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Hydrogen Peroxide Modulates Immunoglobulin Expression by Targeting the 3' *Igh* Regulatory Region through an NFκB-Dependent Mechanism

Eric J. Romer and Courtney E.W. Sulentic*

Department of Pharmacology & Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, Ohio 45435

Abstract

Reactive oxygen species such as hydrogen peroxide (H₂O₂) appear to play a role in signal transduction in immune cells and have been shown to be synthesized upon antigen-mediated activation and to facilitate cellular activation in B and T cells. However an effect of H₂O₂ on B-cell function (i.e. immunoglobulin (Ig) expression) has been less well-characterized. The effects of H₂O₂ exposure on lymphocytes may be partly mediated by oxidative modulation of the NFκB signal transduction pathway, which also plays a role in Ig heavy chain (*Igh*) gene expression. *Igh* transcription in B lymphocytes is an essential step in antibody production and is governed through a complex interaction of several regulatory elements, including the 3' *Igh* regulatory region (3' *IghRR*). Utilizing an in vitro mouse B-cell line model, this study demonstrates that exposure to low μM concentrations of H₂O₂ can enhance 3' *IghRR*-regulated transcriptional activity and *Igh* gene expression, while either higher concentrations of H₂O₂ or the expression of a degradation resistant inhibitory κB (IκBα super-repressor) can abrogate this effect. Furthermore, suppressive H₂O₂ concentrations increased protein levels of the p50 NFκB subunit, IκBα, and an IκBα immunoreactive band which was previously characterized as an IκBα cleavage product exhibiting stronger inhibitory function than native IκBα. Taken together, these observations suggest that exposure of B lymphocytes to H₂O₂ can alter *Igh* transcriptional activity and Ig expression in a complex biphasic manner which appears to be mediated by NFκB and altered 3' *IghRR* activity. These results may have significant implications to disease states previously associated with the 3' *IghRR*.

Keywords

ROS; IκBα; 3' *IghRR*; Ig; B lymphocytes

*Corresponding author: Courtney Sulentic, Ph.D., Department of Pharmacology & Toxicology, Boonshoft School of Medicine, Wright State University, 206 Health Sciences Bldg., 3640 Colonel Glenn Hwy., Dayton, OH 45435; Phone: 937-775-3583; Fax: 937-775-7221; courtney.sulentic@wright.edu. .

DECLARATION OF INTEREST

Furthermore the authors declare that there are no conflicts of interest to disclose.

INTRODUCTION

Reactive oxygen intermediates (ROI) are oxygen containing compounds that readily react with a wide array of biological substrates. There are multiple forms of ROI (e.g. O_2^- , OH^- , H_2O_2) produced from a number of disparate sources including cellular metabolism, enzymatic reactions, xenobiotics, and inducible enzymes [1]. The influence of ROI on cellular function ranges from activation to cytotoxicity. The nature of the effect depends on the concentration and form of ROI, as well as the cellular capacity to neutralize them. H_2O_2 is one of the most predominant forms of ROI. It is a relatively stable intermediate and its electrical neutrality allows it to diffuse through a lipid bi-layer making it a readily available inter-compartmental and/or intercellular oxidant. The concept of ROI acting as a functional element of the cell has been well demonstrated in the immune system. For example, upon antigen-mediated activation, immune cells have been found to synthesize ROI (reviewed in [2]). Furthermore in immune cells such as B and T lymphocytes, H_2O_2 can facilitate cellular activation [3-5]. Moreover, it has been found that xenobiotic-induced ROI can modulate cellular signaling pathways in human lymphocytes [6, 7]. While the molecular basis for H_2O_2 -mediated modulation of lymphocyte signal transduction and gene transcription likely involves oxidative modification of signal transduction proteins and transcription factors, the effects of H_2O_2 on these processes have not been fully explored. One such pathway that is modulated by H_2O_2 and has been found to play a primary role in lymphocyte activation is the NF κ B signaling pathway [8].

NF κ B activation canonically occurs when a stimulus activates an inhibitory κ B kinase (IKK) which targets an I κ B for proteolytic degradation thus liberating the NF κ B transcription factors (e.g. p50/RelA heterodimer) to translocate to the nucleus and facilitate transcription. The effects of H_2O_2 on NF κ B activation have been studied in multiple cell types with results ranging from no effect to a concentration-dependent activation or inhibition [8]. An early study showed NF κ B binding to κ B motifs and activation of a NF κ B-mediated reporter following treatment of a T-cell line with μ M H_2O_2 concentrations [4]. A number of subsequent studies have supported this finding [9] and although not as widely investigated, studies have shown that H_2O_2 treatment of B lymphocytes can modulate NF κ B activation and cellular function at similar (μ M) concentrations [10]. Additionally, NF κ B transcription factors, which were originally discovered in B lymphocytes, are transcriptional regulators of the *Igh* gene [11, 12]. The IgH, an essential protein component of the antibody molecule, facilitates the antibody's ability to recognize and interface with antigen as well as with other immune factors and cells. *Igh* transcription is initiated at the variable heavy chain (V_H) promoter and is regulated by several transcriptional regulatory regions, including the intronic enhancer (E_μ) and a regulatory region 3' of the α constant region termed the 3' *Igh* regulatory region (3' *IghRR*) (Fig. 1). While the E_μ is involved in regulating recombination of the variable region and plays a role in *Igh* transcription, deletion of the 3' *IghRR* severely impairs transcription and class switch recombination of the *Igh* gene underscoring its functional significance in *Igh* gene regulation [13, 14]. The mouse 3' *IghRR* mediates transcriptional control through at least four hypersensitive sites (hs3a; hs1,2; hs3b; hs4) which interact with specific transcription factors including NF κ B [15, 16].

Given the relationship between NF κ B, 3' *Igh*RR, and *Igh* we wanted to test the hypothesis that H₂O₂, either by itself or in conjunction with a B-cell activating stimulus, could modulate *Igh* transcriptional activity via the 3' *Igh*RR in an NF κ B-dependent manner. Utilizing luciferase reporter constructs transiently expressed in the CH12I κ B α AA mouse B-cell line, we determined that H₂O₂ can modulate 3' *Igh*RR-regulated V_H-promoter activity in a concentration-dependent, biphasic manner. This effect appears to be in-part mediated through the NF κ B signaling pathway. When the reporter results were compared to the functional endpoint of *Igh* transcription we found that H₂O₂ can modulate *Igh* transcription in a manner similar to that of the reporter constructs. These results suggest that Ig production and B-lymphocyte function could be altered following exposure to intrinsic (e.g. physiologic) or extrinsic (e.g. xenobiotic) sources of ROI and provide insight into the potential mechanisms for this effect. Furthermore, the 3' *Igh*RR has been associated with several human immune-related diseases that are also associated with increased levels of ROI [17-20], thus the results of the present study support a link between ROI (whether induced physiologically or by xenobiotics), altered 3' *Igh*RR activity, and the initiation and progression of specific disease states.

METHODS

Chemicals and Reagents

Hydrogen peroxide (H₂O₂) solution (30 wt. % in water), IPTG (isopropyl β -D-1-thiogalactopyranoside), and LPS (*Escherichia coli*) were purchased from Sigma Aldrich (Milwaukee, WI). Chemicals were diluted in either water (H₂O₂, IPTG) or 1 $\times$ PBS (LPS).

