Regulation of Activation Induced Deaminase (AID) by estrogen

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Running title: AID regulation by estrogen

Chapter 1

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Summary

Regulation of Activation Induced Deaminase (AID) by the hormone estrogen has important implications for understanding adaptive immune responses as well as the involvement of AID in autoimmune diseases and tumorigenesis. Here I describe the general laboratory techniques for analyzing AID expression and activity induced by estrogen, focusing on the isolation and preparation of cells for hormone treatment and the subsequent analysis of AID responsiveness to estrogen at the RNA level and for determining the regulation of AID activity via estrogen by analyzing Ig switch circle transcripts and mutations in switch region loci.

Key words: Activation induced deaminase, AID, estrogen, adaptive immune responses, splenic B-cells, Ig switch circle transcripts, somatic hypermutations, autoimmune diseases.

1. Introduction

Infection triggers an immune response, which aims to specifically target and remove the source of the infection from the organism while keeping its own tissues unharmed. Interestingly, it has been noted that female mice produce more antibody than male mice after immunization (1, 2) and that the immune responses in human display a similar sexual dimorphism that is characterized by a quicker and stronger humoral immune response in females than in males (3).

The differences in immune responses seem to be partly mediated by hormone levels *in vivo*, since systemic sex hormone treatment or castration in mice converts the immune responses more similar to that of the opposite sex (4, 5). Furthermore, an important factor in the susceptibility of various human autoimmune diseases is the hormonal status of the patient, which can affect the activity of B-lymphocytes (6-8). Female sex biases of over 80% have been reported in various autoimmune diseases including Sjogren's syndrome, lupus and scleroderma (6), while other common diseases such as myasthenia gravis, rheumatoid arthritis (RA) and multiple sclerosis (MS) have a sex distribution of 60–75% women (6, 9). Altogether, the increased prevalence of autoimmune disease in women, the sexual dimorphism of the immune response, and the modulatory effects of sex steroids like estrogen on the immune function *in vitro* suggest their involvement in mediating these sex differences. Indeed, estrogen receptors (ER α and β) are expressed not only in reproductive tissues but also in multiple other cell types, including cells of the immune system such as B-lymphocytes (10-13).

One of the activative effects on B-cells by the hormone estrogen is mediated via its stimulatory effects on Activation Induced DNA deaminase (AID) (14), an enzyme that is necessary for diversification of the antibody repertoire by inducing somatic

hypermutation and Ig class switch recombination via DNA deamination (15, 16). Estrogen has been shown to induce the expression of AID in B-cells and thereby enhance AID-mediated downstream processes including Ig class switching. Hence, AID induction by estrogen is important for understanding adaptive immune responses in physiological conditions but also suggests its involvement in harmful autoimmune processes (14). Due to the mutagenic activities of AID on DNA, these regulatory mechanisms could also have important implications for tumorigenesis in estrogen-responsive tissues other than B-cells (17). Here I describe the application of basic methods for studying the interconnection of estrogen and AID expression and activity in mouse splenic B-cells at the RNA level as well as switch circle transcripts and mutations in Ig switch region loci. I also highlight some of the technical nuances in working with estrogen, which are important to keep in mind for a successful application of these techniques for determining the function of estrogen as a regulator of AID in B-cells.

2. Materials

2.1. Reagents

Reconstitute all reagents using sterile DAPC-treated water or sterile DMSO.

1. Lipopolysaccharide (LPS, Sigma, UK) at 25 µg/ml.
2. Mouse IL4 (R&D Systems Europe Ltd, UK) at 50 ng/ml.
3. Human transforming growth factor (TGF) β1 (R&D Systems Europe Ltd, UK) at 20 ng/ml.
4. The concentrations of estrogen [17-β-estradiol (Sigma, UK)] depend on the experiment and ranges between 1-100 ng/ml (see Table 1), while Tamoxifen

(Sigma, UK) can be used as an antagonist of estrogen at 50 nM. Hormone stocks are prepared in DMSO at a concentration of 100 mM, this is then diluted in DMSO to give 1000 x stock solutions, and final dilutions are made in media (final DMSO concentration is less than 0.1 %). Hormone stocks are kept at -80 °C, other reagents at either -20 °C or 4 °C according to manufacturer's guidance. Prepare 0.1% BSA in PBS and filter sterilize the solution prior to use.

