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Book Chapter

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Author contributions: Nour Ghazzaui, Melissa Ferrad, Hussein Issaoui and Tiffany Marchiol performed experiments. Sandrine Le Noir, Jeanne Cook-Moreau and Yves Denizot analysed data and wrote the paper. Justine Pollet, Sandrine Le Noir and Yves Denizot analyzed transcriptomic data. Yves Denizot obtained financial grants.

Abstract

Numerous B-cell lymphomas feature translocations linking oncogenes with the immunoglobulin heavy chain (IgH) locus. Epigenetic drugs such as histone deacetylase inhibitors (HDACi) have been approved to treat certain T-cell and B-cell lymphomas. Transcription, accessibility and remodelling of the IgH locus are under the control of the potent *cis*-acting 3' regulatory region (3'RR) suggesting that its targeting would be of therapeutic interest to reduce oncogenicity *in vivo*. We thus investigated HDAC recruitment and HDACi effects on 3'RR activation in normal mature mouse B-cells and whether or not results paralleled those obtained with mature mouse B-cell lymphomas. HDAC1 was recruited to the hs1,2 enhancer element in the centre of the 3'RR palindromic structure during mouse B-cell activation. The HDACi SAHA (suberanilohydroxamic acid) reduced B-cell growth and affected B-cell class switch recombination (CSR) in an isotype-dependent

manner (decreased IgG_3 and increased IgG_1) without any obvious effect on complete IgH locus transcription (including 3'RR eRNA production). Results were markedly different in mature mouse B-cell lymphomas with no HDAC1 recruitment to the 3'RR but recruitment of the CBP histone acetyl transferase (HAT) to hs3a and hs3b elements bordering the 3'RR palindromic structure. No corresponding effect of SAHA on *in vitro* growth of freshly isolated B-cell lymphomas was found. In conclusion, differences in HDAC recruitment and HDACi effects exist between normal mature B-cells and mature B-cell lymphomas. The precise mechanism underlining beneficial use of HDACi to treat several B-cell lymphoid malignancies remains to be elucidated but is clearly not mediated by direct action on IgH 3'RR enhancers.

Introduction

After encountering antigen, B-cells undergo class switch recombination (CSR) that substitutes the constant $(C)_\mu$ gene with C_γ, C_ε or C_α, thereby generating IgG, IgE and IgA antibodies with new effector functions but the same antigenic specificity [1]. CSR is controlled in *cis* by the immunoglobulin heavy chain (IgH) 3'regulatory region (3'RR) that is essential to target the DNA-editing enzyme activation-induced deaminase (AID) onto DNA switch (S) acceptor regions [2,3]. The 3'RR is a complex element with four transcriptional enhancers (namely hs3a, hs1,2, hs3b and hs4) encompassed in a unique and functional 3D palindromic architecture [4,5]. The 3'RR has transcriptional activator activity from immature to mature B-cell stages [6,7] and is the conductor for Ig production [8]. Histone deacetylase inhibitors (HDACi) are a class of compounds reported to modulate gene expression by remodelling chromatin accessibility. HDACi are reported to affect *in vivo* and *in vitro* B-cell responses [9,10]. HDACi are approved for treating certain T-cell and B-cell lymphomas [11,12]. Deciphering the molecular events or mechanisms that underlie the B-cell HDACi-induced effect is of interest not only for basic B-cell immune responses but also for a better understanding of the rationale for the use of HDACi in B-cell lymphoma treatment. HDAC1 has been previously reported to bind to the IgH 3'RR during B-cell

activation suggesting a direct role for HDAC recruitment on 3'RR regulatory functions [9]. The aim of this study was to determine if the effect of the HDACi SAHA on B-cell responses is mediated by a direct effect on the IgH locus through a repressive effect on 3'RR enhancer activation or to a more pleiotropic effect on all cell proliferation/activation/survival pathways.

Material and Methods

Mice - 129 *wt* mice and ΔIRIS mice [4] were used. Mice were housed and procedures were conducted in agreement with European Directive 2010/63/EU on animals used for scientific purposes applied in France as the « Décret n°2012-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins scientifiques ». Accordingly, the present project (APAFiS≠13855) was authorized by the « Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche ».

Spleen Cell Cultures for Growth, CSR and Ig Determinations - Single-cell suspensions of $CD43^-$ spleen cells of *wt* mice (8-12 week old, males and females) were cultured 3 days at $1x10^6$ cells/ml in RPMI 1640 with 10% foetal calf serum (FCS), 5μg/ml LPS with or without 20ng/ml IL4 (PeproTech, Rocky Hill, NJ) in the presence or not of various concentrations of SAHA. At day 3, cell proliferation (six replicates) was evaluated using the MTS assay. At day 4, CSR was evaluated by incubating cultured spleen B-cells with anti-B220-SpectralRed (PC5)-labelled antibodies (Biolegend, ref: 103212), anti-IgG_1- (ref: 107020) and anti-IgG_3- (ref: 110002) fluorescein-isothiocyanate (FITC)-labelled antibodies (Southern Biotechnologies) and analyzed on a Fortessa LSR2 (Beckton-Dickinson). At day 3, $1x10^6$ cells were cultured for 24 hours in growth medium without LPS/cytokine/SAHA. Supernatants were recovered and stored at -20°C until used for Ig quantification (ELISA assays specific for IgG_1 and IgG_3) [3,13]. In a separate set of experiments spleen B-cells were directly labelled with anti-B220-, anti-CD19-, anti-IgM-, anti-IgD- and anti-CD138- antibodies to assess the percentage of transitional

B-cells ($B220^+CD19^+IgM^+IgD^-$), mature B-cells ($B220^+CD19^+IgM^+IgD^+$) and plasma B-cells ($B220^+CD19^+CD138^+$) in our experimental conditions.

