Iron Inhibits Activation-induced Cytidine Deaminase Enzymatic Activity and Modulates Immunoglobulin Class Switch DNA Recombination* [S]

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Background: AID is critical for effective immune responses, as it initiates antibody class switching and somatic hypermutation by deaminating deoxycytidines in the immunoglobulin locus switch region and V(D)J DNA.

Results: Fe2+ inhibited AID-mediated deoxycytidine deamination and class switch DNA recombination in B cells.

Conclusion: Iron inhibits the enzymatic function of AID.

Significance: Iron can modulate antibody responses and may dampen AID-dependent autoimmunity and neoplastic transformation.

Immunoglobulin (Ig) class switch DNA recombination (CSR) and somatic hypermutation (SHM) are critical for the maturation of the antibody response. Activation-induced cytidine deaminase (AID) initiates CSR and SHM by deaminating deoxycytidines (dCs) in switch (S) and V(D)J region DNA, respectively, to generate deoxyuracils (dUs). Processing of dUs by uracil DNA glycosylase (UNG) yields abasic sites, which are excised by uracil DNA glycosylase (UNG), as thereby generating a high density of deoxyuracils (dUs) (10). SHM inserts mainly point mutations in Ig V(D)J region DNA at a high rate (2, 3), thereby providing the structural substrate for positive selection of high affinity antibody mutants by antigen (4). CSR substitutes the Ig heavy chain (IgH) constant region, such as Cµ, which is expressed in all naïve B cells, with a downstream Cy, Cα, or Ce region, thereby giving rise to IgG, IgA, or IgE antibodies. Class-switched antibodies display different biological effector functions but maintain the same structure and specificity of the antigen-binding site compared with IgM antibodies produced by unswitched B cells (5).

Both CSR and SHM require activation-induced cytidine deaminase (AID), a member of the AID/APOBEC cytidine deaminase family (6–8). CSR also entails IgH locus germline Iµ-H1, S-Cµ-H transcripts, and elongates through the upstream (donor) S and downstream (acceptor) Cµ-H regions, giving rise to germline Iµ-Cµ, 1γ-Cγ, 1α-Cα, or Iε-Cε transcripts. The “core” of each of S regions contains a tandem array of 5’-AGCT-3’ repeats, which are specifically targeted by 14-3-3 adapter proteins. These adaptors, in turn, recruit AID to S region DNA (9). There, AID will deaminate deoxycytidines (dCs), particularly those within 5’-AGCT-3’ repeats in the core of S regions, thereby generating a high density of deoxyuracils (dUs) (10). These can then be excised by uracil DNA glycosylase (UNG), as recruited to S region DNA by the REV1 DNA polymerase,3 to yield abasic sites, which are excised by apurinic/apyrimidinic endonucleases, leading to single strand DNA breaks. High density single strand DNA breaks in both DNA strands readily form double strand DNA breaks, the obligatory CSR intermediates

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1 This article contains supplemental Figs. S1 and S2 and Table S1.

2 The abbreviations used are: CSR, class switch DNA recombination; 7-AAD, 7-aminoactinomycin D; AID, activation-induced cytidine deaminase; CFSE, carboxyfluorescein diacetate succinimidyl ester; dC, deoxycytidine; dex, dextran; dU, deoxyuracil; H, heavy chain; nt, nucleotides; PTIP, Pax transcription-domain-interacting protein; qRT-PCR, quantitative real-time PCR; S, switch; SHM, somatic hypermutation; UNG, uracil DNA glycosylase.

3 H. Zan, C. A. White, L. M. Thomas, J. Zhang, G. Li, E. S. Yu, Z. Xu, T. Mai, and P. Casali, unpublished data.
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(12, 13). Looping out of the intervening DNA between the donor and acceptor S regions and re-ligation of double strand DNA break free ends result in S-S junctions and recombination. Postrecombination IgH sequences are transcribed, giving rise to postrecombination transcripts, such as \( I_\mu-C_y \), \( I_\mu-C_a \), or \( I_\mu-C_e \) (5).

CSR levels are determined by overall expression level, targeting, and activity of AID (14). AID expression is strictly regulated in a B cell lineage-specific and differentiation stage-specific manner. It is induced by primary CSR-inducing stimuli, for example CD40 or dual engagement of Toll-like receptors (TLRs) and B cell receptor (BCR), through transcription factors such as HOXC4 (15), E2A (16), and NF-κB (16–18), and enhanced by cytokines, such as IL-4 and TGFβ (17). AID levels are also controlled at the post-transcriptional stage (e.g. by microRNAs) and post-translational stage (e.g. by proteasome-mediated degradation) (14). Further, to mediate CSR, AID needs to be targeted to S region DNA by 14-3-3 adaptors through direct protein-protein interaction (9). AID C-terminal truncation mutants cannot bind 14-3-3 and are defective in mediating CSR. Finally, AID dC deamination activity is enhanced by 14-3-3 and regulated by replication protein A and RNA exosomes (19, 20). The important role of 14-3-3, RNA, and RNA exosome components in CSR strongly suggests that the regulation of AID activity constitutes an important step in regulation of CSR.

