. Nine days after primary i.p. immunization, mice receiving JQ1 showed a significant decrease (≈ 4-fold) in specific anti-OVA IgG secreting cells, as evaluated in splenocytes by ELISPOT (Fig. 6A up left). Parallel serum evaluation of circulating antibodies detectable at day 9 showed a significant decrease in specific anti-OVA IgG (Fig 6A up right). As a counterpart of the decreased counts of cells secreting IgG, the number of anti-OVA specific unswitched (IgM) splenocytes by ELISPOT was increased (≈ 1-fold; Fig

6A down left). The latter increase, however, was associated with decreased anti-OVA specific IgM detectable in sera (Fig 6A down right) (although less markedly than for IgG), suggesting that on a "per-cell" basis, IgM secretion also tended to be lower in animals receiving JQ1.

Since secondary responses involve multiple factors in addition to B-cells, we wished to evaluate JQ1 effects over a longer term, in groups of mice kept for 25 days under daily JQ1administration after primary OVA immunization and further booster-immunized at days 7 and 14. In such conditions, mice receiving JQ1 showed decreased specific Abs (Fig 6B), again simultaneously correlated with a CSR defect and a partial defect in plasma cell differentiation affecting IgG and IgE.

Modulation of B-cell intrinsic aspects in a model of Ag-induced asthma in mice

Using BALB/c mice that had been repetitively immunized *i.p*. with OVA, we explored whether the occurrence of allergic manifestations triggered by further *i.n.* administration of the immunizing Ag could be modulated by JQ1. Four groups of mice received either saline, ovalbumin, or ovalbumin + steroid known to prevent the occurrence of allergic asthma (budesonide 3 mg/kg, *i.n.*), or ovalbumin + JQ1 (50 mg/kg, *i.p*).

In such settings, T-cell activation parameters (secretion of IL-4/IL-5, IL-13 and IFN-γ in BALF and lung) were suppressed more strongly by steroids than by JQ1 administration (Fig 8A, 8B). Consequently, functional improvement of asthma (airway resistance and compliance measured by plethysmography (Fig 9A), eosinophil peroxidase activity in lung was unaffected by JQ1 treatment (Fig 9B).

Despite persistent T-cell activation, we observed a significant *in vivo* effect of JQ1 on the biological parameters monitoring antibody-dependent responses. Following the reduced systemic OVA specific IgG1 and IgE response mentioned above (Fig 6B), other biological aspects of airway allergy were locally alleviated in lungs of mice receiving JQ1 compared to mice just receiving ovalbumin: cell infiltration decreased in lung, with reduced numbers of all cell types (except for neutrophils) in broncho-alveolar fluid (BALF) (Fig 7A, B). Moreover, JQ1 reduced total protein levels in BALF. Most of these biological markers of JQ1-mediated immunomodulation reached levels very similar to

those achieved with the standard steroid medication for asthma (budesonide). That this occurred with only marginal changes in cytokine levels suggests that the inhibitory effects of JQ1 on B-lineage cells *in vivo* were direct (similar to those described above *in vitro*) and indirect resulting from defective T-cell help.

Modulation of T cell aspects in a model of Ag-immunized mice

Based on the observation that JQ1 treatment alters cytokines level in asthmatic mice, we assessed its effect *in-vivo* on C57BL/6 mice that had been immunized s.c and i.p with 1W1K1-OVA to explore whether T cells could be modulated by JQ1. Two groups of mice received either 1W1K1-OVA with vehicle or 1W1K1-OVA with JQ1 (50 mg/kg, *i.p*) were assessed. Antigen specific Th cells were assessed by 1W1K1-I pMHCII tetramer staining together with CD44 expression.

In such conditions, draining lymph nodes (dLN) showed less T cellularity in the JQ1 treated group. Regarding Ag-specific T lymphocytes, results showed no global differences in the percentage of Tetra+ cells between the two groups. However, total activated T follicular helper cells (CXCR5+ cells) also were significantly decreased in JQ1 treated cells. In parallel, specific T lymphocyte sub-populations showed a significant increase in GATA3 (Th2 markers), while FOXP3 (T regulatory markers) and Tbet (Th1 markers) were slightly increased (Fig 10).

In spleen, as for dLN, total T cell, absolute W1K1 specific T cells and Tfh numbers were significantly decreased following JQ1 administration. Furthermore, the percentage of antigen-specific Th2 cells was significantly higher following JQ1 injection. Closer examination of the GATA3+ CXCR5 population did not reveal significant differences although there was a tendency towards an increased percentage of Th2 and Th2 like Tfh cells (Fig 11). These results from both dLN and spleen show that JQ1 can differentially impact GATA3+ cells and antigen-specific Tfh while not affecting the Tfr subpopulation.

To assess functional consequences of JQ1 BET bromodomain inhibition, levels of cytokine production after T cells activation were studied. Nine days after s.c and i.p 1W1K1-OVA immunization and i.p JQ1 treatment, spleen CD44+CXCR+ cells were purified and re-stimulated in vitro either with with PMA/Ionomycin (Fig 12A) or Ea2

(Fig 12B). Splenic T cells shown a significant decrease of cells producing CXCR5 and INF-γ by intracellular staining in the JQ1 condition after activation using PMA/Ionomycin. In contrast, no significant changes for these two cytokines were seen with Ea2. IL4 production did not show any change neither with PMA/Ionomycin nor Ea2 stimulation. These results show that JQ1 can alters Thf and Th1 but not Th2 cytokines production.

Finally, to evaluate the impact of JQ1 on the outcome of B cells in vivo, OVA immunized mice were assessed, dLN (Fig 13A) and splenic B cells (Fig 13B) were first count and specific B220+ CD138+ OVA+ cells were analyzed by flow cytometry. We showed a significant increase in dLN and splenic B cellularity in mice treated with JQ1. No significant change in OVA-specific BCR were showed.

Overall, this series of experiments showed that JQ1 induce modulate T cells responses toward a specific antigen and then alters B cells responses and class switching.

Discussion

Inappropriate or exacerbated humoral immune responses are major contributors to immunopathology, notably in conditions where highly pro-inflammatory class-switched Igs such as IgG or IgE are secreted in excess. In addition some of these antibodies can themselves behave as natural adjuvants for Th cells [14], [15], with the risk of immune response runaway. Current therapy of human immunopathology and notably of IgE-mediated allergy relies on a variety of drugs mostly acting through T-cells with broad anti-inflammatory and/or immunosuppressive effects. Besides the drastic use of monoclonal antibodies eliminating B-cells, few compounds can target B-cell function and none are able to directly affect the CSR process. It would thus be of considerable interest to identify drugs acting at the level of CSR and more specifically controlling the production of the most pro-inflammatory class-switched Ig.

CSR in B-cells is modulated by their interactions with other cell lineages, mostly T-cells and by multiple *cis*-regulatory elements, notably 3' IgH enhancers which locally promote AID recruitment [10], [16]. Convergent data have indicated that the molecular process of

CSR is favored by the specific occurrence of single-stranded DNA structures in S-regions. This would notably differentially recruit AID to generate double-strand DNA breaks in S-regions rather than simply mutations as effected in V-regions. On the other hand, several *trans*-regulatory elements are known to promote CSR by regulating germinal transcription and Ig production such as bromodomain protein chromatin readers. These factors might thus have a dual role both on one hand by interacting with enhancers and super-enhancers and on the other hand by participating in the repair complex of broken DNA ends [5].

Based on these elements, we wished to evaluate whether a drug known to i inhibit the function of bromodomain proteins, and notably to alter their role in the process of repair of CSR breaks [5], might directly impact the mechanistic of CSR by acting on the 3'RR superenhancer. BET inhibitors, have been studied in detail for their ability to inhibit tumor growth, they were never explored for their potential impact on immune humoral responses by modulating T cells.

In the present study, we showed that JQ1, a BET inhibitor previously documented for anti-cancer activity [3], can be used at doses up to 40nM without severe toxicity on cultured B cells *in vitro,* or can be administered to mice at the dose of 50mg/kg/day without major toxic effects. Under these conditions we confirmed a direct effect of JQ1 on CSR by following BCR expression on cell membranes of *in vitro* stimulated B-cells. The same was true *in vivo* immunized mice when specific Ig producing cells were quantified by ELISPOT, showing an increase in IgM producing cells *vs* a decrease in IgG producing cells. Consistently, an Ig secretion defect was seen *in vitro* as well as *in vivo* for class-switched Ig.