ChIP Experiments - Single-cell suspensions of $CD43^-$ spleen cells from *wt* mice and ΔIRIS mice were cultured 2 days at $1x10^6$ cells/ml in RPMI 1640 with 10% FCS and 5μg/ml LPS. ChIP experiments were done as previously described [3] with HDAC1 (ab7028, Abcam), HDAC2 (ab7030), HDAC3 (ab7029), CBP (ab2832) and PCAF (ab12188) specific antibodies. PCR primers for quantitative PCR were the following: hs4-Fw-ChIP 5'-CCATGGGACTGAAACTCAGGGAACCAGAAC-3'; hs4-Rev-ChIP 5'-CTCTGTGACTCGTCCTTAGC-3'; hs3b-Fw-ChIP 5'-TGGTTTGGGCCACCTGTGCTGAG-3'; hs3b-Rev-ChIP 5'-GGGTAGGGCAGGGATGTTCA CAT-3'; hs3a-Fw-ChIP 5'-GGGTAGGGCAGGGATGCTCACAT-3'; hs3a-Rev-ChIP 5'-GCTCTGGTTTGGGGCACCTGTGC-3'; hs1,2-Fw-ChIP 5'-AGCATACACTGGGACTGG-3'; hs1,2-Fw-ChIP 5'-CTCTCACTTCCCTGGGGTGTT-3'.

RNAseq Experiments - $CD43^-$ splenocytes were obtained from *wt* mice after 48h of *in vitro* stimulation ($1x10^6$ cells/ml in RPMI 1640 with 10% FCS) with 5μg/ml LPS ± 200 ng/ml SAHA. RNA was extracted using miRNeasy kit from QIAGEN, according to the manufacturer's instructions. Two pooled RNAs (each with three samples) were obtained for each stimulatory condition. RNA libraries were obtained using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina), according to the manufacturer's instructions. Libraries were sequenced on a NextSeq500 sequencer, using NextSeq 500/550 High Output Kit (Illumina) (Nice Sophia-Antipolis Functional Genomics Platform, France). Illumina NextSeq500 paired-end 2x150nt reads were mapped with STAR release v2.4.0a versus mm10 with gene model from Ensembl release 77 with default parameters. RNAseq data were deposited with the accession number GSE169690 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169690) and GSE169691 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169691).

Mature B-Cell Lymphomas - Freshly isolated $B220^+IgM^+IgD^+$ mature B-cell lymphomas were obtained from i*Myc*C_μ, i*Myc*E_μ and i*Myc*C_α mice [14-18]. ChIP experiments and proliferation studies were performed as for mature *wt* B-cells.

Results

HDAC1 is Recruited to the 3'RR hs1,2 Enhancer - A schematic representation of the IgH locus (not to scale) is shown in Figure 1A. The IgH 3'RR palindromic structure (to scale) with its four enhancer elements (hs3a, hs1,2, hs3b and hs4) and the IRIS sequences are represented. In this study we investigated $CD43^-$ mature spleen B-cells (*i.e.*, depleted $CD43^+$ T-cells and monocytes). About 90% of spleen B-cells had a mature $B220^+CD19^+IgM^+IgD^+$ phenotype. The remaining cells consisted of transitional B-cells (IgM^+IgD^-). Less than 1% expressed the CD138 plasmocyte antigen (Figure 1B). Confirming a previous study [9], ChIP assays indicated that HDAC1 binds to the 3'RR hs1,2 enhancer in LPS-stimulated mature B-cell splenocytes (Figure 2A). No significant HDAC1 binding was found for the 3 other 3'RR enhancers (*i.e.*, hs3a, hs3b and hs4). No hs1,2 HDAC1 binding was found in resting B-cell splenocytes (data not shown). In similar LPS-stimulated conditions no significant HDAC2 and HDAC3 binding to hs1,2 was found (Figure 2A). Deconstructing the palindromic 3'RR structure by deleting the 5'IRIS [4] precluded hs1,2 HDAC1 binding in mature B-cell (Figure 2B).

SAHA Effect on In Vitro Mature B-Cell Proliferation, CSR and Ig Synthesis - SAHA selectively inhibits HDAC I (such as HDAC1) and II classes. We thus investigated the role of SAHA on mature B-cell growth, CSR and Ig synthesis. As shown in Figure 3A, MTS assay indicated that SAHA decreased B-cell growth in a dose-dependent manner in two experimental conditions (LPS ± IL4). Flow cytometry analysis indicated that SAHA decreased IgG_3 CSR (LPS stimulation) and increased IgG_1 CSR (LPS+IL4 stimulation) (Figure 3B). ELISA experiments indicated that SAHA effects on IgG_3 and IgG_1 CSR paralleled those on IgG_3 and IgG_1 secretion (Figure 3C)

suggesting that levels of Ig productions depend on the number of switched cells.

