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AID dysregulation in lupus-prone MRL/*Fas*$^{lpr/lpr}$ mice increases class switch DNA recombination and promotes interchromosomal *c-Myc/IgH* loci translocations: modulation by HoxC4

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Abstract

Immunoglobulin (Ig) gene somatic hypermutation (SHM) and class switch DNA recombination (CSR) play important roles in the generation of autoantibodies in systemic lupus erythematosus. Systemic lupus is characterized by the production of an array of pathogenic high-affinity mutated and class-switched, mainly IgG, antibodies to a variety of self-antigens, including nuclear components, such as dsDNA, histones and chromatin. We previously found that MRL/*Fas*$^{lpr/lpr}$ mice, which develop a systemic autoimmune syndrome sharing many features with human lupus, display greatly upregulated CSR, particularly to IgG2a, in B cells of the spleen, lymph nodes and Peyer's patches. In MRL/*Fas*$^{lpr/lpr}$ mice, the significant upregulation of CSR is associated with increased expression of activation-induced cytidine deaminase (AID), which is critical for CSR and SHM. We also found that HoxC4 directly activates the promoter of the AID gene to induce AID expression, CSR and SHM. Here, we show that in both lupus patients and lupus-prone MRL/*Fas*$^{lpr/lpr}$ mice, the expression of HoxC4 and AID is significantly upregulated. To further analyze the role of HoxC4 in lupus, we generated *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice. In these mice, HoxC4-deficiency resulted in reduced AID expression, impaired CSR and decreased serum anti-dsDNA IgG, particularly IgG2a, autoantibodies, which were associated with a reduction in IgG deposition in kidney glomeruli. In addition, consistent with our previous findings that in MRL/*Fas*$^{lpr/lpr}$ mice, upregulated AID expression is associated with extensive DNA lesions, comprising deletions and insertions in the *IgH* locus, we found *c-Myc* to *IgH* (*c-Myc/IgH*) translocations to occur frequently in B cells of MRL/*Fas*$^{lpr/lpr}$ mice. The frequency of such translocations was significantly reduced in *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice. These findings suggest that in lupus B cells, upregulation of *HoxC4* plays a major role in dysregulation of AID expression, thereby increasing CSR and autoantibody production, and promoting *c-Myc/IgH* translocations.

Keywords

Activation-induced cytidine deaminase (AID); B cell lymphoma; cancer; class switch DNA recombination (CSR); c-Myc/IgH translocation; HoxC4; MRL/Fas$^{lpr/lpr}$ mice; systemic lupus erythematosus (SLE)

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Declaration of Interest
The authors report no conflicts of interest.

Introduction

Systemic lupus erythematosus (SLE) is a chronic, inflammatory autoimmune disease characterized by the production of autoantibodies with multiple specificities, including to nuclear antigens, soluble mediators and phospholipids [1, 2]. These autoantibodies are pathogenic and cause damage to specific organs, including kidneys, heart and central nervous system [3–8]. An important feature of pathogenic autoantibodies in lupus is that they are class-switched, mostly to IgG, and somatically mutated [9, 10]. IgG autoantibodies can gain access to the extracellular space, thereby mediating tissue/organ injury. MRL/*Fas*$^{lpr/lpr}$ mice develop a systemic autoimmune syndrome that shares many features with human lupus, such as production of anti-DNA autoantibodies, hypergammaglobulinemia, lymphadenopathy and immune complex glomerulonephritis [11–13].

Immunoglobulin (Ig) class switch DNA recombination (CSR) and somatic hypermutation (SHM) are critical for the maturation of antibody responses to foreign and self-antigens. CSR recombines DNA of two switch (S) regions, each located upstream of different constant heavy chain (C_H) region exon clusters, thereby changing the Ig C_H region and endowing antibodies with new biological effector functions. SHM introduces mainly point-mutations in Ig V(D)J regions, thereby providing the structural substrate for selection of higher affinity antibody mutants by antigen. Both CSR and SHM are highly regulated and require the intervention of activation-induced cytidine deaminase (AID), which is expressed at high levels in activated B cells in germinal centers (GCs) of peripheral lymphoid organs [14, 15]. AID initiates CSR and SHM by deaminating dC residues preferentially within RGYW/WRCY, to yield dU:dG mispairs in DNA [14–23]. dU:dG mispairs trigger DNA repair processes entailing introduction of mismatches (mutations) in V(D)J regions and double-strand DNA breaks (DSBs) in S regions, leading to CSR [14, 24–29]. DSBs in non-*Ig* locus DNA, possibly caused by AID off-targeting, can become substrates for chromosomal translocations [30].

As we showed, lupus-prone MRL/*Fas*$^{lpr/lpr}$ mice display elevated AID expression, leading to increased CSR and SHM, ultimately manifesting in a more than 10-fold increase in serum levels of two major pathogenic Ig isotypes, IgG1 and IgG2a, over healthy mouse controls [11, 31]. Likewise, lupus patients display high levels of circulating IgGs, including pathogenic autoantibodies, and a high proportion of B cells undergoing CSR [32]. In addition to increased CSR to IgG, both SLE patients and lupus-prone mice display a high frequency of mutations in Ig V(D)J DNA sequences [31, 33–39]. Consistent with the role of CSR and SHM in the generation of pathogenic autoantibodies, *Aicda* expression is greatly increased in GC B cells of lupus-prone BXD2 mice [40, 41]. In *Aicda*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice, the absence of AID resulted in a lack of hypermutated and class-switched autoantibodies, such as anti-dsDNA IgG, and led to significant alleviation of glomerulonephritis, mononuclear cell infiltration and immune complex deposition in the kidneys, and dramatically increased survival rates [42–44]. In heterozygous *Aicda*$^{+/-}$ MRL/*Fas*$^{lpr/lpr}$ mice, reduced AID expression resulted in a reduction in the production of high-affinity anti-dsDNA IgG, moderately diminished kidney pathology, temporary decrease in nephritis and increased survival rates [43, 44]. The delayed and reduced symptoms observed in heterozygous *Aicda*$^{+/-}$ MRL/*Fas*$^{lpr/lpr}$ mice suggest that discrete levels of AID expression, and not solely its presence or absence, are important in lupus pathogenesis [43].

Malignancies are associated with systemic lupus and are a significant cause of death in SLE patients [45–53]. Accordingly, hematologic cancers occur more frequently in patients with SLE (~3–4 times greater risk for lymphomas) than in the general population [48, 51, 54]. It is not clear what causes the increased risk of lymphomas [53]. Chromosomal translocations involving the *Ig* locus and a proto-oncogene, such as *c-Myc*, are a hallmark of most B cell

lymphomas and usually result in dysregulated expression of oncogenes brought under the control of the Ig gene enhancers, thereby leading to cell transformation events [55, 56]. AID would mediate chromosomal translocations possibly by introducing DSBs in *IgH* and a proto-oncogene loci [30]. We have shown that in lupus-prone MRL/$Fas^{lpr/lpr}$ mice, increased AID expression was associated with accumulation of a high frequency of deletions and insertions in the *IgH* locus, possibly as a result of AID-mediated DNA cleavages, including DSBs [31]. These could provide the substrate for chromosomal translocations, which may play a significant role in the lymphomagenesis associated with lupus.

