THE IMPACT OF THE NRF2 ACTIVATORS ARSENIC TRIOXIDE AND TERT BUTYLHYDROQUINONE ON B CELL FUNCTION

By

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ABSTRACT

B cells produce immunoglobulins and are essential for robust humoral host defense. B cell function is tightly regulated by interactions with other immune cells and antigens. B cells are subject to rapid clonal expansion and massive production of antibodies, which subjects them to oxidative stress. Many environmental toxicants, such as heavy metals and quinone-type food preservatives, are known to alter the function of other immune cells, such as T cells. The role of environmental toxicants on B cell function has received little investigation so far. Given the pivotal role of antibodies in the context of infection, autoimmunity, and allergy, it is gravely important to understand the impact environmental factors have on B cell function. The food additive tert-Butylhydroquinone and the heavy metal arsenic trioxide have been shown by our laboratory to alter T cell responses to infectious agents and alter cytokine production, partially due to the signaling of nuclear factor erythroid 2-related factor 2 (Nrf2). Due to the reciprocal relationship of B and T cells, this raises the question of the impact on B cell function. In these studies, we describe the impact of the Nrf2 activation compounds arsenic trioxide and tBHQ on human B cell function *in vitro* and on murine B cell function both *in vivo* and *ex vivo*. We isolated human peripheral blood mononuclear cells and exposed them to arsenic trioxide before a challenge with influenza A virus (IAV). After 96 hours, cells were harvested and analyzed using flow cytometry. Cells that were exposed to ATO showed less immunoglobulin surface expression, activation, and effector function. In rodents, we used an *ex vivo* activation model mimicking the T cell- B cell interaction to assess the role of tBHQ on B cell activation in a physiologically significant manner. In this model, we measured a decrease in IgG1 producing cells and a decrease in a variety of activation markers, indicating that tBHQ indeed altered B cell activation.

Interestingly, we also observed a tBHQ dependent decrease in B cell clonal expansion and a diminished inhibition of negative regulators such as CD267. Finally, we measured the effect of tBHQ *in vivo* by exposing mice to 0.0014% tBHQ via their diet, a similar amount as found in commercially used rodent chow. These mice were then sensitized to chicken ovalbumin using a transdermal sensitization model mimicking the pathogenesis of human food allergies. Cells were harvested from lymph nodes to determine whether tBHQ had an impact on the development of allergies. The very early activation marker CD69 was inhibited, while CD25 was not. We also measured an increase in CD80 and CD138, and most importantly, an increase in the key atopic immunoglobulin IgE, both in serum and on the surface of B cells. Collectively, these data are the first descriptions of a change in B cell function upon exposure to NRF2 activating toxicants. Given the rise of atopic disease and the prevalence of infectious disease requiring immunoglobulin mediated immunity, further characterization of the impact of the toxiom on B cell function is urgently needed

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LIST OF ABBREVIATIONS

15d-PGJ2	15-deoxy-Δ12,14-prostaglandin J2
AD	atopic dermatitis
AID	Activation-induced deaminase
ALI	acute lung injury
AP-1	activator protein 1
APC	antigen-presenting cell
ARE	Antioxidant Response Element
ATO	Arsenic Trioxide
B cell	Bursa cell
BALF	broncheoalveolar lavage fluid
Bcl-6	B cell lymphoma 6
BCR	B cell receptor
BHT	butyl hydroquinone
bTRCP	β-transducin repeat-containing protein
BZIP	basic leucine zipper
CD	Cluster of differentiation
CREB	cAMP response element protein
CXCR5	C-X-C chemokine receptor 5 (CD185)
DAI	daily allowable intake
DC	Dendritic cell
DZ	Dark Zone

FDC	Follicular Dendritic cell
FEV1	Forced exhalatory volume 1
FO B	Follicular B cell
GC	Germinal center
GSK3	glycogen synthase kinase 3
GSTP1	glutathione S-transferase Pi-1
HeLa	Helga Lacks derived cells
HIP	hypoxia-induced protein
HSC	Hematopoietic stem cell
HSC	Hematopoietic stem cell
HSC	Hematopoietic stem cells
IAV	Influenza A Virus
IFN-γ	Interferon-gamma
IgM	Immunoglobulin M
ILB	Innate like B cell
ILC	innate lymphoid cell
Keap1	Kelch ECH-associated protein 1
LPS	lipopolysaccharide
LZ	Light Zone
MHC	Major Histocompatibility Complex
NAT2	N-acetyltransferase 2
NF-κB	Nuclear Factor Kappa light chain enhancer B

NKT	Natural Killer T cell
NOAEL	no observed adverse effect level
NQO1	NAD(P)H-quinone oxidoreductase 1
NRF2	Nuclear factor erythroid 2-related factor 2
PARP1	poly(ADP-ribose)polymerase-1
PC	Plasma cell
PD-1	programmed cell death protein 1 (CD279)
PERK	protein kinase RNA-like endoplasmic reticulum kinase
pIgR	Polyimmunoglobulin-Receptor
PKC	protein kinase c
RAG2	Recombination activating gene 2 protein
ROS	Reactive oxygen species
S1P	sphingosine-1-phosphate
SHM	Somatic Hypermutation
SLE	systemic lupus erythematosus
SLO	secondary lymphoid organ
SNP	single nucleotide polymorphism
SUMO	Small ubiquitin-like modifier
T cell	Thymus cell
tBHQ	tert-butylhydroquinone
tBQ	2-tert-butyl-1,4-benzoquinone
TCR	T cell receptor

TFH	T follicular helper cell
TNF-α	Tumor necrosis factor alpha
Trx2	thioredoxin 2
VLRA	Variable lymphocyte receptor A
VLRB	Variable lymphocyte receptor B

Introduction/Literature review

B cells – a view from 30,000 Feet

B cells are the prime mediator of humoral immunity. Their core function in mammals is the production of immunoglobulins of different classes. These immunoglobulins, or antibodies, are small proteins that will bind to specific epitopes on a biological specimen, typically on bacteria, fungi, viruses, or helminths. Immunoglobulins are also involved in pathological processes, such as autoimmunity, allergy, or physical dysfunction through the sheer mass of immunoglobulin fragments. B cells are also professional antigen-presenting cells (APC) that can take up, process, and present antigen on both MHC I and MHCII molecules.

The etymology of the term B cell

B cells are named after the Bursa of Fabricius, an organ found near the cloaca of birds. Hieronymus Fabricius ab Aquapente mentioned this organ first in lectures given at Padova in 1621 [1]. The Latin term *bursa* derives from the Greek word βύρσα [*byrsa*], which can be translated as "purse, bag or sac of leathery material". It was not until 1952 that Bruce Glick discovered in studies at The Ohio State University that surgical removal of the bursa led to the loss of immunoglobulin production in the birds that underwent this procedure [2]. The cells responsible for antibody production were thus dubbed B cells as in "**B**ursa cells". Later anatomical studies indeed revealed that the bursa is indeed a lymphoid organ [3]. Mammals in general, and humans specifically, do not have any organ that could be considered homologous where B cell development occurs in the bone marrow. Fortunately, the acronym remains the same, though considering B cells as "**B**one marrow cells" would likely be unacceptably imprecise and confusing to the wider scientific community.

B cell development

Broadly speaking, all organisms possess some kind of immunological system. Bacteria and protozoa possess multiple mechanisms that provide defense against other microbes [4], and some of the applications derived from those mechanisms have revolutionized biotechnology [5]. Whereas these systems are mostly unspecific in lower life forms, vertebral organisms have evolved to a system that is selective for molecular patterns (antigens) on a cellular level.

Of the cell-based immunities, one can broadly differentiate between the VLRA/VLRB system and the TCR/BCR system. The divergence of these two systems has been pinpointed to the

development of jaws. Our closest relatives that use the VLRA/VLRB system in opposition to the HLA-based system of mammal adaptive immunity are jawless lampreys and hagfish [6-8] .

B cells in mammals, specifically in humans or mice, are derived from hematopoietic stem cells (HSC). These are derived from the embryonic aorta-gonado-mesonephros and thus mesodermal tissue [9], residing in the bone marrow. A broad overview of B cell development is provided in Fig. 1.

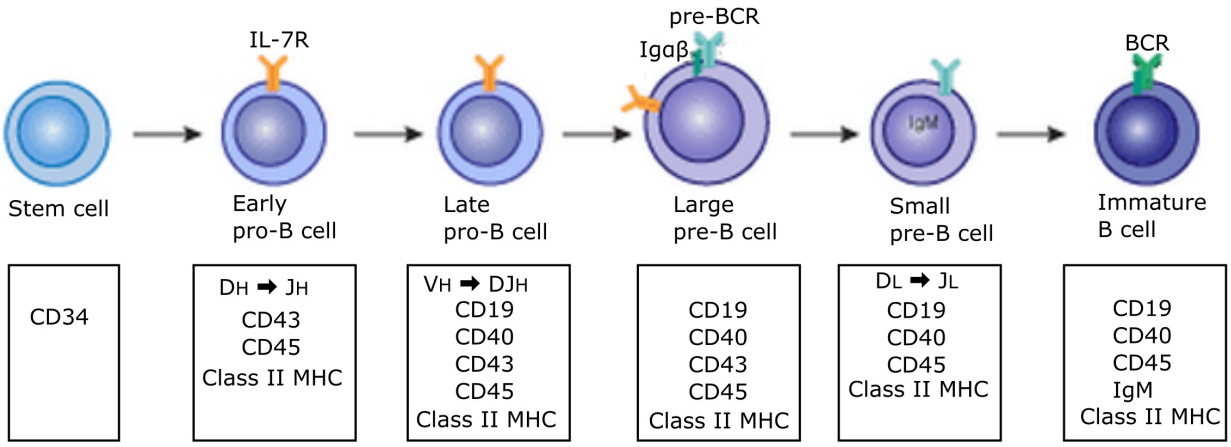


Figure 1. Schematic overview of B cell development. Boxes demarcate surface molecules that can be used to distinguish B cell state. B cells undergo development in the Bone marrow, starting as CD34+ hSC, gradually differentiating into immature B cells, which will disseminate to peripheral tissues and secondary lymphoid organs. Graphic By Mikael Häggström, MD, used with permission.

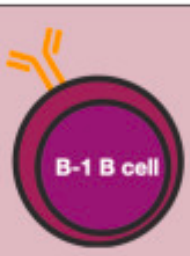
The first step towards B cell fate is the rearrangement of the D and J regions of the heavy chain section (D_h and J_h) in the immunoglobulin gene, leading to a vastly increased variety of immunoglobulin gene variants [10]. At this stage, the cell is considered a pre-B cell. The second

step during the pre-B cell period is the rearrangement of the V_h region and the rearrangement of the light chain V and J regions (V_L and J_L) This V(D)J recombination process is mediated by the Recombination activating gene 2 protein (Rag2)[11], which is equally important in T cell development and used as a genetic target to generate B and T cell-deficient mice. The next step in development is the rearrangement of the **μ**-heavy chains and the κ and **λ** light chains, which ultimately enables the pro-B cell to express an IgM molecule on its surface [12]. At this stage, the cell is considered an immature B cell and leaves the bone marrow to circulate into the spleen and secondary lymphoid organs (SLO)[10]. These immature B cells then reside in SLO as follicular or marginal zone B Cells (FO B cell and MZB cell, respectively) or recirculate. They will remain in this state until they are activated.

Types of B cells

B cells can be classified into different subtypes. There are some significant differences between murine and human cells, and some of those classifications tend to overlap. The subtypes of B cells remain somewhat debated; the following classification is an attempt to synthesize different currently held viewpoints in the literature. Table 1 provides an overview of the most common B cell subtypes.

Table 1. Overview of B cell subtypes. Follicular B cells, Marginal Zone B cells and B-1 B cells differ in function and surface marker expression. Adapted from: DOI.ORG/10.1016/J.JAU

			
Primary localization	Secondary lymphoid organs; mainly in B cell follicles	Secondary lymphoid organs; mainly in the splenic marginal zone	Peritoneal and pleural cavities
Distinguishing surface markers	$B220^{+}$, $CD19^{+}$, $CD1d^{lo}$, $CD21^{+}$, $CD23^{hi}$, $CD43^{-}$, IgM^{lo}, IgD^{hi}	$B220^{lo/+}$, $CD19^{+/hi}$, $CD1d^{hi}$, $CD21^{hi}$, $CD23^{lo}$, $CD43^{-}$, IgM^{hi}, IgD^{lo}	$B220^{-/lo}$, $CD19^{hi}$, $CD1d^{+}$, $CD23^{-}$, $CD43^{+}$, IgM^{hi}, IgD^{lo}
Principal function	Participation in T-dependent immune responses	Housekeeping function (natural IgM), first responders to blood-borne pathogens	Housekeeping function (natural IgM), first responders to mucosal pathogens

Marginal zone B cells

Marginal zone B cells derive their name from being present in the marginal zone of the spleen. This location is particularly well perfused with arterial blood, and thus high concentrations of sphingosine-1-phosphate (S1P) are found. MZ B cells use their S1P receptors to seed these particular areas [13]. MZ B cells are the main producer of IgM and natural autoantibodies (see chapter on Antibodies) [14]. Marginal Zone B cells (MZB) have a strong innate-like function, as they can become activated independently of T cell help [15]. This activation is dependent on

motifs in the antigens. Murine MZB cells are known to be potently activated by LPS [16-18], whereas the T cell-independent activation of human MZB cells is more controversial. It has been reported that human B cells, in contrast to murine B cells, do not react to LPS activation [17]. Newer studies have shown that human B cells, specifically MZB cells, express a wide array of TLR receptors, namely TLRs 1, 6, 7, 9, and 10 [19, 20], indicating that TLR activation can facilitate a polyclonal antibody response in MBZ cells [21]. Overall, MZB cells can be considered innate-like B cells, capable of bridging the innate and adaptive immunity and potentially implicated in pathology due to autoantibodies [22, 23].