Cell Line

The CH12I κ B α AA B-cell line was developed and provided by Dr. Gail Bishop [12] and is a variant of the CH12.LX cell line which was derived from the murine CH12 (surface Ig⁺ and CD5⁺) mature B-cell lymphoma [21]. The CH12I κ B α AA cell line stably expresses an IPTG-inducible I κ B α super-repressor protein (I κ B α AA) which contains a stable insertion of an inducible transgene that expresses a degradation resistant I κ B α super-repressor (I κ B α AA) which constitutively sequesters NF κ B transcription factors in the cytoplasm and is resistant to negative feedback regulation by NF κ B/Rel proteins [12]. Cells were grown as previously described [22]. Cell viability (mean viability from three separate experiments) was determined by trypan blue exclusion using a Beckman Coulter ViCell instrument (Beckman Coulter, Brea, CA).

Transient Transfection and Luciferase Assay

The *Igh* luciferase reporter plasmids were provided by Dr. Robert Roeder (Rockefeller University, New York, NY) and included a promoter alone control containing a V_H-promoter upstream of the luciferase gene and reporters containing the upstream V_H-promoter and either the 3' *Igh*RR or the hs4 enhancer downstream of the luciferase gene (Fig. 1B). Plasmids were constructed using a pGL3 basic luciferase reporter construct (Promega, Madison, WI) as described previously [23]. The 3 $\times$ -NF κ B reporter was provided by Dr. R.T. Hay and contains a promoter with three consensus NF κ B binding motifs derived

from the Ig κ -chain promoter and a Conalbumin transcriptional start site upstream of the luciferase gene [24].

Transient transfections were performed by electroporation at 250 volts, 150 μ F, and 75 ohms as previously described [22]. For each plasmid, multiple transfections were pooled, seeded at 2.0×10^5 cells/ml and then divided into two equal portions. One portion was treated with 100 μ M IPTG for 2 hr to activate the I κ B α AA transgene. The other portion was cultured without IPTG to provide a control that lacked I κ B α AA expression (Fig. 1C). After 2 hr the cells were treated with H₂O₂ (0-200 μ M) in the absence or presence of an LPS (1 μ g/ml) co-treatment, seeded in triplicate into 12-well plates and cultured for 24 or 48 hr in 5% CO₂ at 37°C. After the 24- or 48-hr incubation period, cells were lysed with 1 $\times$ reporter lysis buffer (Promega) and then immediately frozen at -80°C. Measurement of luciferase enzyme activity was performed as previously described [22] and represented as relative light units or fold-change relative to the 0 μ M H₂O₂ control.

RNA Isolation

CH12I κ B α AA cells treated with 1 μ g/ml LPS and/or 0-100 μ M H₂O₂ were plated (2.0×10^5 C/ml; 5.0 ml/well) in triplicate and incubated for 48 hr at 5% CO₂ and 37°C. Cells were harvested and resuspended in 500 μ l of Tri-Reagent (Sigma Aldrich) and stored at -80°C. Samples were thawed on ice then centrifuged at $12\,000 \times g$ for 10 min to remove genomic DNA. The supernatant was mixed with 10% v/v 1-bromo-3-chloropropane (Acros, Geel, Belgium) and the aqueous phase was isolated using phase lock gel tubes (5 Prime, Gaithersburg, MD), then mixed with 250 μ l of isopropanol followed by centrifugation at $12\,000 \times g$ for 8 min. RNA pellets were washed in 75% ethanol, resuspended in nuclease free water and quantified using a NanoDrop ND1000 (Thermoscientific, Wilmington, DE).

cDNA Synthesis and Real-Time PCR

One μ g of total RNA was reverse transcribed into cDNA using the Taqman RT reagent kit (ABI, Branchburg, NJ) as suggested by the manufacturer's instructions. SYBR[®]Green Real-Time PCR was utilized to amplify *μ lgh* and β -actin (endogenous control to normalize cDNA concentrations) transcripts from the reverse transcribed cDNA. The primer sequences were as follows: *μ lgh* forward primer (FP) – 5'TCTGCCTTCACCACAGAAGA3'; *μ lgh* reverse primer (RP) – 5'GCTGACTCCCTCAGGTTCAG3'; β -actin FP – 5'GCTACAGCTTCACCACCACA3'; β -actin RP – 5'TCTCCAGGGAGGAAGAGGAT3'. cDNA (5 ng) was mixed with 2 $\times$ SYBR[®]Green Master Mix, 5.0 μ M of both FP and RP, and RNase/DNase free water to reach a total reaction volume of 25 μ l. The PCR was performed in an ABI 7500 and the cycling conditions were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. A dissociation curve following the PCR reaction verified a single PCR product size and no genomic DNA contamination. The results of the PCR amplification were analyzed using the SDS 2.0 software to determine relative quantification (RQ) values (i.e. fold-change).

Protein Isolation for Western Blot Analysis

CH12I κ B α AA cells treated with 1 μ g/ml LPS and/or 0-200 μ M H₂O₂ were harvested at 24 and 48 hr, washed once with 1 $\times$ PBS, then re-suspended in 150 μ l of mild lysis buffer (150

mM NaCl, 10 mM sodium phosphate pH 7.2, 2 mM EDTA, and 1% Nonidet P-40) and frozen at -80°C for at least 1 hr. Lysates were thawed on ice and centrifuged at 14,000 rpm for 5 min; whole cell lysate was removed from the pelleted cell debris, quantified using the Bio-Rad Protein Assay (Bio Rad, Hercules, CA), and frozen at -80°C .

Western Blot Analysis

Whole cell lysates were thawed on ice and 50 μg of protein was run on a 10% polyacrylamide gel at 200 volts for 30-40 min. The proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) at 1.0 amp for 1 hr. Membranes were blocked overnight at 4°C in 3% BSA (bovine serum albumin)/TTBS (tris-buffered saline with 0.05% tween-20), then incubated overnight at room temperature with either mouse anti- β -actin (Sigma Aldrich) at a 1:10 000 dilution, rabbit anti-I κ B α [sc-371 (C-21), Santa Cruz, Santa Cruz, CA] at a 1:1000 dilution, or mouse anti-p50 (sc-114, Santa Cruz) at a 1:200 dilution. Prior to and after a 1 hr incubation at $\sim 20^{\circ}\text{C}$ with the appropriate horse-radish-peroxidase-conjugated secondary antibody (goat anti-mouse at 1:8000 or goat anti-rabbit at 1:2500), the membrane was washed four times in TTBS at 10 min intervals. All antibodies were diluted in 3% BSA/TTBS and the proteins of interest were detected using the Pierce ECL substrate (Thermoscientific Pierce, Waltham, MA) and a Fuji LAS-3000 Bioimager (Tokyo, Japan).

Statistical Analysis

In Fig. 3, 5, and 7, the mean ($n=3$) was determined for each treatment group and the means generated from several experiments were then averaged and transformed to fold-change (mean $\pm$ SEM) with the 0 μM H_2O_2 control set to 1. A statistical difference in the fold-change between treatment groups and the control was determined by 1-way ANOVA with a Dunnett's post-hoc test. Statistical differences in the fold-effect between the non- and I κ B α AA-expressing cells (Fig. 3 and 5) or between the reporters (Fig. 3 and 7) were determined by a 2-way ANOVA with a Bonferroni post-hoc test. Fig. 2, 4 and 6 represent the mean $\pm$ SEM for each treatment group ($n=3$) of a representative experiment and significance was determined by either a two-tailed t-test (Fig. 2A-D and 6A-D), or a 1-way ANOVA with a Dunnett's post-hoc test (Fig. 2E-H, 6E-H, and 4).

