The Bursting of Activation-Induced Cytidine Deaminase mRNA During B Cell Activation

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Abstract

During B lymphocyte activation and differentiation in the germinal center of secondary lymphoid organs, two processes known as Somatic Hypermutation (SHM) and Class Switch Recombination (CSR) are utilized to optimize the antibody response to antigens. SHM allows for base pair mutations to result in improved affinities for antigens, while CSR precipitates the excision of constant regions (C_H) that are undesired, generating a desired heavy chain constant region, or Ig isotype, for the immunoglobulin antibody that targets an antigen. These two processes are executed by the enzyme Activation-Induced Cytidine Deaminase (AID). The transcription of the gene for AID, on a cellular level, occurs in transcriptional bursts, (i.e. intense periods of activity dispersed amongst long periods of inactivity). We stimulated the naïve (unexposed to an antigen) B cells with lipopolysaccharide (LPS), an endotoxin found on Gram-negative bacteria, and after LPS-exposed incubation, we used the RNA Fluorescent In Situ Hybridization (RNA FISH) technique to check the single molecule level of AID mRNA. We utilized this method to specifically localize and quantify the AID mRNA, allowing us to identify when the bursting of AID transcription occurs and to what extent it was activated over time.

Introduction

In the secondary (peripheral) lymphoid organs, naïve mature B lymphocytes (or Follicular B cells) undergo SHM in the dark zone of the lymphatic germinal center and are referred to as centroblasts (Fig. 1). As a result of SHM, centroblasts undergo mutations and experience either higher affinity or lower affinity for the antigen. These centroblasts enter the light zone of the germinal center and are referred to as centrocytes. The centrocytes with an improved affinity for the antigen undergo CSR to produce various isotypes of immunoglobulin heavy chain constants (Ig isotypes), necessary for the immune response, while the centrocytes with a lower affinity for the antigen undergo apoptosis. The selected centrocytes, after CSR, differentiate into plasma cells and memory B cells.

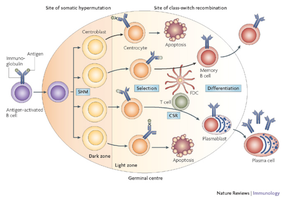


Figure 1. B cell activation and differentiation in the germinal center of a secondary (peripheral) lymphoid organ, including somatic hypermutation and class switch recombination

During SHM and CSR, the enzyme AID plays a crucial role in stimulating improved affinities for antigens and more suitable isotypes for immunoglobulins. In SHM, AID deaminates Cytosine in G:C base pairs into Uracil (i.e. G:U) on the variable segments of immunoglobulin genes, which allows for G:N mutations, A:T mutations, mutations in neighboring A:T base pairs, or normal repair. This results differences in antibody protein structure, and consequently, improved or lower affinities for the antigen. In CSR, AID deaminates dC nucleotides in the top and bottom strands of the switch (S) regions located upstream of each constant region (C_H) in the immunoglobulin heavy chain gene, producing double-stranded DNA breaks (DSB's) in the S region ahead of the Cμ heavy chain constant (Sμ) and the S region ahead of the desired constant C_H region (Fig. 2). This precipitates the excision of undesired C_H regions, prompting transcription for the desired antibody heavy chain. The DSB's made in CSR by AID allow for the production of different immunoglobulin heavy chain constant isotypes (classes), each with various functions in executing the efficient immune response.