2.2. Mouse tissue.

Mouse tissue samples are derived from freshly sacrificed 8-12 week old female unplugged BALB/c mice. Mouse tissue is dissected and passed through cell strainers prior to hormone-depletion and estrogen treatment.

3. Methods

3.1. Isolation of mouse splenic B-cells.

1. Freshly isolated mouse spleens are transferred to a tube containing 30 ml of RPMI 1640+Glutamax with 10% FCS preheated to 18-25 °C.
2. Spleens are sterilized by placing them twice into 70% ethanol for 1 second and then into media.
3. Tissues are then transferred to a wet 70 µm cell strainer on top of a 50 ml tube.
4. Cells are macerated through the filter using the back of a syringe plunger.
5. The filter is rinsed twice with 5 ml RPMI 1640 10% FCS solution.
6. The tube is filled with cold RPMI 1640 10% FCS and centrifuged at 800 rpm at 4 °C for 10 minutes.

7. Supernatant is discarded and cells resuspended in 5 ml room temperature RPMI 1640 1% FCS.
8. The cells suspension is carefully pipetted on 7 ml Lympholyte M (Cedarlane Laboratories Limited, Canada) in a 15 ml tube.
9. The sample is centrifuged at 800 rpm for 20-30 minutes to remove erythrocytes.
10. The interphase with B-cells is collected into a fresh tube (see **Note 1**).
11. After filling the tube with RPMI 1640 1% FCS, cells are centrifuged at 800 rpm for 10 minutes and the supernatant removed, leaving 2-3 ml of media on the cells (see **Note 2**).
12. 120 U of DNase I (Roche, UK) is added per 1 ml cell suspension, mixed gently and incubated on a rotator for 15 minutes at RT. The suspension is filtered through a 70 μ m cell strainer and counted for leucocytes to obtain the total cell number.
13. The tube is filled with cold RPMI 1640 1% FCS and centrifuged at 800 rpm at 4 °C for 10 minutes.
14. Leucocytes are suspended in PBS with 0.1% BSA (1×10^7 per 100 μ l) and transferred to a 15 ml tube.
15. Mouse splenic B-cells are isolated with reagents from the Mouse B-cell Negative Isolation Kit (DynaL Biotech, Norway). 20 μ l heat inactivated FCS per 1×10^7 leucocytes is added followed by the addition of 20 μ l of Antibody Mix per 1×10^7 cells.
16. The mix is incubated for 20 min at 4 °C (see **Note 3**).
17. The cells are then washed in 2ml sterile PBS 0.1% BSA per 1×10^7 leucocytes and centrifuged at 800 rpm at 4 °C for 10 minutes.

18. Supernatant is removed and cells resuspended in 800 μ l PBS 0.1% BSA per 1×10^7 leucocytes.
19. Mouse Depletion Dynabeads are washed in PBS 0.1% BSA before adding 200 μ l per 1×10^7 leucocytes. Cells were incubated for 15 min at RT with gentle rotation.
20. Bead-captured cells are resuspended and 1 ml PBS 0.1% BSA added per 1×10^7 leucocytes.
21. The tube is placed in the magnet for approximately 2 minutes before transferring the supernatant with isolated mouse B-cells into a new tube.
22. Centrifuge cells at 800 rpm at 4 °C for 5 minutes and remove PBS 0.1% BSA from the cell pellet.
23. In the next stage, mouse splenic B-cells are depleted from hormones as described in 3.2.