B1 B cells / Innate-like B cells

B1B B cells are commonly found in mice. B1 B cells are somewhat similar to the MZ B cells in function, though they differ greatly in location – B1 B cells are often found in the periphery, and they line the peritoneal and pleural cavities [24]. Their localization is largely governed by Cxcl13 [24]. B1 cells do not undergo GC reactions and/or somatic hypermutation (SMH), and their BCR repertoire is more limited compared to FO B cells [25]. B1B cells are implicated in the defense against helminths such as *nippostrongylus brasiliensis* [26, 27], spirochetes such as *borrelia hermsii* [28, 29], and Enterobacter such as *e. cloacae* [30]. The existence of B1 B in human cells has been somewhat controversial [26], but one can appreciate the great functional similarity between murine B1B cells and human innate-like, CD5+ B cells [31-33].

Follicular B cells and the germinal center reaction

Follicular B cells are the circulating and lymph node-resident B cells [34]. They are typically found in the cortex region of lymph nodes, Peyer's Patches, or the white pulp of the spleen, and are

instrumental to the generation and expansion of SLOs and follicular dendritic clusters via their secretion of alpha-lymphotoxin [35, 36]. Clusters of naïve FO B cells and follicular dendritic cells (FDC) are coined primary follicles [37] and may give rise to germinal centers (GCs). These areas are particularly close to resident T cells, facilitating the T cell interaction on which FO B cells depend. The interaction between FO B cells and T follicular helper cells (TFH cells) is subject to tight regulation and results in the formation of high-affinity antibodies and isotype switching. This process can be repeated sequentially to increase the affinity of immunoglobulins. A schematic overview of the germinal center reaction is seen in Fig. 3.

The first step of the germinal center reaction is the recognition of a suitable antigen by the FO B cell, either presented by an FDC or a soluble antigen that is taken up directly by the FO B cell [38-41]. This activation sets off a chemotactic sequence: The FO B cells upregulate CD197 (CCR7, C-C chemokine receptor type 7), which recognizes cysteine motifs in its ligands, CCL19 and CCL21 [42]. These are highly expressed in the adjacent T cell zones, thus the activated FO B cell moves to the periphery of the emerging GC (hereby constituting the Light zone of the budding GC) and mingles with the resident TFH cells[43]. This movement happens at surprisingly high speeds of up to 6μm/min [44]. This movement facilitates the “speed dating” between TFH and B cells – the B cell will present fragments of the antigen that activated it on its MHC II molecule, and TFH cells browse through the offered antigens to see if they match the specificity of their TCR. If the antigens indeed match, the T cell will offer costimulatory signals and form a so-called T-B cell entanglement [44-46].

The interaction between FOB cell and TFH cell is initiated by TCR-MHCII-antigen peptide complexes but needs costimulatory signals such as CD40 on the B cell and CD40L on the T cell [47]. This signal activates non-canonical NF-Kappa B signaling and an array of TRAF signals within the B cell [48]. These signals subsequently lead to clonal expansion and activation of the FOB cell, effectively starting the germinal center reaction [46]. At this point, the FOB cells will either commit to the full germinal center reaction leading to high-affinity antibodies or leave the follicle and become short-lived, low-affinity extrafollicular plasma cells [49-51]. Additionally, extrafollicular, T cell-independent memory B cells have been described recently [52]. This explains the comparably rapid first wave of low-affinity antibodies in response to an infection, followed by a larger wave of high-affinity antibodies shortly thereafter [52].

FOB cells that commit to the GC now start expressing high amounts of Activation-induced cytidine deaminase (AID), an enzyme that is essential for the GC process [53]. AID is a cytidine deaminase that converts cytidine bases in the immunoglobulin gene variable regions to uracil bases, effectively turning stable guanine-cytidine matches into mismatched uracil-guanine pairings. These new mismatches are subject to repair processes, which introduce mutations to the variable regions, at a rate about a million times higher than the baseline mutation rate of the genome [54, 55].

This process is termed somatic hypermutation (SHM) and introduces great variability to the variable region of the immunoglobulin gene of each nascent B cell. AID is also implicated in the process of Isotype switching, in which a B cell changes its immunoglobulin constant region, by introducing double-stranded DNA breaks. This was generally believed to be part of the GC

reaction, however, newer studies suggest that this may already be happening in pre-GC B cells, as one of the first actions of AID [56]. Isotype switching changes the BCR immunoglobulin type from the resting IgD/IgM to an IgG, IgA, or IgE type BCR, depending on the signals given during the GC.

SHM and clonal expansion take place in the dark zone (DZ) of the GC. B cells that are actively undergoing these processes are dubbed centroblasts. In the light zone, the B cells present their newly mutated BCR/antigen complexes to TFH cells, which then assess the affinity of the BCR for the antigen. This process is described both in mice [57] and humans [58, 59].

All light zone B cells are in a pro-apoptotic state, and only signals provided by the TFH cell will prevent apoptosis and allow the B cell to either return to the dark zone for further affinity maturation or to become a plasma cell [60]. This process is dubbed positive selection. SHM can also lead to autoreactive immunoglobulins and thus potentially cause antibody-dependent autoimmunity. Considerable debate over the existence of a putative negative selection during the GC reaction is ongoing, but a consensus emerges that it is likely to be an infrequent or apoptosis-independent event [46], e.g. through BCR mutation redemption [61]. BCR redemption is a complex process that involves deactivation of autoreactive BCRs by covering them with carbohydrates, and subsequent re-entry into the GC reaction and further mutation to change the VDJ-product to one that does not bind to auto-antigens [62].

GC B cells, if not undergoing apoptosis, are eventually exported from the germinal center, either as plasmablasts or memory B cells. Plasma cells (PC) tend to have a higher affinity for the antigen,

while memory B cells have a relatively lower affinity [46]. Plasmablasts typically tend to have high expression levels of the transcription factor Blimp-1[60, 63].

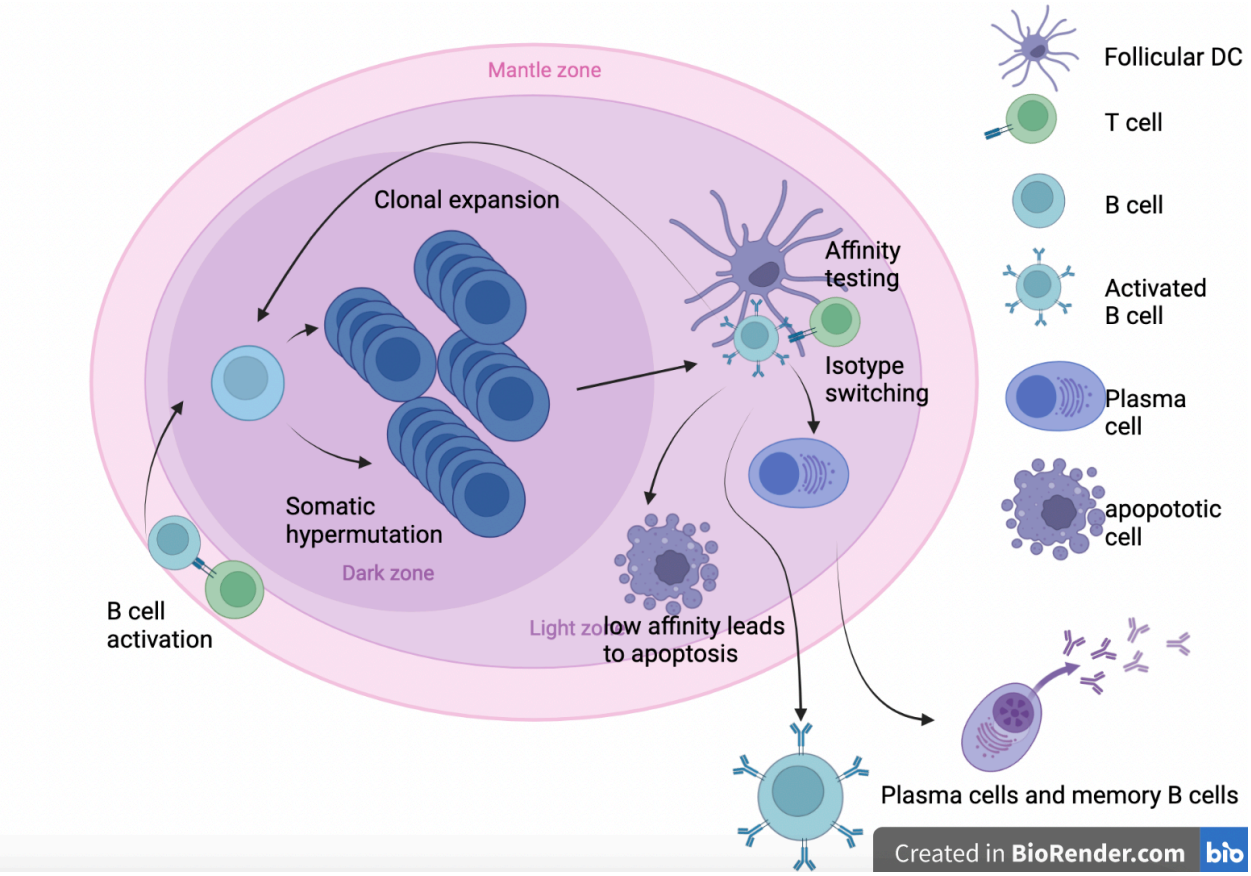


Figure 2. Schematic of the Germinal center reaction. Naïve B cells get activated by T cells specific to an antigen and enter the dark zone. Somatic hypermutation and clonal expansion introduce mutations to IG genes. B cells enter the light zone and test their affinity. Potential outcomes are apoptosis (low affinity), reentering the dark zone for further affinity maturation, or Plasmablast or Memory B cell fate, which then leave the lymph node. Graphic created in Biorender, own work.

Memory B cells

Memory B cells are antigen-experienced B cells that underwent class switching and SHM during a germinal center reaction. An exception to that rule are IgM+ memory B cells, which developed independently of T cells (See B1-B cells). Memory B cells can survive in peripheral tissues for many

years and repeatedly respond to the same antigen if exposure occurs [64]. If they discover an antigen specific to their BCR, they can either traffic back to a SLO and re-enter the germinal center reaction or differentiate into a plasma cell [65]. The factors that decide the fate of GC B cells, once they reach a certain receptor affinity, whether to differentiate into a plasma blast or memory B cell, have been elusive. A generally higher affinity in the case of plasmablasts has often been discussed [46]. Other factors that are thought to distinguish between the memory B cell and plasma cell fates are IL-24 signaling, NF-κB activity, as well as the Blimp1-related transcription factor Bach2 [66]. Blimp-1 and Bach2 signaling is a inhibitor for memory B cell fate and favors plasma cell differentiation, while PAX5 signaling is required for memory B cell differentiation [67, 68].

Plasmablasts/ Plasma cells

Plasma cells are the major antibody producers among B cells. After affinity maturation in the GC reaction, B cells with high affinity receptors express high levels of the transcription factor IRF4 and differentiate into plasma cells [66]. Plasma cells leave the lymph node and preferentially migrate to the bone marrow, where they continuously secrete antibodies. Fully differentiated plasma cells focus solely on the production of antibodies, while immature plasma cells (called plasmablasts) also undergo cell division [67].

Associated cells – TFH cells and follicular dendritic cells

TFH cells are a subtype of CD4 T cells and essential partners in the germinal center reaction [59]. They depend on IL-6, IL-21 and ICOS signaling to commit to the TFH fate [69, 70]. These cells are defined as cells expressing TCR, CD4, CXCR5, Bcl-6, and PD-1. TFH cells participate in the GC

reaction by providing costimulatory signals such as CD40L, CD28, and OX40 [71] to B cells as well as by producing cytokines that determine the resulting antibody isotype and possibly the fate of the resulting B cell [65]. TFH have been classified by the secretion of cytokines into functionally-distinct subtypes including TFH1, TFH2, TFH13 and TFH17 cells [72]. The interaction of TFH with B cells via PD-1 binding is vital for the generation of long-living plasma cells[73]. CD40L-CD40 interactions are essential for the initial formation of GCs and uncontrolled signaling of this pathway can lead to a lupus-like disease [74].

Follicular dendritic cells (FDCs) are functionally dendritic cells, but unlike other DCs, are not derived from HSCs [75]. They form the core of the follicle, where they present antigens to B cells, as a checkpoint for affinity [76]. FDCs canonically do not carry MHCII molecules, however, they have been reported to express microvesicles with MHCII that were passively acquired at their surface [77]. This suggests that their role in phagocytosis and MHCII-restricted antigen presentation is negligible. Instead, they rely on an intricate mechanism where non-antigen specific B cells capture blood or lymph borne antigen, which has been opsonized by other innate immune cells, and transfer these antigens to FDCs [40, 41]. They are also involved in clearing debris from the follicle and SLOs, a mechanism that in rodents has been shown to prevent autoimmune processes [78].

Overview of immunoglobulins

Immunoglobulin production is one of the chief functions of B cells. Immunoglobulins are a considerable part of the protein fraction in serum and are found in most bodily fluids, including saliva, blood, mucus, and feces [59]. Ig molecules can be found on cell surfaces of B cells or as soluble proteins. Human and murine antibodies are Y-shaped proteins of approximately 150 kDa and 10nm in length, consisting of 4 polypeptide chains [79]. The 4 polypeptide chains can be further classified into 2 light chains and two heavy chains, and each chain has both one or more constant and one variable section (Fig 3). Functionally, the antibody molecule can be classified into two regions: The variable region (Fab) and the constant region (Fc).