AID expression is controlled through transcriptional regulation by multiple elements, in particular the conserved helix-loop-helix homeodomain-containing transcription factor HoxC4 [28]. As we have shown, in both human and mouse B cells, *HOXC4/HoxC4* expression is induced by GC differentiation-inducing stimuli, such as CD154 or LPS and IL-4, which are also required for induction of *AICDA/Aicda* expression [57–59]. We showed that HoxC4 binds to a highly conserved HoxC4/Oct site in the *AICDA/Aicda* promoter and activates this promoter in synergy with Oct-1/2, NF-κB and Sp1/Sp3 [59, 60]. In addition, we showed that estrogen, which promotes disease expression in SLE patients and accelerates the appearance of pathogenic autoantibodies and autoimmunity in MRL/$Fas^{lpr/lpr}$ and NZB/NZW F1 mice, enhances AID gene expression, CSR and SHM by directly activating the *HOXC4/HoxC4* promoter, thereby potentiating the induction of HoxC4 [61]. Given the critical role of HoxC4 in regulating AID expression, we have hypothesized that the upregulated AID expression, that leads to the dysregulated CSR and SHM in lupus, results from upregulation of HoxC4.

Here, we have investigated HoxC4-dependent AID expression in lupus and its role in upregulating CSR as well as yielding interchromosomal translocations, as a result of AID off-targeting and emergence of DSBs. We have found that HoxC4 was upregulated in B cells of lupus patients and lupus-prone MRL/$Fas^{lpr/lpr}$ mice. In addition to high levels of anti-DNA IgG autoantibodies and kidney pathology, these mice showed significant levels of interchromosomal translocations between the *c-Myc* and *IgH* loci. To determine the contribution of HoxC4-mediated AID dysregulation to lupus-like autoimmunity and interchromosomal translocations, we have generated $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ mice. These mice displayed decreased levels of autoantibodies and decreased immunopathology, as well as a decreased incidence of interchromosomal *c-Myc/IgH* translocations. Our findings suggest that dysregulated HoxC4 expression and, therefore, AID expression, contributes to the production of pathogenic IgG autoantibodies and interchromosomal *c-Myc/IgH* translocations in lupus B cells.

Materials and Methods

Mice

MRL/$Fas^{lpr/lpr}$ (stock number: 000485), NZB (stock number: 000648) and NZW (stock number: 001058) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). NZB/NZW F1 mice were generated by crossbreeding NZB female mice with NZW male mice. 9-month old female NZB and NZB/NZW F1 mice were used to analyze AID expression by immunoblotting. $HoxC4^{-/-}$ C57BL/6 mice were generated as we described previously [59]. $Aicda^{-/-}$ C57BL/6 mice [62] were provided by Dr. Tasuku Honjo (Kyoto University, Japan). Both $HoxC4^{-/-}$ and $Aicda^{-/-}$ C57BL/6 mice were backcrossed onto the MRL/$Fas^{lpr/lpr}$ background for at least 5 generations. The mice were housed in pathogen-free facilities, and were provided with autoclaved food and deionized water. All animal protocols were approved by the Institutional Animal Care and Use Committee of University of California, Irvine, CA, 92697.

Quantitative real-time PCR (qRT-PCR) analysis of mRNA expression

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy human subjects or SLE patients who were hospitalized because of a recrudescence of the disease. Lymph nodes, thymus, spleen, Peyer's patches and liver cells were isolated from MRL/ *Fas*$^{lpr/lpr}$ and non-autoimmune C57BL/6 mice. Total RNA was extracted from 2–10 × 10^6 cells using Trizol® Reagent (Invitrogen Corp., Carlsbad, CA) according to manufacturer's instructions. Residual DNA was removed from the extracted RNA with gDNA eliminator columns (Qiagen, Valencia, CA). The first strand cDNAs were synthesized from equal amounts of total RNA (2 μ*g*) using the SuperScript™ III First-Strand Synthesis System (Invitrogen Corp.) with Oligo dT-primer. The expression of *HoxC4/HoxC4, AICDA/Aicda*, germline I_H-C_H, circle I_H-Cμ and post-recombination Iμ-C_H transcripts was quantified by real-time quantitative RT-PCR (qRT-PCR) using the appropriate primers ([59, 63] and Table 1) and Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA) to measure SYBR-green (DyNAmo HS SYBR Green, New England Biolabs Inc., Beverly, MA) incorporation with the following protocol: 95°C for 15 min, 40 cycles of 94 °C for 10 sec, 60°C for 30 sec, 72°C for 30 sec. Data acquisition was performed during 72°C extension step. Melting curve analysis was performed from 72°C–95°C. The ΔΔCt method was used for data analysis.

Immunoblotting

Cell extracts containing equal amounts of protein (25 μ*g)* were fractionated through 10% SDS-PAGE. Separated proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories) overnight (30 V) at 4 °C. The membranes were blocked and incubated overnight at 4 °C with mouse mAb to AID (Clone ZA001, Invitrogen) or mouse mAb to β-actin (Clone AC-15, Sigma). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary Abs. After washing with 0.05% PBS-Tween 20, bound HRP-conjugated Abs were detected using Western Lightning® Plus—Enhanced Chemiluminescence reagents (PerkinElmer Life and Analytical Sciences).

Detection of serum anti-dsDNA IgG antibodies

Mouse serum anti-dsDNA IgG1 and IgG2a were measured by ELISA. 96-well ELISA plates (MICROLON™ 600 flat bottom clear polystyrene plate, high binding, chimney well; USA Scientific Inc., Ocala, FL) were coated with 50 μl of 500 μg/ml protamine sulfate in PBS at room temperature for 1 h, followed by another overnight incubation at 4°C with dsDNA at 10 μg/ml in 10 mM Tris. The plates were treated with blocking buffer (0.25% bovine serum albumin in 0.05% PBS-Tween 20) at room temperature for 45 min. Mouse sera was serially two-fold diluted, starting from 1:1,000 in blocking buffer, and 100 μl was added to each of the wells and incubated at 37°C for 1 h, then washed three times with 0.05% PBS-Tween 20. Biotinylated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) was then added to each well at 1:5,000 dilution and incubated at room temperature for 2 h, then washed 3 times with 0.05% PBS-Tween 20. Streptavidin-HRP (Sigma Aldrich, St. Louis, MO) was added to the plates at 1:2,500 dilution. After 2 h at room temperature, plates were washed three times with 0.05% PBS-Tween 20. After addition of substrate solution (1x phosphate citrate, OPD, hydrogen peroxide), the plates were incubated for 30 min at room temperature in the dark, before stopping the enzymatic reaction with 1 M H_2SO_4 and measuring O.D. at 492 nm with a Luminescence Detector 400 (Beckman Coulter, Brea, CA). Titers were expressed in the 50% effective concentration (EC_{50}) units. An EC_{50} was defined as the number of dilutions needed to reach 50% of binding saturation, as calculated using GraphPad® Prism software (GraphPad Software Inc., La Jolla, CA). All assays were performed in triplicates. Differences in Ig titers were analyzed using paired *t*-tests.

Immunofluorescence and kidney histology

Kidneys from 16 week-old mice in Tissue-Tek® OCT™ Compound (Sakura Finetek USA Inc., Torrance, CA) were frozen on dry ice and sectioned. Sections were then stained with hematoxylin and eosin (H&E) or processed for immunofluorescence. H&E images were taken with the use of a 20x objective on a light microscope. Sections prepared for immunofluorescence were fixed in cold acetone and stained with FITC-conjugated anti-mouse IgG1 (clone A85-1) or anti-mouse IgG2a (clone R19-15) mAb (BD Biosciences, San Jose, CA) at 1:1,000 dilution. Immunofluorescent images were taken with the use of a 60x objective on a fluorescent microscope.

Ig CSR analysis

The number of $B220^+$ or PNA^{hi} $B220^+$ cells expressing IgG1, IgG2a, IgG2b, IgG3 or IgA was determined by flow cytometric analysis. Single cell suspensions were prepared from the spleen and lymph nodes of 11-week-old MRL/*Fas*$^{lpr/lpr}$ mice and stained with PE-conjugated anti-mouse CD45R (B220) mAb (clone RA3-6B2) (eBioscience, San Diego, CA) and Alexa Fluor® 647-conjugated PNA (Invitrogen Corp.), as well as rat anti-mouse IgG1 (clone A85-1), anti-mouse IgG2a (clone R19-15), anti-mouse IgG3 (clone R40-82), or antimouse IgA-FITC (clone C10-3) mAb (BD Biosciences). Cells were fixed with 1% paraformaldehyde in PBS and analyzed using a FACSCalibur™ flow cytometer (BD Biosciences).