Iron is a crucial metal element. It mediates many metabolic pathways and is required for proliferation of cells, including B and T lymphocytes (21). B lymphocyte proliferation is inhibited by iron chelators, such as deferoxamine and salicylaldehyde isonicotinoyl hydrazone, or depletion of ferritin, a ferrous iron (Fe\(^{2+}\)) transporter (21, 22). Despite the importance of iron in B cell proliferation, iron overload is associated with impaired immune defense to viruses and bacteria, including Mycobacterium tuberculosis, Candida albicans and Pasteurella septic, in humans and experimental animals (23–26), possibly due to impaired antibody responses. Accordingly, patients with hemochromatosis display iron overload, as caused by excess absorption of iron, and a reduced level of class-switched antibodies (27). Finally, heme, an iron-containing molecule essential for diverse organisms, has been implicated in inhibiting CSR (28), suggesting a role of iron in modulating this important B cell differentiation process.

Here, we have tested the hypothesis that iron suppresses CSR, thereby inhibiting the generation of class-switched B cells and antibodies. We have used a well defined B cell culture system to analyze the role of Fe\(^{2+}\) in CSR and B cell plasmacytoid differentiation, which underpin the high rate production of class-switched antibodies. We have further measured core CSR parameters, such as levels of germline \( I_\mu-S-C_H \) transcription, AID, and other CSR factors. Finally, we have used \textit{in vitro} dC DNA deamination assays involving purified recombinant AID to analyze Fe\(^{2+}\)-mediated inhibition of CSR at the molecular level.

**EXPERIMENTAL PROCEDURES**

\textit{B Cells}—Preparation and purification of mouse spleen and lymph node B cells were as described (18). B cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with penicillin-streptomycin and amphotericin B (1% v/v), FBS (10% v/v; Hyclone), and 50 mM β-mercaptoethanol (RPMI-FBS). To induce CSR, B cells were stimulated with LPS (5 μg/ml, from \textit{Escherichia coli}, serotype 055:B5 and deproteinized by chloroform extraction; Sigma-Aldrich) alone for CSR to IgG3 or with IL-4 (3 ng/ml; R&D Systems) for CSR to IgG1 or with IL-4, TGFβ (3 ng/ml; R&D Systems) plus rat anti-mouse IgG chain monoclonal antibody (clone 11–26) conjugated to dextran (anti-β mAb/dex, 30 ng/ml; Fina Biosolutions; IgG is expressed in all naïve B cells) for CSR to IgA. FeCl\(_2\) and FeSO\(_4\) (ACS grade; Sigma-Aldrich) dissolved in PBS were freshly prepared and added at a final concentration of 80 μM or 20 μM and 40 μM as indicated.

**Surface Ig Analysis**—After 4 days of stimulation, B cells were harvested for flow cytometry analysis of surface expression of Ig (Igμ, and/or slgγ3, slgγ1, and slgα for CSR to IgG3, IgG1 and IgA, respectively), B220 (clone RA3-6B2, ebioscience) and/or CD138 (after 5 days of stimulation), as we described (18). Briefly, B cells were centrifuged at 400 × g for 5 min and then stained with fluorochrome-conjugated mAbs in Hanks’ buffered salt solution (HBSS) containing BSA (1%, w/v) for 15 min. After washing, cells were resuspended in HBSS-BSA buffer and analyzed using a FACScalibur (BD Biosciences). Data were analyzed by using the Flowjo® software (Tree Star). Dead (7-AAD\(^{+}\)) cells were excluded from analysis.

**B Cell Proliferation and Viability Analysis**—CFSE-labeled B cells were stimulated for 4 days and harvested for flow cytometry analysis of CFSE intensity (which halves in two daughter cells when a cell divides) and surface expression of Ig, as described above. To analyze B cell proliferation, individual cell divisions were first determined by the cell proliferation platform of Flowjo; and CSR to IgG3, IgG1, or IgA as a function of division number was analyzed by the ratio of IgG3\(^{+}\), IgG1\(^{+}\), or IgA\(^{+}\) B cells, respectively, in each division over total B cells in that division. For B cell viability analysis, cells were stained with 7-AAD, which enters apoptotic and necrotic cells, but not intact cells, to intercalate into DNA, and analyzed by flow cytometry.