The variable regions contain paratopes, which are the areas of an antibody that bind to epitopes on an antigen. The variable region thus determines the binding affinity to epitopes on antigens, while the constant region determines the isotype of the antibody and provides the structural integrity of the protein [80]. While there is immense variability in the Fab region, due to VDJ recombination and SHM, the Fc region has 5 different possible isotypes, of which some have further subtypes. These isotypes are IgD, IgM, IgG, IgA, and IgE [59]. Each one has a specific biological function and differs from the other subtypes.

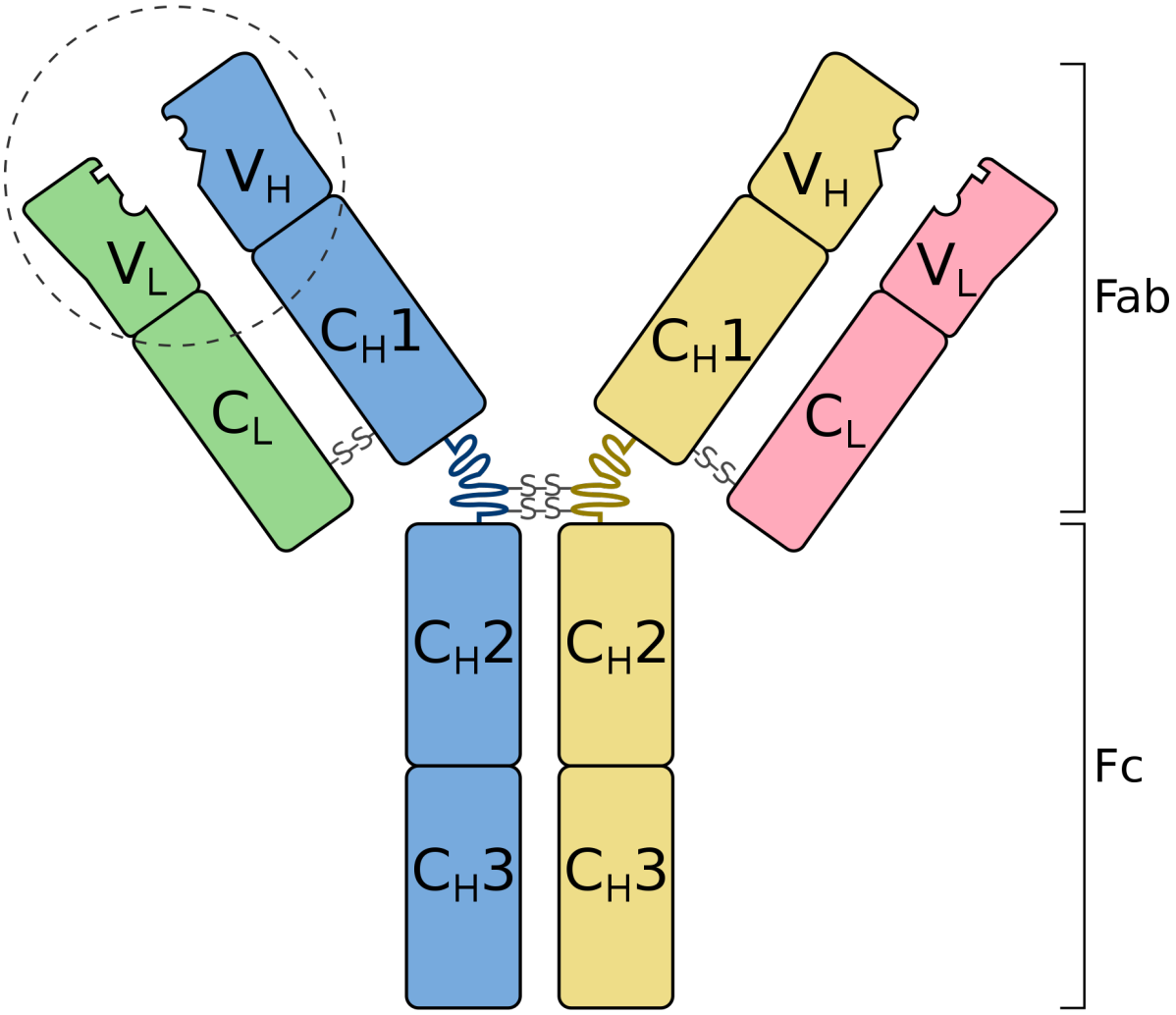


Figure 3. Antibody structure. Simplified cartoon of an immunoglobulin. The heavy chains are shown in yellow and blue, the light chains in green and pink. The left antigen binding site is circled. The heavy chains contain a variable domain (VH), followed by a constant domain (CH1), a hinge region, and two more constant domain (CH2, CH3). The light chains have a variable domain (VL) and a constant domain (CL). The CH2 and CH3 regions constitute he variable and CH1, CL regions form a pair of antigen-binding fragments (Fab). Disulfide bonds between the

Figure 3 (cont'd)

chains are drawn as S-S. Their exact number and location vary for different isotypes. Used under CC BY-SA 4.0, created by User TokenZero

IgD is one of the rarer Ig isotypes, expressed on immature and resting B cells, usually together with IgM. IgD is also found in serum in very low concentrations, compromising less than 0.25% of Ig proteins. It also has a comparably short serum half-life of ~2.8 days [81]. IgD does not seem to be essential to B cell function, as IgD deficient mice have a largely normal B cell phenotype [82]. IgD seems to have some functions in communication between basophils and B cells [83]. In addition, serum IgE levels and the number of peripheral B cells are moderately reduced in IgD-deficient mice [82]. Secreted IgD usually is found as a monomeric molecule[59].

IgM, which is co-expressed with IgD on resting B cells, B1B cells, and marginal zone B cells, is usually the first antibody produced in large numbers in response to an antigen challenge, making it a useful clinical indicator to detect recent diseases in contrast to an older resolved or chronic pathogenic process. IgM can be translocated from the bloodstream into the gut, mucosal surfaces, or breastmilk using the polyimmunoglobulin receptor (pIgR)[84]. While B cells that produce IgM have undergone VDJ recombination, they may or may not have been subject to SHM, and thus the average affinity of IgM molecules to target antigens is lower than other isotypes, like IgA, IgG, and IgE [85]. IgM is also a potent opsonin, marking antigens tagged by IgM for phagocytosis by macrophages and other innate immune cells. Uptake of these antigens via Fc receptors on APCs can also lead to the increased presentation of the antigen on MHC molecules,

further amplifying the immune response to the antigen [59]. IgM typically forms pentamers connected to each other using the J chain subunit [86].

IgA is the immunoglobulin most often found in mucus, inside the gut canal, and in saliva. It is also present in breast milk and serum [59]. It is a weak opsonizer[59]. IgA is produced after a B cell undergoes isotype switching. The interaction with TFH cell with B cells, as described previously, is essential for IgA class switching.IL-5 and TGF- β1 are the key cytokines produced by TFH cells that promote IgA production [87]. There is, however, a T-cell independent pathway to IgA production [88]. IgA molecules are heavily glycosylated to be more resistant in the environments in which they are often found [89]. IgA often forms a dimer that is held together by a J chain, similar to IgM, but also by a crucially important secretory component. This protein is part of the pIgR and cleaved off together with IgA dimers during the translocation of IgA to its destination compartment [90]. IgA has two subforms, IgA1 and IgA2. They differ structurally, as IgA1 has the classic structural components shared with the other antibody subtypes, while IgA2 differs as it is not held together by disulfide bonds, but by non-covalent interactions instead[91]. While IgA is not the most common Ig molecule class in the bloodstream, it is the most produced overall [59].

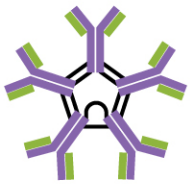
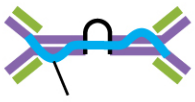
IgG is the immunoglobulin class most abundant in serum. IgG is an excellent opsonin and neutralizing antibody [59]. The key cytokine released by TFH cells to induce an IgG fate is interferon-gamma (IFN-γ)[92, 93]. IgG has 4 subclasses, IgG1, IgG2, IgG3, and IgG4, which are biochemically almost homologous, but differ in function. IgG1 is the most common subclass but can also be an intermediate in the class switching towards an IgE fate [94, 95], a process called sequential switching. IgG1 is almost exclusively the result of the GC reaction and mostly targets

protein epitopes[92]. IgG2 can be generated in a T cell-independent manner in an extrafollicular reaction, and often targets bacterial polysaccharides [96-98]. IgG3 antibodies tend to have a stronger pro-inflammatory effector function and are therefore especially potent in fighting off infectious pathogens [99]. They also have a remarkably lower half-life than the other IgG subtypes, perhaps as a biological function to limit the time course of a strong inflammatory reaction [100]. IgG4 antibodies are considered anti-inflammatory and tolerance-inducing [101]. IgG4 is the least common IgG subtype, comprising only about 5% of all IgG antibodies. It is a non-opsonin, curiously, it can even prevent the formation of complement immune complexes using its "Fab-arm exchange" [101, 102]. In this process, the IgG4 heavy chains disassociate from each other and reassemble with another IgG4 half-molecule that underwent the same process, yielding a bispecific antibody, which is unable to form complement immune complexes for the lack of accessible Fc region [102].

IgE is implicated in the immune response to helminths and parasites, but also in allergy and atopic disease. IgE is mostly a product of the GC reaction, but in rare instances can be generated extrafollicularly[103]. IgE is found in either a high-affinity or low-affinity form. Low-affinity IgE antibodies are associated with defense against helminths, as those pathogens do not elicit high-affinity IgE antibodies [95, 104, 105]. A likely explanation is that helminths and protozoic parasites often have carbohydrate-covered antigens at their outer surface, and often undergo antigenic shift, explaining the need for low-affinity, less specific IgE [106]. This has been postulated as the reason why people infected with helminths suffer less from allergies and anaphylactic shock, one of the tenets of the hygiene hypothesis [107]. Only high-affinity IgE can prime and subsequently

facilitate the degranulation of mast cells, the crucial step in anaphylaxis [108-110]. The production of high-affinity IgE is believed to require an intermediate class switch to IgG1, before switching into IgE [105]. The longstanding dogma of IgE generation states that Th2 cells and IL-4 are essential factors in IgE generation [111-114]. Newer studies paint a more nuanced picture, in which TFH cells secrete IL-4, IL-5, IL-13, and low amounts of IL-21 to induce IgE fate in the GC reaction. Loss of IL-13 abrogated the IgE production in this peanut-antigen model [108, 110].

Table 2. Overview of Immunoglobulin classes. Depicted are 5 common classes of Immunoglobulins and some of their key functions. Not depicted are subclasses. Graphic taken with permission under CC-BY-SA 4.0, by unknown user.

The Five Immunoglobulin (Ig) Classes					
Properties	**IgG monomer**	**IgM pentamer**	**Secretory IgA dimer**	**IgD monomer**	**IgE monomer**
Structure					
Heavy chains	γ	μ	α	δ	ε
Number of antigen-binding sites	2	10	4	2	2
Molecular weight (Daltons)	150,000	900,000	385,000	180,000	200,000
Percentage of total antibody in serum	80%	6%	13% (monomer)	<1%	<1%
Crosses placenta	yes	no	no	no	no
Fixes complement	yes	yes	no	no	no
Fc binds to	phagocytes				mast cells and basophils
Function	Neutralization, agglutination, complement activation, opsonization, and antibody-dependent cell-mediated cytotoxicity.	Neutralization, agglutination, and complement activation. The monomer form serves as the B-cell receptor.	Neutralization and trapping of pathogens in mucus.	B-cell receptor.	Activation of basophils and mast cells against parasites and allergens.

The role of B cells in Pathology

As the sole producer of antibodies, the role of B cells in pathology cannot be overstated. B cell-derived antibodies are involved in defense against all infectious diseases, autoimmune processes, atopic disease, and even in wound healing [115]. While protective against most infectious agents, autoreactive antibodies and altered B cell phenotypes are found in autoimmune diseases such as scleroderma [116], lupus [117], multiple sclerosis [118], allergic rhinitis[119], rheumatoid arthritis [120], celiac disease [121], type 1 diabetes [122] and even potentially in type 2 diabetes [123], among many other autoimmune processes. B cell inhibitors, such as rituximab (Rituxan), ocrelizumab (Ocrevus), ofatumumab (Kesimpta), and obinutuzumab (Gazyva) are used for many autoimmune pathologies[124], often off-label [125] as a last resort. The prominent role of B cells in cancer immunology is beyond the scope of this thesis and covered in many excellent reviews [126-129]. B cells themselves can be the source of malignancies, especially for lymphomas, myelomas, and leukemia subtypes [58].

Toxicological studies in B cells

Given the pivotal role of B cells in physiology and pathology, they have received comparatively little investigation in toxicological studies. Early studies in a chicken model showed that the chemotherapeutic and immunosuppressant cyclophosphamide inhibits B cell formation and humoral immunity even at low doses [130]. A common antihistamine, chlorpheniramine, conversely induced an expansion of the B cell population in a rat model [131]. Benzo(a)pyrene, a polycyclic aromatic hydrocarbon amplified the expression of phase two enzymes, but not AHR in rainbow trout B cells [132]. The heavy metals mercury and lead have been shown to affect IgM secretion in chicken-derived B cell lines [133, 134]. Interestingly, mercury decreased IgM production [134], while lead induced an intracellular accumulation of IgM, suggesting a secretion defect [133]. In human B cells, mercury modified the Lyn pathway [135]. While the physiological implications are somewhat unclear, this modification has been suggested as a biomarker for mercury exposure. Toluene diisocyanate exposure can change B cell phenotype with pathophysiological consequences. B cells exposed to toluene diisocyanate can induce asthma even if the exposure ceases, as evidenced by a murine adoptive transfer model [136]. Addressing popular toxicological concerns over environmental radiation, a study examined whether 900mhz GSM band waves influenced murine lymphocytes and found while there were minor, short-term alterations in T cell cytokine secretion, B cells were not influenced by this form of radiation [137]. In human cell lines, different translational inhibitors, such as trichothecene mycotoxin, ricin, and Shiga-toxin were evaluated for their cytotoxicity and found to be generally more potent in mature B cells than immature ones, though some mycotoxins showed no discrimination between

maturity levels [138]. Studies in the mouse B cell line CH12IκBαAA showed that low doses of hydrogen peroxide decreased Ig production in an NF-KB-dependent manner [139].