We showed that IgH constant gene transcripts specific for the pre-CSR stage were globally increased while switched transcripts decreased. Noticeably, AID loading on S-regions was also clearly decreased, supporting that not only the repair step, but also the initiation of DNA lesions was affected, while CSR junctions globally occurred in DNA recovered from activated B-cells. The positions of breaks within S regions also became

more closely dependent on AID consensus motifs. This altogether indicated a specific B-cell maturation defect targeting CSR.

To evaluate the functional effects of this CSR blockade, we followed specific Ag responses in mice in the ovalbumin sensitization scheme classically used for inducing allergic airway inflammation. Importantly, objective biological markers of allergic asthma triggered by intranasal administration of the Ag in ovalbumin-immunized mice were significantly reduced in mice receiving JQ1, with a global efficiency close to that of steroids, and a notable reduction in all inflammatory cell counts in BALF. Importantly, markers from the T-cell branch of inflammation were in parallel affected.

To better understand the overall impact of bromodomain inhibition on the immune response, and also on T cells, we also performed immunizations of animals receiving doses of 50 mg/kg/day JQ1. The final analyses showed strong modulation of T responses, modifying their equilibrium in favor of Th2 polarization. These data suggest that the decrease in CSR following inhibition of bromodomain proteins is related to B-intrinsic modifications with a decrease in accessibility of the switch regions due to a functional alteration of the 3'RR superenhancer, but also integrates modulation of interactions with TFH cells during the development of the immune response.

However, both *in vivo* and *in vitro* experiments concur to demonstrate that BET contributes to CSR in intact cells. The CSR defect imposed by JQ1 *in vitro*, translated *in vivo* with lowered numbers of cells undergoing CSR after immunization, defective secretion of class-switched Ig, and reduced development of allergy biological parameters.

The present study with JQ1 indicates that bromodomain ligands can decrease the functional interactions of AID with S-regions and with the 3'RR SE which controls CSR. Looking for bromodomain ligands with improved specificity for immune cells and CSR now appears as a promising goal in order to lower toxicity and more specifically target the production of class-switched Ig, an option that would be of interest in many diseases such as in auto-immune or immuno-allergic conditions.

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Figures

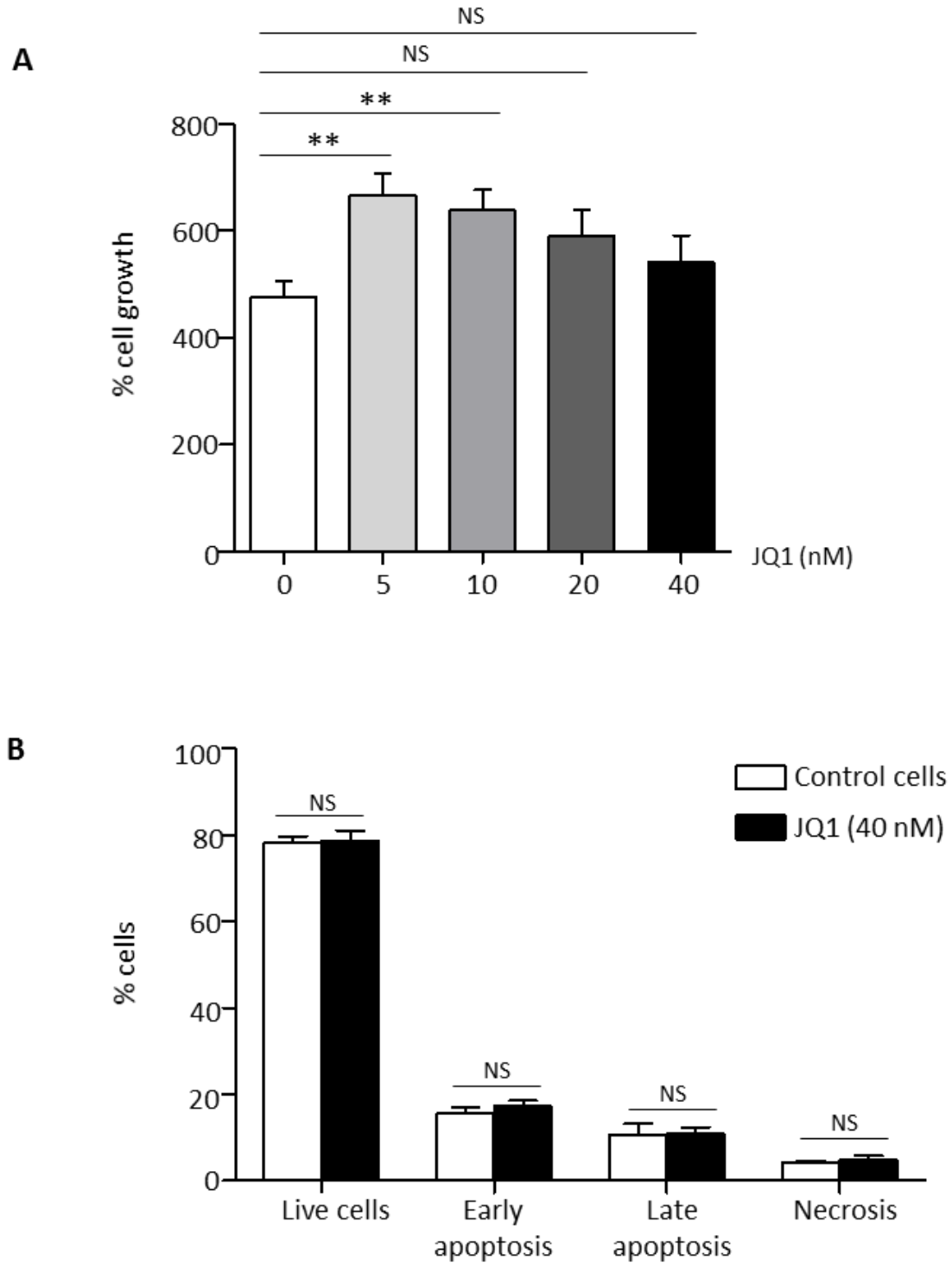

Figure 1: Effect of JQ1 on cell growth and viability of mouse primary B cells in vitro.

(A) Mouse primary B cells stimulated by LPS-IL-4 for 4 days and treated with 0, 5, 10, 20 or 40 nM JQ1. Proliferation was measured by MTS assay. (B) Cell survival and death were analyzed by staining with AnnexinV-7AAD after 4 days culture as in A and in the presence of 40 nM JQ1. Data represent mean % cell growth ± SEM for 6 mice. NS: not significant. $^{**}P<0.01$ compared with untreated cells.