3.2. Hormone depletion from cells.

1. Opti-MeM Reduced Serum Medium is supplemented with: Charcoal Stripped Fetal Bovine Serum (Invitrogen Ltd, UK); Non-essential amino acids (final concentration and Sigma, UK catalog number): L-Alanine (8.9 mg/ml - A7469); L-Asparagine (15.0 mg/ml - A4159); L-Aspartic Acid (13.3 mg/ml - A4534); L-Glutamic Acid (14.7 mg/ml - G8415); Glycine (7.5 mg/ml - G7126); Proline (11.5 mg/ml - P5607); L-Serine (10.5 mg/ml - S2600). Filter-sterilize the media before use (see **Note 4** and **Note 5**).
2. Pre-warm media at 37 °C before resuspending cells in it.
3. Cells are resuspended gently in sterile Opti-MeM Reduced Serum Medium (Invitrogen Ltd, UK) supplemented with 10% Charcoal Stripped Fetal Bovine

Serum and non-essential amino acids.

4. Cells are incubated for 24 - 72 h (see 3.3) in hormone-depleted serum at a concentration of 250,000 cells/ml (see **Note 6** and **Note 7**).

3.3. Time and concentration of estrogen used during various experiments.

A list of different concentrations of estrogen and the time of cell treatment for various experiments is indicated in Table 1 (see **Note 8**). It is recommended to add estrogen freshly to media (see **Note 9**).

Table 1. Treatment of cells with hormones.

Experiment	Cells	Depletion (h)	[nM]	Treatment (h)
AID mRNA	B-cells	24	0.1-1,000	1-8
Mutations in switch regions	B-cells	24	10-50	6 days
CSR (switch circle)	B-cells	24	1-50	48

3.4. Analysis of AID mRNA induction in response to estrogen treatment.

1. RNA is extracted from splenic B-cells using the RNeasy Mini kit (Qiagen, Germany) according to manufacturer's instructions. RNA is eluted with 30 µl of RNase-free water by centrifuging the column at 13,000 rpm for 1 minute (see **Note 10**).
2. RNA concentration is measured and the samples stored at -80 °C until cDNA synthesis (see **Note 11**).

3. cDNA synthesis is performed as follows: 2.5 µg of total RNA is denatured at 70 °C for 2 min and then placed on ice, before adding 5x first strand buffer, DTT to a final concentration of 5 mM, dNTPs to a final concentration of 0.4 mM each, 0.1 µg oligo dT primer (Invitrogen Ltd, UK) and/or 2.5 µg d(N)₁₀ random primers (Promega, UK), 8 U RNase OUT (Invitrogen Ltd, UK), 200 U Superscript III reverse transcriptase (Invitrogen Ltd, UK) and the appropriate quantity of RNase free H₂O to a 20 µl reaction. This is incubated at 37 °C for 10 min, 50 °C for 40 min and then 55 °C for 10 min. 80 µl of sterile H₂O is added to the cDNA before incubating at 100 °C for 2 min.
4. Analyse gene expression by quantitative real time PCR (Q-PCR), first by confirming the responsiveness of cells to estrogen is determined by Q-PCR of GREB1 (gene regulated by breast cancer 1), a known estrogen-responsive gene (18) (see **Note 12**). The Q-PCR reaction is as follows: 10 µl of SYBRGreen PCR (Qiagen, Germany) reaction mixture is added to 9 µl of RNase free water and primers (GREB1_forward 5'-TCCGAGTTCAGAGGTCGGC-3' and GREB1_reverse 5'-GTCCTACCTGTTGAGCTCCCACT-3') with a final concentration of 0.5 µM. 1 µl of cDNA is added to the PCR reaction. First, reaction is incubated at 50 °C for 2 min, then at 95 °C for 15 min followed by 40 cycles of melting at 95 °C for 30 seconds, primer annealing at 56 °C for 30 seconds and extension at 72 °C.
5. AID expression is analysed by Q-PCR primers Aid_Forward 5'-AACCCAATTTTCAGATCGCG-3' and Aid_Reverse 5'-AGCGGTTCTGGCTATGATAAC-3'.
6. GAPDH housekeeping gene is amplified with Q-PCR primers GAPDH_Forward 5'-GCACAGTCAAGGCCGAGAAT-3' and GAPDH_Reverse 5'-

GCCTTCTCCATGGTGGTGAA-3' for normalizing gene expression across all samples (Figure 1).