Large observational studies, albeit lacking in toxicological specificity, showed that exposure to a variety of pesticides over 15 years decreased B cells, regulatory B cells, and plasmablasts in the peripheral blood of Brazilian farmers, concurrent to an increase in serum IL-6 [140]. Another human observational study in Germany showed that exposure to lowly chlorinated biphenyls modestly decreases CD19+ B cells [141].

One of the major areas of toxicological investigation into B cell function has been over the persistent organic pollutant 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), colloquially referred to as dioxin. Dioxin is a contaminant in the herbicide agent orange, and in concentrated form has been used for political assassination attempts [142]. TCDD effects on B cells were described in birds [143] and mice [144], reducing the Ig response [145]. TCDD's effects on human cells via the aryl-hydrocarbon receptor (AHR) were also shown. In vitro, most of those donor-derived cells showed an impaired IgM response to CD40 stimulation [146]. TCDD interruption of the mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) pathway in primary human B cells led to decreased expression of CD80, CD86, and CD69 after stimulation with CD40 [147]. Overall, though AHR signaling is relatively well conserved between species, AHR stimulation by TCDD shows species-specific effects [148]. While both mice and men have diminished IgM secretion after TCDD exposure, the human impairment seems to be due to failure in IgM secretion, while mice have reduced mRNA expression [149], and there is considerable variation between individual humans regarding the magnitude of these effects [150]. Mechanistically, mouse TCDD

AHR signaling involves the BLIMP-1 pathway in CD40-dependent activation [151] and PAX5 in TLR-mediated activation [152]. SerpinB has emerged as a key molecule in TCDD injury, with SerpinB deficient mice having a more profound response to TCDD [153].

In humans, experiments with cells derived from CD34+ umbilical cord-derived cells have shown impaired B cell lymphopoiesis upon exposure to TCDD with involvement of early B cell factor 1 (EBF1) and paired box 5 (PAX5) [154] and impaired B cell lymphopoiesis upon exposure to TCDD [155]. Studies with peripheral blood human B cells have identified Src homology phosphatase 1 (SHP-1) as a mediator of B cell repression, potentially due to an interaction with Bcl-6 [156]. These effects seem to involve a reduction of STAT3 phosphorylation and are reversible by the addition of IFN-γ [157].

Interestingly, CD5+ B cells, which are considered innate-like B cells, seem to be maybe even more sensitive to TCDD in vitro, as measured by IgM suppression after TCDD challenged and basal AHR expression [158]. In these cells, lymphocyte tyrosine kinase (LCK) and PD-1 seem to mediate these effects [159]. In contrast, a recent study in mice suggested that follicular B cells are more sensitive to TCDD and that B cell suppression by TCDD improves the outcomes in experimental autoimmune encephalitis, a mouse model of multiple sclerosis, via IgG inhibition [160].

Similarly to the TCDD studies, Delta-9 hydro cannabinol causes a reduction in IgM secretion via a decrease in STAT3 phosphorylation [161].

Very little is known about the impact of tBHQ on B cell function. Our group showed that tBHQ increased IgM production by LPS-activated B cells *ex vivo* in a Nrf2-dependent manner [162]. In

contrast, expression of CD69, CD25, CD22, and CD138 were decreased by tBHQ via a Nrf2-independent mechanism.

Nuclear factor erythroid 2-related factor 2 (Nrf2)

Discovered in 1994, Nrf2 is a transcription factor that is activated by oxidative stress [163]. Its basic structure is part of the cap'n'collar (CNC) basic leucine zipper (BZIP) group. The evolution of Nrf2 correlates with the rise of atmospheric oxygen levels and its first orthologues were found in fungi 1.5 GA (~1.5 billion years) ago [164]. Nrf2 is ubiquitously expressed in mammalian cells and highly conserved among species [165, 166]. In homeostasis, Nrf2 is bound to its repressor protein, Keap-1, and the total amounts and ratios of Nrf2 and Keap1 differ between tissues [167], which may hint at differing susceptibility of tissues to oxidative stress. Nrf2 was initially discovered as a transcription factor binding to a locus that controlled the expression of the hemoglobin beta-subunit, though it turned out that Nrf2 was not essential for the generation of hemoglobin or survival in mice [165]. Since its initial discovery, several polymorphisms of Nrf2 have been described in humans [168].

Biochemical properties of Nrf2

Under homeostatic conditions, Nrf2 is bound to Keap-1 [167]. Keap 1 catalyzes the ubiquitination of bound Nrf2 by E3 ubiquitin ligase (Fig 4A), directing it to the 26S proteasome for degradation [169-171]. Under conditions of oxidative or electrophilic stress, Nrf2 induces the transcription of an array of genes in the nucleus [172]. The precise mechanism of activation of Nrf2 remains somewhat unclear, though it was evident that Nrf2 inducers alter sulfhydryl groups on Keap1 [173]. One mechanistic hypothesis of Nrf2 activation postulates that these changes to Keap1, alter the binding interactions in the Keap1-Nrf2 complex (Fig 4B). The interaction of the Nrf2 DLG complex with Keap1 (latch) is released, while the interaction of the Nrf2 ETGE complex (hinge)

remains stable. Thus, Nrf2 is still attached to Keap1, but these changes hinder the ubiquitination and degradation of bound Nrf2 [174] [175, 176]. In this “hinge and latch” theory, freshly synthesized Nrf2 does not bind to the already saturated Keap1-Nrf2 complexes, and instead freely translocates to the nucleus. A competing, but very similar model suggests a conformational change in Keap1, making the Nrf2-Keap1 complex unsuitable for polyubiquitination [177]. An alternative mechanism for Nrf2 degradation involves the Neh6 domain of Nrf2, which contains a redox-insensitive degron [178]. This area of Nrf2 can be phosphorylated by glycogen synthase kinase 3 (GSK-3) [179], which makes it a binding target for beta-transducin repeat-containing protein (bTRCP), which then, in turn, ubiquitinates cytosolic Nrf2 and targets it for degradation [180]. This degradation pathway is Keap1 independent.

However derived, free Nrf2 forms a heterodimeric complex with small Maf proteins, which induces phase II enzymes through the antioxidant response element (ARE) [181-183]. Complexes of Nrf2 with jun proteins have also been reported, inducing a similar group of genes [184].

Non-canonical mechanisms of Nrf2 signaling involve defects in autophagy, where p62 proteins aggregate to Keap1, inhibiting Nrf2 degradation [185-187]. Nrf2 itself can induce the production of p62 proteins, effectively creating an anti-oxidative positive feedback loop [188].

Nrf2 has been described to undergo post-translational modifications. Particularly important for this project, Nrf2 is phosphorylated by casein kinase 2 in the Neh4/5 domain, which amplifies Nrf2 activation by tBHQ [189]. While this phosphorylation of Nrf2 is important for its activation, high amounts of sequential phosphorylation lead will accelerate the degradation of Nrf2 [190]. Another study has shown that phosphorylation in a different region of Nrf2 by PKC will lead to

diminished Keap1-Nrf2 interaction, enhancing Nrf2 signaling [191, 192]. Nrf2 can also be subject to an activating phosphorylation by protein kinase RNA-like endoplasmic reticulum kinase (PERK), which was observed under conditions of endoplasmatic reticulum stress [193, 194]. Nrf2 is also to some extent regulated via its acetylation status, as Nrf2 deacetylation by SIRT1 activators stunted Nrf2 target gene expression [195]. One pathway of acetylation involves the Neh4 and Neh5 domains, which bind to cAMP response element protein (CREB), which then in turn acetylates Nrf2 [196]. While this leads to increased expression of ARE-regulated genes, the expression of heme oxygenase 1 seems to be exempt from this activation [195, 197].

Mechanisms that interrupt Nrf2 signaling

The Neh3 domain of Nrf2 is important for the induction of transcription by Nrf2. When small deletions at the C terminal were introduced, the Nrf2 variant could still bind the antioxidant response element and maf proteins, but no actual transcription of Nrf2 target genes occurred. Curiously, this deletion also increased the half-life of Nrf2 [198]. Apart from these mechanisms, a number of other proteins are involved in the regulation of Nrf2. Insulin has been shown to decrease the expression of Nrf2 in renal cells by using a specific DNA-responsive element binding heterogeneous ribonucleoprotein particle F/K [199]. WDR23 is an E3 ligase that can ubiquitinate Nrf2 completely independent of Keap1 and does not depend on the DLG or ETGE motifs in Nrf2 [200]. Under hypoxic conditions, such as a perfusion reperfusion injury, the hypoxia-induced protein siah2 abrogated Nrf2 signaling in a Keap1 independent fashion [201]. PAQ3 regulates Nrf2 signaling by tethering both Nrf2 and Keap1 to the Golgi-apparatus, facilitating the degradation of Nrf2 [202]. Hrd1, a protein involved in ER stress during liver cirrhosis, has been

shown to negatively regulate Nrf2. Hrd1 itself is an E3 ubiquitin ligase, capable of enhancing Nrf2 ubiquitylation and degradation [203]. There is also evidence for a process that degrades intranuclear Nrf2 instead of cytosolic Nrf2. Small ubiquitin-like modifiers (SUMO) 1 and 2 target nuclear Nrf2 for a process called SUMOlyation [204], which then leads to polyubiquitination and degradation of Nrf2 in nuclear structures called promyelocytic leukemia nuclear bodies. This process seems to be particularly important in the pathology of viral and cancerous diseases [205].

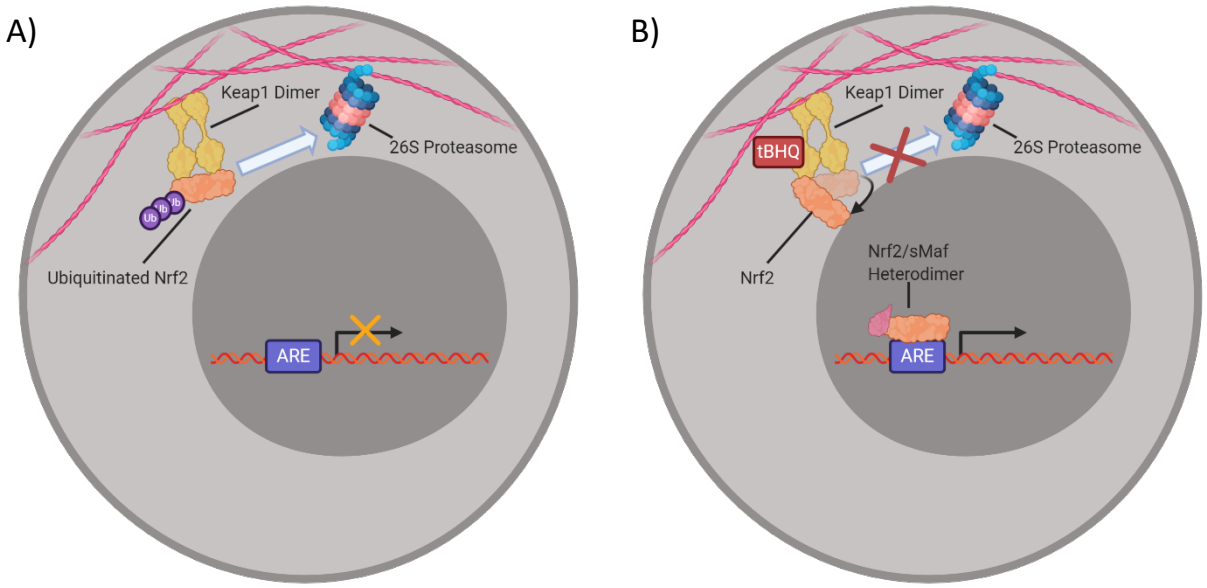


Figure 4. Simplified scheme of Nrf2 activation by tBHQ. A. In homeostasis, Nrf2 is tethered to a Keap1 and promptly ubiquitinated and directed to the 26S proteasome. B. Under conditions of oxidative stress, the binding of Nrf2 to Keap1 is altered and it is no longer directed to the proteasome. De novo synthesized Nrf2 can translocate to the nucleus, dimerize with small maf proteins, and activate Nrf2 target gene transcription. Figure taken with permission from Freeborn et al [206].

Auxiliary Nrf2 activating proteins

Nrf2 binding to the ARE was enhanced by the protein poly(ADP-ribose)polymerase-1 (PARP1), surprisingly without physically touching or poly(ADP-ribosyl)ating, but instead by forming a complex with small maf proteins and the ARE, which results in increased Nrf2 target gene expression [207]. Another unexpected facilitator of Nrf2 was RAC3/AIB1/SRC3, an oncogene that

is commonly involved in human breast cancers. Overexpression of RAC3 in HeLa cells induced Nrf2 and its target gene HMOX1 [208].