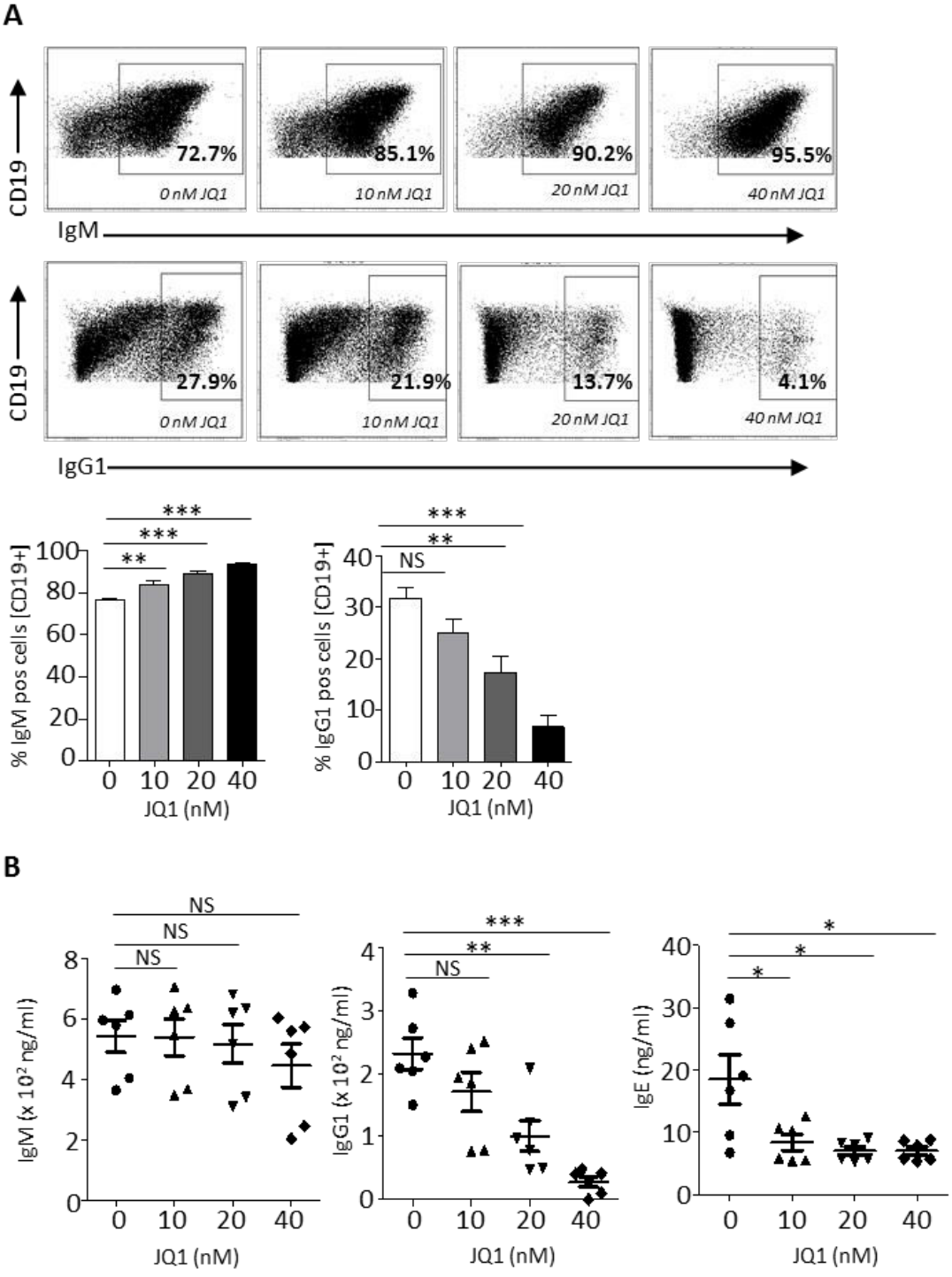

Figure 2: JQ1 decreases CSR in stimulated primary mouse B cells.

(A) Primary mouse B cells were stimulated with LPS and IL-4 and treated with 10, 20 and 40 nM JQ1. Cells were collected after 4 days culture and stained for IgM, IgG1 and CD19 for flow cytometry analysis. Histograms (bottom) represent mean % ± SEM for 6 mice. ***P*<0.01, ****P*<0.001 compared to untreated cells. Gates from a representative cytogram are shown (Top). (B) Supernatants from *in vitro* stimulated splenocytes (treated 4 days with LPS and IL4 in the presence of 10, 20 or 40 nM JQ1) were quantified by ELISA for the production for IgM, IgG1 and IgE secretion. Data represent mean % cell growth ± SEM for 6 mice. NS: not significant. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with untreated cells.

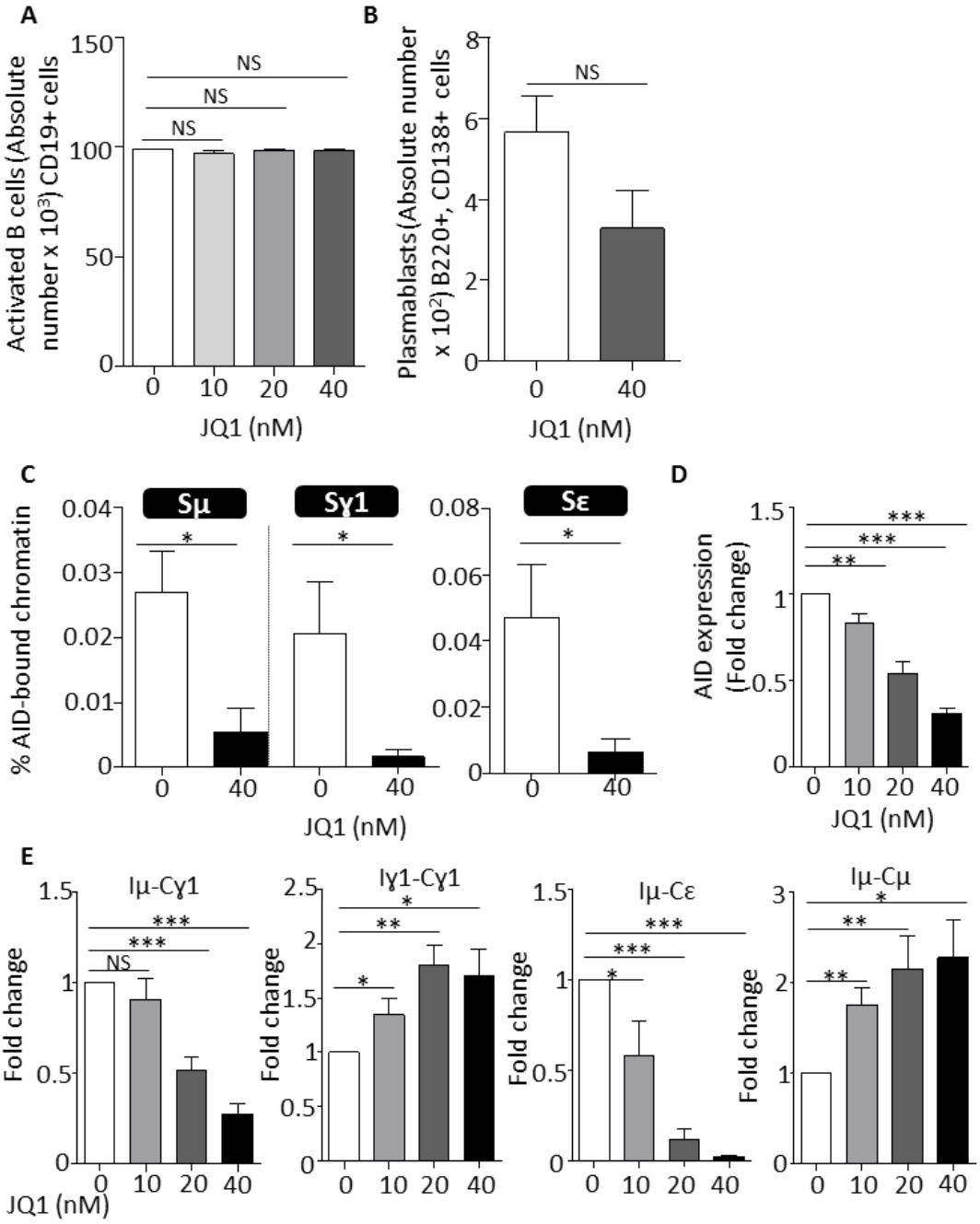

Figure 3: JQ1 decreases post switch IgG1 and IgE heavy chain transcripts while increasing IgM and IgH germline transcripts in primary mouse stimulated B cells

(A) Absolute numbers of B lymphocytes in day 4 LPS-stimulated cultures and of (B) plasmablasts, with or without JQ1 treatment. (C) ChIP experiments with anti-AID Ab and DNA evaluation by specific QPCR, showing AID-recruitment to Sμ, Sε and Sγ1-regions in cells stimulated with LPS+IL-4. (D) AICDA gene expression (encoding AID) by splenocytes stimulated by LPS+IL4 for 3 days and treated with 10, 20 and 40 nM JQ1. (E) Germline Cγ1 and Cμ transcripts and post-switch IgG1 and IgE (Iμ-Cγ1 and Iμ-Cε) transcripts were analyzed by RT-qPCR in B cells stimulated by LPS-IL-4 for 4 days in the presence of 10, 20 or 40 nM JQ1. Histograms represent mean fold change ± SEM for 6 mice. NS: not significant. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the control untreated cells.