3.5. Circle transcript detection by RT-PCR in mouse B-cells upon estrogen treatment.

1. Place isolated splenic B-cells into 12-well plates in 1 ml of hormone-free medium with supplements.
2. Stimulate isolated splenic B-cells for up to 72 h with LPS + IL4 for inducing switching to IgG1 and IgE, LPS + TGF- β for switching to IgA and LPS for switching to IgG3.
3. Hormones are added to the cells together with LPS and cytokines in fresh media.
4. After hormone treatment, collect cells, isolate RNA and synthesize cDNA as described above.
5. Use Q-PCR for analyzing the relative amounts of circle transcripts. Primers that are used for detecting IgG1 (19), IgG3, IgA (20) and IgE (21), are listed in Table 2.

Table 2. Primers used for detecting circle transcripts.

Subclass	Orientation	Sequence
IgG1	Forward	5'-TCGAGAAGCCTGAGGAATGTG-3'
IgG1	Reverse	5'-GAAGACATTTGGGAAGGACTGACT-3'
IgG3	Forward	5'-TGGGCAAGTGGATCTGAACA-3'
IgG3	Reverse	5'-AATGGTGCTGGGCAGGAAGT-3'
IgA	Forward	5'-CCAGGCATGGTTGAGATAGAGATAG-3'
IgA	Reverse	5'-AATGGTGCTGGGCAGGAAGT-3'
IgE	Forward	5'-TTGGACTACTGGGGTCAAGG-3'

IgE	Reverse	5'-CAGTGCCTTTACAGGGCTTC-3'
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6. Use Q-PCR for analyzing circle transcripts as follows: 10 µl of SYBRGreen PCR (Qiagen, Germany) reaction mixture is added to 9 µl of RNase free water and forward/reverse primers with a final concentration of 0.5 µM. 1 µl of cDNA is added to the PCR reaction. First, reaction is incubated at 50 °C for 2 min, then at 95 °C for 15 min followed by 40 cycles of melting at 95 °C for 30 seconds, primer annealing at 56 °C for 30 seconds and extension at 72 °C.

3.6. Analysis of mutations in switch regions in response to estrogen treatment.

1. Genomic DNA is isolated from cells using PUREGENE products for Genomic DNA Isolation (Gentra Systems, USA) according to manufacturer's recommendations. 50 µl of TE (pH 8.0) is added to DNA pellets and left to rehydrate overnight at RT before storing the samples at -20 °C.
2. The switch region loci (the S γ 3 region is described here as an example) from genomic DNA are PCR amplified as follows: 50 ng of DNA is added to a 50 µl PCR reaction that included the forward primer for S γ 3 region g3.2F (5'-gcgaattcTTGCAACTCC-TAAGAGGAAAGATCCC-3') and reverse primer for S γ 3 region g3.2R (5'-gcggatcCAGCCTGGTCC-CTACACTCCTAACAAAC-3') (22) at 20 µM, 10x buffer, 10x PCR enhance, dNTP's each at 0.4 mM and 2.5 U of Pfu Turbo R^oDNA polymerase.
3. The reaction is initially denatured at 94 °C for 1 min, followed by 9 cycles of denaturation at 94 °C for 30 seconds, decreasing annealing temperature from 68 °C by 1 °C every cycle, extension at 72 °C for 2 min. Thereafter, 35 cycles denaturation at 94 °C for 30 seconds, annealing at 63 °C and extension at 72 °C for

2 min is performed, before a final extension at 72 °C for 5 min to generate a 750 bp PCR product.