Xeno- and endobiotics as Nrf2 activators

Nrf2 activation by xenobiotic compounds is plentiful and has received broad public interest. Nrf2 activation by edible supplements has been deemed as a "hack for health" by prominent podcasters like Joe Rogan [209] and Dr. Andrew Huberman [210]. These sensationalist claims usually refer to isothiocyanate sulforaphane, an Nrf2 activating compound found in *Brassicaceae spec* and some other plants. Sulforaphane has received great academic and public interest, which has resulted in over 3000 peer-reviewed publications and 50 clinical trials over the years [211]. However, many of these studies are problematic, as animal studies have used dosages magnitudes higher than human studies, among other problems. From a toxicological perspective, Nrf2 activators of interest can be grouped into endogenous compounds such as prostaglandins, reactive electrophiles like tBHQ [212], dimethyl fumarate [213], and heavy metals, such as cadmium[214], lead [215] and arsenic [216]. These compounds can also be grouped via their mechanism of Nrf2 activation – either by inducing oxidative stress, phosphorylation of Nrf2, or sulfhydryl modifications of Keap1 [217, 218]. Multiple synthetic Nrf2 activators (which often act as Keap-1 inhibitors), such as CDDO-me, omovalexolone and oltipraz are currently in clinical trials [219].

tBHQ, an Nrf2 activator of particular interest in this thesis, induces mitochondrial stress via thioredoxin-2 (Trx2) in HeLa cells [220]. tBHQ also interacts with cysteine residues on Keap1 at C151, similarly to sulforaphane, diethyl maleate, and arsenic [221, 222]. tBHQ and other

hydroquinones need to be oxidated by divalent copper ions to 2-tert-butyl-1,4-benzoquinone (tBQ) before they can modulate C151 on Keap1 [223]. Arsenic, another model toxicant in this thesis, can generate H2O2 in cells [224], which triggers Nrf2 to induce expression of target genes, such as NAD(P)H-quinone oxidoreductase 1 [225]. Like tBHQ, arsenic also modulates C151 on Keap1, and other reactive cysteine sensors, namely C273 and C288 [222]. However, arsenic appears to activate Nrf2 through multiple mechanisms, including release of p62 through autophagy, promoting Nrf2 dimerization, dissociation of Nrf2 from Keap1 and polyubiqutination/degradation of Keap1 [225]. It is also important to note that arsenic has a multitude of Nrf2 independent effects on mammalian cells, including epigenetic changes [226], metabolic shift towards glycolysis [227] and disruption of zinc finger mediated DNA repair [228]. Endogenous activators of Nrf2, like 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ2), activate Nrf2 by altering the sulfhydryl group on C288 on Keap1 [229]. Zinc Ions, released by intracellular damage, are also sensed by Keap1, leading to a halt of Nrf2 degradation and subsequent activation of Nrf2 signaling as described above [230].

Tert-butylhydroquinone

tBHQ (IUPAC: tert-butyl-1,4-benzenediol) (Fig 7) is a synthetic aromatic phenol derivative. It is mainly used as an antioxidant type food preservative in vegetable oils and goods containing fats, such as meats, fish, and baked goods [231, 232]. In addition to foods, it is added to industrial organic peroxides, varnishes, lacquers, and biodiesel [233]. The World Health Organization's and United Nations' Food and Agriculture Organization's Joint Expert Committee on Food Additives (JECFA) established a daily allowable intake (DAI) of 0.7mg/kg/day for humans [234]. Currently, tBHQ is limited by the FDA to be less than 0.02% of fat or oil (200mg/kg)[235]. The European Food Safety Authority sets the same regulation and designates tBHQ as food additive E219 [236]. The toxicological studies cited by the opinion of the EFSA rely on unpublished data by BD Astille from 1968, which was quoted by the WHO JECFA in their meetings [237]. The EFSA justifies their DAI with a non-peer reviewed feeding study in dogs [238], which showed a NOAEL at 72/mg/kg/day and the LOAEL at 220mg/kg/day, to which they applied a 100-fold safety factor. Model diets assume that in western diets, 33-55% of all calories are derived from fat, which translates to 86-194g of fat per day [236]. Adults consuming such a diet, assuming that tBHQ is present in consumed fats at 200mg/kg would therefore consume up to 38.8mg of tBHQ, or about 0.6mg/kg/day. The JEFCA suggests that in many western countries, such as New Zealand, Australia, and the United States, consumers may exceed the ADI of 0.7mg/kg/day [239]. These regulatory decisions are mostly based on studies performed before western authorities formulated guidelines on immunotoxicological testing [240]. In a proof of concept for a high throughput immunotoxicity screening, tBHQ was identified as a substance of concern [241]. It is

important to note that in the *in vivo* studies later described in this dissertation, the mice were exposed to 0.0014% tBHQ, which is roughly tenfold lower than the ADI for humans. This is also the dose of tBHQ routinely found in commercially available mouse chow.

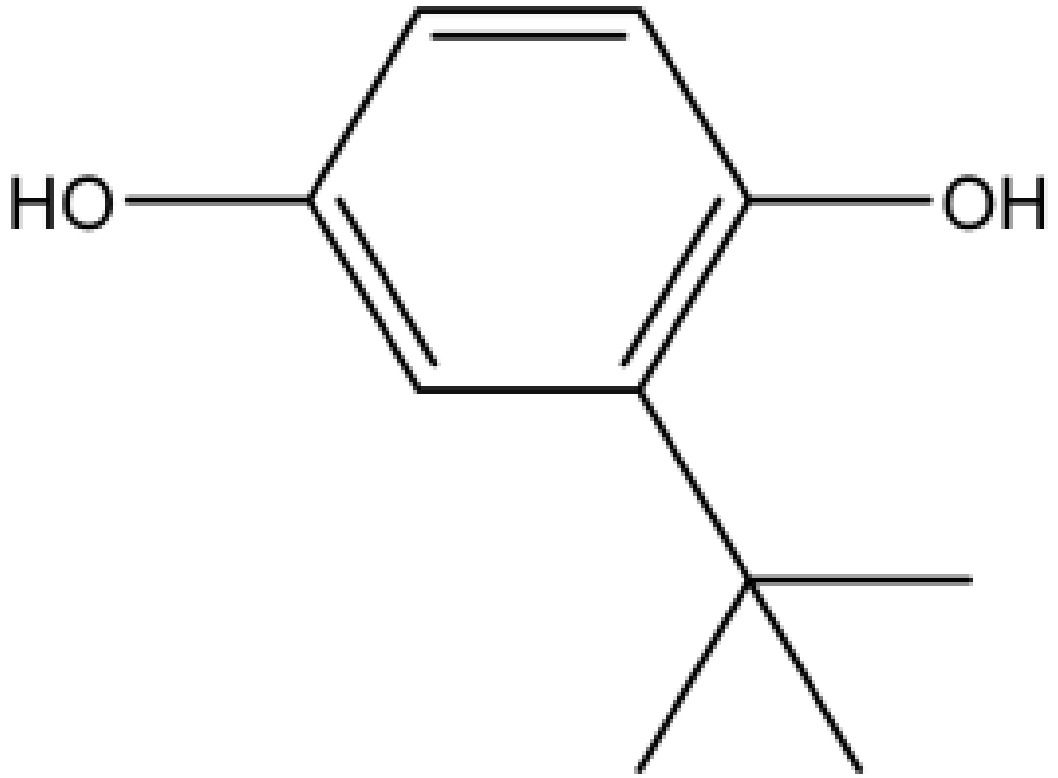


Figure 5. Chemical Structure of tBHQ. tBHQ is an antioxidant with a basic phenolic structure. It is used under the code E319 in the European Union. It is typically used as a food preservative in human and rodent food products.

Nrf2 off-target signaling

The promiscuity of many Nrf2 activators complicates the identification of Nrf2-dependent effects. When designing studies, it is important to consider indirect effects of Nrf2 on other

signaling pathways that may also induce Nrf2 target genes. C-myc negatively regulates Nrf2 signaling by complexing with Nrf2 and c-jun [242]. A cross-activation of Nrf2 and its target gene NAD(P)H-quinone oxidoreductase 1 (NQO1) has been reported after a challenge with TCDD, a ligand for AHR [243]. Mice genetically ablated for Nrf2 showed alterations in their NF-κB pathways [244]. There can be significant overlap between Nrf2, AP-1, and NF-κB signaling. For example, depending on the mode of activation [245], Nrf2, AP-1 and NF-κB can each independently induce expression of the Nrf2 target gene HMOX1 [246]. There is also evidence to suggest that Nrf2 and the Blimp-1/Bach2 axis negatively regulate each other [247]. While Nrf2 is generally considered a transcriptional activator, Bach2 is considered a transcriptional repressor. Because Bach1 and Nrf2 modulate gene expression through the same cis-elements of the ARE, the two transcription factors antagonize the activity of one another [248]. A similar mutual repression pattern has been observed between Nrf2 and NF-κB [249]. FOSL/FRA1 is a protein of the JUN family and part of the AP-1 signaling pathway that exerts some control over Nrf2 signaling. Fra1 ablation leads to abrogated mitochondrially induced apoptosis in response to oxidative stress and increased Nrf2 target gene products, extended Nrf2 half-life, and intranuclear Nrf2 accumulation [250]

Human Nrf2 polymorphisms

As an area of emerging research, not much is known about Nrf2 polymorphisms in humans. The central importance of Nrf2 in oxidative homeostasis suggests the potential for polymorphic impacts on autoimmunity, cancer, and allergy. A study comparing single nucleotide polymorphisms (SNP) of Nrf2 in juvenile systemic lupus erythematosus (SLE) found that the G653A SNP did not influence the incidence of SLE itself, but the incidence of lupus-associated nephritic syndrome [251]. A study looking at patients with lung cancer found a decreased forced exhalatory volume in 1 second (FEV1) for the intron SNP rs2364723 [252]. Similar findings with reduced FEV1 were found in current smokers in a Japanese population [253]. The SNP C617A correlated with a major increase in acute lung injury after trauma in an ethnically diverse US study [254]. The A653A SNP was correlated with elevated systolic/diastolic blood pressure, elevated albumin, and higher mortality in female hemodialysis patients [255].

A series of case studies by the University Hospital in Göttingen, Germany described 4 patients with extremely rare Nrf2 isoforms that were constitutively active. Three of these patients had SNPs involving the ETGE motif of Nrf2 (the “hinge”), namely G81S, T80K, and E79K. The remaining patient had SNP involving the DLG motif (the “latch”), G31R. All patients were pediatric and suffered from hypogammaglobulinemia, recurrent infections, failure to thrive, and developmental delay. After diagnosing patient one, the other three were detected around the globe using GeneMatcher [256]. This incredibly rare genetic aberration highlights the wholistic importance of Nrf2-mediated homeostasis and that uncontrolled Nrf2 signaling has devastating effects on human health.

Nrf2 in Allergy and Autoimmunity

Our group has published two reviews on the role of Nrf2 in autoimmunity [206] and allergy [257] recently. The primary evidence for a role of Nrf2 in the regulation of the immune system was found in Nrf2-deficient mice, which developed an SLE-like syndrome in female mice, including hallmarks like generation of anti-dsDNA antibodies and nephritic syndrome [258, 259]. This observation in mice dovetails with later observation in human cohorts that Nrf2 SNP increases the risk for lupus exacerbations[251]. EAE, a model for human MS, is exacerbated in the absence of Nrf2 [260, 261]. Currently, there are three Nrf2 activators available as FDA-approved treatments for MS, dimethyl fumarate (Tecfidera) [213], monomethyl fumarate (Bafiertam) [262], and diroximel fumarate (Vumerity) [263]. Other Nrf2 activators, such as sulforaphane, A-1396076, 3H-1,2-dithiole 3-thione, and dimethyl itaconate can improve outcomes in a host of different rodent models of autoimmunity [264-267].

Mouse studies with ablation of Nrf2 typically used Nrf2 -/- mice, which had a genetic insertion of a lacZ gene into exon 4 and 5 of the *Nfe2l2* gene. This disrupted expression of wildtype Nrf2, and the newly generated fusion gene is not expressed at the protein level in any tissue [165].

In the context of atopic disease and asthma, whole body ablation of Nrf2 in mice increases airway inflammation and hypersensitivity to OVA, with IL-4 and IL-13 increases in BALF [268]. Nrf2 protected against these pathological changes [269]. Human studies have shown that lower expression profiles of Nrf2 correlate with asthma severity [270]. Sulforaphane, an Nrf2 activator, paradoxically relieved asthma-related bronchoconstriction while decreasing Nrf2 target gene expression in a human study for 60% of patients in the cohort, while 20% of patients experienced

clinical exacerbation [271]. In a rodent model of response to the chemical warfare and terrorism agent chlorine gas (Cl_2), Nrf2 was shown to be protective of airway irritation, and the use of GSH inhibitors and Nrf2 activators elegantly showed a direct Nrf2 effect that was independent of Nrf2-mediated induction of glutathione synthesis genes [272].

The mainstay of therapy in all airway hypersensitivity pathologies are inhaled glucocorticoids [58], which can lose efficaciousness over prolonged usage. In a mouse asthma model, response to inhaled steroids was restored via Nrf2 activation [273], likely due to the signaling of Nrf2 target gene aldehyde oxidase, which directs the formation of tight junctions and adherent junction in the epithelium of airways [274]. Human epidemiological studies uncovered a link between isoforms of the gene encoding for Nrf2, N-acetyltransferase 2 (NAT2), and glutathione S-transferase Pi-1 (GSTP1) and the use of acetaminophen in the pathogenesis of asthma [275]. This study leaves the question of whether the Nrf2 SNPs impacted are rooted in the metabolism of acetaminophen and reactive metabolites, immune cell function, or something entirely different. Nrf2 has been investigated as a therapeutic target for airway disease [276] and mouse studies have shown some success in suppressing asthma models by decreasing type two innate lymphoid cells (ILCs) [277].

The pathogenesis of atopic dermatitis (AD), a skin condition with abnormal epidermal barrier function and immune cell infiltration [58], is dependent on Nrf2 in a rodent model of AD using 2,4,6-trinitro-1-chlorobenzene as an AD inducer [278]. This study showed that genetically Nrf2 deficient mice have subdued expression of type II cytokines, decreased IgE, and lower dermal infiltration of immune cells [278]. There have been a lot of tentative studies suggesting a

protective role of Nrf2 activation in atopic dermatological models, however, those studies were not sufficient to establish a clear causal role of Nrf2 [279-283].