Detection and sequencing of c-Myc/IgH translocations

Genomic DNA was prepared from spleens of mice using DNeasy® Blood and Tissue Kit (Qiagen). NestedPCRs for translocations were performed on genomic DNA from 10^5 cells with Phusion® High Fidelity DNA Polymerase using the following primers: 5′-TGAGGACCAGAGAGGGATAAAAGAGAA-3′ and 5′-GGGGAGGGGGTGTCAAATAATAAGA-3′ for the first round PCR; 5′-CACCCTGCTATTTCCTTGTTGCTAC-3′ and 5′-GACACCTCCCTTCTACACTCTAAACCG-3′ for the second round PCR. PCR conditions were as follows: 98°C for 2 min followed by 25 cycles (98°C, 10 s; 58°C, 45 s; and 72°C, 7 min) for the first round and 30 cycles (98°C, 10 s; 58°C, 45 s; and 72°C, 7 min) for the second round. Amplified DNA was fractionated through 1.2% agarose, blotted onto Hybond-N^+ membranes (Amersham Biosciences Inc., Piscataway, NJ) and hybridized to [γ-^{32}P]-ATP-labeled *c-Myc*-specific oligonucleotide probes (5′-GCAGCGATTCAGCACTGGGTGCAGG-3′). To further confirm *c-Myc/IgH* translocation sequences, the PCR products were then cloned into pCR-Blunt II-TOPO vector (Invitrogen Corp.) and sequenced. Sequence alignment was done by comparing the sequences of PCR products with germline *c-Myc* and *IgH* genomic sequences using NCBI *BLAST* http://www.ncbi.nih.gov/BLAST.

Results

HoxC4 and AID are upregulated in SLE patients and lupus-prone mice

We have demonstrated that AID expression is increased in B cells of lupus-prone MRL/*Fas*$^{lpr/lpr}$ mice [31]. Because of the important role of HoxC4 in activating the *AICDA/Aicda* promoter and modulating AID expression, we analyzed *HOXC4/HoxC4* and *AICDA/Aicda* expression in SLE patients and MRL/*Fas*$^{lpr/lpr}$ mice, and their corresponding healthy controls. As measured by real-time qRT-PCR, *HOXC4* and *AICDA* expression in the PBMCs from SLE patients was significantly increased as compared to healthy subjects (p = 0.0050 for *HOXC4* and p = 0.0019 for *AICDA*)(Fig. 1a). Accordingly, lupus-prone MRL/*Fas*$^{lpr/lpr}$ mice displayed a significant increase in *HoxC4* and *Aicda* expression in the lymph

nodes, Peyer's patches and spleen, which contain a large proportion of hypermutating and class switching B cells, but not in the thymus or liver, as compared to healthy C57BL/6 mice (Fig. 1b).

Consistent with the increased *Aicda* transcript level in MRL/$Fas^{lpr/lpr}$ mice ([31] and Fig. 1b), the amount of AID protein in lymph nodes of lupus-prone MRL/$Fas^{lpr/lpr}$ mice was also significantly higher than in healthy C57BL/6 mice; this was also true of NZB/NZW F1 mice (Fig. 2a), which, like MRL/$Fas^{lpr/lpr}$ mice, also generate somatic hypermutated and class switched autoantibodies, and develop lupus [64]. The abundance of AID in these mice was much greater than in their parental NZB strain, which also develop some autoimmune symptoms, albeit much less severe and at an older age than NZB/NZW F1 mice. Thus, in humans and mice with systemic autoimmunity, upregulation of B cell AID expression is associated with upregulation of HoxC4.

Decreased AID expression in HoxC4-deficient MRL/$Fas^{lpr/lpr}$ mice

We have shown that HoxC4 deficiency significantly impaired *Aicda* expression in the spleen, lymph nodes and Peyer's patches of immunized C57BL/6 mice, as well as LPS-/CD154- and IL-4-stimulated spleen B cells from unimmunized C57BL/6 mice [59, 61]. To determine the role of HoxC4 in the production of autoantibodies in lupus, we generated $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ mice by backcrossing $HoxC4^{-/-}$ C57BL/6 mice onto MRL/$Fas^{lpr/lpr}$ mice for 5 generations. The proportions of $B220^+$ B cells and $PNA^{hi}B220^+$ (GC) B cells in the spleen or lymph nodes of $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ mice were comparable to those in $HoxC4^{+/+}$ MRL/$Fas^{lpr/lpr}$ mice (not shown). The level of AID protein in lymph nodes of $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ mice, however, was decreased (Fig. 2a). This was associated with a 40–55% reduction in *Aicda* transcripts (measured by real-time qRT-PCR in spleen and lymph nodes) in $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ mice as compared to their $HoxC4^{+/+}$ MRL/$Fas^{lpr/lpr}$ counterparts (Fig. 2b), further suggesting that HoxC4 upregulation is responsible for the dysregulated AID expression in MRL/$Fas^{lpr/lpr}$ mice.

HoxC4 deficiency reduces serum IgG1 and IgG2a autoantibody levels in MRL/$Fas^{lpr/lpr}$ mice

Production of anti-dsDNA IgG autoantibodies is characteristic of human and mouse lupus. These autoantibodies contribute to glomerular damage by depositing in glomeruli, as components of immune complexes. In lupus-prone mice, including the MRL/$Fas^{lpr/lpr}$ strain, IgG2a is the dominant IgG subclass of anti-dsDNA autoantibodies [11, 31]. To address the contribution of HoxC4 to the generation of autoantibodies in lupus, we analyzed anti-dsDNA IgG1 and IgG2a autoantibodies in the serum of $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ and $HoxC4^{+/+}$ MRL/$Fas^{lpr/lpr}$ littermates. $HoxC4^{+/+}$ MRL/$Fas^{lpr/lpr}$ mice displayed abundant circulating anti-dsDNA IgG, particularly IgG2a (Fig. 3a). Consistent with their reduced AID expression, $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ mice displayed a 57% reduction ($p = 0.04$) in circulating anti-dsDNA IgG1 (Fig. 3a) and an over 69% reduction ($p = 0.035$) in circulating anti-dsDNA IgG2a autoantibodies. Thus, HoxC4 deficiency results in reduced AID expression and decreased levels of autoantibodies in lupus mice.

HoxC4 deficiency reduces kidney IgG2a deposition and glomerular damage in MRL/$Fas^{lpr/lpr}$ mice

Glomerular damage characterized by enlarged, hypercellular glomeruli, with increased numbers of both resident cells and infiltrating leukocytes, as well as mesangial matrix expansion, is a hallmark of nephritis in the kidney of MRL/$Fas^{lpr/lpr}$ mice. To characterize the impact of HoxC4 deficiency on lupus-like disease, we studied histological signs of glomerulonephritis in representative H&E stained kidney sections from 16-week-old $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ and $HoxC4^{+/+}$ MRL/$Fas^{lpr/lpr}$ mice. While $HoxC4^{+/+}$ MRL/

Fas$^{lpr/lpr}$ mice developed some kidney pathology, as revealed by typical features of glomerulonephritis, *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice displayed reduced glomerular damage, as indicated by much less severe proliferative glomeruli, reduced hypercellularity, and only moderate destruction of glomerular structure (Fig. 3b). To evaluate glomerular deposition of autoantibodies, kidney sections from the same mice were stained for IgG1 and IgG2a. While sections from both *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ and *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice exhibited only weak granular staining for IgG1, kidney sections from *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice displayed a strong glomerular deposition of IgG2a. Consistent with the reduced level of circulating anti-dsDNA IgG2a and reduced glomerular damage, *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice displayed reduced deposition of IgG2a in kidney glomeruli. Thus, in *HoxC4*$^{-/-}$ lupus mice, the reduced AID expression and reduced levels of circulating anti-dsDNA IgG autoantibodies result in reduced kidney pathology and IgG2a deposition.