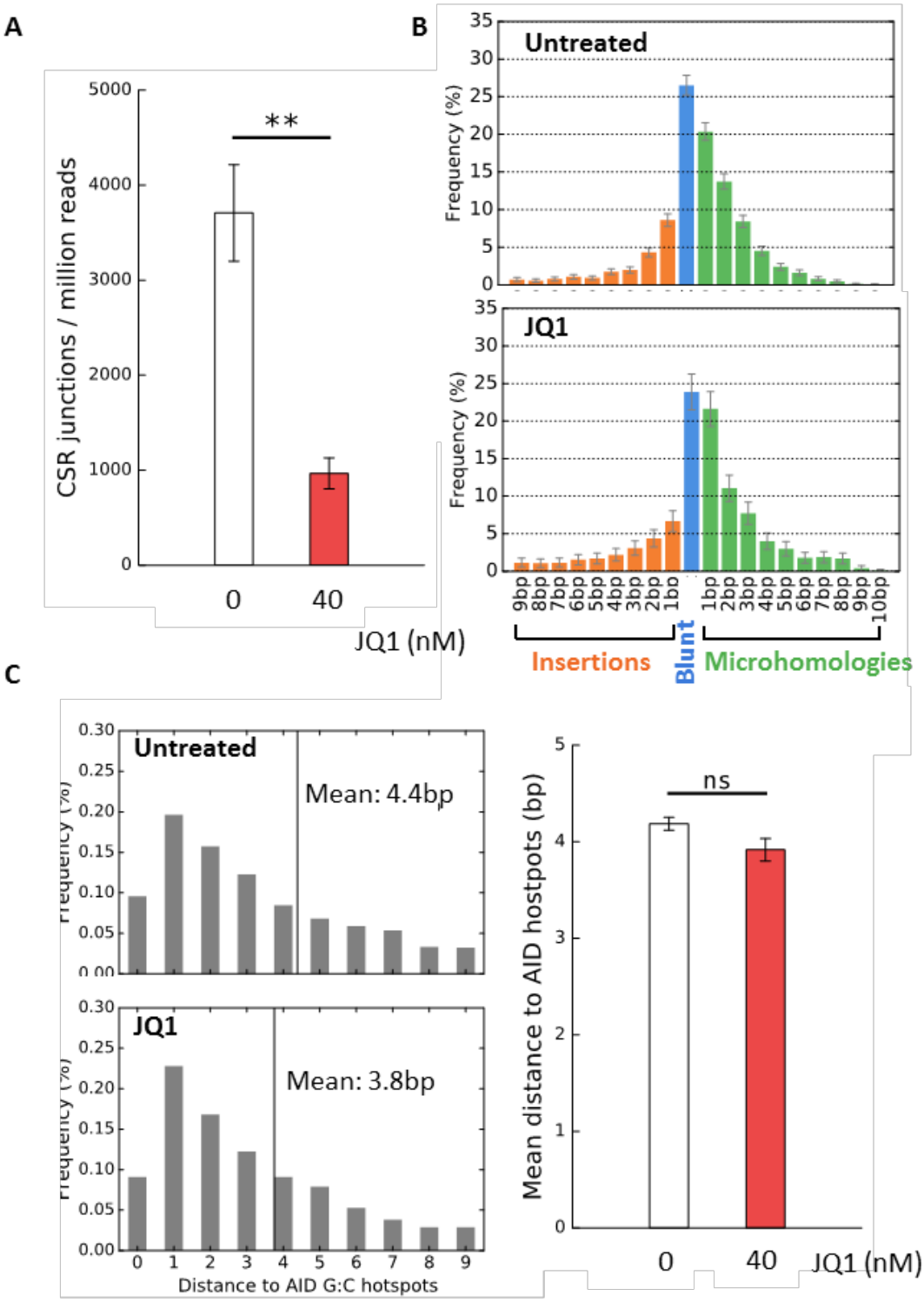

Figure 4: JQ1 impeded CSR and affected AID-induced break localization.

CSR junctions from stimulated primary mouse B cells were amplified by PCR and sequenced by NGS. Counts of identified junctions (A), junction structures (B) and relative position of breaks in Sγ1 to AID hotspots (C) were obtained from CSReport sequence analysis. Data represent mean ± SEM for 6 mice (A, C) or one representative sample (B, C). NS: not significant, **P<0.01 compared with untreated control cells.

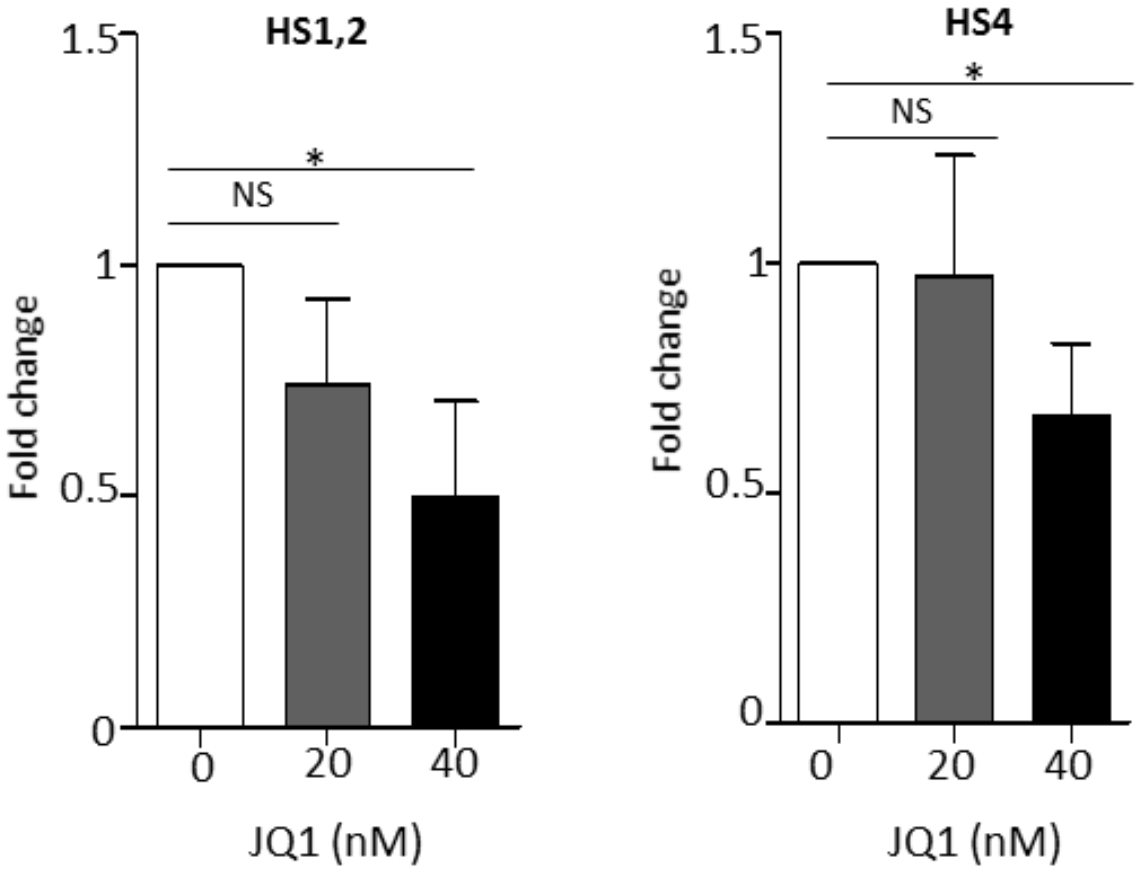

Figure 5: JQ1 affected 3'RR transcription.

Hs1,2 and Hs4 transcripts were evaluated by RT-qPCR of RNA from splenocytes stimulated with LPS and IL4 for 4 days. (Data represent mean signal ± SEM from 6 mice. NS: not significant, *P<0.05.