**RNA Isolation and Transcript Analysis by Quantitative Real-time PCR (qRT-PCR)**—Total RNA was extracted from 5 × 10\(^6\) B cells using a RNaseasy Mini Kit (Qiagen) according to the manufacturer’s instruction. First strand cDNA were synthesized from 2 μg of total RNA using the SuperScript\textsuperscript{TM} III system with oligo(dT) primer (Invitrogen) and measured by qRT-PCR using appropriate primers (supplemental Table S1) and SYBR Green (Dyno HS kit; New England Biolabs). PCR was performed in the MyiQ Single-color RT-PCR Detection System (Bio-Rad Laboratories) according to the following protocol: 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s. Melting curve analysis was performed at 72–95 °C. The ΔΔCt method was used to analyze levels of transcripts, and data were normalized to the level of \textit{Cd79b}, which encodes the B cell receptor \textit{Igβ} chain, as constitutively expressed in B cells.

**Secreted Ig Analysis**—To analyze titers of IgG3, IgG1, or IgA secreted from cultured B cells, culture supernatants from B cells stimulated for 4 days were diluted 4-, 10-, and 4-fold, respectively, with PBS plus 0.05% (v/v) Tween 20 (PBST). 2-fold serially diluted samples and the standard for each Ig isotype (IgG3, IgG1, or IgA) were incubated in a 96-well plate pre-
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FIGURE 1. Fe$^{2+}$ suppresses CSR. A, surface expression of B220 and IgG3 (induced by LPS), IgG1 (induced by LPS plus IL-4), or IgA (induced by LPS, IL-4, TGFβ plus anti-μ mAb/dex) in the absence (nil, black) or presence of 80 μM FeCl$_2$ (plum) or FeSO$_4$ (teal). The number inside each rectangle indicates the percentage of B220$^+$ cells that are switched to the indicated Ig isotypes. Ratios of the proportion of switched B cells in B cells stimulated in the presence of Fe$^{2+}$ to that of B cells stimulated in the absence of Fe$^{2+}$ are depicted in histograms (mean ± S.E. of data from three independent experiments). B, titers of IgG3, IgG1, or IgA in supernatants of B cells stimulated with LPS, LPS plus IL-4, or LPS, IL-4, TGFβ plus anti-μ mAb/dex in the absence (nil, black) or presence of 80 μM FeCl$_2$ (plum) or FeSO$_4$ (teal) (mean ± S.D. of data from three independent experiments). C, levels of postrecombination μ-γ, μ-γ', and μ-μ transcripts in B cells stimulated with LPS, LPS plus IL-4, or LPS, IL-4, TGFβ plus anti-μ mAb/dex in the absence (nil, black) or presence of 80 μM FeCl$_2$ (plum) or FeSO$_4$ (teal). Data were normalized to the expression of CD79b and are expressed as ratios of values in B cells stimulated in the presence of Fe$^{2+}$ to those in B cells stimulated in the absence of Fe$^{2+}$ (mean ± S.E. of data from three independent experiments). **, p < 0.05 (p values: t test).

Blocked with PBST and BSA and coated with a preadsorbed goat Ab to mouse IgG to capture IgG3 and IgG1 or goat Ab to mouse Ig to capture IgA (all goat Abs were from Southern BioTech). Captured Igs were reacted with biotin-labeled rat mAb to mouse IgG3, IgG1, or IgA (BD Biosciences), followed by incubation with horseradish peroxidase (HRP)-labeled streptavidin (Sigma-Aldrich) and development with o-phenylenediamine. A$_{492 \text{ nm}}$ was measured with a Luminescence Detector 400 (Beckman Coulter). Ig concentrations were analyzed using Excel® software (Microsoft).

**AID dC DNA Deamination Assay**—Purified recombinant GST-AID (1 ng) and UNG (0.2 unit, in excess to convert all AID-generated dUs to abasic sites; New England Biolabs) were incubated with the HPLC-purified single strand oligonucleotide 5'–AGGCTAAAAGGCTAAAAGCTAAA–3' labeled with Alexa Fluor 647 at the 3' end (Alexa-5'–AGGCTAAAGGCTAAAAGCTAAA–3' (dUs are underlined) was used as the substrate for UNG. This oligonucleotide (termed Alexa-[5'-AGU/(C)T-3']$_{1,24}$ nt) was generated by incubating 1 pmol of Alexa-[5'-AGGCTAAAGGCTAAAAGCTAAA–3']$_{1,24}$ nt with GST-AID (1 ng) in a 20-μl reaction mixture containing Tris-HCl (25 mM, pH 7.5), MgCl$_2$ (50 mM), DTT (0.5 mM), CHAPS (0.01% v/v), and glutathione (1 mM) at 37 °C for the indicated time. RNAse A (Qiagen) and FeCl$_2$, ZnCl$_2$, MnCl$_2$, MgCl$_2$, or NiCl$_2$ (ACS grade; all from Sigma-Aldrich) were freshly added and prepared, within 30 min of preparation, at final concentrations as indicated. After the reaction, heat-labile abasic sites were cleaved to completion by heating at 95 °C for 10 min in the presence of NaOH (200 mM). The intact substrate Alexa-[5'-AGGCTAAAGGCTAAAAGCTAAA–3']$_{1,24}$ nt and cleavage products were fractionated by denaturing 15% PAGE containing 8 M urea and visualized using a Typhoon™ 9410 scanner (GE Healthcare). The intensity of substrate and cleavage product bands was quantified using the ImageQuant™ TL software (GE Healthcare). AID deamination activity was defined as the percentage of DNA substrate showing deamination (sum of the three cleavage products over the sum of the substrate and cleavage products).