The role of Nrf2 in food allergy has received very little exploration. Our laboratory has shown that tBHQ in mouse chow exacerbated anaphylactic reactions to OVA in a dermal sensitization model [284, 285]. This effect was abrogated when mice received Nrf2 deficient T cells in an adoptive transfer model, indicating a key role of Nrf2 in the pathogenesis of food allergy. The studies in this dissertation are further characterizing this role, focusing on the contribution of B cells.

The role of Nrf2 in different immune cells

Stem cells

Hematopoietic stem cells (HSC) are to some extent regulated by Nrf2. In Nrf2-deficient mice, the ratios of different progenitor cell subtypes are altered and Nrf2 slowed the proliferation of cells in the T cell lineage, skewing the ratio of generated white blood cells towards myelocytes [286]. Nrf2 mRNA was highly expressed in cells developing into granulocytes but was expressed in much lower levels in cells of the B cell lineage. In addition, genetic activation of Nrf2 led to a decreased ratio of lymphocytes to granulocytes [287].

Natural Killer cells

Nrf2 activation via tBHQ diminishes the activation and expression of cytotoxic proteins on NK cells. In a murine *ex vivo* model [288] and an *in vivo* model using IAV as a challenge, Nrf2 activation inhibited FasL and CD107a expression [289]. Nrf2 activation induces the production of IL-27 by myeloid cells, which recruits and activates NK cells in an influenza challenge model [290]. In a mouse model of cancer, topical administration of tBHQ reduced tumor growth in lymphocyte-deficient mice and increased NK cell infiltration into the tumor. This study also showed that Nrf2 induced an IL17D response that was required for this infiltration [291]. The regulation of IL17D by Nrf2 has been suggested as a potential therapeutic target for cancer therapy [292].

The Nrf2-Keap1 signaling pathway for metabolic control in natural killer T cells (NKT), which have a constitutively high metabolism generating large amounts of ROS. Interruption of Nrf2-Keap 1 complexes and therefore untethered Nrf2 signaling delayed NKT development and decreased

NKT numbers. They observed both an increase in NKT cell proliferation and NKT cell apoptosis, and the effect of apoptosis on the NKT cell numbers dominated. A concurrent ablation of Nrf2 rescued the NKT phenotype [293].

Dendritic cells

Dendritic cells (DC) are professional antigen-presenting cells (APC) and thus have a major role in the pathogenesis of allergy. Nrf2-deficient DCs had higher amounts of ROS than wildtype DC when exposed to the common allergen ragweed [294]. Nrf2-deficient DCs show increased expression of MHC-II and CD86 and adapt a Th2-like phenotype when challenged with ambient particulate matter [295]. Similarly, the loss of Nrf2 in bone marrow-derived DCs led to redox dysregulation resulting in higher intracellular ROS and lower GSH expression and higher activation potential for cytotoxic T cells [296]. Additionally, Nrf2-deficient DCs proliferated more, yielding higher overall cell numbers, and had a stronger IFN-γ - CD8 T cell response in a cancer model [297]. When TLR agonists such as LPS were used, Nrf2-deficient DCs produced greater amounts of IFN- γ than the wild-type controls [298]. The direct challenge of dendritic cells with crude preparations of *Helicobacter pylori* outer membrane vesicles activates Nrf2 and induces the Nrf2 target gene HMOX [299]. In a rodent *in vivo* study where mice were challenged with non-typeable *Haemophilus influenza*, absence of Nrf2 in DCs resulted in greater induction of activation markers and a generally stronger humoral response [300]. Arsenic, one of the challenge models used in this thesis, can suppress human DC secretion of IL-12 in an Nrf2-dependent manner [301].

Macrophages

Macrophages are a subtype of APC closely related to DCs. In addition to their APC function, Macrophages are phagocytic and therefore have a high basal amount of intracellular ROS. Nrf2-deficient macrophages are more susceptible to oxidative damage [172, 302]. Clearance of bacteria by phagocytosis is directly dependent on Nrf2 signaling [303, 304]. Macrophages are highly sensitive to TLR agonists such as bacterial polysaccharides, which typically results in induction of proinflammatory genes[305]. Nrf2 counteracts the effects of TLR agonists on macrophages by inhibiting the transcription of IL-6 and Il-1b [306]. Curiously, Nrf2 signaling in macrophages is protective in atherosclerosis and facilitates the polarization of macrophages to a phenotype (MOX macrophages) suited to the containment of atherosclerotic lesions [307-309]. Nrf2 has been suggested to interfere with macrophage polarization, favoring the M2 phenotype, however, the abundance of signaling pathways involved, including Nrf2, NF-κB, PPARγ, and autophagy, make this determination difficult to prove [310].

T cells

T cells have been the primary focus of our lab over the last decade. Seminal was the discovery that the activation of Nrf2 polarized CD4 T cells *ex vivo* to a Th2 phenotype via GATA3 while Nrf2-deficient cells polarized towards a Th1 phenotype [212]. In contrast to this observation, Nrf2-knockout mice had greater number of Th2 cells than wild-type mice in a model of bleomycin-induced lung fibrosis [311]. Nrf2 activation in T cells protected from acute kidney injury in mice by inhibiting IFN-γ and TNF-α secretion by CD4 T cells with a concomitant increase in regulatory T helper cells [312, 313]. Our group showed impaired activation of Jurkat T cells (a human T cell

line) in the presence of the Nrf2 activators tBHQ and CDDO-Im, though some of the effects were at least partially Nrf2-independent [314, 315]. Our lab also showed impaired activation of primary human CD4+ T cells in the presence of tBHQ *ex vivo*, though the role of Nrf2 was not explored in this study [316]. A study in human T cells that used CRISPR/CAS9 to knock down Keap1 causing highly activated Nrf2 signaling, found that the proportion of CD4 T cells expressing activation markers and T cells expressing IL-10 was increased while the proportion of CD8 and IL-17-expressing cells was decreased [317]. In stark contrast, *in vivo* genetic activation of Nrf2 specifically in FOXP3+ T cells reduced the population of this T cell subtype [318]. This model showed increased inflammatory T cell responses in the lung and liver at baseline, resembling autoimmune processes. This finding highlights the highly divergent outcomes of increased Nrf2 signaling depending on cell type, compartment, species, and model and the difficulties in accurately measuring, characterizing, and reporting the effects of Nrf2. One of the current challenges investigators in the field of Nrf2 in immune populations are facing is the reconciliation of these complex and sometimes seemingly contradictory observations.

B cells

B cells are understudied in general, and specifically in the context of Nrf2. Studies on a lymphoblast-derived B cell line have suggested that Bisphenol A is cytotoxic to B cells and sensitivity depends on the level of Nrf2 expressed [319]. Another study suggested that Nrf2 deficiency increases the immunoglobulin response to the intracellular bacteria *Haemophilus influenza* [300]. Sulforaphane, an Nrf2 activator, inhibits B cell function in a mouse model of arthritis, decreasing IL-6, and IL-17, leading to less collagen-specific IgG1 and IgG2a and inhibiting

the activation of murine splenocytes after LPS challenge [320]. Nrf2 overexpression in vitro increased CXCR4 expression and increased the survival of a B cell line under hypoxic conditions [321]. In humans, a cohort study of people suffering from SLE and age-matched healthy controls showed that in SLE patients, cytosolic ROS were decreased and mitochondrial ROS were increased in B cells, and expression levels of Nrf2 and Keap1 were significantly increased in B cells of SLE patients [321].

Our lab has shown recently that B cells, when challenged with TLR agonist LPS, showed impairment of the activation markers CD69, CD25, CD22, and CD138 when tBHQ was present, independent of whether the cells had intact Nrf2 signaling. In contrast, tBHQ increased IgM secretion in wild-type, but not Nrf2-null, mice, indicating this effect is Nrf2-dependent [162]. This effect on T cell-independent B cell activation underlines the need for studies that investigate T cell-dependent B cell activation, which are shown in Chapters 3 and 4 of this thesis. I hypothesize that in addition to these T cell-independent effects, there are effects on the T cell-dependent activation of B cells. In chapter 2, I will measure the impact of arsenic trioxide on human peripheral blood B cells after challenge with influenza A. In chapter 3, I will show the effect on B cell activation in a murine model of T-cell dependent B cell activation, and additionally I will be able to determine whether these effects are Nrf2 dependent. In chapter 4 and 5 I will show the impact of dietary tBHQ on the development of allergies against chicken ovalbumin in a transdermal sensitization model. Collectively, these studies will elucidate the impact of the Nrf2 activators tBHQ and ATO on B cell activation and some elements of B cell function.

Chapter 2: Arsenic trioxide inhibits the response of primary human B cells to influenza virus A in vitro

Abstract

Arsenic compounds are common environmental toxicants worldwide and particularly enriched in the Northeast and the Southwestern United States, the Alps, and Bangladesh. Exposure to arsenic is linked with various detrimental health outcomes, including cancer, cognitive decline, and kidney damage. Our group has previously shown that arsenic trioxides alter T cell cytokine production. In this study, we demonstrate that exposure to arsenic compounds alters B cell function in an *in vitro* influenza model. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood and cultured with arsenic trioxide (As_3O_2, ATO) and subsequently challenged with Influenza A virus. B cells showed a decreased expression level of CD267, surface IgG and CD80 when treated with arsenic trioxide. Taken together, the data suggest that arsenic trioxide affects the activation and surface antibody expression of human peripheral B cells. Overall, this suggests that arsenic trioxide exposure could cause impaired humoral immunity.

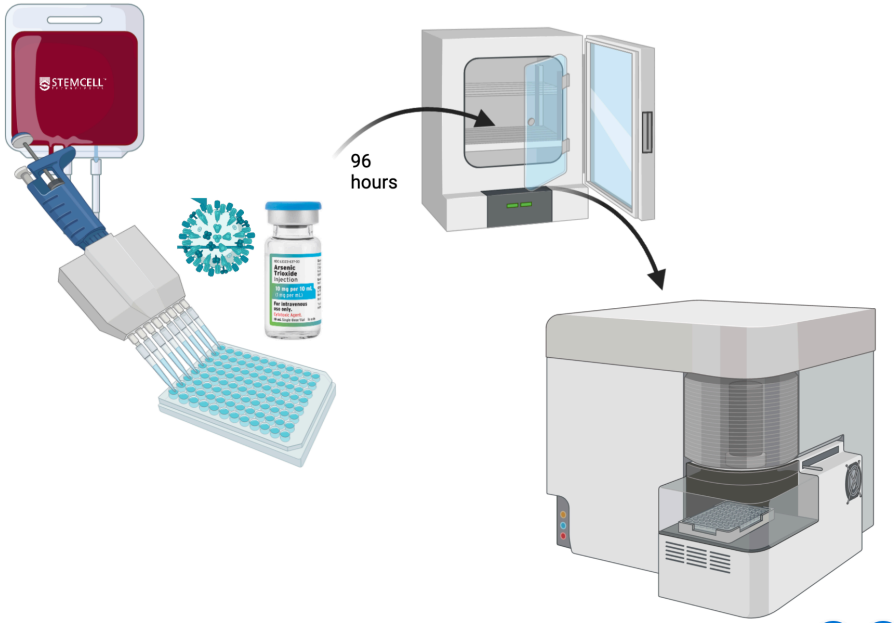


Figure 6. Schematic of the experimental setup. Human PBMCs were isolated and treated with either VEH (0.04% PBS) or 0.5µM or 1µM of As_2O_3. 30 min later, PBMCs were challenged with 0.5 HAU of influenza virus A. Cells were collected at 96 h, washed, and labeled for FACS analysis.

Introduction

Arsenic is a naturally occurring contaminant in aquiferous water reservoirs, on which a significant minority of US households rely [322]. In the United States, the Southwest, Northeast, and upper Midwest are prone to high levels of arsenic in well water. The EPA recommends arsenic mitigation or alternative water sources if the arsenic concentration in drinking water exceeds 10mg/l [322]. Globally, Bangladesh and the Austrian Styr have chronically elevated arsenic contamination in water [323, 324]. Most arsenic compounds found in water are inorganic AS(III) or AS (V) species [325], which are the most acutely toxic variants of arsenic [326]. Acute arsenic toxicity, rarely seen due to environmental exposure, usually manifests as fulminant multi-organ failure [327]. The gastrointestinal system is typically affected first within hours of oral or intravenous administration of high amounts of inorganic arsenic, followed by the liver, lungs, and kidneys. Death often ensues within hours to days following exposure [328]. Chronic exposure to arsenic is much more common and is associated with an increased lifetime risk for multiple cancers, cardiovascular disease, intellectual decline, dermatological, endocrinological, and neurological impairment, and miscarriages [329]. Our group has previously shown that arsenic trioxide impairs the release of cytokines from T cells [330]. These findings raised the question of whether exposure to arsenic changes the response of immune cells to pathogens, such as viruses and bacteria. In this study, we are using an *in vitro* model of influenza A exposure to measure the response of B lymphocytes exposed to arsenic trioxide. We have previously shown that T cells respond differently and produce fewer type 1 cytokines in the same model [331].

Viral infections are subject both to the cellular and humoral response of the immune system. The cellular response is driven by T cells, while the humoral response relies on antibodies produced by B cells. Both pathways are intimately intertwined. On the one hand, B cells require T cells for isotype switching and antibody production but conversely can serve as antigen-presenting cells to activate T cells.

Successful clearing of viral infections often requires the generation of antigen-specific immunoglobulins. B cells produce the low-affinity pentamer immunoglobulin M (IgM) first, before undergoing somatic hypermutation, affinity maturation, and isotype switching [332]. In an antiviral scenario, the result is a high-affinity immunoglobulin G (IgG) which binds with great specificity and affinity to viral antigens [332, 333]. B cells also undergo clonal expansion during this time, an energy-intensive process resulting in a marked increase in the number of active, antigen-specific B cells [334].