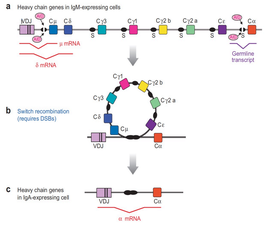


Figure 2. Class switch recombination with activation-induced cytidine deaminase in heavy chain gene

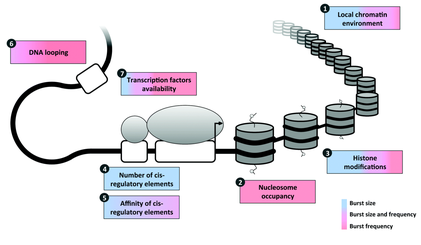
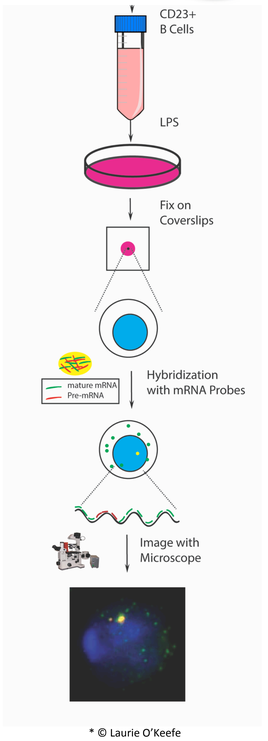


Figure 3. Factors affecting burst size and burst frequency in transcriptional bursting

The transcription of mRNA, including AID mRNA, occurs in transcriptional bursts or pulses, which may be a result of closed/open chromatin formation, transcription factors, cell cycle effects, cell size extracellular signaling, etc. (Fig. 3). Transcription factors regulate mRNA activation by alternating between a repressive, inactive state, and a permissive, active state, generating surges of intense transcription. The stochastic nature of this occurrence produces variation in quantity and location of mRNA within tissues. Single molecule Fluorescent In Situ Hybridization (smFISH) can identify both mature and pre-mRNA transcripts of endogenous genes. This method provides us with more insights on the activation of AID transcription and its transcriptional bursting.

Methods



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1. Use slides' rough edges to mash a mouse (*Mus musculus*) spleen to yield a spleen cell suspension
2. Upon filtering the suspension, add 2 μg Anti-CD23 Biotin Antibodies and 30 μL Anti-Biotin MicroBeads
3. Pass the mixture through a magnetic filter with 12 mL of MACS buffer
4. Suction out the mixture attached to the magnetic filter, yielding naïve B cells, and add 100 μg/mL lipopolysaccharide (LPS)
5. Incubate in 37.0°C and 5.0% CO_2 for 17 hours
6. Put 50 μL of cells on a coverslip and incubate for 20 minutes
7. Place coverslips in 2 mL PBS, then 1 mL 4% PFA for fixation, then PBS 2 mL thrice, then 2 mL 70% Ethanol
8. Store overnight in 4°C
9. Wash with 2 mL 10% Formamide/2xSSC washing buffer, then add 2 pL mRNA probes
10. Hybridize for 12 hours in 37 degrees Celsius
11. Wash with 2 mL 10% Formamide/2xSSC, then 2 mL 2xSSC, then stain nucleus with DAPI fluorescent stain
12. Image under wide-field epifluorescence microscope
13. Repeat steps 5-12 with B cells incubated for 24 hours between LPS stimulation and fixation, as well as 48 hours

Results

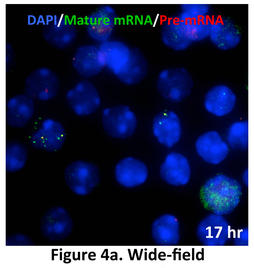

Figure 4a. Wide-field epifluorescence microscopy imaging of B cells incubated for 17 hours LPS stimulation. Minimal mRNA transcriptional bursting activity was detected, indicated by low presence of mature mRNA (green) and pre-mRNA (red/yellow)

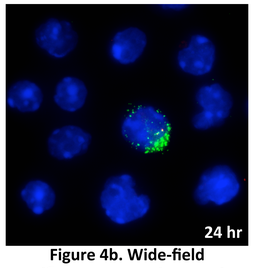

Figure 4b. Wide-field epifluorescence microscopic imaging of B cells incubated for 24 hours LPS stimulation. Mild mRNA transcriptional bursting activity was detected, indicated by moderate presence of mature mRNA (green) and pre-mRNA (red/yellow)