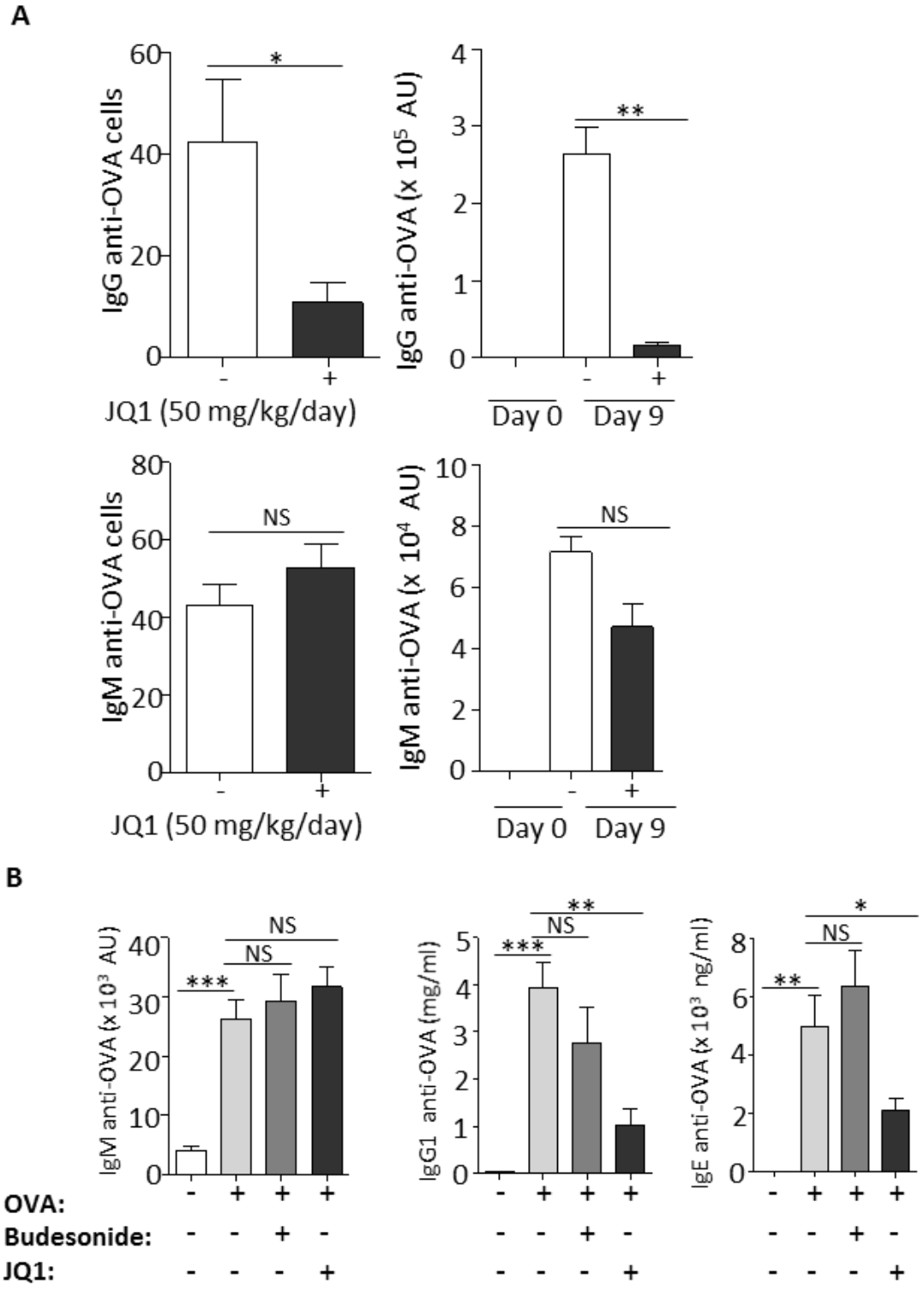

Figure 6: JQ1 inhibited class-switching *in vivo* and decreased production of specific antibodies.

(A) Mice administered daily with 50 mg/kg JQ1 were assayed after primary immunization for numbers of splenocytes producing Ag-specific IgM or IgG cells (upper and lower left panels) in ELISPOT assays, and for levels of Ag-specific Abs (upper and lower right panels) in ELISA assays. (B) Mice administered daily with JQ1 were assayed after repeated rounds of i.p. and i.n. immunization by ovalbumin for levels of Ag-specific IgM, IgG1 and IgE Abs after 25 days. NS: not significant. **P*<0.05, ***P*<0.01, ****P*<0.001.

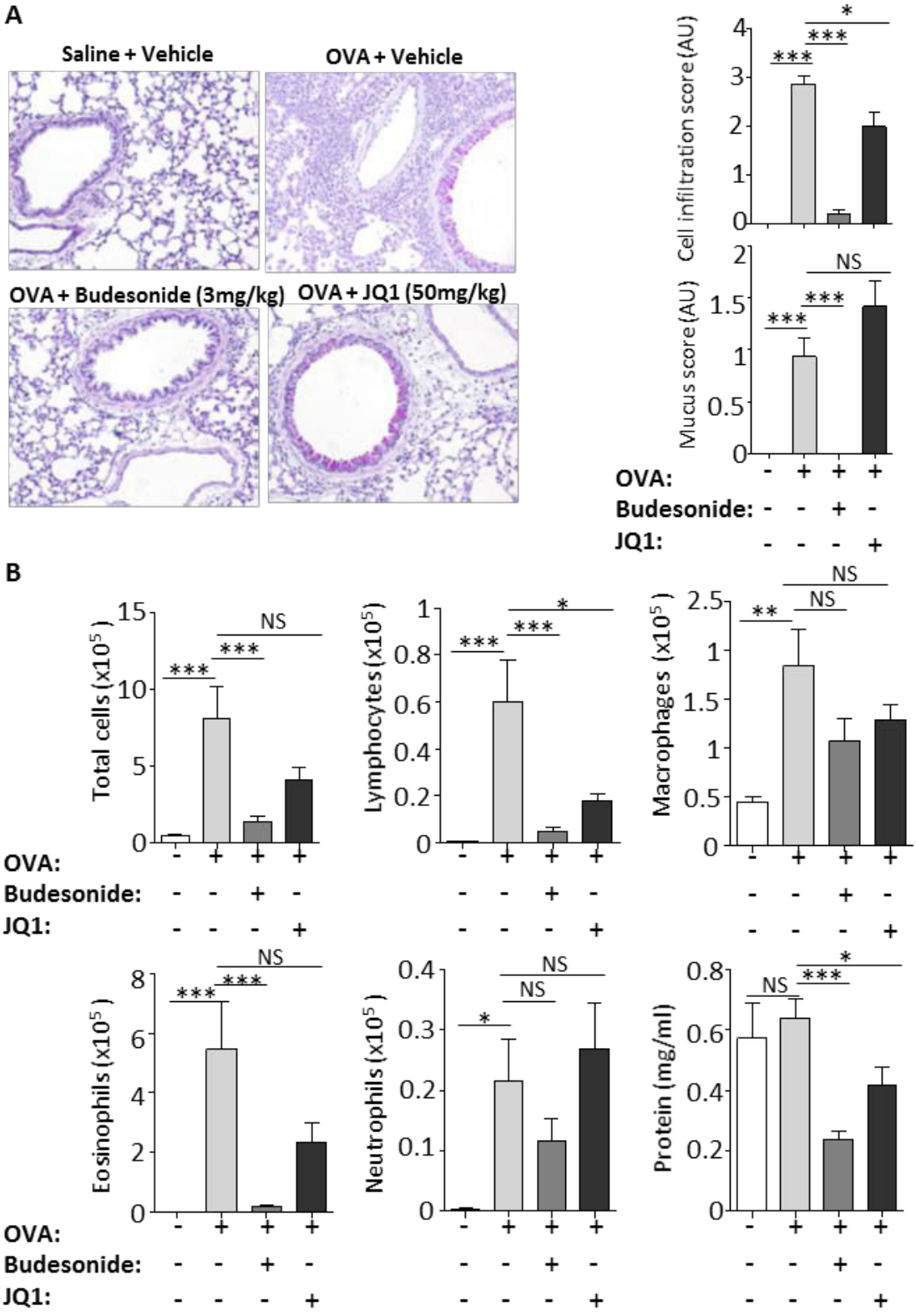

Figure 7: Lung tissue inflammation is lowered by JQ1 in mice with OVA-induced allergic asthma.

In an allergic asthma model after repeated *i.p.* and *i.n;* antigen administration, mice administered daily with 50 mg/kg JQ1 for 4 weeks displayed (A) a less abundant cell infiltration of lung, lower inflammation and (B) lower counts of total cells, macrophages, eosinophils and lymphocytes, together with lower protein levels, in broncho-alveolar fluid (BALF).