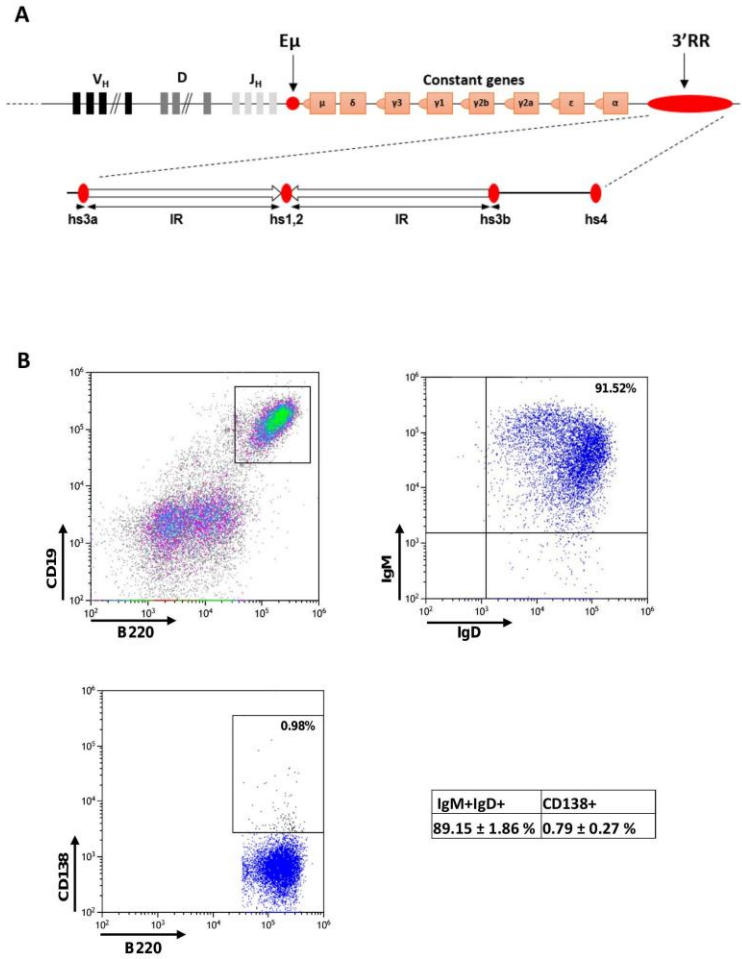

Figure 1: IgH locus and mature spleen B-cells. A: IgH locus (not to scale). The locations of variability (V), diversity (D), junction (J) and constant segments are indicated as well as 5'Eμ and 3'RR enhancers. The palindromic structure of the IgH 3'RR (to scale) with its four enhancer elements (hs3a, hs1,2, hs3b and hs4) and the IRIS sequences are represented.
B: Surface phenotype of $CD43^-$ spleen B-cells. % of IgM^+IgD^+ and IgM^+IgD^- cells were determined after gating on $B220^+CD19^+$ cells. The following labelled antibodies were used: B220-bv510, CD19-PE, IgM-FITC, IgD-bv421 and CD138-APC. Results are representative of 4 mice. Results are reported as mean ± SEM of 4 experiments.

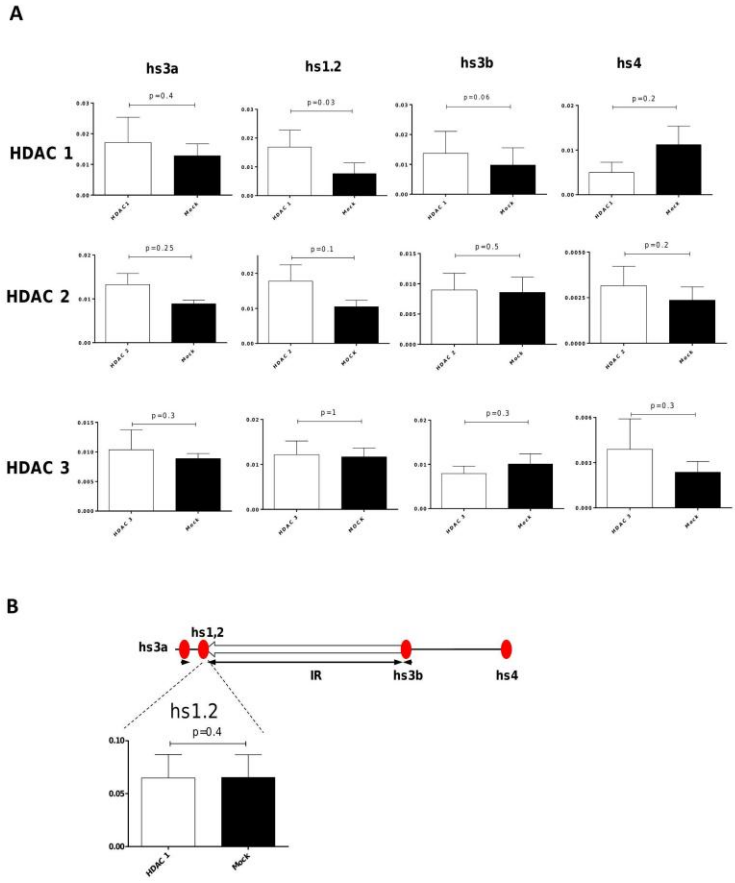

Figure 2: HDAC and 3'RR enhancers. A: HDAC1, HDAC2 and HDAC3 fixation on enhancer elements of the 3'RR. ChIP experiments were performed on 2-day LPS-stimulated spleen B-cells. Mean ± SEM of 5 experiments (significance with the student-*t*-test for paired data). The mock IP during ChIP is the control IP to avoid nonspecific antigen-antibody reactions. Another antibody (anti mouse rabbit IgG) which does not specifically bind HDAC was used. Quantitative PCR experiments were compared between samples treated with HDAC antibodies (white bars) and unspecific antibodies (mock, black bars). B: The 3'RR in ΔIRIS mice (to scale). HDAC1 fixation on the hs1,2 enhancer was studied on 2-day LPS-stimulated spleen ΔIRIS B-cells. Mean ± SEM of 6 experiments (significance with the student-*t*-test for paired data).