RESULTS

I κ B α inhibits transcriptional activity of the 3' *Igh*RR-regulated V_H -promoter

To determine if H_2O_2 modulates *Igh* transcriptional activity via the 3' *Igh*RR in an NF κ B-dependent manner, we utilized a luciferase reporter transcriptionally regulated by the V_H -promoter and the 3' *Igh*RR as well as the CH12I κ B α AA mouse B-cell line which contains a stable insertion of an inducible transgene that expresses a degradation resistant I κ B α super-repressor (I κ B α AA). The I κ B α AA constitutively sequesters NF κ B transcription factors in the cytoplasm [12]. Since our previous studies have shown that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a known disrupter of B-cell function (reviewed by [25] and an inducer of ROI [26, 27]), can enhance NF κ B binding within the hs4 enhancer as well as transcriptional activity of an hs4 luciferase reporter [28, 29], we also investigated the effects of H_2O_2 on the hs4 enhancer. Furthermore, to express Ig and secrete antibodies, B lymphocytes must first

be stimulated. Therefore, we utilized the polyclonal B-cell activator and TLR-4 ligand LPS which is a major component of bacterial cell walls.

CH12I κ B α AA cells were transiently transfected with reporter constructs regulated by a V_H-promoter alone (V_H-Luc) or the V_H-promoter in conjunction with either the 3' *Igh*RR (V_H-Luc-3' *Igh*RR), or the hs4 enhancer (V_H-Luc-hs4) (Fig. 1B). The transfected cells were cultured for 48 hr in the absence or presence of I κ B α AA and/or cellular activation (Fig. 1C). A 48 hr treatment period was previously determined to be an optimal time to achieve LPS-induced activation of the 3' *Igh*RR luciferase reporter [22, 29]. Utilizing the same treatment conditions (Fig. 1C), the level of NF κ B activity in our cell line model was also assessed by measuring the transcriptional activity of a 3 $\times$ -NF κ B reporter plasmid (3 $\times$ -NF κ B-Luc) (Fig. 1B).

In the non-activated cells, I κ B α AA expression significantly inhibited the activity of the regulated (3' *Igh*RR and hs4) and unregulated promoter (V_H) and the 3 $\times$ -NF κ B reporter (Fig. 2A-D). In LPS-activated cells, 3' *Igh*RR activity increased 15.2 fold over the NA control group, and consistently exhibited higher over-all levels of luciferase activity compared to LPS-induced V_H and hs4 reporter activity (Fig. 2E-G). I κ B α AA expression decreased the LPS activation of the 3' *Igh*RR reporter by 57.9% (Fig. 2E). Interestingly, V_H and hs4 were only modestly activated (<2-fold) by LPS. Furthermore, I κ B α AA expression induced a modest, non-significant inhibition of LPS-induced V_H activity and no effect on LPS-induced hs4 activity which contrasted with the significant inhibition of both V_H and hs4 basal activity (compare Fig. 2F and 2G to 2B and 2C). Consistent with LPS induction of NF κ B-mediated transcriptional activity, LPS significantly activated the 3 $\times$ -NF κ B reporter (7.5 fold), which was inhibited by 79.8% with the co-expression of I κ B α AA (Fig. 2H). Taken together, these results demonstrate that LPS induction of transcriptional activity is primarily mediated through the 3' *Igh*RR not the V_H-promoter alone and that the hs4 enhancer is not sufficient to optimally mediate LPS-induced activation of the 3' *Igh*RR. There also appears to be a significant NF κ B component to these effects since I κ B α AA expression partially inhibits LPS-induced activation of the 3' *Igh*RR-regulated and unregulated V_H-promoter.

H₂O₂ differentially modulates the activity of the regulated and unregulated V_H-promoter and *μ*lgh transcription

To explore the effects of oxidative stress (i.e. H₂O₂) on the regulated and unregulated V_H-promoter, we transiently expressed either, the 3' *Igh*RR, hs4, or V_H reporters in the CH12I κ B α AA cells, and treated with varying concentrations of H₂O₂ for 48 hr. Luciferase activity was represented as fold-change compared to the 0 μ M H₂O₂ control. Lower concentrations of H₂O₂ (30-50 μ M H₂O₂) resulted in a significant enhancement of 3' *Igh*RR activity in both the non-activated and LPS-activated cells, albeit to a lesser degree in the non-activated cells, i.e. 2-fold above basal activity versus 3-fold above LPS-induced activity (Fig. 3A and 3B). Non-activated cells also had a much lower luciferase activity compared to LPS-activated cells (Fig. 2). Increasing the concentration above 50 μ M H₂O₂ resulted in a concentration-dependent decrease in reporter activity in both the non-activated and LPS-activated cells (Fig. 3A and 3B). Interestingly, V_H did not exhibit H₂O₂-mediated enhancement in the non-activated and LPS-activated cells, but did exhibit a concentration-

dependent inhibition by H₂O₂ (Fig. 3A and 3B). Since enhancement of transcriptional activity by lower concentrations (30-50 μ M) of H₂O₂ occurs only with the regulated V_H-promoter and inhibition of promoter activity by higher concentrations of H₂O₂ occurs with the regulated and unregulated V_H-promoter, these results suggest that the 3' *IghRR* mediates the H₂O₂-induced activation and higher concentrations of H₂O₂ directly inhibit V_H-promoter activity.

H₂O₂ is known to cause oxidative stress and cell death, therefore it is important to consider the effects of H₂O₂ on cell viability. Following the 48 hr treatment, there was a concentration-dependent effect of H₂O₂ on cell viability of the non-activated cells as measured by trypan blue exclusion. The 40 μ M H₂O₂ treatment resulted in 87.0 $\pm$ 2.2% cell viability while the 100 and 200 μ M H₂O₂ treatments resulted in cell viabilities of 57.6 $\pm$ 2.8% and 46.2 $\pm$ 4.9%, respectively. The effect of H₂O₂ treatment on cell viability of the LPS-activated cells was minimal with 94.8 $\pm$ 0.6% cell viability following the 40 μ M H₂O₂ treatment, 85.8 $\pm$ 2.5% viability with the 100 μ M H₂O₂ treatment, and 79.1 $\pm$ 3.2% viability with the 200 μ M H₂O₂ treatment. Clearly, due to the marked decrease in cell viability, cytotoxicity cannot be ruled out as a major contributor to the inhibitory effect of the 100 and 200 μ M H₂O₂ treatments on reporter activity in non-activated cells. In contrast, the effect of 100 and 200 μ M H₂O₂ treatments on the viability of LPS-activated cells is much less pronounced and most likely does not play a significant role in the inhibitory effect of higher H₂O₂ concentrations on reporter activity; however, we cannot rule out the initiation of early apoptotic events not detectable by trypan blue exclusion that could influence reporter activity.