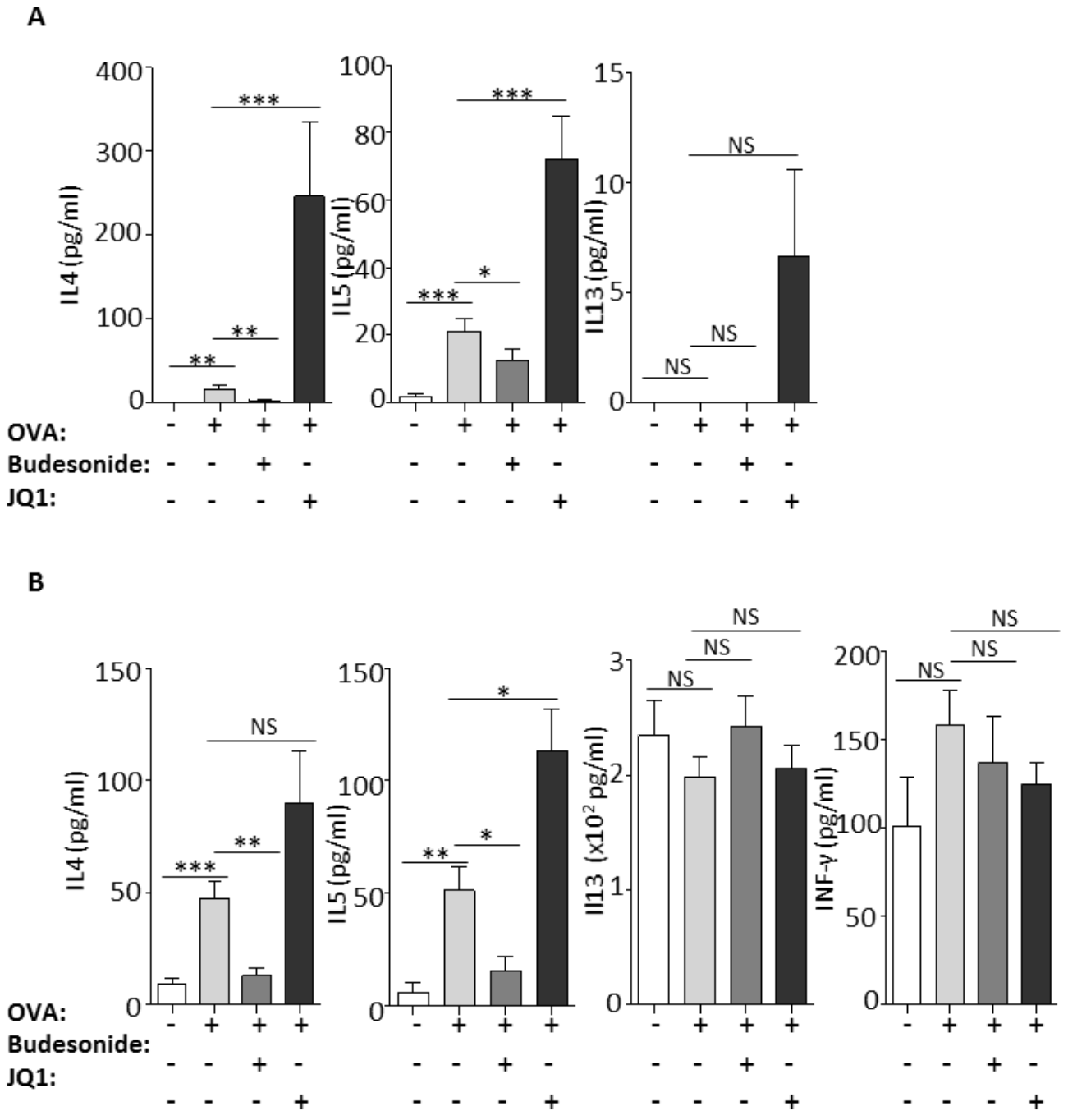

Figure 8: Th cytokines in mice with OVA-induced allergic asthma.

In an allergic asthma model after repeated *i.p.* and *i.n.* antigen administration, mice administered daily with steroids, and to a lesser extent mice receiving JQ1, displayed (A) higher levels of IL-4, IL-5, and IL-13 in broncho alveolar fluid (BALF), and (B) higher levels of IL-4 and IL-5 but no significant change IL-13 and IFN-γ in lungs.

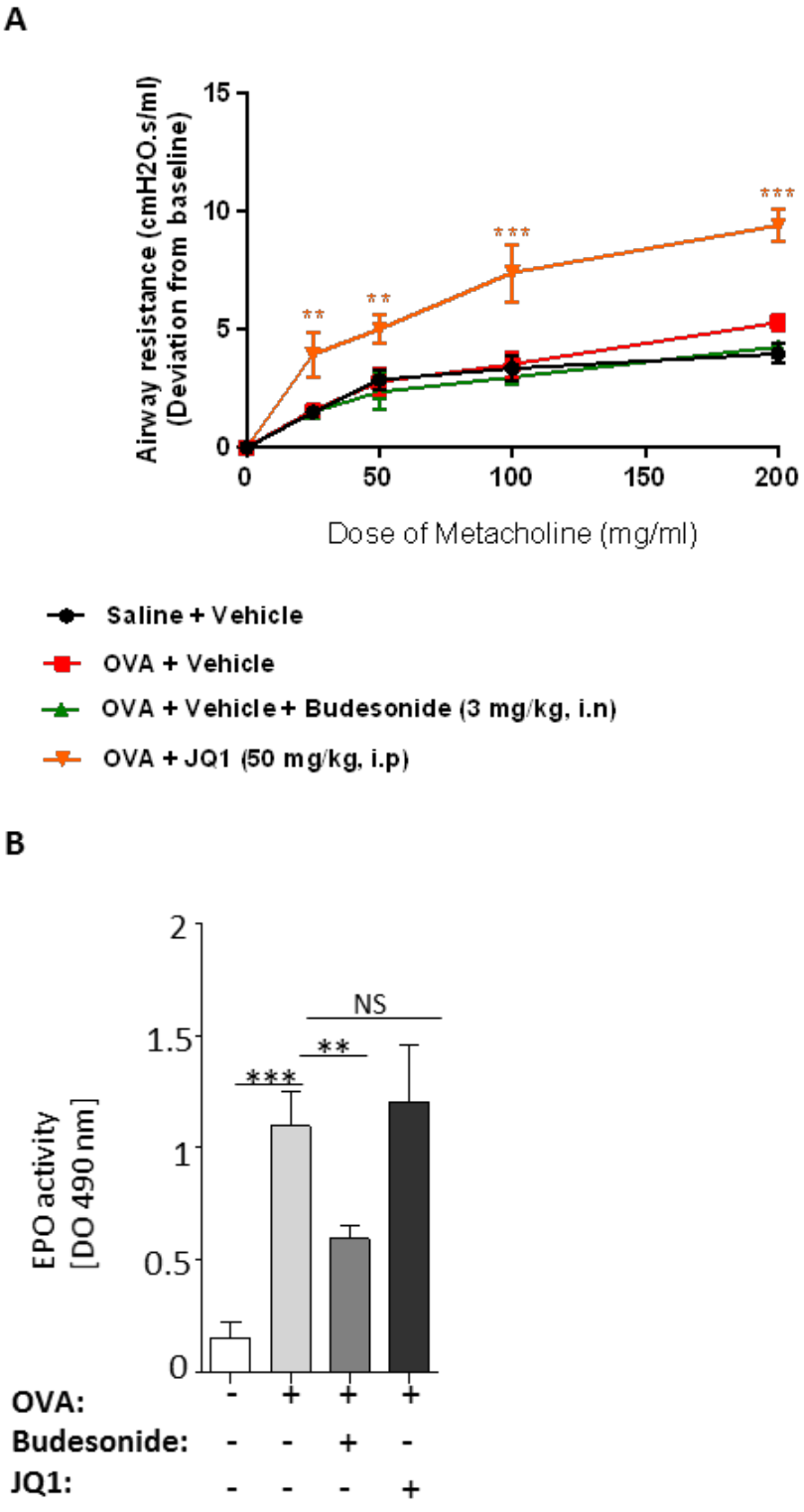

Figure 9: Functional parameters of asthma and EPO activity in mice with OVA-induced allergic asthma.

After repeated *i.p.* and *i.n.* antigen administration triggering allergic asthma and in comparison to those simply receiving antigen, mice receiving daily steroids showed significantly improved (A) airway resistance (no effect of JQ1 on airway resistance).(B) JQ1 had no effect on lung eosinophil peroxidase activity while steroids improved EPO activity.