HoxC4 deficiency impairs CSR in MRL/Fas$^{lpr/lpr}$ mice

We have shown that HoxC4 is important for AID expression, and, therefore, CSR and SHM [59]. Because of the high "background" CSR in unstimulated B cells from MRL/*Fas*$^{lpr/lpr}$ mice [31], we did not analyze *in vitro* induced CSR in MRL/*Fas*$^{lpr/lpr}$ B cells here. Rather, to determine whether the reduced anti-dsDNA autoantibodies in *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice resulted, at least in part, from an impairment of CSR, we analyzed the expression of the surface Ig isotypes in B220^{+} B cells from the spleen and lymph nodes of *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ and *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice. Consistent with our previous findings [31], B cells from *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice effectively switched to all secondary isotypes analyzed (Fig. 4a), particularly IgG2a (19%), the dominant isotype of autoantibodies in murine lupus, and IgA (29%). Further, the proportions of switched cells among PNA^{hi} B220^{+} GC B cells in spleens and lymph nodes of *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice were even higher than that among total B220^{+} B cells. Like in *HoxC4*$^{-/-}$ C57BL/6 mice [59], in MRL/*Fas*$^{lpr/lpr}$ mice, HoxC4 deficiency resulted in a significant reduction of CSR to IgG1, IgG2a, IgG3 and IgA (Fig. 4a). In *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice, the proportion of IgG2a^{+} B cells in the spleen and lymph nodes was decreased by 77% and 88%, respectively, as compared to their *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ counterparts. In the spleen and lymph nodes of *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice, the proportion of IgG1^{+}, IgG2a^{+}, IgG3^{+} or IgA^{+} cells among PNA^{hi} B220^{+} GC B cells was also reduced at a level comparable to that of total B220^{+} B cells (Fig. 4b). The reduced proportion of switched B cells in *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice did not stem from any alteration of the B cell compartment, as in these mice, the spleen size, and proportions of B220^{+} B cells and PNA^{hi} B220^{+} GC B cells in both the spleen and lymph nodes were comparable to those in *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice (data not shown).

Further, lower CSR in B cells from *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice was not due to impairment of germline I_H-C_H transcription, which is necessary for CSR. As shown by real-time qRT-PCR, the amount of germline Iγ1-Cγ1, Iγ2a-Cγ2a, Iγ2b-Cγ2b, Iγ3-Cγ3 and Iα-Cα transcripts in B cells from *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice was comparable to their *HoxC4*$^{+/+}$ counterparts (Fig. 5), while circle Iγ1-Cμ, Iγ2a-Cμ, Iγ2b-Cμ, Iγ3-Cμ and Iα-Cμ transcripts, and post-recombination Iμ-Cγ1, Iμ-Cγ2a, Iμ-Cγ2b, Iμ-Cγ3 and Iμ-Cα transcripts, which are generated by CSR, were significantly lower in the cells from *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice. Thus, in lupus-prone MRL/*Fas*$^{lpr/lpr}$ mice, HoxC4 deficiency impairs CSR without affecting germline I_H-C_H transcription.

c-Myc to IgH translocations occur frequently in B cells from MRL/Fas$^{lpr/lpr}$ mice, are reduced in the absence of HoxC4 and abrogated in the absence of AID

Patients with lupus display an elevated risk for developing B cell malignancies, particularly B cell lymphomas [45–53]. Reciprocal chromosomal translocations involving *IgH* genes are a hallmark of mature B cell lymphomas and generally result in dysregulated expression of

oncogenes brought under the control of Ig enhancers [55]. AID introduces DSBs in the *IgH* locus during CSR and SHM; these DSBs would lead to translocations involving the *IgH* locus and a proto-oncogene, such as *c-Myc*[30]. We showed that MRL/$Fas^{lpr/lpr}$ mice accumulate a high frequency of deletions and insertions in the *IgH* locus [23], suggesting that the increased AID expression in these mice results in abnormal rates of DNA lesions, leading to frequent DSBs. We sought to determine whether such DSBs led to interchromosomal translocations in autoimmune MRL/$Fas^{lpr/lpr}$ mice. To this end, we analyzed *c-Myc/IgH* translocations in spleen B cells from MRL/$Fas^{lpr/lpr}$ mice by specific nested PCR followed by Southern blotting [65, 66]. We detected a high frequency (6.4×10^{-6} translocations/cell) of *c-Myc/IgH* translocations in spleen B cells from $Aicda^{+/-}$ MRL/$Fas^{lpr/lpr}$ mice (Fig. 6). The identity of these translocations was further verified by DNA sequencing (Fig. 7). *c-Myc/IgH* translocations were not detectable in the absence of AID, suggesting that the *c-Myc/IgH* translocations occurring in MRL/$Fas^{lpr/lpr}$ mice were mediated by AID. Accordingly, $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ mice, which showed reduced AID expression, displayed a 53% reduction in the frequency of *c-Myc/IgH* translocations. Thus, AID dysregulation in autoimmune MRL/$Fas^{lpr/lpr}$ mice results in high levels of interchromosomal translocations involving the *IgH* locus. HoxC4 deficiency results in decreased AID expression and reduction of *c-Myc/IgH* translocations.

Discussion

In lupus patients and mice, multi-organ damage and pathology are associated with the emergence of pathogenic autoantibodies [4–7]. Lupus autoantibodies are highly specific for dsDNA, histones, DNA-histone complexes, soluble Sm nuclear proteins, cytoplasmic RNA-protein conjugates or ribosomal constituents, and are possibly elicited by apoptotic nucleic bodies [12]. An important feature of these autoantibodies is that, like high affinity antibodies elicited by microbial pathogens, they are heavily mutated and class-switched, mostly to IgG [9, 10]. A strong causative link between dysregulated CSR/SHM and lupus has been established by the “dose-dependent” effect of AID expression on the emergence of specific class-switched autoantibodies and immunopathology in lupus-prone MRL/$Fas^{lpr/lpr}$ and BXD2 mice [31, 41–44]. As we showed, lupus-prone MRL/$Fas^{lpr/lpr}$ mice display increased AID expression, increased CSR and SHM and extensive DNA lesions [31]. As we also showed, HoxC4 directly activates the promoter of the AID gene to induce AID expression, CSR and SHM [59]. Here, we have provided evidence that in autoimmune lupus-prone mice, upregulated AID expression likely results from upregulation of HoxC4 and leads to CSR dysregulation and emergence of interchromosomal *c-Myc/IgH* translocations. HoxC4 was upregulated in the lymphoid tissues of MRL/$Fas^{lpr/lpr}$ mice, but not in non-lymphoid tissues, further indicating that HoxC4 expression is tightly regulated and important for the immune/autoimmune response. Deletion of HoxC4 in MRL/$Fas^{lpr/lpr}$ mice reduced AID expression, CSR, autoantibody production, glomerular damage and *c-Myc/IgH* translocations.