We examined hs4 reporter expression under the same H₂O₂ treatment conditions. In a manner similar to the 3' *IghRR*, LPS activation of the hs4 reporter was enhanced by lower concentrations of H₂O₂ (40-75 μ M) and this enhancement appeared to be abrogated by higher concentrations of H₂O₂ (100-200 μ M) (Fig. 3B). Notably the enhancement of the hs4 reporter occurred at a slightly higher H₂O₂ concentration range (Fig. 3B). Furthermore in marked contrast with the 3' *IghRR* reporter, the non-activated hs4 reporter was not activated by H₂O₂ (Fig. 3A). However, similar to the 3' *IghRR* reporter the basal activity of the hs4 reporter was significantly inhibited by the 75-200 μ M H₂O₂ treatments (Fig. 3A). Taken together, these results suggest that the hs4 enhancer plays a significant but partial role in the H₂O₂ enhancement of 3' *IghRR*-mediated transcriptional activity in LPS-activated cells and has no role in non-activated cells. Therefore, other enhancer elements (i.e. hs3a, hs1,2 or hs3b) within the 3' *IghRR* appear to play a part in the enhancement of 3' *IghRR*-mediated transcriptional activity in non-activated cells and to contribute to the greater sensitivity of 3' *IghRR* as compared to hs4 to H₂O₂.

The effect of H₂O₂ treatment on the expression of the endogenous μ *Igh* (encodes the heavy chain protein of IgM) was measured by real-time PCR in the CH12I κ B α AA cell line to assess whether or not the 3' *IghRR* reporter constructs exhibited an expression profile representative of an endogenous locus. A 40 μ M H₂O₂ co-treatment significantly enhanced the 2-fold LPS-mediated induction of μ *Igh* expression, while the 100 μ M H₂O₂ co-treatment resulted in a modest inhibition. These results correlate well with the biphasic effect of H₂O₂ observed with the 3' *IghRR* and hs4 reporters (compare Fig. 4 and 3). Furthermore, with the

expression of I κ B α AA the 40 μ M H₂O₂ co-treatment did not result in an enhancement of LPS-induced μ Igh expression (Fig. 4B). However in non-activated cells, H₂O₂ treatment did not generate a significant enhancing effect, but there was a significant inhibition of basal expression with the higher concentrations of H₂O₂ (compare Fig. 4 and 3). A lack of a stimulatory effect on μ Igh expression in the non-activated cells is not altogether surprising considering the very low overall activity of the 40 μ M H₂O₂-induced 3'IghRR reporter in non-activated cells compared to activated cells. For example, LPS induced an ~20-fold increase (above basal) in 3'IghRR activity (~500 RLU to ~10,000 RLU) which corresponded to an ~2-fold increase in endogenous μ Igh levels (Fig. 2 and 4). In comparison 40 μ M H₂O₂ only induced an ~2-fold increase (above basal) in 3'IghRR activity (~500 RLU to ~1000 RLU) in non-activated cells (Fig. 3). Therefore, it is unlikely that this low induction in transcriptional activity would produce a biologically significant effect in endogenous μ Igh expression.

Inhibition of NF κ B/Rel activity by I κ B α AA abrogates the stimulatory but not the inhibitory effect of H₂O₂ on the 3'IghRR- and hs4-regulated V_H-promoter

NF κ B proteins can be functionally modulated by changes in the redox state of the cell and may also be involved in regulating 3'IghRR activity through interactions with NF κ B DNA binding sites within the 3'IghRR [15]. Correspondingly, I κ B α AA expression suppressed the H₂O₂-mediated enhancement of 3'IghRR and hs4 in LPS-activated cells (Fig. 3D) and eliminated the H₂O₂-enhanced 3'IghRR activity in the non-activated cells (Fig. 3C), suggesting a prominent role of NF κ B/Rel proteins in these effects. I κ B α AA expression did not appear to significantly alter the inhibitory profile of the regulated and unregulated V_H-promoter activity observed with higher concentrations of H₂O₂ (Fig. 3).

To further characterize the role of NF κ B/Rel proteins in the H₂O₂-mediated enhancement of the hs4 and 3'IghRR luciferase reporters, we evaluated the effect of H₂O₂ on the 3 $\times$ -NF κ B reporter under the same treatment conditions. Similar to the hs4 and 3'IghRR reporter constructs, lower concentrations (30-50 μ M) of H₂O₂ enhanced LPS activation of the 3 $\times$ -NF κ B reporter; whereas, higher concentrations (75-200 μ M) either lacked an effect or inhibited LPS activation (compare Fig. 5B and 3B). In contrast with the 3'IghRR but similar to the hs4, the 3 $\times$ -NF κ B expression profile in non-activated cells did not exhibit an H₂O₂-mediated enhancement but did show a concentration-dependent inhibition, albeit, the 3 $\times$ -NF κ B expression was inhibited by a slightly wider range of concentrations (compare Fig. 5A and 3A). Taken together these results suggest that during LPS activation, H₂O₂ can elicit a concentration-dependent biphasic effect on 3'IghRR and hs4 transcriptional activity that is in-part directly mediated by NF κ B. Conversely, the contrast between the 3 $\times$ -NF κ B and 3'IghRR profiles suggests that the H₂O₂-mediated enhancement of 3'IghRR activity in non-activated cells may not involve direct regulation by NF κ B or may require additional transcription factors to facilitate H₂O₂-mediated NF κ B binding. Indeed there are binding sites for several other transcription factors within the 3'IghRR enhancers [16] which may mediate the transcriptional activity in non-activated cells.

H₂O₂ and LPS exhibit temporally-dependent effects on transcriptional activity

Interestingly, NFκB activation of B lymphocytes by LPS occurs rapidly and is likely initiated by LPS-induced IκBα degradation, which occurs in 1-1.5 hr ([30] and data not shown). Therefore, optimal induction of 3' *Igh*RR by LPS at 48 hr may involve secondary effects. Subsequently, we evaluated the temporal effects of IκBαAA expression on the basal and LPS-induced transcriptional activation of the regulated and unregulated V_H-promoter. Similar to the 48 hr time point, IκBαAA expression inhibited the basal activity of all the reporters at 24 hr (Fig. 6A-D and Fig. 2A-D). LPS activation resulted in a less than 2-fold activation of V_H and hs4 at 24 hr which was comparable with the expression profile of those plasmids at 48 hr (compare Fig. 6F-G to 2F-G). Expression of IκBαAA inhibited LPS-activation of the V_H reporter by 36.9% at 24 hr versus 25.6% at 48 hr (compare Fig. 6F and 2F). Notably, LPS activation of the hs4 reporter was not inhibited by IκBαAA expression at 24 or 48 hr (compare Fig. 6G and 2G). The most prominent difference between the two time points is with the 3' *Igh*RR reporter which exhibited only a 2-fold activation by LPS at 24 hr versus the 21.1-fold activation at 48 hr (compare Fig. 6E and 2E). Interestingly, at 24 hr IκBαAA expression did not inhibit the modest LPS-induced activation of the 3' *Igh*RR reporter but instead trended toward a non-significant enhancing effect, which contrasted with the 72.2% inhibition observed at 48 hr (compare Fig. 6E and 2E). For the 3×-NFκB reporter, LPS induced a 2.6-fold activation at 24 hr versus a 7.5-fold activation at 48 hr. At both time points IκBαAA expression significantly inhibited LPS-induced 3×-NFκB reporter activity, i.e. 88.1% for 24 hr and 79.8% for 48 hr (compare Fig. 6H and 2H). In summary, LPS does not appear to optimally activate 3' *Igh*RR activity at 24 hr and given the diverging effects of IκBαAA on the 3' *Igh*RR and hs4 reporters at 24 versus 48 hr, the 48 hr transcriptional effects are likely influenced by delayed or secondary IκBα/NFκB signaling processes.