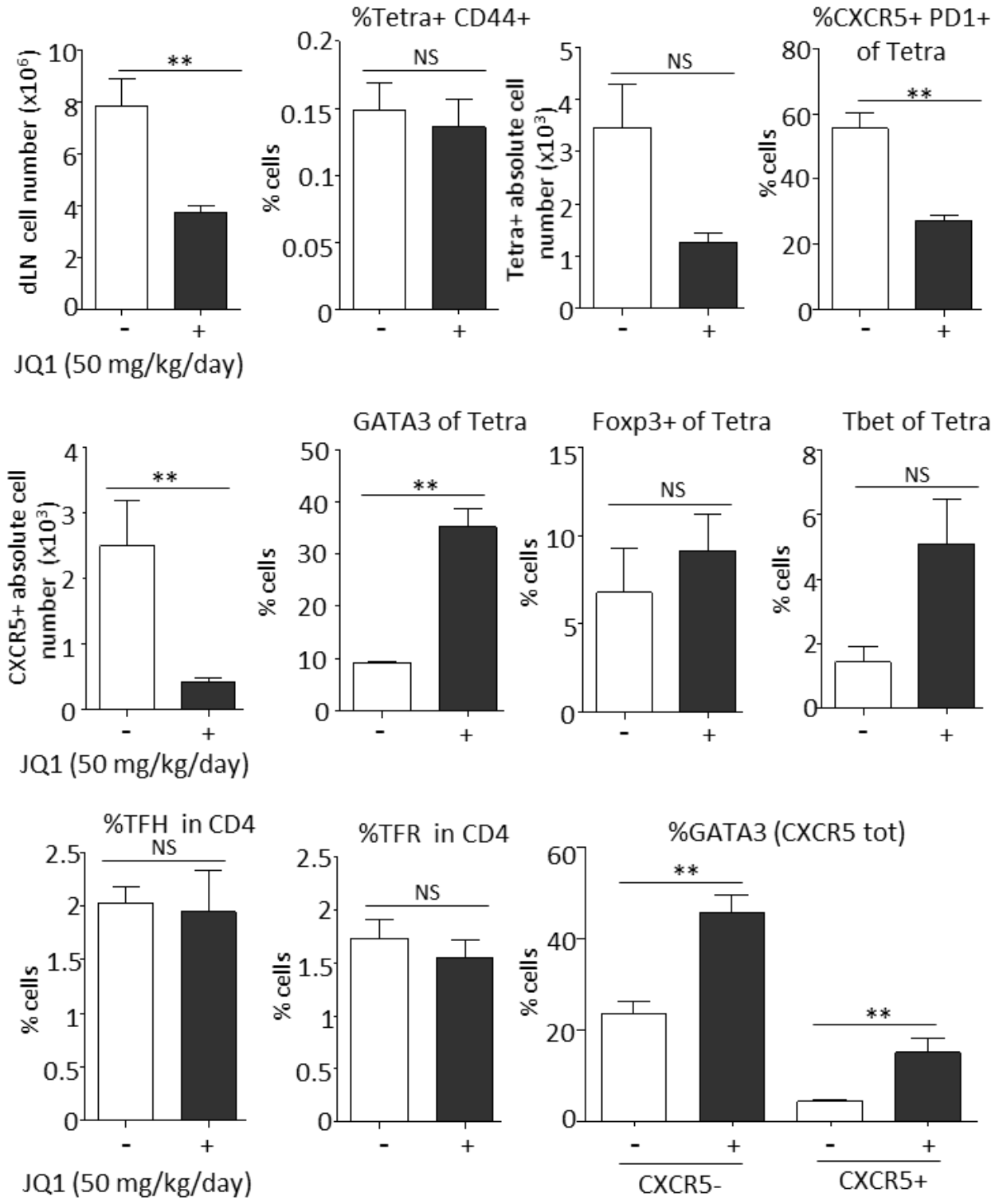

Figure 10: Effect of JQ1 on T cells in mice draining lymph nodes (dLN).

Total cell number in dLN was determined. Expression of T cell markers (GATA3, Foxp3, Tbet, CXCR5, TFH, TFR) were assessed by flow cytometry. NS: not significant. ***P*<0.01 compared with control mice.

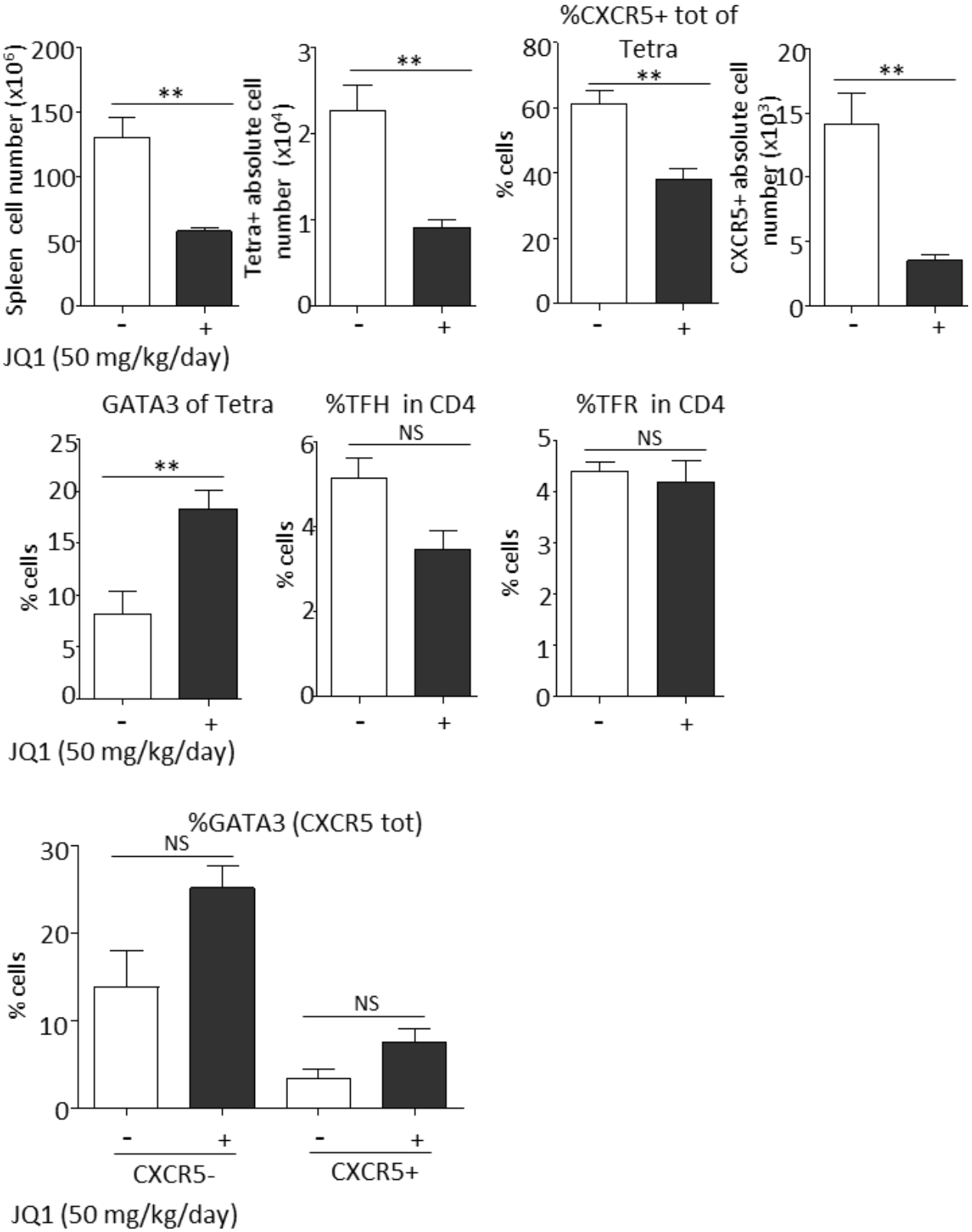

Figure 11: Effect of JQ1 on T cells in mice spleen.

Total cell numbers in mouse spleens were determined. Expression of T cell markers (GATA3, CXCR5, TFH, TFR) were assessed by flow cytometry. NS: not significant. ***P*<0.01, compared with control mice.