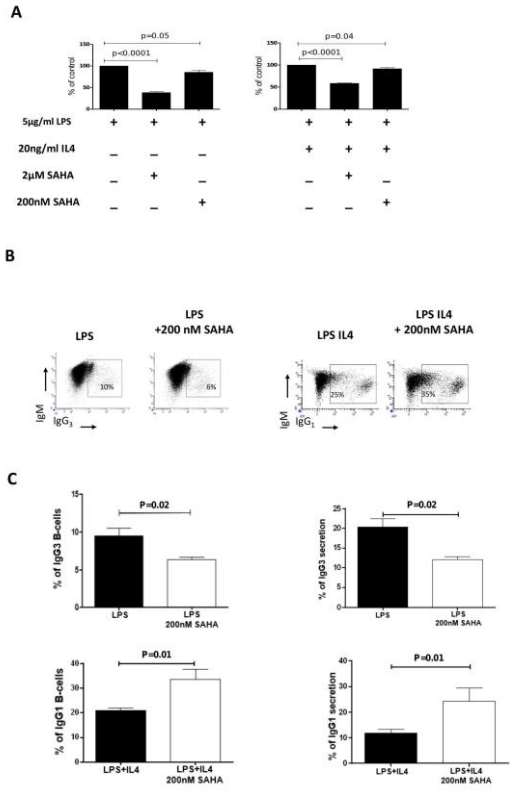

Figure 3: Influence of SAHA on *in vitro* mature B-cell growth, CSR and Ig secretion. A: Influence of SAHA on *in vitro* mature B-cell growth. Proliferation (six replicates) was evaluated by the MTS assay after 3 days stimulation with LPS (5 µg/ml) ± IL-4 (20 ng/ml) in the presence of various SAHA concentrations. Results (mean ± SEM of 4 independent experiments) are reported as % of variations as compared to stimulated cells without SAHA. Mann-Whitney *U*-test for significance. B: Influence of SAHA on *in vitro* mature B-cell CSR. Spleen B-cells were stimulated as in A. IgG_3 CSR (LPS stimulation) and IgG_1 CSR (LPS+IL4 stimulation) were evaluated by flow cytometry. Cells gated on $B220^+$ and/or $CD138^+$ cells were labelled with anti-IgM, anti-IgG_3 and anti-IgG_1 antibodies. A typical flow cytometry phenotyping was reported for each stimulatory condition. Results are reported as mean ± SEM of 4 and 5 independent experiments for IgG_3 and IgG_1, respectively. Mann-Whitney *U*-test for significance. C: Influence of SAHA on *in vitro* Ig secretion. Spleen B-cells were stimulated as in A and Ig secretion was evaluated at day 4 by specific ELISAs. Results are reported as mean ± SEM of 4 and 5 independent experiments for IgG_3 and IgG_1, respectively. Mann-Whitney *U*-test for significance.

SAHA Effect on IgH Locus Transcription - CSR and Ig synthesis are controlled through IgH locus transcription with 3'RR as a conductor [8, 13]. We then determined if the effect of SAHA on B-cell responses was mediated through an overall down regulation of IgH locus transcription and/or a specific targeting of 3'RR activation assessed through generation of enhancer RNA (eRNA). RNAseq experiments indicated that SAHA treatment had no effect on 3'RR activation judging by the expression of 3'RR eRNA (both sense and antisense) (Figure 4). Since the 3'RR controls IgH locus transcription, the lack of SAHA effect on 3'RR activation was consistent with the absence of obvious effects of SAHA on $I_{\gamma 3}$-$C_{\gamma 3}$ transcription (LPS stimulation) and $I_{\gamma 1}$-$C_{\gamma 1}$ transcription (LPS+IL4 stimulation) of the IgH locus (Figure 4). We next analyzed genome-wide gene expression between LPS- and LPS+SAHA-treated mature spleen B-cells. Data indicated 112 down-regulated genes by SAHA (log2 fold change threshold > 1, adjusted p value <0.05) (Table 1). Numerous genes were implicated in growth processes, in epigenetic-related processes (Smyd2, Nek2, Trmt10a, Rnmtl1) and histone markers (Hist1h2ai/2an/2ah/2ag/2af/2ab/3b/3g) highlighting efficiency of SAHA treatment. Seventy seven genes were up-regulated in response to SAHA but without evident links to HDAC/AID pathways and switch processes (Table 2).

Table 1: Down-regulated genes in response to SAHA. $CD43^-$ spleen B-cells were stimulated for 2-day with LPS ± SAHA. Genes implicated in growth processes are colored in yellow. Genes implicated in epigenetic-related processes and histone markers are colored in bleu. Log2 fold change threshold > 1 and adjusted p value <0.05.