Arsenic trioxide is also used therapeutically to treat cancer, Specifically, it is used together with all-trans retinoic acid as the first-line therapy for promyelocytic leukemia [335]. New oral formulations are currently in trial to replace the inconvenient intravenous administration [335]. However, most human exposure to arsenic trioxide occurs in the context of naturally occurring or inadvertent environmental contamination of drinking water.

In this study, we report a direct effect of arsenic trioxide on B cell activation, using our unique *in vitro* influenza infection model by which we can monitor the response of primary human immune cells (Fig. 6).

Methods

Materials

Arsenic trioxide was purchased from Sigma Aldrich (St. Louis, MO). All fluorescent antibodies used in this study were purchased from Biolegend, (San Diego, CA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

Human Peripheral Mononuclear Cell Preparation

PBMCs were isolated from Leukapheresis packs (Gulf Coast Blood Center, Houston, TX) using Lymphoprep gradient separation media (Stem Cell Technologies, BC). Cells were cultured in complete RPMI (1640 RPMI, 10% Fetal Bovine Serum (Biowest, MO), 25mM HEPES, 1mM Sodium Pyruvate, 1x nonessential amino Acids, 100 U/ml Penicillin, 100 U/ml streptomycin) at 2 x 10^5 cells/well in a 96-well round bottom plate. Cells were either incubated without further treatment (BKG) or treated with vehicle (VEH, 0.004% PBS), 0.5µM, 1µM, or 2µM arsenic trioxide. After 30 min, cells in the VEH and arsenic groups were exposed to 0.5 hemagglutination units (HAU) of influenza A/PR/8/34 (H1N1). The number of hemagglutination units influenza virus achieving optimal PBMC activation was determined in pilot studies and found to be 0.5 HAU. No virus was added to the BKG cells, BKG cells were neither treated with ATO nor IAV, and corresponding amounts of cell culture media were added instead. Cells were cultured for 96 h at 37˚C and 5% CO2 and subsequently harvested for FACS analysis and supernatant collection (Fig 6).

Flow Cytometry

96 h after treatment, supernatants were collected, and cells were prepared for flow cytometry. Cells were washed with phosphate-buffered saline, labeled with Zombie Aqua Fixable Viability

Dye (BioLegend, San Diego, CA), washed in FACS buffer (1% FBS in PBS), labeled with surface marker antibodies (Table 3), washed in FACS buffer, fixed and permeabilized with Foxp3/Transcription factor staining kit (Invitrogen, Waltham, MA), then labeled with antibodies against Immunoglobin M (IgM) and Immunoglobulin G (IgG). After labeling, cells were washed twice in permeabilization buffer, resuspended in FACS buffer, then immediately analyzed on a 4-laser Attune NxT (ThermoFisher, Waltham, MA). UltraComp eBeads (Invitrogen, Waltham, MA) were used to determine laser gains and compensation matrices.

Table 3. Fluorescent antibodies used in Chapter 2.

Target	Label	Clone	Manufacturer
CD22	BV421	S-HCL-1	Biolegend
CD86	PE-Cy7	BU63	Biolegend
CD40	AF-700	5C3	Biolegend
CD25	BV711	M-A251	Biolegend
CD80	PE-Cy5	2D10	Biolegend
CD19	PE	HIB19	Biolegend
IgG	APC	M1310G05	Biolegend
CD267	PE-Dazzle 594	1A1	Biolegend
CD69	BV-605	FN50	Biolegend
IgM	BV-605	RMM-1	Biolegend
CD122	PerCP Cy5.5	TU27	Biolegend
B220	FITC	RA36B2	Biolegend
Live/Dead	Fixable Aqua	/	Thermo Fisher

Statistical Analysis

The data were compiled from three different donors and are presented as the mean ± standard error of the mean. One-way ANOVA was used to determine statistical differences between treatment groups, and Dunnett's multiple comparisons test was used to compare each treatment group to VEH. Calculations and graphical visualization of the results were done using PRISM Graphpad 9.20 software (La Jolla, Ca).

Results

Arsenic Trioxide decreases B cell viability and size increase following activation

In flow cytometry, the forward scatter (FSC) parameter provides an estimate of the size of cells being measured. The FCS is a function of the voltage recorded of a photodiode located behind, but slightly transposed to the travel path of the blue laser (408nm wavelength). Larger cells with diffract the blue laser beam more so than smaller cells and thus increase the fraction of light hitting the transposed photodiodes, increasing the measurement of FCS. B cells upon activation will expand in cell size and accordingly, the FSC intensity will increase proportionately. To facilitate interpretation of FCS data, which typically ranges from $1x10^{\wedge 6}$ to $3x10^{\wedge 6}$, we normalized the measured FCS values to that of the vehicle group, thus comparing the all other groups to it. We observed a statistically significant increase in FSC intensity 96 h after exposure to influenza (Fig 7B). At 1µM ATO this effect was diminished. As ATO is known to be cytotoxic, we wanted to control for potential changes in cell viability. Using an amine-reactive dye, we measured the percentage of amine-nonreactive cells (live cells) of total cells after debris and doublet exclusion. We observed a non-statistically relevant decrease, but no significant changes in the number of viable cells with ATO in concentrations up to 1 mM compared to the vehicle group (Fig. 7a). Significant changes were seen at 2µM. The data at 2µM is presented in the following figures to show a concentration response, however the results at 2µM may be partially due to unspecific cytotoxicity.

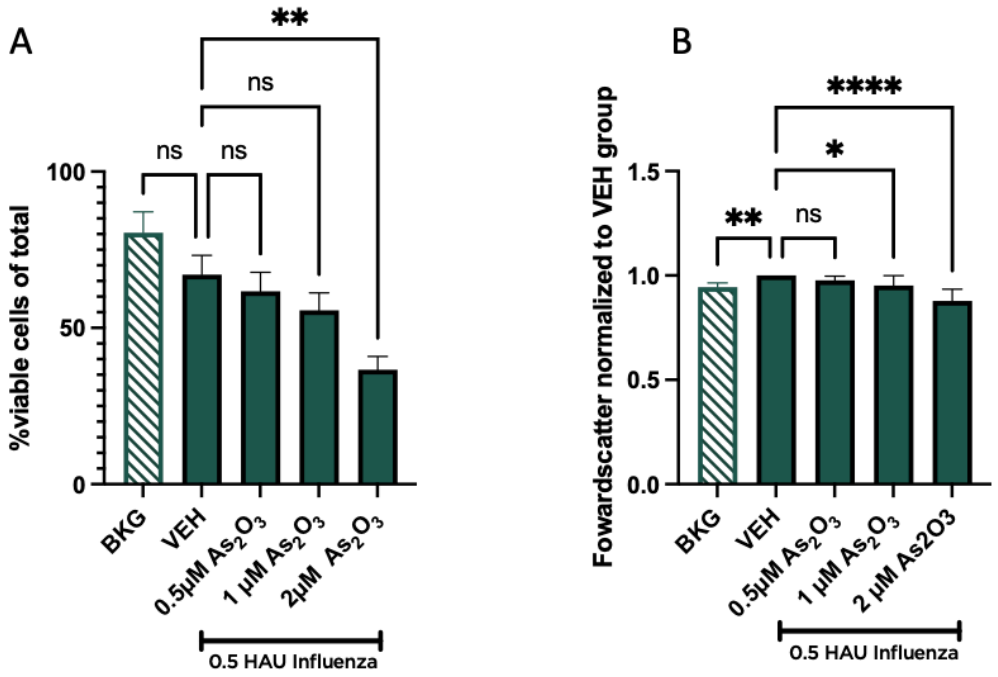


Figure 7. ATO has a minor impact on viability and cell size of human PBMCs. Human PBMCs were isolated and treated with either VEH (0.04% PBS) or 0.5µM, 1µM or 2µM of As_2O_3. 30 min later, PBMCs were challenged with 0.5 HAU of influenza virus A. Cells were collected at 96 h, washed, and labeled for FACS analysis. A: Viability was quantified by use of a fixable, viability dye and detected by flow cytometry. Flow cytometric gates were set on FCS/SSC and doublet exclusion prior to live/dead analysis. B: Cell size was ascertained by measuring forward scatter by flow cytometry. Data were normalized to the VEH group. Data are presented as the mean ± SE of three donors. ** represents $p < 0.01$, *** represents $p < 0.001$? and ns = not significant.

Arsenic trioxide decreases the percentage of IgG+ B cells but does not change the number of B cells

Among the many roles a B cell can play during an infection, the production of high-affinity immunoglobulins is arguably the most important. Mature but resting B cells express IgM and IgD type B cell receptors on their surface. After activation, B cells undergo affinity maturation and isotype switching. Typically, during a viral infection, IgM-producing B cells undergo class switch recombination to produce IgG antibodies. Circulating B cells that express IgG on their surface are antigen-experienced cells that have previously undergone isotype switching.

In this study, to determine B cell phenotype we used CD45R/B220 as a marker for B cells. In Fig 3, we observe no statistically significant change in the percentage of B cells among PBMCs after exposure to either ATO, influenza virus, or both.

Among B cells, the number of cells that expressed IgG on the cell surface was significantly increased upon exposure to influenza. The addition of ATO diminished this effect in a concentration-dependent manner, with statistically significant decreases in the 1μM group (P=0.0069) (Fig 8B).

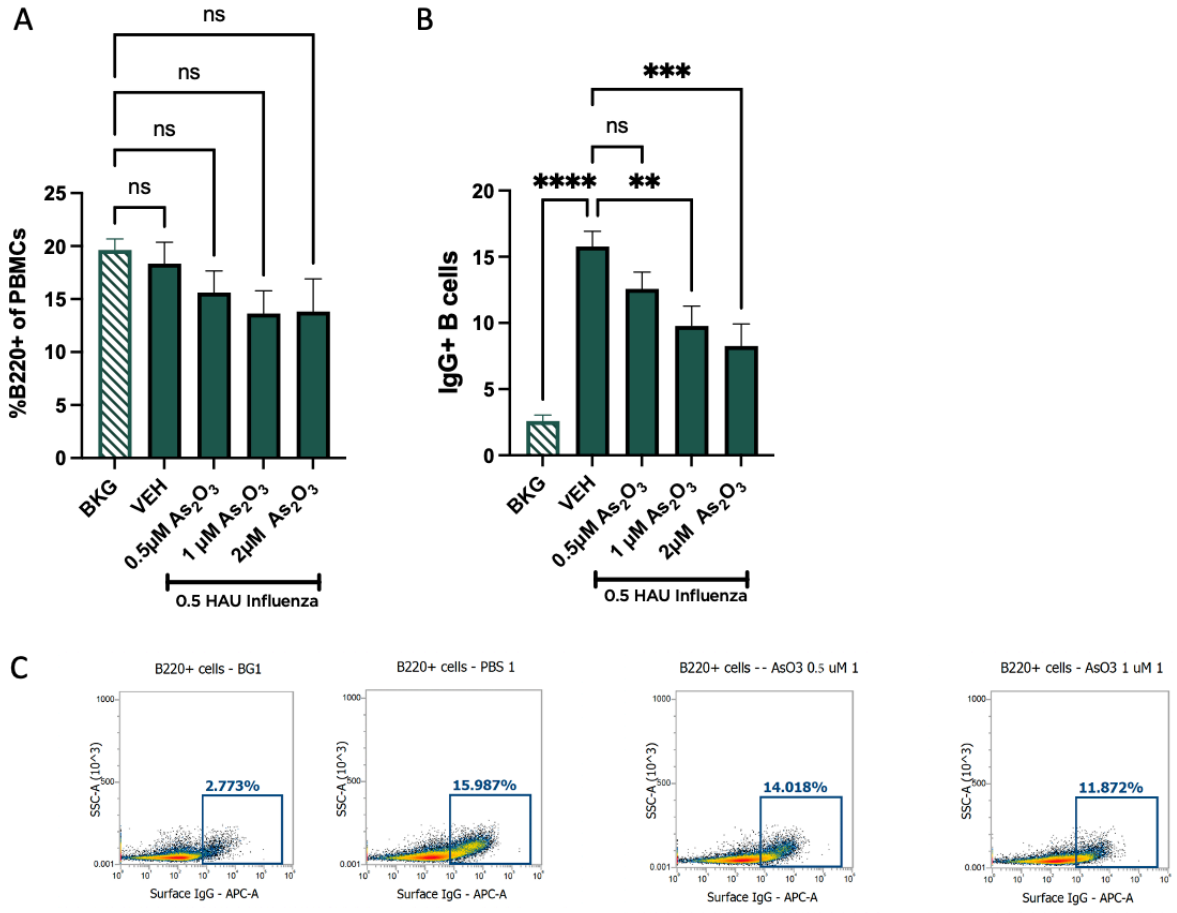


Figure 8. Surface IgG is decreased in the presence of ATO while B cell populations do not significantly change. Human PBMCs were isolated and treated with either VEH (0.04% PBS) or 0.5µM, 1µM or 2µM of As_2O_3. 30 min later, PBMCs were challenged with 0.5 HAU of influenza virus A. Cells were collected at 96 h, washed, and labeled for FACS analysis. Viability dyes for live/dead cell discrimination as well as fluorescently conjugated antibodies for B cell identification and surface IgG, among other targets, were used to identify and quantify protein expression on B cells using an Attune NxT 4 Laser cytometer. A. B cells were

Figure 8 (cont'd)

identified by expression of B-220 and are represented as a percentage of the total PBMC population. B. IgG-expressing B cells (B-220+) were identified and quantified by flow cytometry. Data was normalized to VEH group due to inherent physiological variation in PBMC donors. C. Representative pseudocolor plots for single samples in the BKG, VEH and 1μM As2O3 groups. Data are presented as the mean ± SE of three donors. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

Arsenic trioxide decreases the expression of CD25 and CD80 but does not affect CD86 expression

CD25 is a marker for mature B cells with increased antigen-presenting capacity and a marker of activation. The addition of influenza virus increased the percentage of CD25-expressing B cells in the PBMC population. The addition of ATO caused a concentration-dependent decrease in the relative amounts of CD25+ B cells (Fig. 9). CD80 and CD86 are costimulatory proteins expressed on professional antigen-presenting cells that interact with CD28 and CTLA-4 on T cells. Both molecules are typically expressed simultaneously with overlapping functions; however, CD80 shows a much higher affinity for CD28 [336]. CD86 co-stimulation is implicated in a skew towards a TH2 response in T cells, while CD80 has been associated with TH1 responses [337]. In our assay, PBMCs increased the expression of both CD80 and CD86 in response to challenge with Influenza A virus (Fig 10). We observed a concentration-dependent decrease in CD80 expression in the

presence of arsenic trioxide. CD86 on the other hand did not show a comparable effect. (Fig 10). Collectively, the data suggest differential effects on markers of activation and co-stimulation.