Since the above results clearly demonstrate time-dependent effects, reporter activity following H₂O₂ treatment was also analyzed at 24 hr. In marked contrast to the 48 hr results (Fig. 3 and 5), there was no H₂O₂-mediated enhancement of basal or LPS-induced activity of the 3' *Igh*RR, hs4, or 3×-NFκB reporter constructs at 24 hr (Fig. 7A and B). However, an H₂O₂ concentration-dependent inhibition was observed for all reporter constructs. Notably there was significantly weaker inhibition of the 3' *Igh*RR when compared to the V_H in the non-activated (40 μM H₂O₂) and LPS-activated (40-100 μM H₂O₂) cells. The hs4 and 3×-NFκB reporters also demonstrated a weaker inhibition by H₂O₂ as compared to V_H but only in the non-activated cells (Fig. 7A and B). Additionally, H₂O₂ induced a slightly greater but non-significant decrease in cell viability in non-activated cells at 24 hr compared to 48 hr (77.6±7.7% versus 87.0±2.2% for 40 μM H₂O₂; 48.0±5.4% versus 57.6±2.8% for 100 μM H₂O₂; and 42.1±4.9% versus 46.2±4.9% for 200 μM H₂O₂). However, in LPS-activated cells at 24 hr versus 48 hr, H₂O₂ treatment either had little effect on cell viability (94.4±1.4% versus 94.8±0.6 for 40 μM H₂O₂; 93.6±0.9% versus 85.8±2.5% for 100 μM H₂O₂; and 86.5±2.1% versus 79.1±3.2% for 200 μM H₂O₂). For all of the reporters IκBαAA expression did not alter the inhibitory effect of H₂O₂ at 24 hr (data not shown). Therefore, it appears that the H₂O₂-mediated enhancement versus inhibition are differentially regulated with the enhancement being specifically mediated by the 3' *Igh*RR and hs4 in a temporal and IκBα/NFκB-dependent fashion as further supported by similar

results obtained with the 3 $\times$ -NF κ B reporter. Furthermore, cytotoxicity may play a role in the inhibitory effect of H₂O₂ on luciferase activity in non-activated cells. However, it is unlikely that the inhibitory effect of H₂O₂ in LPS-activated cells is mediated by cytotoxicity though initiation of early apoptotic events cannot be ruled out.

H₂O₂ temporally modulates I κ B α and p50 protein expression

The H₂O₂-mediated enhancement of 3' *IghRR* and *hs4* expression and its inhibition by I κ B α AA suggests that NF κ B plays a role in this effect which is further substantiated by a similar H₂O₂-mediated enhancement of LPS-activated 3 $\times$ -NF κ B transcriptional activity. These observations led us to explore the effects of H₂O₂ on I κ B α , as it is a key regulator of NF κ B activity and its expression can be modulated by ROI. The phosphorylation of I κ B α and subsequent activation of NF κ B by H₂O₂ at the 1 to 2 hr time point has been demonstrated but little is known about the effects of H₂O₂ exposure on I κ B α expression at later time points [9]. Given that the H₂O₂-mediated enhancement of 3' *IghRR*, *hs4*, and 3 $\times$ -NF κ B expression was not observed until the 48 hr time point and that the inhibitory effect at higher concentrations of H₂O₂ observed at 24 hr was maintained through the 48 hr time point, I κ B α expression was examined at 24 and 48 hr to determine how I κ B α expression correlates with the effect on reporter activity.

Western blot analysis with an anti-I κ B α antibody yielded a doublet with the bottom band representing I κ B α (~37 kDa) and the top band most likely I κ B β since the top band appears to migrate at the correct size for I κ B β (~43 kDa) and the anti-I κ B α antibody is cross reactive with the β isoform (Fig. 8A and B). The co-treatment of LPS with 100 or 200 μ M H₂O₂ resulted in increased I κ B α at 48 hr (Fig. 8B). A third, faster migrating I κ B α immunoreactive band (~34 kDa) appeared prominently in both the non-activated and LPS-activated cells at 24 hr and in only the LPS-activated cells at 48 hr (Fig. 8A and B). It was not present in any treatment group at 1 hr (data not shown). Additionally, there was a similar but much lighter band with the 40 μ M H₂O₂ treatment which was comparable in intensity to the respective 0 μ M H₂O₂ control (Fig. 8A and B). This ~34 kDa band may represent N-I κ B α , a caspase-mediated cleavage product of I κ B α . Interestingly, N-I κ B α has been shown to act as a degradation resistant form of I κ B α and to inhibit NF κ B/DNA interactions and NF κ B-mediated transcriptional activity [31]. Furthermore, the expression of N-I κ B α appears to be stronger at both the 24 and 48 hr time points in all treatment groups with LPS stimulation (Fig. 8A and B). Moreover, expression of the N-I κ B α is greater in the LPS co-treatments with higher concentrations of H₂O₂ (100 and 200 μ M) compared to a 40 μ M H₂O₂ concentration or LPS alone (Fig. 8A and B). Notably, a prominent anti-I κ B α immunoreactive band appeared at 48hr and inconsistently at 24 hr in the non-activated 100 and 200 μ M H₂O₂ treatments which migrated between 17.5 and 29.4 kDa (Fig. 8A and B). I κ B α cleavage products of this size have not been well characterized; however, the appearance of this band corresponds with the decreased cell viability seen in non-activated cells treated with 100 and 200 μ M H₂O₂ contrasting with the more viable 0 and 40 μ M H₂O₂ treatments.

In addition to I κ B α , we also evaluated the expression levels of the p50 NF κ B subunit due to its potential to form inhibitory p50/p50 homodimers. Interestingly, p50 expression was

noticeably reduced at 24 hr and markedly reduced at 48 hr in response to LPS activation which was not significantly altered by the 40 μ M H₂O₂ co-treatment (Fig. 8C and D). In contrast, p50 protein levels were clearly greater in the LPS-activated cells co-treated with 100 and 200 μ M H₂O₂ at both 24 and 48 hr (Fig. 8C and D). Taken together, these results suggest that higher concentrations of H₂O₂ can potentially result in a delayed, concentration-dependent increase in p50 and I κ B α levels (LPS-activated cells only) as well as an increase in N-I κ B α levels. Based on previous studies, the 34 kDa N-I κ B α protein can serve as a degradation resistant inhibitor of NF κ B [31], and its appearance at 24 hr temporally correlates with the concentration-dependent decrease in reporter activity by H₂O₂ (compare Fig. 8A and 7). If the combined effect of increased I κ B α and N-I κ B α and p50 are indeed mediating the inhibitory effect on the 3' *Igh*RR and 3 $\times$ -NF κ B reporters, the concentration-dependent and temporal effect of H₂O₂ on reporter activity and I κ B α expression may define the balance between enhancing and inhibitory signals on reporter activity.

DISCUSSION

We have previously identified chemical-induced modulation of 3' *Igh*RR activity that mirrored the effects on endogenous heavy chain mRNA and protein expression [22, 28, 29, 32]. Similarly, the current study demonstrated an H₂O₂-induced concentration-dependent and biphasic effect on LPS-mediated 3' *Igh*RR reporter activity that highly correlated with the effects of H₂O₂ on LPS-induced μ *Igh* RNA transcript production (i.e., functional endpoint of endogenous *Igh* transcription). These results also correlated with the biphasic effect of H₂O₂ on LPS-induced Ig protein levels ([22] and data not shown). Therefore, ROI appears to target *Igh* transcriptional activity through the 3' *Igh*RR. There are a host of sources for ROI, including IgM biosynthesis, other cells in the inflammatory process, xenobiotics, ionizing radiation, or pharmacologic agents such as the redox cycling anthracyclines (reviewed in [1] and [33]), all of which could target the 3' *Igh*RR and modulate antibody production and B-lymphocyte function.