IgG autoantibodies form immune complexes that are deposited in the renal capillaries and are important in the pathogenesis of lupus, whereas unswitched IgM autoantibodies may play a protective role by enhancing apoptotic cell clearance and minimizing inflammation [44, 67–70]. Consistent with the findings that reduced AID expression in $Aicda^{+/-}$ MRL/$Fas^{lpr/lpr}$ mice results in decreased levels of serum anti-dsDNA IgG [42], we have shown here that in HoxC4-deficient MRL/$Fas^{lpr/lpr}$ mice, together with decreased AID expression, the serum levels of anti-dsDNA IgG1 and IgG2a are both decreased. The significant decrease in anti-dsDNA IgG2a correlated with a decrease in kidney deposition of IgG2a and mild pathology. The absence of IgG1 deposition in kidneys of both $HoxC4^{-/-}$ MRL/$Fas^{lpr/lpr}$ and $HoxC4^{+/+}$ MRL/$Fas^{lpr/lpr}$ mice likely reflects the lower levels of circulating anti-dsDNA IgG1. Indeed, the percentage of $IgG1^{+}$ B cells was almost 15-fold lower than

IgG2a$^+$ B cells in these mice. Thus, our findings indicate that HoxC4 plays a significant role in the mechanistic events that underlie the generation of class-switched autoantibodies in lupus. These form immune complexes that are important in recruiting inflammatory cells and mediators of tissue damage.

Upregulation of HoxC4 expression can result from increased estrogen levels. As we showed, estrogen activates the *HoxC4* promoter through three highly conserved estrogen receptor responsive elements to enhance HoxC4 expression and potentiate HoxC4-mediated AID induction, CSR and SHM [61]. A female predominance of autoimmunity involving pathogenic autoantibodies, such as anti-dsDNA autoantibodies, and lupus, is well documented [71]. Estrogen promotes disease expression in lupus patients, of whom over 90% are females, and accelerates the appearance of pathogenic autoantibodies and disease development in lupus-prone MRL/*Fas*$^{lpr/lpr}$ and NZB/NZW F1 mice [71, 72]. This together with our demonstration of decreased AID expression, autoantibody levels and pathology in *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice further outlines the possible role of estrogens and HoxC4 in the chain of events leading to the emergence of autoantibodies in lupus. HoxC4 might be upregulated and play a role in other autoimmune disorders with female prevalence, in which high affinity or class-switched autoantibodies are important in disease pathogenesis, such as Sjogren's syndrome or *myasthenia gravis*[73]. Whether other hormones or mediators are involved in inducing HoxC4 upregulation in lupus patients and MRL/*Fas*$^{lpr/lpr}$ mice, and whether there are other pathways that lead to increased AID expression remains to be determined.

HoxC4 expression is upregulated in estrogen-responsive neoplastic cells, leading to secondary upregulation of AID, which may contribute to DNA lesions and widespread chromosomal damage, and, ultimately, tumorogenesis [61]. Interestingly, lupus and other autoimmune diseases are linked to an increased risk of certain cancers. Specifically, lupus patients may experience an elevated risk of B cell lymphomas [48, 53, 74, 75]. These, like most B cell neoplasia, are derived from GC B cells, where HoxC4 and AID are preferentially expressed, or from post-GC cells, indicating that HoxC4 can be involved in the generation of these malignant B cells [57, 59, 76]. This notion is strengthened by the demonstration that malignant B cells of Burkitt's lymphoma, B cell chronic lymphocytic leukemia and mantle B cell lymphoma, display elevated expression of HoxC4 and AID [77–80]. By generating staggered DSBs in *Ig* or non-*Ig* loci through cleaving intact dsDNA, or as we have shown, by processing blunt DSB ends generated independently of AID [81–83], AID dysregulation could lead to increased nucleotide insertions and deletions in the *IgH* locus, as we showed to occur in MRL/*Fas*$^{lpr/lpr}$ mice [31]. Our present demonstration of frequent interchromosomal *c-Myc/IgH* translocations in autoimmune MRL/*Fas*$^{lpr/lpr}$ mice but not in healthy C57BL/6 mice strongly suggests that the DSBs required for such insertions and deletions also promote the emergence of translocations. Interestingly, interchromosomal *c-Myc/IgH* translocations were shown to occur in transgenic mice that overexpressed AID under control of *Igκ* regulatory elements [30]. In addition, mutation of the *miRNA-155*-binding site in AID mRNA resulted in a 2–3 fold increase in AID mRNA and protein levels and led to about a 5-fold increase in *c-Myc/IgH* translocations [84]. Finally, AID expression levels determined the extent of *c-Myc/IgH* translocations and the incidence of tumor development in a plasmacytoma mouse model [85]. Further characterization of the effect of HoxC4 on other B cell neoplasia translocation partners, such as *miR-142*, may outline a broader role for HoxC4 in the generation of B cell malignancies [30].

In conclusion, our findings define an important role for HoxC4 in the upregulation of AID expression and CSR dysregulation in the generation of class-switched autoantibodies in lupus. They also show that HoxC4-mediated AID upregulation leads to interchromosomal

translocations between the *c-Myc* protooncogene and the *IgH* locus, thereby providing a first possible mechanistic insight into the increased frequency of hematological malignancies in lupus.

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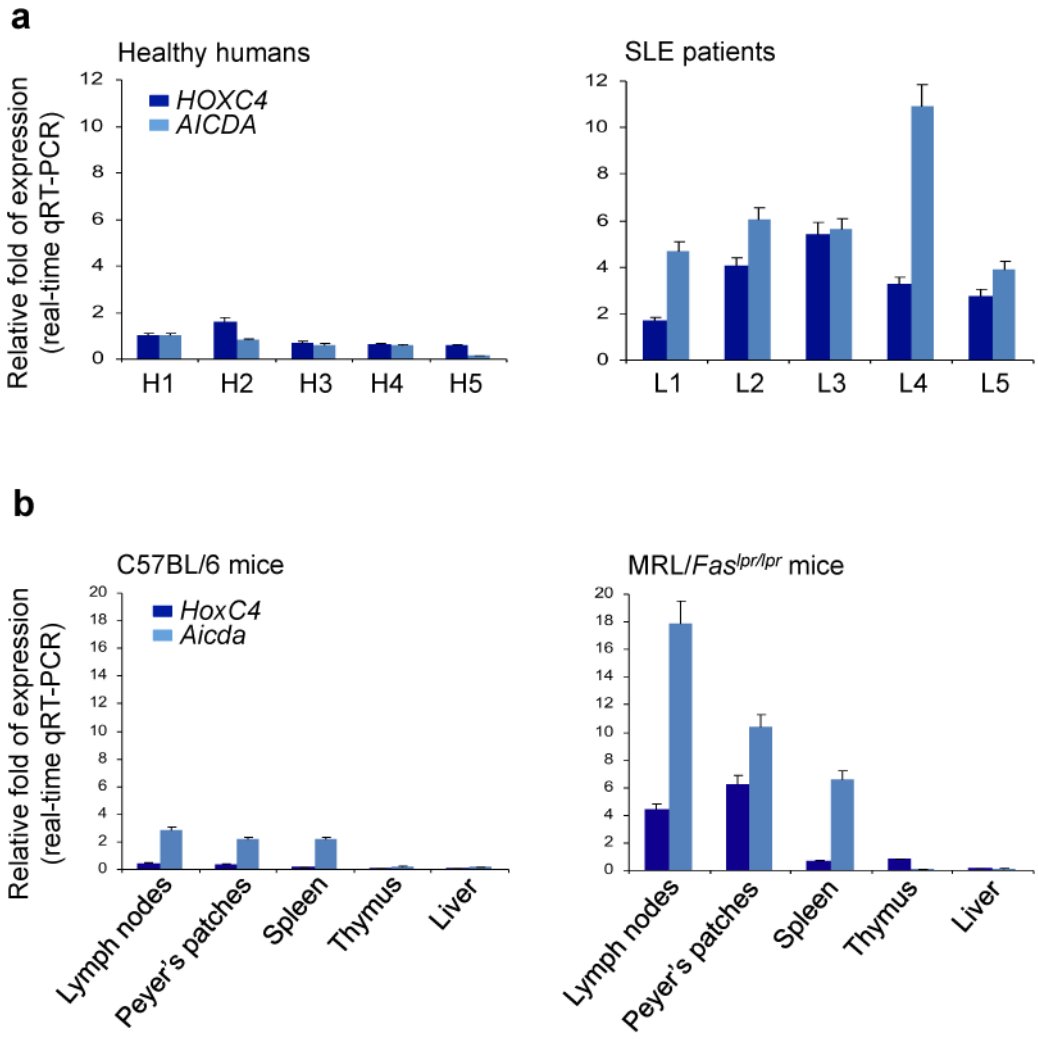

Figure 1.