Down regulated genes								
Genes	log2 fold change	P adjusted	Genes	log2 fold change	P adjusted	Genes	log2 fold change	P adjusted
Hspd1	-1.11	4.15e-6	Cdca8	-1.05	7.90e-4	Kpna2	-1.07	4.63e-6
Sgol2a	-1.03	0.001	Rcc1	-1.05	3.07e-5	Birc5	-1.07	9.03e-5
Ctla4	-1.71	0.004	Sesn2	-1.27	0.006	Idi1	-1.03	1.99e-4
Il10	-1.28	3.07e-5	Hmgn2	-1.10	5.94e-5	Hist1h2ai	-1.00	0.002
Aspm	-1.08	6.00e-5	Alpl	-1.15	0.001	Hist1h2an	-1.18	8.18e-4
Nuf2	-1.01	0.001	Cldn12	-1.10	0.036	Hist1h2ah	-1.29	1.89e-4
Cenpf	-1.06	1.55e-4	Dbf4	-1.00	1.45e-4	Hist1h2ag	-1.12	0.002
Smyd2	-1.26	0.001	Ncapg	-1.10	6.00e-5	Hist1h2af	-1.34	6.22e-6
Nek2	-1.01	0.001	Adap1	-1.01	0.002	Hist1h3g	-1.00	0.001
Suv39h2	-1.12	0.011	Asns	-1.41	9.08e-8	Hist1h2ab	-1.21	1.14e-4
Sapcd2	-1.07	0.034	RP23-5D6.6	-1.59	0.048	Hist1h3b	-1.25	3.07e-5
Snora17	-1.00	0.047	Ptgir	-1.35	4.06e-4	Eef1e1	-1.15	1.64e-4
Pkp4	-1.26	0.008	Slco3a1	-1.24	0.001	Cenpp	-1.13	0.011
Kif18a	-1.12	1.06e-4	Prc1	-1.19	2.51e-5	Nfil3	-1.64	0.001
Aven	-1.05	0.013	Lrrc32	-1.22	0.025	Trip13	-1.13	9.03e-5
Chac1	-2.14	1.14e-4	Plk1	-1.09	0.001	Ccnb1	-1.33	2.09e-8
Oip5	-1.03	0.010	Fbxo5	-1.13	4.94e-4	Ppap2a	-1.03	0.021
Nusap1	-1.09	3.07e-5	Prdm1	-1.43	1.65e-5	Rrm2	-1.05	8.49e-5
Bub1	-1.13	2.03e-5	Ppa1	-1.22	1.31e-6	Id2	-1.10	0.033
Eif2s2	-1.00	9.03e-5	Dna2	-1.06	0.002	Mis18bp1	-1.25	2.03e-5
Fam83d	-1.16	0.013	Cdk1	-1.21	4.72e-7	Cdc45	-1.05	2.79e-4
Mybl2	-1.08	3.18e-4	Hsp90b1	-1.04	1.08e-4	Tfrc	-1.03	3.07e-5
Ube2c	-1.10	4.20e-5	Parpbp	-1.01	0.016	Sgol1	-1.32	7.60e-5
Pfdn4	-1.08	0.005	Nup37	-1.05	0.001	Kif20a	-1.15	7.85e-4
Aurka	-1.21	9.24e-5	Hmgb2	-1.11	6.66e-4	Tubb6	-1.33	0.014
Cfp	-1.48	4.44e-6	Neto2	-1.09	0.031	Ska1	-1.07	0.006
Kif4	-1.01	4.10e-4	Cenpn	-1.07	0.009	Kif11	-1.09	1.03e-5
Slc7a3	-1.60	3.73e-6	Slc7a5	-1.11	0.001	Slc35g1	-1.16	0.036
Siah1b	-1.15	0.015	Cdkn3	-1.24	0.010	Psmc3ip	-1.15	0.003

Fabp5	-1.19	0.002	Ska3	-1.22	0.001	Mfsd2a	-1.18	0.004
Ect2	-1.15	2.39e-5	Pbk	-1.08	0.001			
Ccna2	-1.06	8.66e-6	Spc24	-1.08	2.79e-4			
Plk4	-1.02	1.48e-4	H2afx	-1.06	2.66e-4			
Fam46c	-1.60	9.08e-8	Kif23	-1.24	1.01e-5			
Slc16a1	-1.04	3.54e-5	Ccnb2	-1.01	4.67e-4			
Cenpe	-1.02	3.40e-4	Traip	-1.01	0.006			
Trmt10a	-1.00	0.006	Pno1	-1.02	4.68e-4			
Depdc1a	-1.05	0.002	Hmmr	-1.07	9.03e-5			
Ccne2	-1.02	0.001	Rnmtl1	-1.10	0.013			
Kif2c	-1.08	0.001	Tmem97	-1.13	2.06e-4			
Cdc20	-1.37	4.19e-6	Top2a	-1.02	8.66e-6			

Table 2: Up-regulated genes in response to SAHA treatment. Same cells as in Table 1. Log2 fold change threshold > 1 and adjusted p value <0.05.

Up-regulated genes					
Genes	log2 fold change	P adjusted	genes	log2 fold hange	P adjusted
Dst	1.51	0.012	Tsc22d1	1.45	0.006
Bmpr2	1.75	0.015	Ddx25	1.45	0.036
Mroh2a	2.35	0.010	Cyp4f18	1.50	2.72e-7
Mr1	1.11	0.047	Lpcat2	1.78	0.004
Pbx1	2.23	0.026	Fam65a	1.53	0.023
Cr2	1.22	9.08e-8	Cbfa2t3	1.32	0.045
Ralgps1	1.29	0.037	Zfhx2	1.26	0.025
Gsn	1.53	6.22e-6	Slc9a9	2.54	2.796e-4
Cers6	1.06	0.015	Abhd14b	1.63	0.045
Ttn	2.20	0.023	Fbxw10	1.87	0.048
Accs	1.10	0.033	Cacnb1	1.83	0.041
Slpi	1.52	0.005	Kif19a	2.55	0.004
Arhgap6	1.28	0.033	Rab37	1.56	0.030
Maml3	1.13	0.033	Sox4	1.29	0.049
Cd1d1	1.22	0.001	Pdlim7	1.09	0.037

Tspan2	1.02	0.008	Naip5	1.20	0.016
Rwdd3	1.29	0.037	Zfp36l1	1.02	8.89e-5
Sit1	1.19	0.031	Ift43	2.29	0.037
Mir5120	1.43	0.032	Lifr	2.35	0.040
Whrn	1.52	0.001	Mapk11	1.03	0.005
Rnu11	1.11	0.005	Iglc4	1.99	0.048
Ahdc1	1.13	0.031	Lamp3	1.70	0.035
Padi2	1.50	0.044	Bcl6	1.15	9.493e-4
Tnfrsf18	1.94	0.003	BC051142	1.61	0.026
Gsap	1.33	8.52e-4	Msh5	1.24	0.035
Afap1	2.09	0.018	Tnf	1.12	7.90e-4
Antxr2	1.02	0.034	Lta	1.24	0.041
Rasgef1b	1.13	7.46e-4	Gabbr1	1.23	0.028
Gbp8	2.32	3.99e-4	Crisp3	1.49	7.90e-4
Clip2	1.65	0.018	Cxxc5	1.01	0.006
Hip1	1.48	0.002	Clcf1	1.03	0.002
Vamp5	1.40	0.032	Ifit1bl1	1.06	0.017
Pex26	1.30	0.048	Grk5	1.26	0.044
Tmem147os	1.49	0.043	mt-Tq	1.39	0.016
Arrb1	1.13	0.001	Evi5l	1.21	0.032
Trim3	1.29	0.048			
Sbf2	1.12	0.003			
Rassf7	1.35	0.040			
Marcks	1.24	2.03e-5			
Smpdl3a	1.07	0.012			
Gdf11	1.04	0.001			
Fcer2a	1.17	1.67e-6			