**UNG dU Excision Assay**—The Alexa Fluor 647-labeled dU-containing oligonucleotide 5'-AGU/(C)TAAAAGU/(C)TA-AAAAGU/(C)TAAA-3' (dUs are underlined) was used as the substrate for UNG. The oligonucleotide (termed Alexa-[5'-AGU/(C)T-3']$_{1,24}$ nt) was generated by incubating 1 pmol of Alexa-[5'-AGGCTAAAGGCTAAAAGCTAAA–3']$_{1,24}$ nt with GST-AID (1 ng) in a 20-μl reaction mixture containing Tris-HCl (25 mM, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.01% (v/v) CHAPS, 1 mM glutathione, and 10 ng/ml RNase A) at 37 °C for 30 min. Incubation of Alexa-[5'-AGU/(C)T-3']$_{1,24}$ nt with UNG was carried out in reaction buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.01% (v/v) CHAPS, 1 mM glutathione, and 10 ng/ml RNase A) in the absence or presence of 20 μM FeCl$_2$, ZnCl$_2$, MnCl$_2$, MgCl$_2$, or NiCl$_2$ at 37 °C for 20 min. After the reaction, heat-labile abasic sites were cleaved to completion by heating at 95 °C for 10 min in the presence of 200 mM NaOH. The intact substrates Alexa-[5'-AGU/(C)T-3']$_{1,24}$ nt and cleavage products were fractionated by denaturing 15% PAGE containing 8 M urea and visualized using a Typhoon™ 9410 scanner. The intensity of the substrate and cleavage product bands was quantified using the ImageQuant™ TL software. The UNG dU excision activity
was defined as the percentage of substrates showing cleavage (sum of the three cleavage products over the sum of the substrate and cleavage products).

Mice—C57BL/6 mice were maintained in a pathogen-free vivarium at the University of California, Irvine. The mice used in all experiments were 8–12 weeks old and without any apparent infection or disease. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Irvine.

Statistical Analysis—Statistical analysis was performed using the Excel software to determine p values by paired Student t test. p values < 0.05 were considered significant.