HOXC4/HoxC4 and *AICDA/Aicda* are upregulated in PBMCs from SLE patients, and in lymph nodes, Peyer's patches and spleens of autoimmune MRL/*Fas*$^{lpr/lpr}$ mice. Total RNA was prepared from (**a**) the PBMCs from 5 female SLE patients (L1, L2, L3, L4 and L5) and 5 age- and sex-matched healthy subjects (H1, H2, H3, H4 and H5), or from (**b**) the spleen, lymph nodes, Peyer's patches, thymus and liver of non-immunized 8-week old MRL/*Fas*$^{lpr/lpr}$ or healthy C57BL/6 mice. Expression of *HOXC4/HoxC4* and *AICDA/Aicda* transcripts was analyzed by real-time qRT-PCR using SYBR green and normalized to *GAPDH*/*Gapdh* expression. Data are from 3 independent experiments (mean values ± SD).

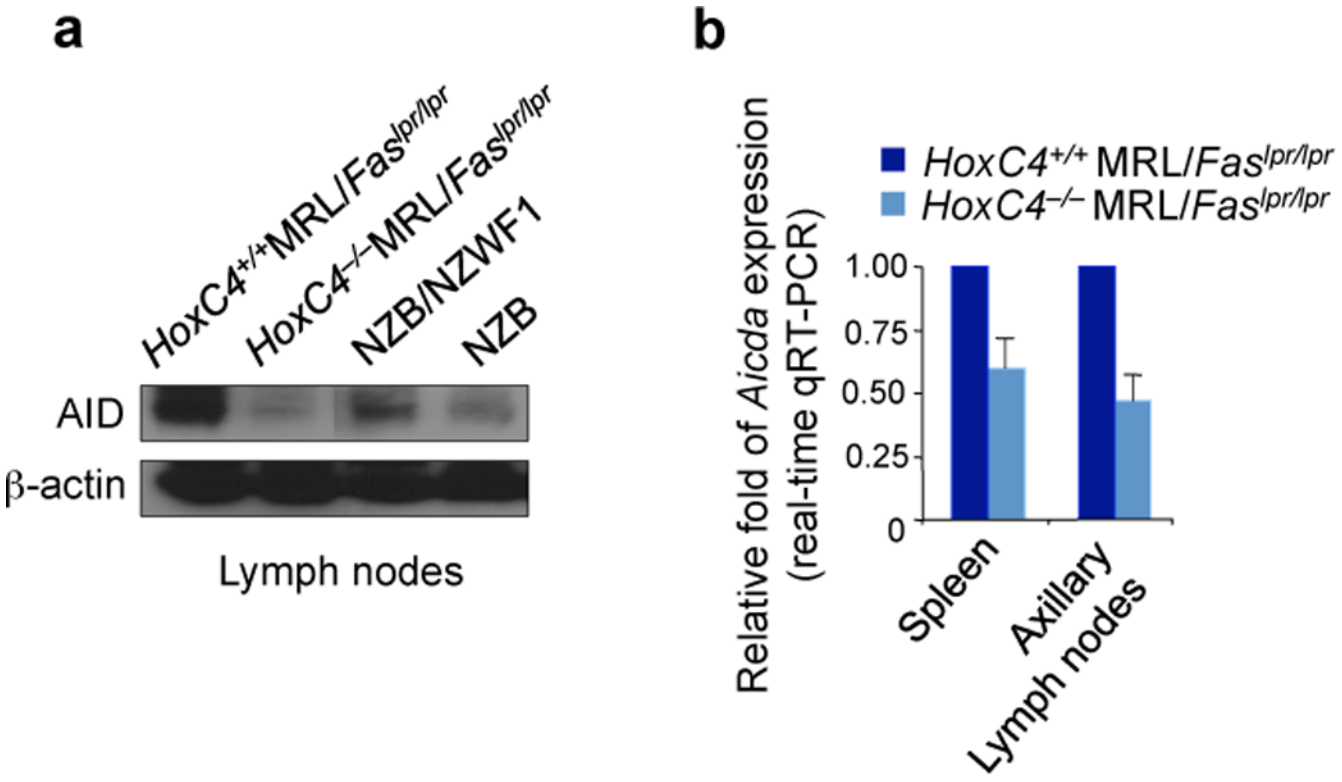

Figure 2.

HoxC4 deficiency impairs AID expression in MRL/*Fas*$^{lpr/lpr}$ mice. (**a**) AID and β-actin proteins in lymph nodes of non-immunized 12-week old female *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice and *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ littermates, as well as non-immunized 9-month old female NZB/NZW F1 and NZB mice were detected by immunoblotting. Data are representative of 2 independent experiments. (**b**) Total RNA was prepared from the spleen and lymph nodes of *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ and *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ littermates. *Aicda* transcripts were measured by real-time qRT-PCR using SYBR green and normalized to *Gapdh* expression. Data are from 3 independent experiments (mean values ± SD).

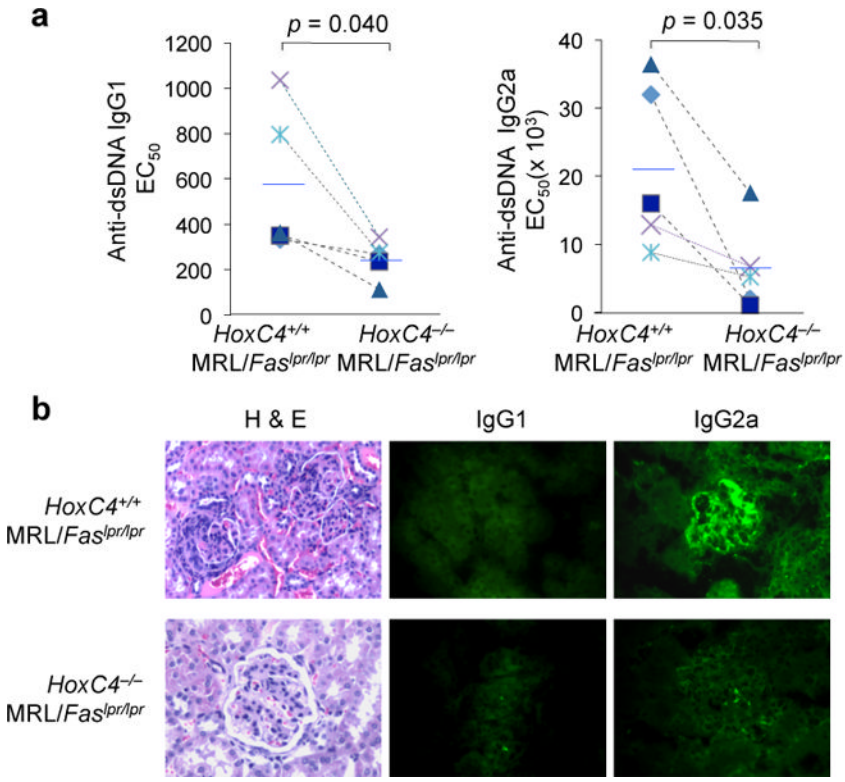

Figure 3.