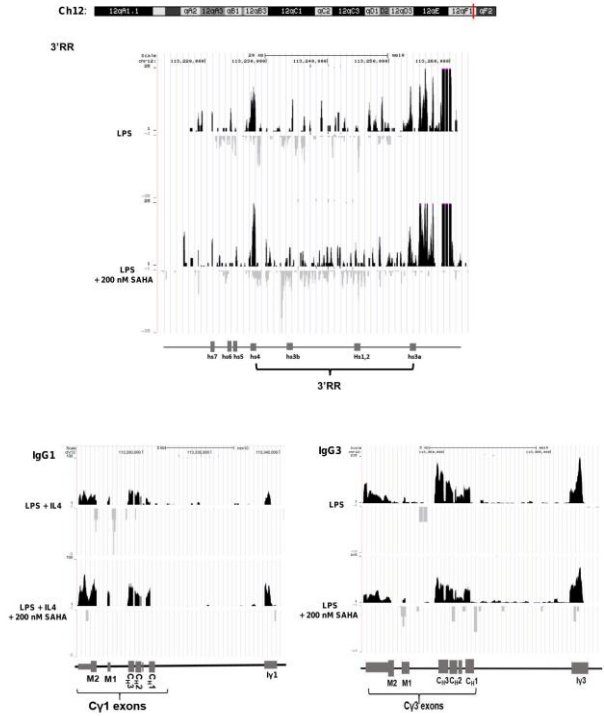

Figure 4: Influence of SAHA on IgH locus transcription. Upper panel: Effect of SAHA on 3'RR eRNA. Sense (in black) and antisense (in grey) transcription in two-day LPS (5μg/ml) ± SAHA (200 nM) stimulated mature B-cell splenocytes from *wt* mice. One representative experiment out of 2 is reported (pooled cells from three mice per group). The locations of hs3a, hs1,2, hs3b and hs4 enhancer elements of the 3'RR are shoxn. Lower panels: Effect of SAHA on $I_{\gamma 3}$-$C_{\gamma 3}$ transcription (LPS stimulation) and $I_{\gamma 1}$-$C_{\gamma 1}$ transcription (LPS+IL4 stimulation). One representative experiment out of 2 is reported.

SAHA and in vitro B-Cell Lymphoma Proliferation - Convincing demonstrations of the essential contributions of the 3'RR in mature B-cell lymphomagenesis have been provided by knock-in (KI) animal models which bring the oncogene *c-myc* under 3'RR transcriptional control (such as for i*Myc*C_{μ}, i*Myc*E_{μ} and i*Myc*C_{α} mice) [14-18]. We next examined the effect of SAHA on growth of mature mouse B-cell lymphomas ($B220^{+}CD19^{+}IgM^{+}IgD^{+}$) induced after c-myc insertion into the IgH locus. As shown in Figure 5A, and in contrast to normal mature B-cells, results clearly indicated an inconsistent SAHA

effect on mature B-cell lymphoma growth; some lymphoma samples increased their LPS stimulated growth in response to SAHA, some had unchanged growth and some reduced their growth (despite the same $IgM^{+}IgD^{+}$ mature B-cell phenotype). ChIP assays indicated no HDAC1 binding to the 3'RR hs1,2 enhancer in these freshly isolated mature mouse B-cell lymphomas (Figure 5B).

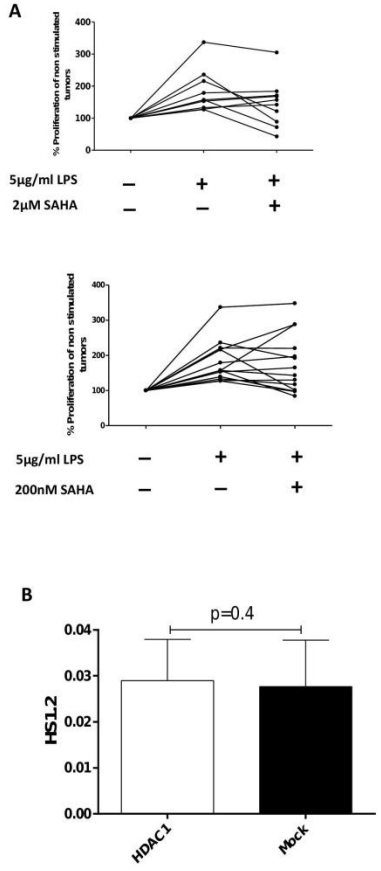

Figure 5: Influence of SAHA on proliferation of c-myc-induced mature B-cell lymphomas. A: Freshly isolated mature B-cell lymphomas from $iMycE_{\mu}$, $iMycC_{\mu}$ and $iMycC_{\alpha}$ mice were used. Each line represents one B-cell lymphoma. Each point represents the % proliferation in response to LPS with or without SAHA (10 and 14 different mouse B-cell lymphomas for 2mM SAHA and 200 nM SAHA, respectively). Each point is the mean of six

replicates. Proliferation of $B220^{+}IgM^{+}IgD^{+}$ B-cell lymphomas was evaluated with the MTS assay The first part of each graph shows the effect of LPS on B-cell lymphoma proliferation (to ensure that B-cell lymphomas remain alive after 3 days of *in vitro* growth). Thus all tested lymphomas had a percentage of proliferation higher than their controls without LPS (indicated as 100%). Only B-cells lymphomas with a higher proliferation in response to LPS were investigated for SAHA treatment to withdraw lymphomas unable to survive under the experimental conditions. B: hs1,2 HDAC1 binding in mature mouse B-cell lymphomas. ChIP experiments were performed on freshly isolated B-cell lymphomas from $iMycE_{\mu}$, $iMycC_{\mu}$ and $iMycC_{\alpha}$ mice. Mean ± SEM of 10 experiments (no significance with the Wilcoxon matched paired test). See legend to Figure 1 for mock explanation.