4. PCR fragments are cloned into TOPO vector using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, UK) according to manufacturer's guidance. Cells are plated on LB plates with kanamycin and grown at 37 °C overnight.
5. Individual bacterial colonies from LB plates with antibiotics are picked into separate wells of the 96-well plates containing 1.2 ml of LB medium.
6. The plates are covered with an adhesive sealing film (ThermalSeal, Sigma, UK) with aeration holes and incubated overnight at 37 °C with constant rocking at 1300 rpm.
7. The bacteria are pelleted at 3,000 rpm for 15 min, the supernatant decanted and plates stored at -80 °C until mini-preparation and sequencing.
8. Mini-preparation is performed with QIAprep Spin Miniprep Kit according to manufacturer's guidelines.
9. Sequencing is performed with M13 forward and M13 reverse primers, followed by sequence alignment to the parental switch region sequence (see **Note 13**).
10. Mutations in the switch region sequence are validated by their presence in both forward and reverse sequencing reactions.

4. Notes

1. The efficiency of separating erythrocytes from other cells can be visually assessed by the color of the interphase cell pellet, which will be white in the

absence of erythrocytes, while the inefficient separation of erythrocytes will maintain its red color.

2. After collecting the cells from the interphase, it is important to wash the cells with media as described, for removing the lympholyte M. Otherwise, the cell survival will be decreased.
3. Rotating at 4 °C improves antibody binding to the cells and hence the total purity of splenic B-cells.
4. Do not use phenol red as a pH indicator in the media, because this reagent can activate estrogen receptors and hence will mask the specific effects of estrogen stimulation.
5. Do not use conventional serum in your media, because it will contain hormones including estrogen that will mask the specific effects of estrogen stimulation in your samples. It is recommended to use Charcoal Stripped Fetal Bovine Serum for analyzing the effects of estrogen stimulation in B-cells and other tissues.
6. Usually, hormone depletion of mouse splenic B-cells should be performed for 24 hours, followed by treatment with estrogen in combination of LPS, IL4 or other cytokines for activating B-cells. A convenient format for such experiments is a 24-well plate and using 250,000-500,000 cells / well in 1 ml of media.
7. If hormone depletion is performed samples other than primary cells such as mouse or human cell lines, the cells can be depleted from hormones for up to 72 h prior to estrogen treatment. Since each cell line has different characteristics of estrogen receptor alpha and beta expression levels, a longer than 24 h hormone depletion is recommended particularly for cancer lines

with high levels of estrogen receptor expression. This should be determined with experimental optimization of the treatment conditions.

8. Aliquot estrogen stocks to smaller volumes (e.g. 25ul) in separate tubes and store them at -80 °C. Avoid repeated freezing and thawing of the stocks.
9. Add estrogen to media freshly every day since estrogen is not stable in media for a prolonged time. Tamoxifen can be used as an antagonist of estrogen in the experiments.
10. To avoid any possible degradation of RNA in samples, wipe the bench and pipettes with RNase Away (Life Technologies) prior to starting the process. Use RNase free tubes and RNase free water. The integrity of RNA can be confirmed by running it on a 1% agarose gel and visualized by Ethidium bromide with UV.
11. Avoid repeated freezing and thawing of RNA samples, which will decrease RNA integrity.
12. The primers for Q-PCR should be designed, using 60 °C as the T_m and 150 bp as the length of the PCR product.
13. The switch region locus should be sequenced also in the isolated splenic B-cells prior to cytokine stimulation, to obtain the unmutated parental sequence that can be used as a template for sequence alignments.

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Figure legends

Figure 1. The effects of estrogen on AID mRNA in splenic B-cells. (A) Changes in AID mRNA in response to estrogen treatment in stimulated B-cells. Isolated mouse splenic B-cells were stimulated with LPS and IL4 and treated with different physiological concentrations of estrogen for 8 h. (B) AID mRNA in response to estrogen treatment in un-stimulated B-cells, after 8 h treatment with physiological concentrations of estrogen. Data is representative of three independent experiments and error bars indicate standard deviations from the average. DMSO is set to 1, and treatments are represented as relative change to DMSO. NT - not treated.