HoxC4 deficiency decreases levels of serum IgG1 and IgG2a autoantibodies, IgG2a kidney deposition and glomerular damage in MRL/*Fas*$^{lpr/lpr}$ mice. (**a**) Titers of circulating anti-dsDNA IgG1 and anti-dsDNA IgG2a in 5 pairs of 16-week-old *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice and *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ littermates (each symbol represents an individual mouse) depicted as the number of dilutions needed to reach 50% of saturation binding (EC_{50}). *P* values, paired *t*-test. (**b**) Kidney sections from 3 *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ and 3 *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice were stained with H&E (left panels) or with FITC-labeled anti-IgG1 or anti-IgG2a mAb (middle and right panels). Depicted are kidney sections from one representative pair of *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ and *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice.

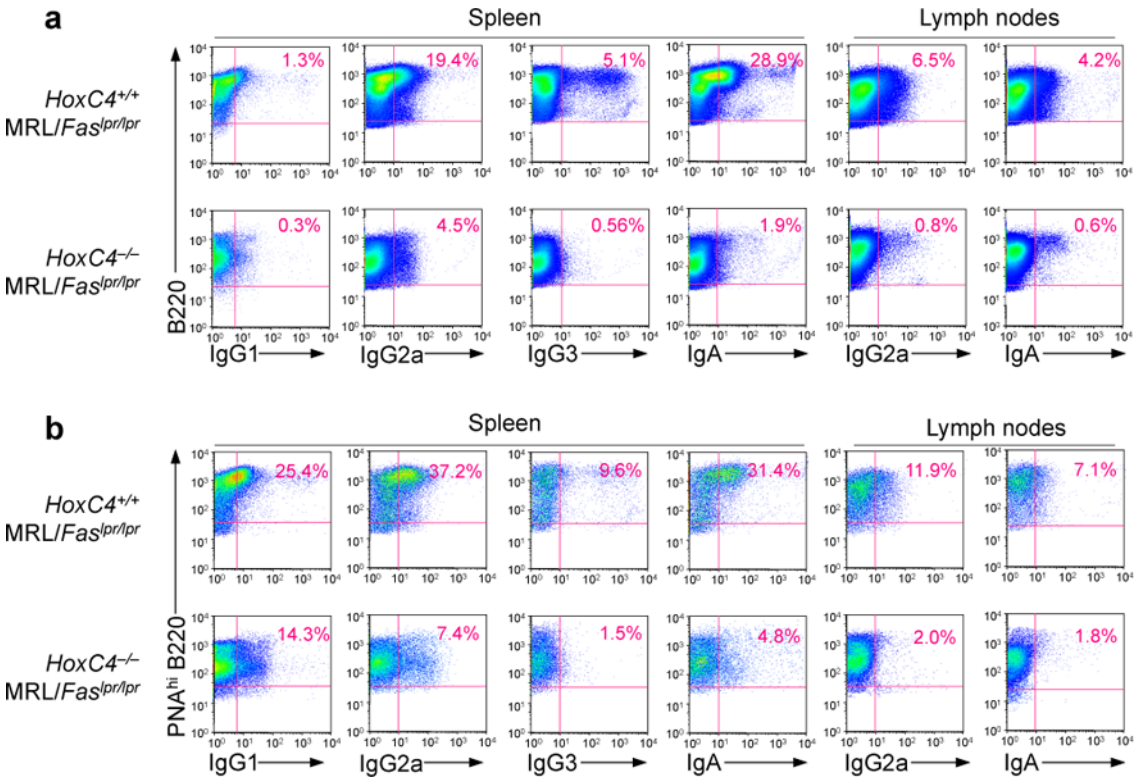

Figure 4.

HoxC4 deficiency impairs CSR in MRL/*Fas*$^{lpr/lpr}$ mice. (**a**) B220^{+} B cells from the spleen or lymph nodes of *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ and *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ littermates were analyzed for surface IgG1, IgG2a, IgG3 and IgA expression by flow cytometry. (**b**) B220^{+}PNA^{hi} GC B cells from the spleen or lymph nodes of *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ and *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ littermates were analyzed for surface IgG1, IgG2a, IgG3 and IgA expression by flow cytometry.

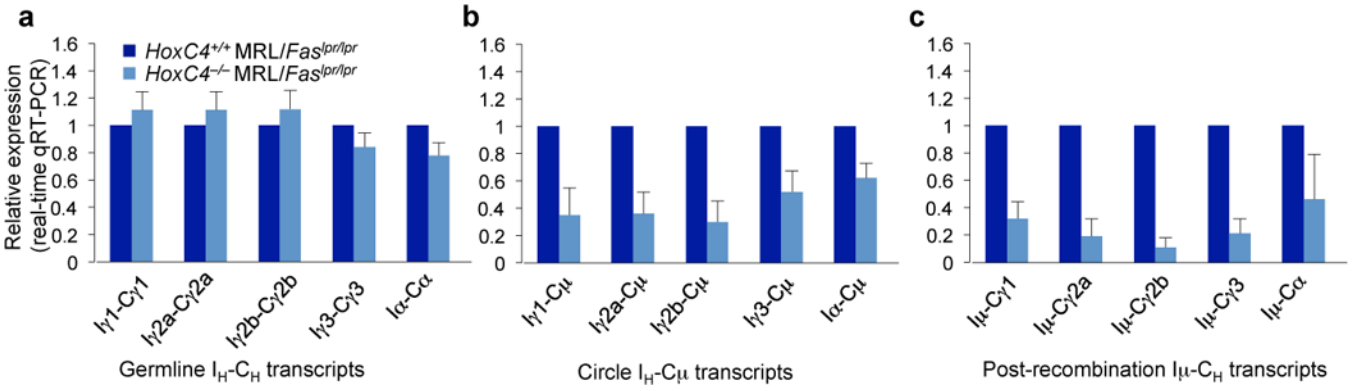

Figure 5.

HoxC4 deficiency does not alter germline I_H-C_H transcripts but results in lower expression of circle I_H-Cμ and post-recombination Iμ-C_H transcripts in MRL/*Fas*$^{lpr/lpr}$ mice. Total RNA was prepared from spleens of non-immunized *HoxC4*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ and *HoxC4*$^{-/-}$ MRL/*Fas*$^{lpr/lpr}$ mice. Germline I_H-C_H transcripts (**a**), circle I_H-Cμ transcripts (**b**) and post-recombination Iμ-C_H transcripts (**c**) were measured by real-time qRT-PCR using SYBR green and normalized to *Gapdh* expression. Data are from 3 independent experiments (mean values ± SD).