We further investigated the mechanisms by which ROI (i.e. H₂O₂) exposure can modulate *Igh* transcriptional activity through the 3' *Igh*RR. In contrast to the concentration-dependent biphasic effect of H₂O₂ on 3' *Igh*RR reporter expression and on endogenous μ *Igh* transcript levels, the V_H reporter only exhibited an inhibitory effect. These differences suggest that 3' *Igh*RR regulation confers not only a higher concentration-dependent threshold for H₂O₂-mediated inhibition but an enhancement of transcriptional activity at low H₂O₂ concentrations which likely leads to the previously demonstrated enhancement of endogenous Ig protein levels at low H₂O₂ concentrations [22]. However, it should also be noted that the activation of 3' *Igh*RR by H₂O₂ in the absence of stimulation did not appear to translate into an effect on endogenous μ *Igh* transcription which is not surprising considering the markedly lower basal activity of the 3' *Igh*RR reporter compared to the LPS-induced activity. Therefore a stimulatory signal in addition to H₂O₂ exposure is likely required to reach a threshold of 3' *Igh*RR activation to induce transcriptional effects on endogenous *Igh* transcription. Such a threshold effect was also observed in a sub-clone of the Jurkat T-cell line which required co-stimulation with phorbol myristate acetate for 30-50 μ M H₂O₂ to enhance NF κ B-mediated expression [5].

The ability of I κ B α AA expression to suppress the H₂O₂-mediated enhancement of the 3'*IghRR* and *hs4* reporters suggests a significant role for NF κ B which is further supported by an H₂O₂-mediated enhancement of the 3 $\times$ -NF κ B reporter and inhibition of this enhancement by I κ B α AA expression. A significant role of NF κ B corroborates a host of studies demonstrating that a wide concentration range of H₂O₂ can induce NF κ B activation. Studies with lymphocytes and HeLa cells have demonstrated H₂O₂-induced NF κ B DNA binding and/or NF κ B-mediated transcriptional activity with H₂O₂ concentrations (30–60 μ M) similar to those used in our study [4, 34].

Interestingly, the biphasic effect of H₂O₂ on reporter activity and on endogenous *μ Igh* expression was limited to the later time point (i.e. 48 hr versus only inhibition at 24 hr) which was also the ideal time frame for optimal LPS-induced 3'*IghRR* transcriptional activity. Delayed activation is consistent with the requirement of 2–5 days to achieve optimal Ig secretion in LPS-activated B lymphocytes [35]. The mechanism of this delayed activation may involve a prolonged activation of NF κ B/Rel proteins which has been shown to persist for up to 24 hr (later time points were not examined) in pre-B cells and immature dendritic cells treated with LPS [36, 37]. Furthermore, following the initial rapid activation of p50/RelA NF κ B dimers, by 24 hr the NF κ B proteins p52, RelB, and c-Rel, were also activated [36]. Krappman and coworkers also demonstrated a delayed, NF κ B-dependent activation of AP-1 and Oct-2 which appeared to be necessary for the NF κ B-dependent activation of Ig κ light chain; the authors postulated that rapid induction of the p50/RelA NF κ B dimer was important to immediate early gene induction but the delayed activation of the p52/RelB NF κ B dimer and of AP-1 and Oct-2 are required to maintain high expression levels of persistently activated genes such as the Ig κ light chain [36, 37]. Since previous studies have demonstrated a prominent role of NF κ B, Oct, and AP-1 in the activation of the 3'*IghRR* [15, 38], a similar mechanism as described above for the Ig κ light chain may be mediating the delayed NF κ B-dependent activation of the 3'*IghRR* and endogenous *μ Igh* as seen in the current study with LPS-activated CH12I κ B α AA cells.

Since NF κ B activation canonically occurs with rapid (30 min to 2 hr) proteolytic degradation of I κ B α [30], NF κ B activation in the presence of renewed and sustained endogenous I κ B α expression at the 24 and 48 hr time points appears paradoxical. However, I κ B α is upregulated by activation of NF κ B and, as mentioned above, a prolonged activation of NF κ B/Rel proteins that was sensitive to inhibition by I κ B α AA, was demonstrated up to 24 hr in pre-B cells and immature dendritic cells treated with LPS despite endogenously expressed I κ B α [36, 37]. This effect may relate to a delayed activation of other NF κ B dimers and NF κ B-dependent transcription factors that are only sensitive to I κ B α inhibition during the early, transient NF κ B activation phase [36, 37]. Interestingly, tyrosine phosphorylation of I κ B α has been found to decrease its association with RelA in the absence of I κ B α degradation [39]. Likewise, H₂O₂ was shown to induce tyrosine phosphorylation of I κ B α in the Jurkat T-cell line as well as nuclear translocation of RelA without I κ B α degradation thus potentially explaining how endogenous I κ B α expression could occur simultaneously with NF κ B activation [9].

Results with the 3 $\times$ -NF κ B reporter and expression of the I κ B α AA strongly support a role of I κ B α -regulated NF κ B proteins in the H₂O₂-mediated enhancement of the reporters but the

mechanism behind the inhibitory effect is less clear. Consistent with the previously reported inhibition of CD40-induced IgM expression by 100 μ M H₂O₂ [10], inhibition of V_H, 3'*IghRR* and hs4 reporter activity at 24 and 48 hr was maximal at the 100 and 200 μ M treatments and was most pronounced at the 24 hr time point. Interestingly, LPS abrogated the inhibitory effect of the 100 and 200 μ M H₂O₂ treatments on 3'*IghRR* and hs4 reporter activity at 48 hr and to a lesser extent at 24 hr. However, H₂O₂ concentrations greater than 50 μ M reversed the enhancing effect of 40 μ M H₂O₂ and also resulted in a decrease of H₂O₂-enhanced and LPS-induced Ig expression. The mechanism behind the H₂O₂-mediated reduction in reporter activity and Ig expression is likely multifaceted and may involve a combination of increased I κ B α expression, expression of N-I κ B α , and decreased cellular viability. Additionally, the inhibition of the unregulated (V_H) and regulated (3'*IghRR* and hs4) promoter by H₂O₂ at 24 hr suggests a common mechanism of action. We speculate that the V_H-promoter is directly targeted for the inhibitory effect whereas the transcription factor milieu induced by low concentrations of H₂O₂ induces enhancer activity that overrides the inhibitory effect on the V_H-promoter (results summarized in Table 1). It is also important to note that the 3'*IghRR* has been shown to physically interact with the V_H-promoter creating a unique promoter/enhancer DNA complex that regulates *Igh* transcription [40]. Therefore, the effects on the V_H may be artificial due to the absence of the 3'*IghRR* and may not be relevant in a functional 3'*IghRR*-regulated *Igh* locus. Additionally, the initial inhibitory effects of H₂O₂ observed at 24 hr may involve p50/p50 homodimer formation, which is substantiated by a study demonstrating that low μ M H₂O₂ treatments of a Jurkat T-cell line resulted in protein binding to κ B motifs within the κ light chain enhancer that was entirely composed of p50 [4]. Correspondingly, our studies with LPS-activated cells demonstrate increased p50 protein levels relative to the LPS control with the 100 and 200 μ M H₂O₂ treatment at 24 and 48 hr. Therefore an H₂O₂-mediated induction or stabilization of p50 may contribute to the inhibitory effect on reporter activity and Ig expression due to the well-established inhibitory effect of p50 homodimers on transcription.