CPB is recruited to the hs3a and hs3b Enhancer in Freshly Isolated Mature Mouse B-Cell Lymphomas - Histone acetyl transferases (HATs) and HDACs act in concert to remodel chromatin and alter gene expression. CBP HAT binding to the hs3 enhancer was reported in Raji cells (a human Burkitt's cell line) [19]. As reported in Figure 6A, CBP was recruited to hs3a and hs3b in freshly isolated B-cell lymphomas. However, PCAF, another HAT, was not (data not shown). In contrast to B-cell lymphomas, no significant CBP binding was found in normal resting B-cell splenocytes or after 2-days LPS-stimulation (Figure 6B).

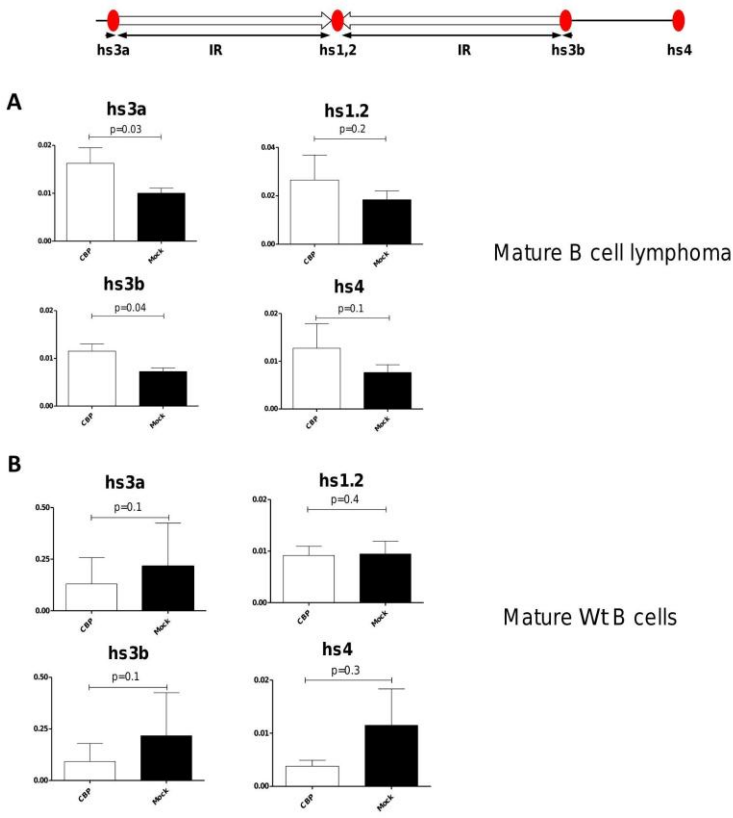

Figure 6: CBP fixation on 3'RR in freshly isolated mature B-cell lymphomas and *wt* B-cells. A: CBP ChIP experiments were performed on freshly isolated B-cell lymphomas from transgenic mice bearing c-myc in various locations of the IgH locus (iMycE$_\mu$, iMycC$_\mu$ and iMycC$_\alpha$ mice). Mean ± SEM of 4 experiments. Significance with the student-*t*-test for paired data. B: CBP ChIP experiments were performed on 2-day LPS-stimulated spleen B-cells. Mean ± SEM of 5 experiments (significance with the student-*t*-test for paired data). See legend to Figure 1 for mock explanation.

Discussion

The balance between acetylation and deacetylation of chromatin histone proteins and non-histone proteins controls gene expression regulation. HATs and HDACs are chromatin-modifying enzymes. Their interplay regulates the action of numerous signal transducers and activators of transcription with,

in the end, potent effects on a wide range of cell processes such as cell cycle, cell death, differentiation, immune response and cancer. HDACi have been approved as additional treatment for lymphomas [11,12]. HDACi are reported to affect B-cell responses both *in vivo* and *in vitro.* We studied the relationship between the HAT/HDAC pathway and 3'RR enhancers of the IgH locus during normal mature B-cell responses and mature B-cell lymphoma growth.

As previously reported [9], we observed that in LPS-activated mature B-cell splenocytes HDAC1 was recruited to the hs1,2 element of the 3'RR as determined by ChIP assays. In similar experimental conditions, other members of HDAC class I with ubiquitous tissue distribution such as HDAC2 and HDAC3 were not recruited to the 3'RR. The mouse 3'RR contains four enhancer elements with hs1,2 flanked by IRIS sequences and the center of a 25-kb palindrome bordered by two hs3 enhancer inverted copies (hs3a and hs3b). Evolution maintained this unique palindromic arrangement in mammals suggesting that it is functionally significant [20]. Deconstructing the palindromic IgH 3'RR in ΔIRIS mice strongly impacts its function [4]. In agreement with this latter result we report that HDAC1 is not efficiently recruited to the hs1,2 enhancer in LPS-stimulated ΔIRIS mature spleen B-cells.