RESULTS

Fe^2+ Suppresses CSR to Multiple Ig Isotypes—Decreased levels of class-switched antibodies and impaired immune responses in human and mice with iron overload prompted us to hypothesize that CSR is inhibited by iron. To test this hypothesis, we analyzed CSR in B cells stimulated with LPS (for induction of CSR to IgG3, which mediates the antibody response to many pyogenic bacteria); LPS plus IL-4 (to IgG1, which is the most abundant IgG subclass and provides neutralizing antibodies to blood-borne viral and bacterial pathogens); or LPS, IL-4, and TGFB plus anti-δ mAb/dex (to IgA, which mediates the front-line immune defense on respiratory and digestive mucosa) in the presence of Fe^2+ (FeCl_2 or FeSO_4). The proportion of surface IgG3^+ B cells was significantly decreased in the presence of FeCl_2 or FeSO_4, compared with B cells cultured in the absence of Fe^2+ (Fig. 1). Fe^2+ inhibition of generation of IgG3^+ B cells was dose-dependent, starting at 20 μM Fe^2+, further at 40 μM, and by as much as 77 and 57% at 80 μM FeCl_2 and FeSO_4, respectively (supplemental Fig. S1). Iron concentrations range from 10 to 30 μM in the serum of healthy adults and from 30 to >40 μM in the serum of patients with hemochromatosis (29). Likewise, in the presence of Fe^2+, the proportion of surface IgG1^+ and IgA^+ B cells was reduced by as much as 50% (FeCl_2) and 84% (FeSO_4), respectively. FeCl_2 or FeSO_4 did not alter B cell surface expression of Igμ (Fig. 2A), showing that Fe^2+ did not interfere with expression of membrane Igs; rather, it specifically inhibited the generation of class-switched (IgG3^+, IgG1^+, and IgA^+) B cells. Further, titers of IgG3, IgG1, and IgA antibodies secreted from stimulated B cells were decreased in the presence of FeCl_2 (by 91, 68, and 66%, respectively) or FeSO_4 (by 75, 54, and 79%, respectively) (Fig. 1B). Finally, Fe^2+–mediated impairment in the generation of surface and secreted class-switched Igs was associated with decreased levels of postrecombination μ-Cy3 (53 and 83% lower by FeCl_2 and FeSO_4, respectively), μ-Cy1 (40 and 24% lower), or μ-Cx (46 and 90% lower) transcripts, which are direct indexes of DNA recombination involving Sμ and Sγ3, Sγ1, or Sα (Fig. 1C). Thus, Fe^2+ suppresses CSR to multiple Ig isotypes, thereby inhibiting the generation of class-switched antibodies.
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Fe²⁺ Does Not Impair B Cell Proliferation or Viability—B cell proliferation is required for efficient CSR. We next analyzed whether B cell proliferation or viability was affected by Fe²⁺ under the same stimulation conditions as those used for CSR induction. B cells stimulated with LPS (for CSR to IgG3), LPS plus IL-4 (to IgG1), or LPS, IL-4, TGFβ plus anti-δ mAb/dex (to IgA) for 4 days and cultured with FeCl₂ or FeSO₄ completed virtually the same number of cell divisions as their counterparts cultured without the Fe²⁺ treatment (Fig. 2, B and C). Furthermore, the proportion of class-switched (IgG3⁺, IgG1⁺, and IgA⁺) B cells within each cell division was decreased in the presence of FeCl₂ or FeSO₄, showing that Fe²⁺-mediated CSR inhibition was independent of B cell proliferation. Finally, B cells stimulated with LPS, LPS plus IL-4, or LPS, IL-4, TGFβ plus anti-δ mAb/dex in the absence (nil, black) or presence of 80 μM FeCl₂ (plum) or FeSO₄ (teal). Data were normalized to the level of Cd79β and are expressed as ratios of values in B cells cultured with LPS, LPS plus IL-4, or LPS, IL-4, TGFβ plus anti-δ mAb/dex in the presence of Fe²⁺ to those in B cells cultured in the absence of Fe²⁺ (mean ± S.E. of data from three independent experiments).

Fe²⁺ Does Not Affect Plasmacytoid Differentiation—During the antibody response, unswitched (IgM⁺) and switched (IgG⁺, IgA⁺, and IgE⁺) B cells differentiate into short lived plasmablasts and long lived plasma cells (plasmacytes), respectively, thereby generating secreted IgM Abs or class-switched (IgG, IgA, and IgE) Abs. Both plasmablasts and plasma cells express high levels of surface CD138 (syndecan-1), the hallmark of plasmacytoid differentiation (30). Accordingly, B cells stimulated in vitro by LPS, LPS plus IL-4, or LPS, IL-4, TGFβ plus anti-δ mAb/dex expressed surface CD138. Generation of CD138⁺ cells was not affected or even slightly enhanced in the presence of FeSO₄ (Fig. 3A).

Plasmacytoid differentiation requires B lymphocyte-induced maturation protein 1 (BLIMP1), which activates expression of secreted Ig protein chains and, by down-regulating PAX5, depresses expression of X-box binding protein 1 (XBP1) (31–33). XBP1 is a member of the CREB/ATF transcription factor family and activates transcription of genes encoding proteins that function in secretory pathways. Transcription of both the Prdm1 (encoding BLIMP1) and Xbp1 gene is also regulated by the IRF4 transcription factor, which plays a role in both CSR and plasmacytoid differentiation. In stimulated B cells, expression of If4, Prdm1, or Xbp1 was not suppressed by FeCl₂ or FeSO₄ (Fig. 3B). In fact, expression of Prdm1 and Xbp1 was up-regulated by FeSO₄ in B cells stimulated with LPS plus IL-4 or LPS, IL-4, TGFβ plus anti-δ mAb/dex, largely in agreement with the role of Fe²⁺ in the generation of CD138⁺ plasmacytoid cells and plasmacytes. Thus, Fe²⁺ does not suppress plasmacytoid differentiation, indicating that the significant decrease in titers of secreted class-switched antibodies in the presence of Fe²⁺ is due to Fe²⁺-mediated CSR inhibition.