The wide array of potential oxidative forces that can arise from chemical exposure, drug treatment regimens, and components of the physiological and pathophysiological immune responses may significantly modulate Ig expression through NF κ B regulation of the 3'*IghRR* and ultimately affect local or systemic humoral immune function. Furthermore, the 3'*IghRR* has been associated to date with several human immune-related disorders including Burkitt's lymphoma, and rheumatoid arthritis [17, 18]. Moreover, these same diseases have been associated with oxidative stress and ROI. For example, exogenous or induced H₂O₂ has been found to modulate cell growth and viability of Burkitt's lymphoma cells in vitro [19]. Additionally, a decrease in intracellular reductants (e.g. the reduced form of glutathione-GSH) has been identified in lymphocytic cells of rheumatoid arthritis patients [20]. Inappropriate 3'*IghRR*-mediated increases in Ig expression initiated by H₂O₂ or other forms of ROI could be involved in the initiation and progression of autoimmune-related diseases or lymphomas whereas inhibition of Ig expression may lead to inadequate immunity against pathogens. Understanding ROI's capacity to alter *Igh* expression and potentially Ig-related immune responses will contribute to our understanding of how the exposure to a variety of ROI or ROI-producing compounds may affect immune function and ultimately human health.

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Nonstandard abbreviations

Igh	immunoglobulin heavy chain gene
3'IghRR	3'Igh regulatory region
IκBαAA	I κ B α super-repressor with two serine to alanine mutations
IKK	I κ B kinase
N-IκBα	I κ B α lacking the N-terminal
TCDD	2,3,7,8-tetracholorodibenzo-p-dioxin
V_H	variable heavy chain

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Figure 1. Schematic of the *Igh* locus, luciferase reporter constructs, and experimental design

A) Simplified diagram of a rearranged mouse *Igh* locus which includes the variable heavy chain promoter (pV_H) and the four enhancers (hs3a; hs1,2; hs3b; hs4) of the 3'*Igh*RR.

“VDJ” represents the variable region of *Igh* which encodes the antigen binding site. “C” represents the constant regions of *Igh*; only C_μ and C_α are depicted which encode the heavy chain for the IgM and IgA isotypes, respectively. B) Schematic of luciferase (Luc) reporters for the unregulated *Igh* promoter (V_H -Luc), the regulated *Igh* promoter (V_H -Luc-3'*Igh*RR and V_H -Luc-hs4), and 3 $\times$ -NF κ B-Luc which contains a Conalbumin A (ConA) promoter sequence with three NF κ B DNA binding consensus sequences (κ B). C) Schematic of the experimental design for the transfection studies.

Figure 2. NF κ B-mediated activity and 3'*IghRR*-regulated and unregulated V_H -promoter activity is activated by LPS and inhibited by IkB α AA expression in CH12IkB α AA cells

Cells transiently transfected with either the V_H -Luc-3'*IghRR* (A and E), V_H -Luc (B and F), V_H -Luc-hs4 (C and G), or 3x-NF κ B-Luc (D and H) reporter plasmids were cultured at 2.0×10^5 cells/ml for 2 hr in media alone or with IPTG to activate the IkB α AA super-repressor. The cells were then cultured for an additional 48 hr in the absence (A-D) or presence (E-H) of LPS (1.0 μ g/ml). "NA" denotes naïve (untreated) cells. Luciferase enzyme activity is represented on the y-axis as relative light units (mean $\pm$ SEM). The results are representative of 3-7 separate experiments (n=3 for each treatment group). Percent inhibition by IkB α AA relative to NA (A-D) or LPS (E-H) controls is indicated. Statistical differences between NA and IkB α AA (A-D) were determined by a 2-tailed t-test and between LPS and LPS + IkB α AA or NA (E-H) were determined by a 1-way ANOVA with a Dunnett's post-hoc test; * $P < 0.05$.

Figure 3. Hydrogen peroxide modulates 3'*IghRR*-regulated V_H -promoter activity in an $\text{I}\kappa\text{B}\alpha$ -dependent manner

CH12 $\text{I}\kappa\text{B}\alpha\text{AA}$ cells were transiently transfected with the V_H -Luc-3'*IghRR* (■), or V_H -Luc-hs4 (○), or V_H -Luc (▼) reporter plasmids were cultured at 2.0×10^5 cells/ml for 2 hr in media alone (A and B) or with IPTG to activate the $\text{I}\kappa\text{B}\alpha\text{AA}$ super-repressor (C and D). The cells (with or without IPTG) were then cultured for 48 hr with varying concentrations of H_2O_2 (0–200 μM) in the absence (A and C) or presence (B and D) of LPS (1.0 $\mu\text{g/ml}$). “C” denotes the non-activated control. Results are represented as fold-change (mean $\pm$ SEM) relative to the respective 0 μM H_2O_2 control and were generated from 3–7 separate experiments ($n=3$ for each treatment group). Statistical differences of the H_2O_2 treatment groups compared to 0 μM H_2O_2 were determined by a 1-way ANOVA with a Dunnett’s post-hoc test and resulted in significance ($P < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: A) V_H -Luc-3'*IghRR*: 30, 40 and 200 μM ; V_H -Luc: 5 and 40–200 μM ; V_H -Luc-hs4: 75–200 μM . B) V_H -Luc-3'*IghRR*: 30–50 μM ; V_H -Luc: 100–200 μM ; V_H -Luc-hs4: 50 μM . C) V_H -Luc-3'*IghRR*: 100–200 μM ; V_H -Luc-hs4: 50–200 μM . D) V_H -Luc-3'*IghRR*: 30, 40 and 200 μM ; V_H -Luc: 50–200 μM ; V_H -Luc-hs4: 40 and 200 μM . Statistical differences between treatment groups of no $\text{I}\kappa\text{B}\alpha\text{AA}$ (A and B) and $\text{I}\kappa\text{B}\alpha\text{AA}$ (C and D) expressing cells were determined by a 2-way ANOVA with Bonferroni post-hoc test.

* $P < 0.05$ for V_H -Luc-3' *IghRR* and † $P < 0.05$ for V_H -Luc-hs4 relative to corresponding treatment group. Statistical differences compared to the V_H -Luc reporter were determined by a 2-way ANOVA with a Bonferroni post-hoc test and resulted in significance ($P < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: A) V_H -Luc-3' *IghRR*: 30-50 μM ; V_H -Luc-hs4: 50-75 μM . B) V_H -Luc-3' *IghRR*: 30-50 μM ; V_H -Luc-hs4: 30-50 μM . D) V_H -Luc-3' *IghRR*: 30-50 μM .

Figure 4. Hydrogen peroxide affects μ IgH transcript levels

Total RNA was extracted from CH12I κ B α AA cells (2.0×10^5 cells/ml) that were cultured for 48 hr. Prior to RNA isolation cells were A) treated for 48 hr with 0, 40, or 100 μ M H₂O₂ in the absence or presence of 1.0 μ g/ml LPS or B) pretreated for 2 hr in media alone or with IPTG to activate the I κ B α AA super-repressor then treated for 48 hr with 0, 40, or 100 μ M H₂O₂ in the presence of 1.0 μ g/ml LPS. One μ g of total RNA was reverse transcribed to cDNA and 5 ng of total cDNA was utilized to amplify μ IgH and β -actin via SYBR[®]Green real-time PCR. The results are expressed as relative quantification (RQ) value compared to the 0 μ M H₂O₂ control. The data is representative of at least 2 separate experiments (n=3 for each treatment group). Statistical differences compared to the respective 0 μ M H₂O₂ control were determined by a 1-way ANOVA with a Dunnett's post-hoc test; * $P < 0.05$ or ** $P < 0.01$.