Histone acetylation has been associated with key remodelling events of the IgH locus including CSR [21]. We examined the role of the HDACi SAHA in B-cell growth, CSR, Ig production and IgH locus transcription. SAHA decreased *in vitro* mature B-cell growth in a dose dependent manner and in response to various stimulatory conditions. In contrast, SAHA decreased CSR toward IgG_3 and stimulated CSR toward IgG_1. The effect of SAHA on Ig production (stimulation for IgG_1 and inhibition for IgG_3) paralleled its effect on CSR. Thus the elevated production of IgG_1 in response to SAHA was due to an increased level of IgG_1 switched cells. In turn, reduced production of IgG_3 in response to SAHA was due to decreased amounts of IgG_3 switched cells. This result differs markedly from previously reported results with primary spleen B-cells from MRL-lpr mice (a mouse strain prone to develop an autoimmune disease

resembling systemic lupus erythematosus) where the HDACi Trichostatin A (TSA) inhibited both germline and post-switch γ1 and γ2a transcription suggesting that inhibition of HDAC activity can downregulate gene transcription [9]. RNAseq analysis of transcription at the IgH locus of *wt* mature spleen B-cells in response to LPS revealed no evident effect of SAHA on transcription at the IgH locus in $I_{\gamma 3}$-$C_{\gamma 3}$ regions (nor in $I_{\gamma 1}$-$C_{\gamma 1}$ regions in response to LPS+IL4 stimulation). Furthermore, 3'RR eRNAs were not affected by SAHA treatment suggesting no direct effect on 3'RR activation. The effect of SAHA on CSR and Ig production is thus not through a direct effect on 3'RR activation but through another mechanism (such as isotype-dependent acetylation/deacetylation of S regions) that remains to be determined. Genome-wide gene expression analysis in response to SAHA indicated several down-regulated genes implicated in growth processes, a result that fitted well with the effect of SAHA as an inhibitor of cell proliferation. Similarly several down-regulated genes linked to epigenetic-related processes were found highlighting efficiency of SAHA treatment on mature B-cell responses.

Deciphering the mechanism that underpins the B-cell HDACi-induced effect is also of interest for a better understanding of the rationale for use of HDACi to treat B-cell lymphomas. Our results indicated that HDC1 does not bind to the hs1,2 enhancer element in freshly isolated mature mouse B-cell lymphomas with a c-myc insertion in the IgH locus; a major difference compared to results of stimulated normal mature B-cells. Another major difference was the binding of the HAT CBP to hs3a and hs3b enhancers that bounder the 3'RR palindrome indicating that the HAT/HDAC pathway is not identically regulated in stimulated normal mature B-cells and freshly isolated mature B-cell lymphomas. Furthermore if normal mature B-cell growth was consistently down regulated *in vitro* by SAHA, this was not the case in freshly isolated mature B-cell lymphomas where SAHA sometimes had no effect or a decreased/stimulatory effect without evident links to a B-cell lymphoma phenotype. This effect is consistent with the fact that despite similar "primom movens" (the insertion of c-myc in the IgH locus) B-cell lymphomas arise with different kinetics, various KI67 indices

and different locations due to the numerous different oncogenic hits favouring lymphoma emergence. Our results clearly indicated that even though SAHA affects normal mature B-cell growth, this effect is not consistently found in mature B-cell lymphomas and that trying to use HDACi to down regulate 3'RR activation in order to down regulate its effect on oncogene transcription (c-myc in our animal models) is utopic.

Important differences thus exist concerning the effect of SAHA on proliferation of *wt* mature B-cells *vs* mature B-cell lymphomas. Differences are also documented concerning the recruitment of CBP/HDAC1 to the IgH 3'RR. We report an isotype-effect of SAHA on B-cell CSR and no effect of SAHA on IgH locus transcription. SAHA effects are not mediated by down-regulation of 3'RR transcriptional activity in normal mature B-cells. Previous studies with transgenic mice bearing an IgH with an inserted c-myc (*i.e.* under transcriptional dependence of the 3'RR) have suggested that targeting the 3'RR would be of interest in order to down regulate c-myc deregulated transcripts leading to B-cell lymphomagenesis [14,18,22]. Our present results argue against this hypothesis. Translocations in B-cell lymphomas undoubtedly induce epigenetic changes [23] and epigenetic drugs targeting histone acetylation (HDACi) and histone methylation (EZH2 inhibitors) are already used to treat several B-cell lymphoid malignancies [11,12]. The precise mechanism underlining their beneficial use remains to be elucidated but is clearly not mediated by direct action on IgH 3'RR enhancers.

Only one HDACi (*i.e.*, SAHA, a pan-HDACi) was investigated in this study, which might limit the generalization of our results across different HDACi (particularly pan-HDACi *vs* class I-specific inhibitors). HDACi are approved for treatment of certain lymphomas but their narrow therapeutic index limits their use. Inconsistent effects of HDACi treatments have been reported [12] and HDACi are found to synergize with other treatments such as PD1 blockade [24]. As the HAT CBP, rather than HDAC1, was recruited to the 3'RR in mature B-cell lymphomas but not normal mature B cells, it will be interesting to see whether targeting CBP would be active against mature B-cell

lymphomas. It is possible that recruitment of CBP, instead of HDAC1, causes the intrinsic resistance to HDACi in some types of B-cell lymphomas.

In this study only *wt* mature spleen IgM^+IgD^+ B-cells were specifically considered because the 3'RR is the major driver of CSR and Ig synthesis. It is also why we explored HDAC binding and HDACi effects only in mature B-cell lymphomas ($B220^+IgM^+IgD^+$). It is evident that similar studies on the entire spectrum of B-cell subsets (from pro-B cells to plasmocytes) and B-cell lymphomas would be of interest. Finally, despite wide functional/structural similarities between mouse and human 3'RRs [20], our results cannot be directly translated into human counterparts requiring further studies to clarify this point.

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