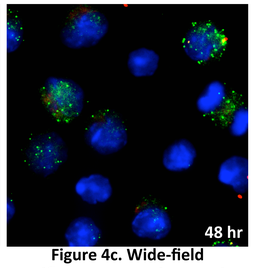

Figure 4c. Wide-field epifluorescence microscopic imaging of B cells incubated for 48 hours LPS stimulation. High mRNA transcriptional bursting activity was detected, indicated by high presence of mature mRNA (green) and pre-mRNA (red/yellow)

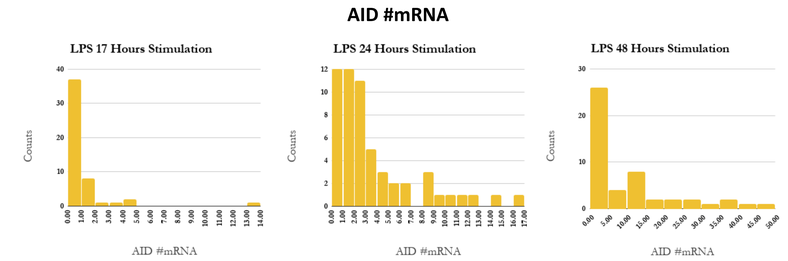

Fig. 5a **Fig. 5b** **Fig 5c.**

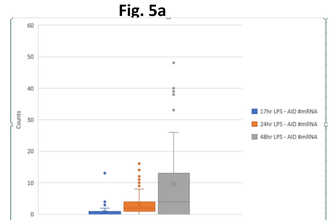

Fig. 5d

Figure 5a. Cell counts per AID #mRNA 17 hours after LPS stimulation

Figure 5b. Cell counts per AID #mRNA 24 hours after LPS stimulation

Figure 5c. Cell counts per AID #mRNA 48 hours after LPS stimulation

Figure 5d. Box plot of AID #mRNA with 17, 24, and 48 hour stimulation, respectively

Analysis and Conclusions

Analyzing the images, we identified an increase in AID mRNA quantities from the 17 hour LPS stimulated to the 48 hour LPS stimulated cells, illustrated by the surge in bright green points (mature mRNA). In the 17 hours LPS stimulated cells, 74% (37 out of 50) of cells exhibited zero AID mRNA molecules, and another 16% exhibited only one AID mRNA molecules. In contrast, in the 24 hours LPS stimulation cells, approximately 89% (44 out of 56) exhibited AID mRNA molecules, while over 5 molecules of AID mRNA were detected in 23% of cells. Using the wide-field epifluorescence microscopy, we were able to visualize B cell transcriptional bursting of AID mRNA as a phenomenon that is significantly activated more than 24 hours after LPS stimulation. After 48 hours of LPS stimulation, a staggering majority of cells experienced intense transcriptional bursting activity, with 38% of cells (19 out of 50) displaying AID mRNA molecule quantities greater than 10. The experimental results illustrated a two-state, repressive and permissive transcriptional bursting model, in which at any given point in time, the AID mRNA quantity would exhibit significant standard deviation, with large differences between the mean and maximum. This was demonstrated, as the standard deviation for the 17, 24, and 48 hour LPS stimulation cells was approximately 1.99, 3.75, and 12.35, with a significant difference between the mean and the upper limit of the third quartile, let alone outliers. The strong degree of variability in AID mRNA transcriptional bursting activation, as seen in the two-state transcriptional bursting model, is a result of the innate variability of individual cells in bursting activation. The discrepancies between cells in cell size, cell cycle stage, extracellular signals, transcription factors, and varied chromatin configuration influence the activation of mRNA transcription. It is well established that AID mRNA expression level increases with B cell activation, due to the need for AID enzyme to execute SHM and CSR. In addition to this knowledge, we were able to identify that significant activation for AID, and consequently, significant mRNA transcription, occurs more than 24 hours after antigen (LPS) stimulation. In the future, we can better understand the mechanism of AID activation in B cells by observing live-imaging transcriptional bursting. The purpose of this research is to gain more insight on B cell biology.

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