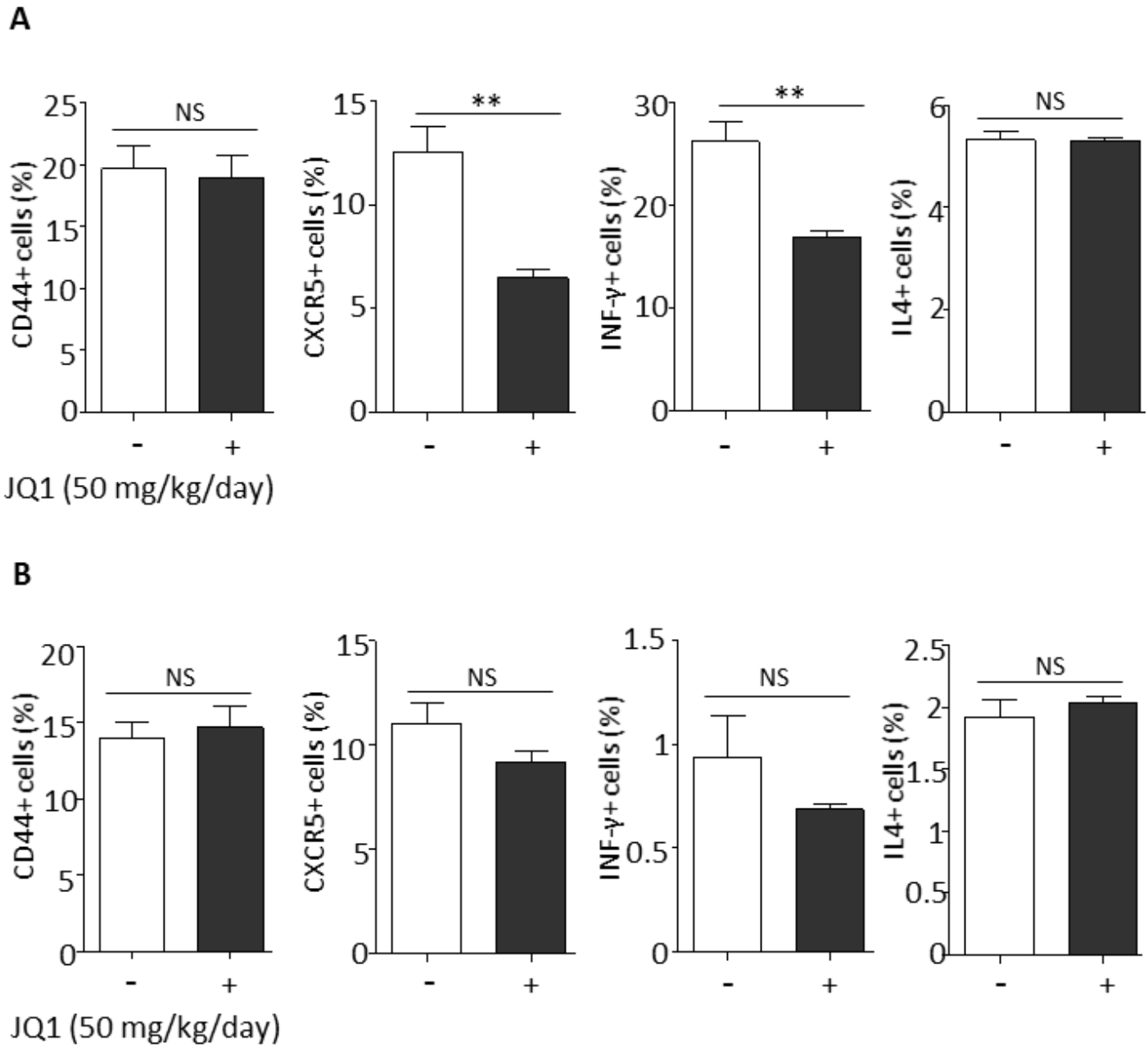

Figure 12: Effect of JQ1 on T cell cytokines.

T cell cytokines were measured after PMA Ionomycin (A) and Ea2 (B) stimulation. CD44, CXCR5, INF-γ and IL4 were assessed by flow cytometry. NS: not significant. ***P*<0.01 compared with control mice.

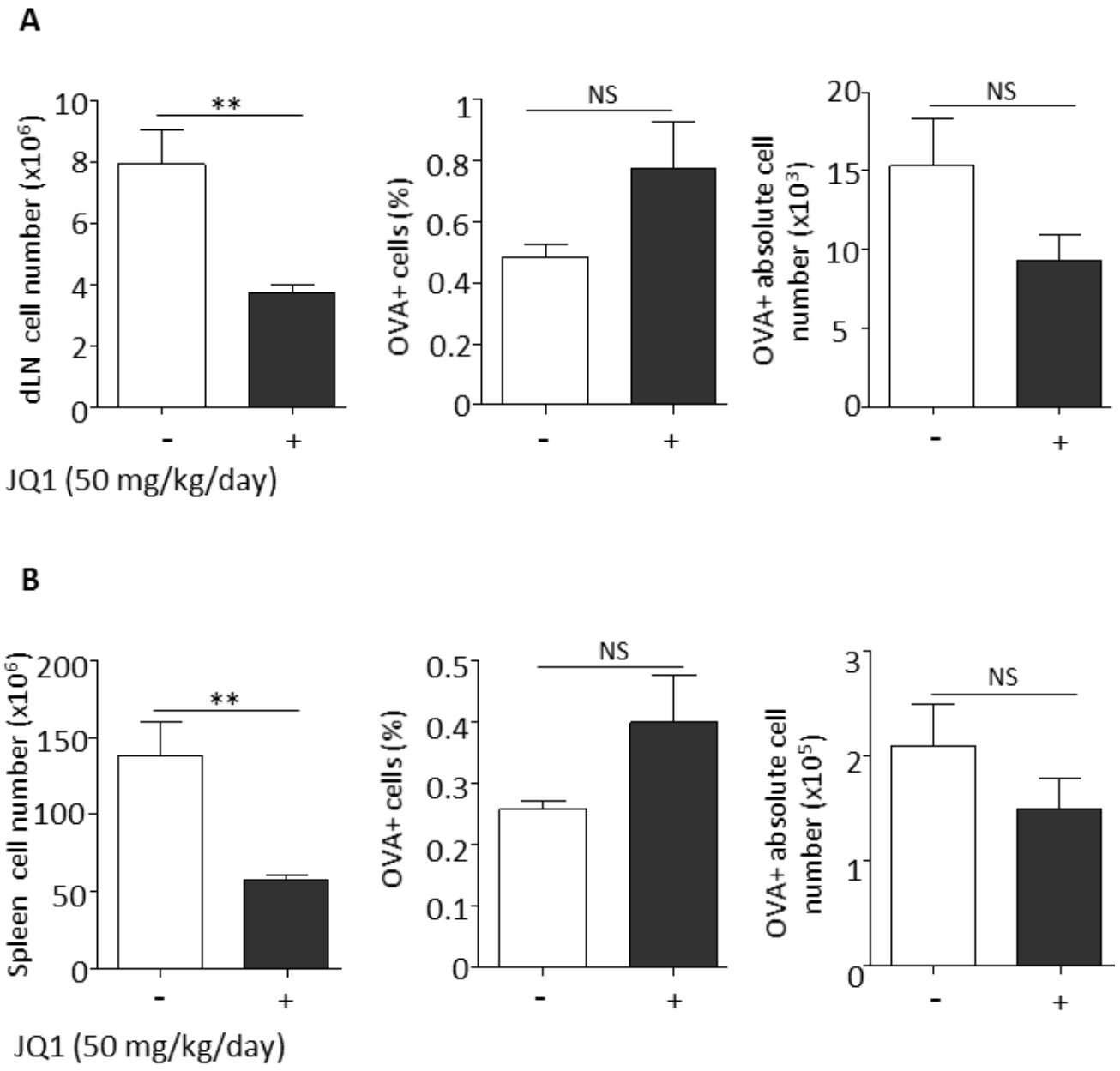

Figure 13: Effect of JQ1 on B cells.

dLN (A) and spleen (B) B cells were assessed for cellularity and OVA specific cells in mice treated or not with JQ1 50 mg/kg/day. NS: not significant. ***P*<0.01 compared with control mice.