Figure 5. Hydrogen peroxide modulates 3x-NF κ B-Luc reporter activity

CH12IkB α AA cells transiently transfected with the 3x-NF κ B-Luc reporter plasmid were cultured at 2.0×10^5 cells/ml for 2 hr in media alone (■ No IkB α AA) or with IPTG to activate the IkB α AA super-repressor (▲ + IkB α AA). The cells (with or without IPTG) were then cultured for 48 hr with varying concentrations of H_2O_2 (0-200 μM) in the absence (A) or presence (B) of LPS (1.0 $\mu\text{g/ml}$). Results are represented as fold-change (mean $\pm$ SEM) relative to the respective 0 μM H_2O_2 control and were generated from 5 separate experiments for non-activated cells and 3 separate experiments for the LPS-activated cells (n=3 for each treatment group). Statistical differences of the H_2O_2 treatment groups compared to 0 μM H_2O_2 were determined by a 1-way ANOVA with a Dunnett's post-hoc test and resulted in significance ($P < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: A) "No IkB α AA": 50-200 μM H_2O_2 ; "+ IkB α AA": no significance. B) "No IkB α AA": 40, 50, and 200 μM H_2O_2 ; "+ IkB α AA": 50-200 μM H_2O_2 . * $P < 0.05$ represents statistical differences between "No IkB α AA" and "+ IkB α AA" as determined by a 2-way ANOVA with a Bonferroni post-hoc test.

Figure 6. LPS activation and IkBαAA expression result in differential effects on reporter activity at 24 hr

CH12IkBαAA cells transiently transfected with the V_H-Luc-3'IgHRR (A and E), V_H-Luc (B and F), V_H-Luc-hs4 (C and G), or 3x-NFκB-Luc (D and H) reporter plasmids were either cultured at 2.0×10^5 cells/ml for 2 hr in media alone or with IPTG to activate the IkBαAA super-repressor. The cells were then cultured for 24 hr in the absence (A-D) or presence (E-H) of LPS (1.0 μg/ml). "NA" denotes naïve (untreated) cells. Luciferase enzyme activity is represented on the y-axis as relative light units (mean ± SEM). The results are representative of 3 experiments for each reporter (n=3 for each treatment group). Percent inhibition by IkBαAA relative to NA (A-D) or LPS (E-H) controls is indicated. Statistical differences between NA and IkBαAA (A-D) were determined by a 2-tailed t-test and statistical differences between LPS and LPS + IkBαAA or NA (E-H) were determined by a 1-way ANOVA with Dunnett's post-hoc test; * $P < 0.05$.

Figure 7. Hydrogen peroxide inhibits reporter activity at 24 hr

CH12I κ B α AA cells transiently transfected with either the $V_H\text{-Luc-3'IgHRR}$ (■), $V_H\text{-Luc-hs4}$ (○), $V_H\text{-Luc}$ (▼), or $3x\text{-NF}\kappa\text{B-Luc}$ (□) reporter plasmids were cultured at 2.0×10^5 cells/ml for 2 hr in media alone (A and B) or with IPTG to activate the I κ B α AA super-repressor (data not shown). The cells were then cultured for 24 hr with varying concentrations of H_2O_2 (0-200 μM) in the absence (A) or presence (B) of LPS (1.0 $\mu\text{g/ml}$). "C" denotes the non-activated control. Results are represented as fold-change (mean $\pm$ SEM) relative to the respective 0 μM H_2O_2 control and statistical differences of the H_2O_2 treatment groups compared to 0 μM H_2O_2 were determined by a 1-way ANOVA with a Dunnett's post-hoc test and resulted in significance ($P < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: A) $V_H\text{-Luc-3'IgHRR}$: 40-200 μM ; $V_H\text{-Luc}$: 30-200 μM ; $V_H\text{-Luc-hs4}$: 40-200 μM ; $3x\text{-NF}\kappa\text{B-Luc}$: 40-200 μM . B) $V_H\text{-Luc-3'IgHRR}$: 100-200 μM ; $V_H\text{-Luc}$: 30-200 μM ; $V_H\text{-Luc-hs4}$: 30-200 μM ; $3x\text{-NF}\kappa\text{B-Luc}$: 40-200 μM . Statistical differences compared to the $V_H\text{-Luc}$ reporter were determined by a 2-way ANOVA with a Bonferroni post-hoc test and resulted in significance ($P < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: A) $V_H\text{-Luc-3'IgHRR}$: 40 μM ; $V_H\text{-Luc-hs4}$: 30 and 40

μM ; 3 $\times$ -NF κ B-Luc: 30 and 40 μM . B) V_H-Luc-3'*Igh*RR: 40-100 μM ; 3 $\times$ -NF κ B-Luc: 30 μM . Results were generated from 3 separate experiments (n=3 for each treatment group).

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Figure 8. Hydrogen peroxide alters I κ B α and NF κ B/p50 protein expression

CH12I κ B α AA cells (2.0×10^5 cells/ml) were cultured for 24 hr (A and C) or 48 hr (B and D) with varying concentrations of H_2O_2 (0, 40, 100 and 200 μM) in the absence or presence of LPS (1.0 $\mu\text{g/ml}$). Cells were harvested in mild lysis buffer and 50 μg of total protein from each treatment group was subjected to 10% SDS-PAGE electrophoresis, transferred to a PVDF membrane and probed with anti-I κ B α (sc-371) (A and B), anti-p50 (sc-114) (C and

D) or anti- β -actin antibody (A-D). The immunoreactive bands for I κ B α and N-I κ B α (~34kDa band) are indicated by arrows.

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Table I
Summary of H₂O₂-mediated transcriptional activity

		Non-activated		LPS-activated	
		Low [H ₂ O ₂] ^b	High [H ₂ O ₂]	Low [H ₂ O ₂]	High [H ₂ O ₂]
Transcriptional Activity	24 hr	↓ 3' <i>Igh</i> RR ^a	↓ 3' <i>Igh</i> RR	– 3' <i>Igh</i> RR	↓ 3' <i>Igh</i> RR
		↓ hs4	↓ hs4	↓ hs4	↓ hs4
		↓ V _H	↓ V _H	↓ V _H	↓ V _H
		↓ NFκB	↓ NFκB	↓ NFκB	↓ NFκB
	48 hr	↑ 3' <i>Igh</i> RR	↓ 3' <i>Igh</i> RR	↑ 3' <i>Igh</i> RR	↓ 3' <i>Igh</i> RR
		– hs4	↓ hs4	↑ hs4	↓ hs4
		↓ V _H	↓ V _H	– V _H	↓ V _H
		↓ NFκB	↓ NFκB	↑ NFκB	↓ NFκB

^a Activity (↑, increased; ↓, decreased; – no change) of luciferase reporters.

^b Low [H₂O₂], approximately 30-50 μM H₂O₂; High [H₂O₂], approximately 100-200 μM H₂O₂