Fe²⁺ Does Not Alter Induction of AID, 14-3-3γ, or PTIP—To address the molecular mechanisms underlying Fe²⁺-mediated CSR inhibition, we analyzed the effect of FeCl₂ and FeSO₄ on AID, 14-3-3γ, and PTIP. In B cells stimulated by LPS, LPS plus IL-4, or LPS, IL-4, TGFβ plus anti-δ mAb/dex, FeCl₂ or FeSO₄ did not affect the induction of AID (encoded by the Aicda
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gene), which is essential for CSR, 14-3-3-γ, which is rapidly induced, and recruit AID to the S regions that will undergo recombination, or PTIP (encoded by the Paxip1 gene), which mediates multiple histone modifications and germline transcription in acceptor S regions (34) (Fig. 4). Likewise, FeCl₂ or FeSO₄ did not affect the induction of (spliced) germline Ig-μ, Ig-3-μ3, Ig-1-γ1, and/or Igα-Cα transcripts, with the exception of some reduction in Igμ-Cμ and Igα-Cα transcript levels in B cells stimulated with LPS, IL-4, TGFB plus anti-δ mAb/dex in the presence of FeCl₂ to those in B cells stimulated in the absence of FeCl₂ (mean ± S.E. of data from three independent experiments). **, p < 0.05 (t test).

**FIGURE 4. FeCl₂ does not alter induction of Aicda, 14-3-3-γ, or Paxip1, or germline Ig-5-C₅ transcription in general.** Levels of Aicda, 14-3-3-γ, and Paxip1 transcripts in B cells were stimulated with LPS, LPS plus IL-4, or LPS, IL-4, TGFB plus anti-δ mAb/dex in the absence (nil, black) or presence of 80 μM FeCl₂ (plum) or FeSO₄ (teal) as well as germline Ig-μ, Ig-3-μ3, Ig-1-γ1 or Igα transcripts in those B cells. Data were normalized to the level of Cd79b and are expressed as ratios of values in B cells stimulated in the presence of FeCl₂ to those in B cells stimulated in the absence of FeCl₂ (mean ± S.E. of data from three independent experiments). **, p < 0.05 (p values: t test).

DISCUSSION

Here, we have shown that FeCl₂ efficiently inhibited the generation of class-switched B cells and antibodies by interfering with the intrinsic CSR mechanisms without altering B cell proliferation or viability. FeCl₂ inhibition of CSR, such as from IgM to IgG3 (by FeCl₂) or IgA (by FeSO₄), was profound and concomitant with normal plasmacytoid differentiation of B cells. It was associated with normal expression levels of important CSR factors, including AID, 14-3-3, and PTIP, and mainly reflected FeCl₂ inhibition of AID enzymatic activity (dC DNA deamination). Such inhibition was specific, as emphasized by the failure of other bivalent metal ions (Zn²⁺, Mn²⁺, Mg²⁺, or Ni²⁺) to inhibit AID enzymatic activity and the inability of FeCl₂ to inhibit UNG dU excision activity (whether FeCl₂ inhibits enzymatic or scaffold functions of other CSR factors remains to be determined). By inhibiting AID enzymatic activity, FeCl₂ not only inhibits CSR but would also interfere with AID-mediated SHM and generation of high affinity antibody mutants, which are positively selected by antigen in germinal centers of peripheral lymphoid organs (e.g. lymph nodes and spleen).

FeCl₂ inhibition of enzymatic function of AID in CSR is further emphasized by our findings that FeCl₂ did not generally

nases can be bound by Zn²⁺ or Fe²⁺ (36). To test our hypothesis, we performed in vitro AID dC deamination assays, in which dCs within the three 5’-AGCT-3’ motifs of a 24-nt single strand oligonucleotide Alexa-5'-AGCT-3'₁₄₂₄₄₄ was converted by AID to dUs. These were, in turn, excised by UNG to yield labile abasic sites, which were then cleaved by alkaline and heat treatment to generate 18-nt, 10-nt, and 2-nt cleavage products (Fig. 5A). FeCl₂ inhibited AID-mediated deamination of dCs within Alexa-5’-AGCT-3’₁₄₂₄₄₄₄ in a dose-dependent fashion and within a narrow range, i.e. from 2 μM (7% inhibition of the AID enzymatic activity) to 8 μM (87% inhibition) (Fig. 5B).

RNase A has been shown to enhance AID-mediated dC DNA deamination in vitro (37–39), possibly by degrading an RNA molecule that binds tightly to recombinant AID and inhibits its enzymatic activity. To ascertain that FeCl₂ does not merely interfere with the activity of RNase A used in our assays, we performed AID deamination assays without RNase A. In the absence of RNase A, AID-mediated dC deamination plateaued at 60% deamination activity within 15 min compared with 80% deamination activity within 5 min in the presence of RNase A. The (RNase A-independent) intrinsic AID dC deamination activity was efficiently inhibited by FeCl₂ (Fig. 5, C and D). In contrast to FeCl₂, bivalent metal ions Zn²⁺, Mn²⁺, Mg²⁺, and Ni²⁺ exhibited little, if any, inhibitory effects on AID-mediated deamination (Fig. 6).