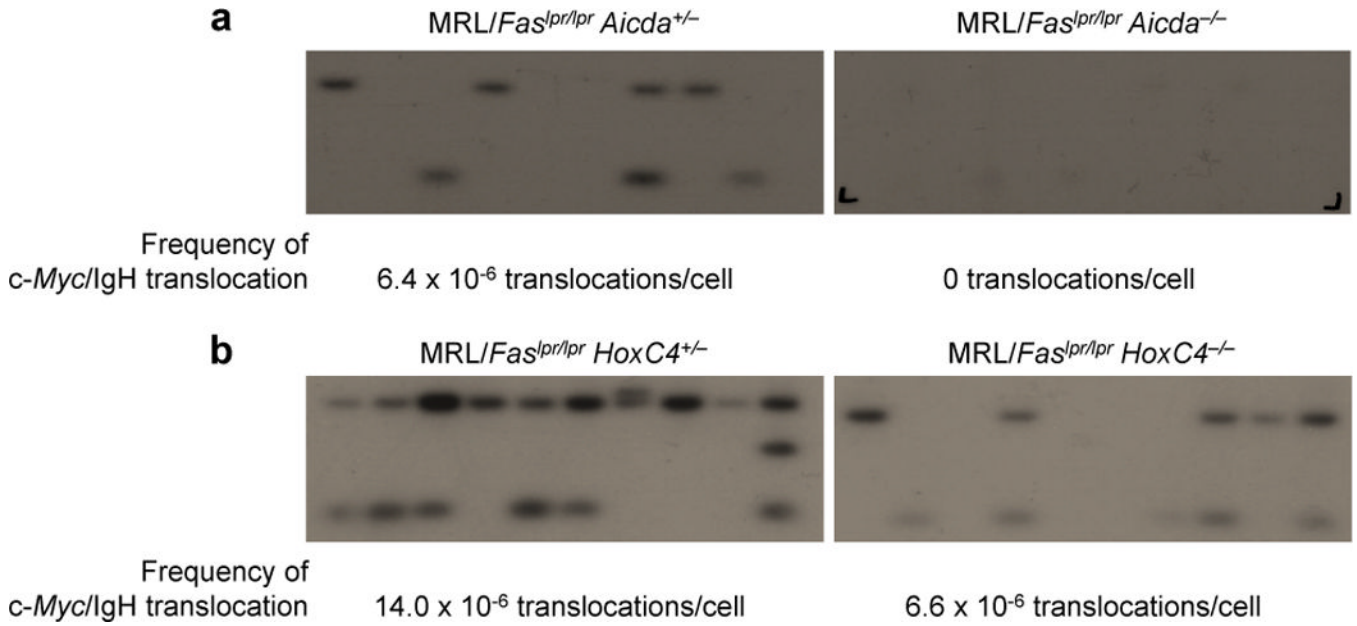

Figure 6.

c-Myc to *IgH* translocations are frequent events in B cells from MRL/$Fas^{lpr/lpr}$ mice, are reduced in the absence of HoxC4, and eliminated in the absence of AID. Genomic DNA was prepared from spleen cells of (**a**)$HoxC4^{+/+}$ $Aicda^{+/-}$ MRL/$Fas^{lpr/lpr}$, $HoxC4^{+/+}$ $Aicda^{-/-}$ MRL/$Fas^{lpr/lpr}$ mice, and (**b**)$HoxC4^{+/+}$ $Aicda^{+/+}$ MRL/$Fas^{lpr/lpr}$ and $HoxC4^{-/-}$ $Aicda^{+/+}$ MRL/$Fas^{lpr/lpr}$ mice. *c-Myc/IgH* translocations were detected by nested PCR using primers specific to the *IgH* and *c-Myc* locus, and further specified by Southern blotting with a [γ-^{32}P]-labeled *c-Myc* specific probe. Each individual PCR assay was performed using a DNA template corresponding to 5×10^5 cells. The corresponding frequency of translocations per cell is indicated underneath each panel. The data are representative of independent experiments with 3 sets of mice.

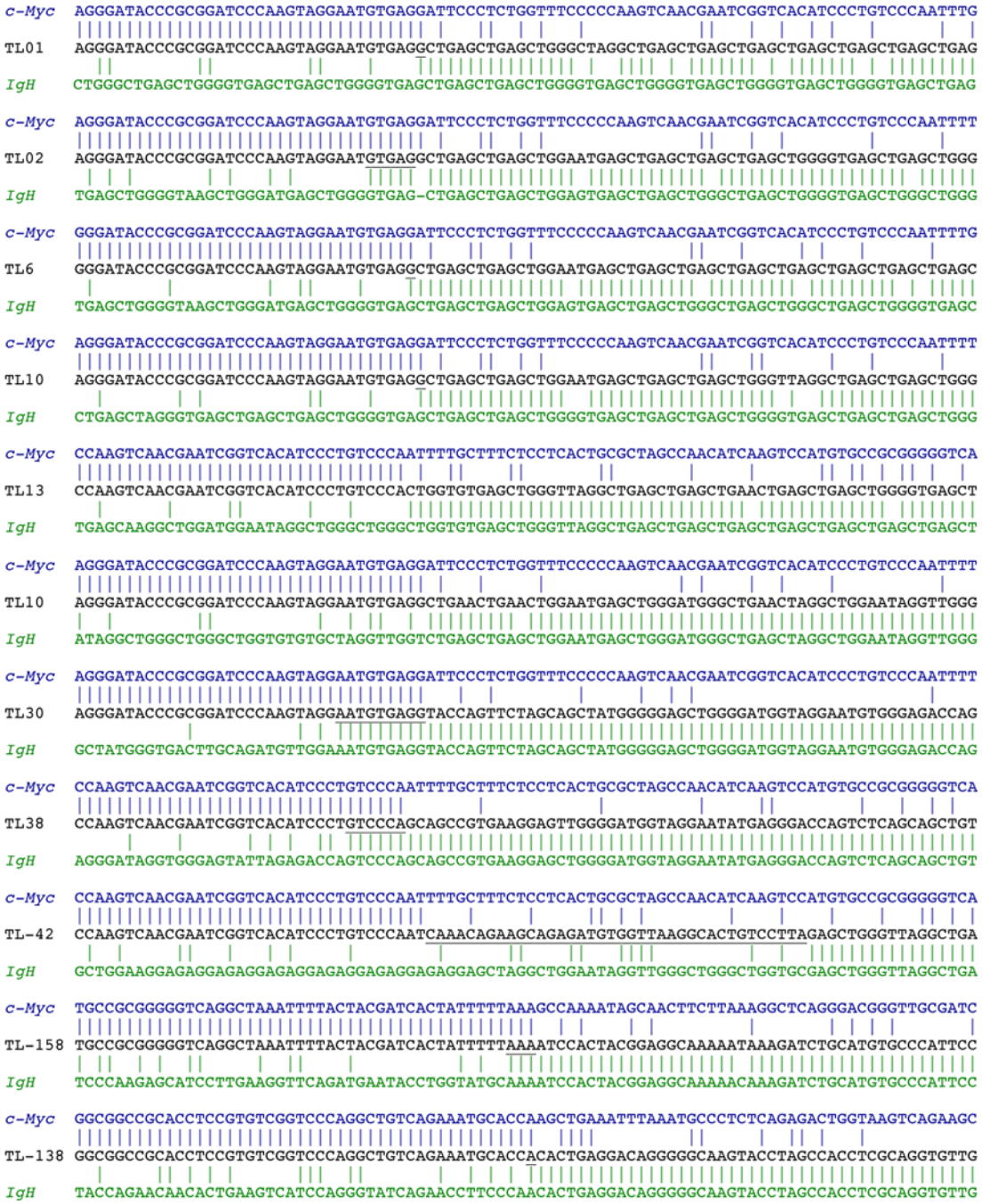

Figure 7.

Sequences of *c-Myc/IgH* translocation junctions. Fourteen *c-Myc/IgH* junction DNAs from spleen B cells of *HoxC4*$^{+/+}$ *Aicda*$^{+/+}$ MRL/*Fas*$^{lpr/lpr}$ mice were amplified, cloned and sequenced. Each sequence is compared with germline *c-Myc* (blue) and germline *IgH* (green) sequences. Microhomologies or insertions are underlined.

Table 1

Primers for real-time qRT-PCR detection of circle transcripts.

Circle transcript	Forward primer	Reverse primer
Iγ1-Cμ	5’-TCGAGAAGCCTGAGGAATGTG-3’	5’-TGGTGCTGGGCAGGAAGT-3’
Iγ2a-Cμ	5’-GCTGATGTACCTACCGAGAGA-3’	5’-TCTGAACCTTCAAGGATGCTCTTG-3’
Iγ2b-Cμ	5’-CACTGGGCCTTTCCAGAACTA-3’	5’-TGGTGCTGGGCAGGAAGT-3’
Iγ3-Cμ	5’-GAGGTGGCCAGAGGAGCAAGAT-3’	5’-TGGTGCTGGGCAGGAAGT-3’
Iα-Cμ	5’-CAAGAAGGAGAAGGTGATTCAG-3’	5’-TGGTGCTGGGCAGGAAGT-3’