To analyze whether FeCl₂ also affects UNG dU excision activity, we generated dU-containing oligonucleotides that could give rise to abasic sites for cleavage by alkaline and heat treatment only after UNG-mediated dU excision (Fig. 7, A and B). This was not affected by FeCl₂, Zn²⁺, Mn²⁺, Mg²⁺, or Ni²⁺, as shown by >90% of the substrates showing cleavage (Fig. 7C).

Thus, FeCl₂, but not other bivalent metal ions (Zn²⁺, Mn²⁺, Mg²⁺ or Ni²⁺), effectively inhibits the intrinsic AID dC DNA deamination activity in a dose-dependent fashion. FeCl₂ does not affect UNG-mediated dU excision.
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Affect germline \( I_\delta - S - C_\mu \) transcription. The only exception was the reduction of germline \( I_\mu - S - C_\mu \) (by 30%) and \( I_\alpha - S_a - C_\alpha \) (by 70%) transcription in B cells stimulated with LPS, IL-4, TGF\( \beta \) plus anti-\( \delta \) mAb/dex by FeSO\( _4 \). Such inhibition likely resulted from FeSO\( _4 \)-mediated interference with TGF\( \beta \)-induced activation of transcription factors that are critical for germline \( I_\alpha - S_a - C_\alpha \) transcription, such as RUNX and SMAD. Reduction in germline \( I_\mu - S_a - C_\mu \) and \( I_\alpha - S_a - C_\alpha \) transcription compounded the Fe\( ^{2+} \) inhibition of AID-mediated dC DNA deamination, leading to the profound decrease (90%, as assessed by postrecombination \( I_\mu - C_\alpha \) transcripts) of CSR to IgA. Lack of inhibition of germline \( I_\alpha - S_a - C_\alpha \) transcription by FeCl\( _2 \) suggests that FeCl\( _2 \) does not modulate TGF\( \beta \) signaling and extends previous findings showing the differential ability of FeSO\( _4 \) and FeCl\( _2 \) in modulating selected biological processes, such as xylanase enzymatic activity and production of homopolymer \( \epsilon \)-poly-L-lysine in Kitasatospora kifunense (40, 41).

Our findings that the generation of class-switched and, likely, high affinity antibodies can be suppressed by iron (21, 22) strongly suggest that cytoplasmic/nuclear iron levels need be
temporarily down-regulated in antigen-activated B cells, perhaps as signaled by CSR-inducing stimuli, such as CD154:CD40 engagement and/or B cell receptor cross-linking, which, as we have shown, is critical for Toll-like receptor-dependent and T cell-independent CSR in vivo and in vitro (18). Our findings suggest that in antigen-primed B cells, such as those engaged in the germinal center reaction, a lower level of iron than that steadily maintained in naive B cells would enable efficient class switching while maintaining proper B cell proliferation. Downregulation of iron levels in germinal center B cells could result from reduced iron uptake dependent on transferring receptor 2, as suggested by down-regulation of transferring receptor 2 expression in those B cells (42). B cells existing the germinal center reaction (after undergoing CSR and SHM) would replenish their iron pool in the transition to memory B cells or plasma cells and might do so by “siphoning” iron from macrophages, a major iron reservoir in peripheral lymphoid organs, via long-range “nanotubes” (43) that function as conduits for molecular exchange between cells (44).

Due to the high mutagenicity of AID, expression and targeting of this cytidine deaminase are tightly regulated (45). Our findings that Fe$^{2+}$ suppresses AID enzymatic activity outline another level of control (by endogenous metabolites, i.e. iron and factors that regulate iron transport and uptake) of overall AID activity, in addition to the regulation of AID expression. Genetic defects, infectious agents, or tissue injuries can perturb the B cell-intrinsic homeostatic mechanisms that regulate intracellular iron level and lead to iron overload. Consistent with the principle outlined here by our in vitro findings, high iron levels are associated in different contexts with impaired antibody responses (25, 46). Most notably, patients with hemochromatosis, a genetic disease characterized by iron overload (ranging from 30 μM to >40 μM), have reduced level of class-switched IgG and IgA antibodies, which can be readily explained by our findings that CSR was substantially decreased by Fe$^{2+}$ at 40 and 80 μM, and lowered defenses with increased susceptibility to viral and, more frequently, bacterial infections (26, 27). Once established, infections could in turn interfere with antibody class switching and the immune response by further up-regulating B cell iron levels. For instance, by inducing macrophage expression of ferritin, HIV-1 accessory protein Nef would lead to up-regulation of B cell intracellular iron levels, leading to decreased AID and inhibition of class-switching of HIV-1-specific antibodies (22, 44, 47, 48); Nef can also directly inhibit CSR (49).

Our data showed that the AID enzymatic activity was highly sensitive to Fe$^{2+}$ concentrations in vitro (2−8 μM) and likely in switching B cells, which, as we contend, would temporarily down-regulate intracellular iron levels to enable efficient CSR. Notably, the narrow range of concentrations at which Fe$^{2+}$ inhibited AID enzymatic functions fell within the putative range of intracellular free iron levels (0.5–10 μM) in many cell types, likely including B cells (50, 51). Fe$^{2+}$ would inhibit AID enzymatic activity by displacing Zn$^{2+}$ in this enzyme catalytic site by virtue of the similar chemical coordination properties of these two metal ions (supplemental Fig. S2). Accordingly, the endogenous cytosine deaminase purified from E. coli contains mostly Fe$^{2+}$ but also Zn$^{2+}$, whereas the cytosine deaminase isolated from yeast contains mostly Zn$^{2+}$ but also Fe$^{2+}$. The subtle coordination differences of Fe$^{2+}$ and Zn$^{2+}$ would, however, result in significantly different catalytic rates (36, 52). Fe$^{2+}$ can inhibit the activity of Zn$^{2+}$-containing yeast cytosine deaminase, and conversely, Zn$^{2+}$ can inhibit the activity of Fe$^{2+}$-containing E. coli cytosine deaminase (52, 53). How Fe$^{2+}$ would displace Zn$^{2+}$, which generally coordinates catalytic residues in metalloenzymes, likely including AID, with a high affinity (54) from AID remains to be elucidated. An alternative possibility is that Fe$^{2+}$ binds to residues different from those of Zn$^{2+}$, thereby inducing allosteric changes that lead to impairment of AID enzymatic activity. Oxidation of Fe$^{2+}$ to Fe$^{3+}$ within the E. coli cytosine deaminase over the course of hours results in inactivation of that enzyme (53). It is, however, unlikely that AID-mediated dC DNA deamination and CSR were inhibited by Fe$^{2+}$ oxidized from Fe$^{2+}$, as B cells maintain a reducing environment in the cytoplasm and the nucleus. In addition, all Fe$^{2+}$ solutions used in our assays were freshly prepared and used within 30 min in the presence of glutathione. Finally, catalytic residues in UNG are not coordinated by metal ions (55).

Fe$^{2+}$-mediated inhibition of AID and CSR can be exploited by the body to inhibit unwanted class switching of autoantibodies associated with chronic inflammation, as suggested by increased iron levels in peripheral lymphoid organs in patients with chronic inflammatory diseases (56). Patients with systemic

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**Iron Inhibits AID Activity and CSR**

![Figure 7](image)

**FIGURE 7.** Fe$^{2+}$ does not alter UNG dU excision activity. A, schematics of UNG-mediated dU excision assay. B, omission of UNG yielded no cleavage products. C, UNG-mediated dU excision in the absence or presence of FeCl$_3$, ZnCl$_2$, MnCl$_2$, MgCl$_2$, or NiCl$_2$ is shown. Numbers below each lane indicate the percentage of the substrate showing dU excision. Data are representative of three independent experiments.
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lupus erythematosus and lupus-prone MRL/Fas<sup>lpr/lpr</sup> mice, however, would overcome the iron inhibition of AID by expressing abnormally high levels of AID, resulting from estrogen up-regulation of HoxC4 induction (57). In these autoimmune patients and mice, increased AID expression leads to high levels of class-switched and high affinity pathogenic autoantibodies, mainly to double strand DNA, increased organ and tissue damage, and increased mortality (58–61). These could be reversed in MRL/Fas<sup>lpr/lpr</sup> mice by dietary iron (62), suggesting that the effects of high levels of AID can be overridden by iron supplement, thereby pointing to a potential iron-based therapeutic intervention in lupus.

High AID levels, perhaps compounded by potential systemic decrease of iron levels associated with loss of red blood cells in lupus, would lead to significantly increased insertion/deletions in VDJ region DNA and chromosomal translocations involving the IgH and c-Myc loci (59, 61, 63). Thus, iron might also protect from AID-mediated genome-wide damage, mutations, generation of double strand DNA breaks, and chromosomal translocations (64), particularly in proto-oncogenes, such as c-Myc, and tumor suppressor genes, such as p53 (11, 65), thereby inhibiting tumorigenesis. In conclusion, our findings provide an important mechanistic underpinning to the association between iron overload and defective antibody responses. They also suggest a therapeutic application of iron in autoimmunity and neoplasia.

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