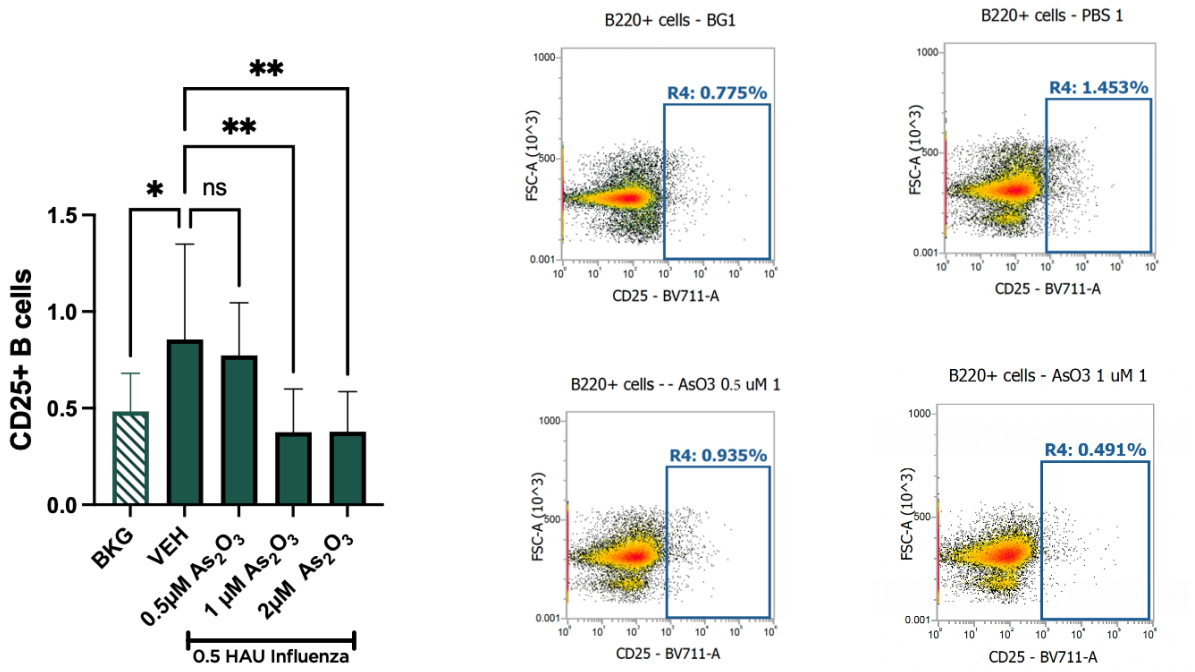


Figure 9. Expression of CD25 decreases with ATO treatment. Human PBMCs were isolated and treated with either VEH (0.04% PBS) or 0.5μM, 1μM or 2μM of As_2O_3. 30 min later, PBMCs were challenged with 0.5 HAU of influenza virus A. Cells were collected at 96 h, washed, and labeled for FACS analysis. Viability dyes for live/dead cell discrimination as well as fluorescently conjugated antibodies for B cell identification and CD25, were used to identify and quantify protein expression on B cells using an Attune NxT 4 Laser cytometer. Data are presented as the mean ± SE of three donors. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates

significance relative to the VEH group, p<0.001, **** indicates significance relative to the VEH group, p<0.0001). The figure depicts the compiled data from 4 donors.

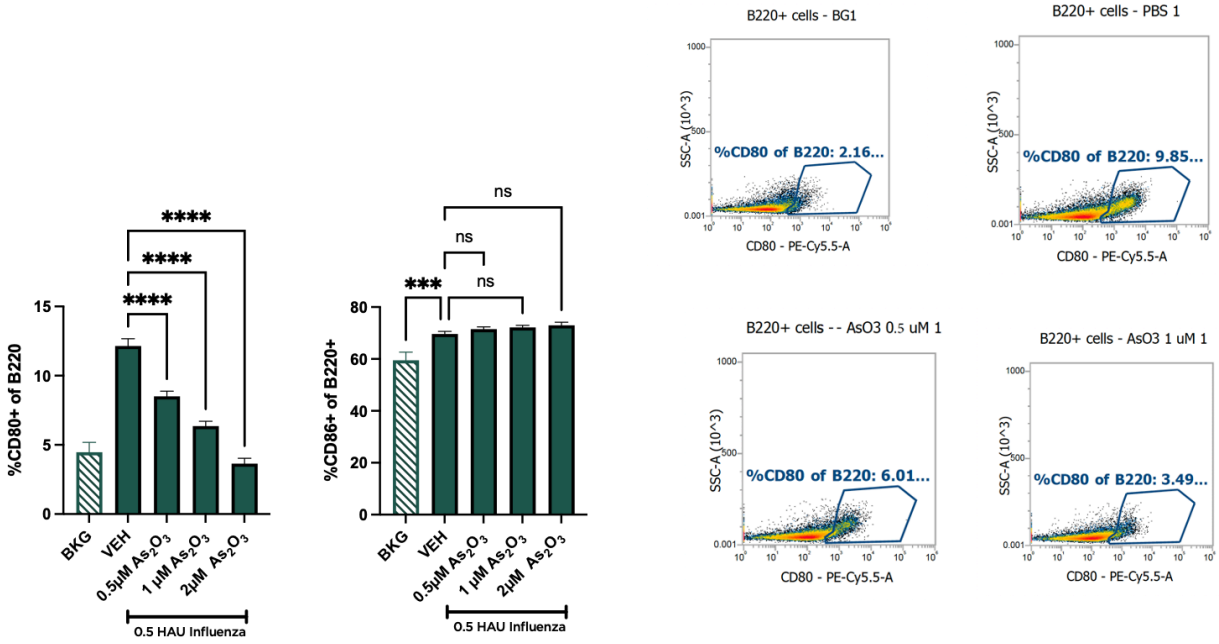


Figure 10. ATO decreases the expression of CD80 but not CD86. Human PBMCs were isolated and treated with either VEH (0.04% PBS) or 0.5µM, 1µM or 2µM of As_2O_3**.** Cells were collected at 96 h, washed, and labeled for FACS analysis. Viability dyes for live/dead cell discrimination as well as fluorescently conjugated antibodies for B cell identification, CD80 and CD86, were used to identify and quantify protein expression on B cells using an Attune NxT 4 Laser cytometer. Data are presented as the mean ± SE of three donors. (ns indicates not significant, * indicates significance relative to the VEH group, p<0.05, ** indicates significance relative to the VEH group, p<0.01, *** indicates significance relative to the VEH group, p<0.001, **** indicates significance relative to the VEH group, p<0.0001). The figure depicts the compiled data from 4 donors.

Arsenic trioxide decreases the expression of CD22 and CD267 on B cells in a concentration-dependent manner.

B cell differentiation and activation are tightly regulated. Overactive B cells and autoantibodies are implicated in most autoimmune diseases, chiefly in SLE (lupus), Mixed Connective Tissue Disease, Sjogren's Disease, and autoimmune hepatic disorders [338]. Synchronizing affinity maturation, clonal expansion, and isotype switching is a delicate balance regulated by many different proteins [332]. We next tested the hypothesis that arsenic trioxide alters the expression of some of these regulatory proteins, such as CD267 and CD22.

CD267, also known as Transmembrane activator and CAML interactor (TACI) is a transmembrane protein associated with T-cell independent B cell activation and negative regulation of B cell proliferation [339]. CD267/TACI was decreased by influenza infection in our model and was further decreased in the ATO-treated groups (Fig. 11). CD22, a member of the immunoglobulin superfamily and has a similar physiological role to CD267 in B cell activation. It works as an autoregulatory protein, preventing hyperactivation of the B cell receptor (BCR). In our in vitro model, we saw an induction of CD22 during the influenza challenge, likely to prevent an overreaction by B cells. This induction was abrogated in a concentration-dependent manner by arsenic trioxide (Fig 11). The results suggest that ATO has a suppressive effect on the expression of regulatory proteins on the cell surface of B cells.

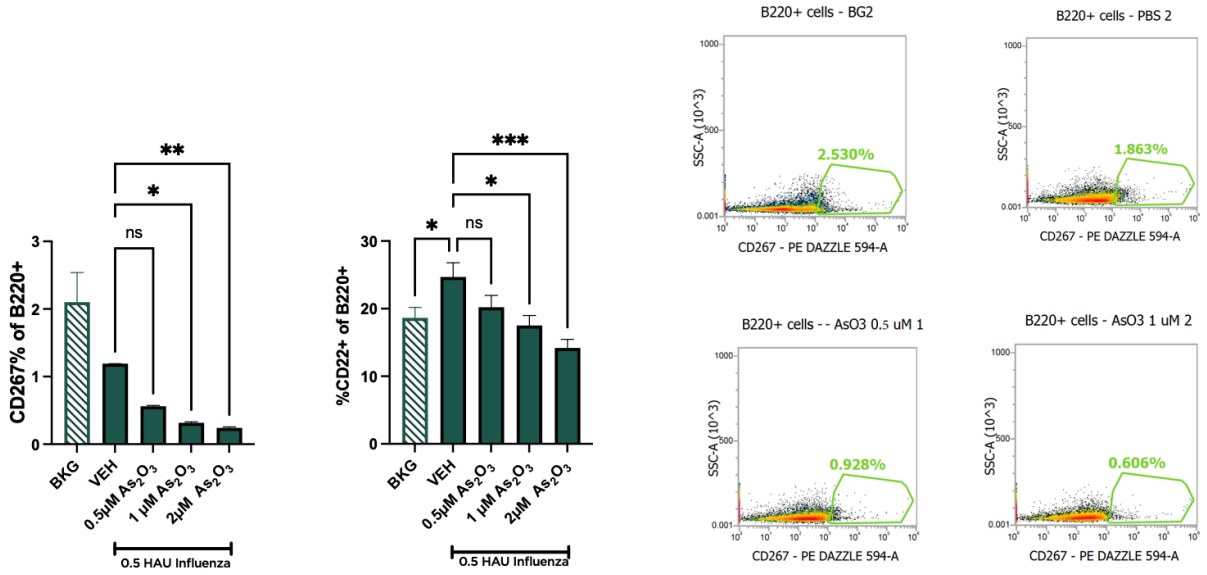


Figure 11. ATO decreases the expression of CD267 and CD22. Human PBMCs were isolated and treated with either VEH (0.04% PBS) or 0.5µM, 1µM or 2µM of As_2O_3. 30 min later, PBMCs were challenged with 0.5 HAU of influenza virus A. Cells were collected at 96 h, washed, and labeled for FACS analysis. Viability dyes for live/dead cell discrimination as well as fluorescently conjugated antibodies for B cell identification, CD267 and CD22, were used to identify and quantify protein expression on B cells using an Attune NxT 4 Laser cytometer. Data are presented as the mean ± SE of three donors. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

Discussion

We used an in vitro infection model to determine whether the presence of arsenic trioxide affected the activation of human B cells in response to influenza in isolated PBMCs. We demonstrated a decrease in activation markers, a decrease in surface IgG+ B cells and a change in the expression pattern of the costimulatory molecules CD80 and CD86. The effects demonstrated here were observed in a concentration-dependent fashion. I acknowledge that arsenic trioxide started to show a cytotoxic effect at 2µM and thus, nonspecific cytotoxic effects may confound results. However, even if the 2µM point were omitted, there is a significant effect in most markers at 1µM. Taken together, the data suggest that arsenic trioxide affects the activation and surface antibody expression of human peripheral B cells.

The activation marker CD25 is expressed in humans after activation. It's expression levels correlate with a larger cell size and indicate readiness to expand clonally, as CD25 the high-affinity subunit of the IL-2 receptor [340] and is also a marker for memory B cells [341]. Though it would be premature to infer from the markers used in this study to say that the memory B cell pool expanded, overall the data suggest the B cell population as a whole showed a higher activation status, which was diminished in the presence of ATO.

CD80 and CD86 are costimulatory molecules that interact with T cells and can send stimulatory signals to T cells. Although CD80 and CD86 are often considered to be immunologically equivalent, they share only 26% sequence homology and have different affinities for their receptors CD28 and CTLA-4. CTLA-4 is considered canonically to be an inhibitory signal while CD28 canonically is a stimulatory signal. It has been hypothesized in the past that CD80 signaling

preferentially polarizes T cells to a Th1 phenotype, while CD86 polarizes towards a Th2 phenotype [342]. This is particularly interesting in the present study, as I would expect immune cells to react to an intracellular pathogen such as IAV with a Th1 polarized, IgG-producing response. Indeed, we observed a marked increase in CD80-expressing cells, and a less pronounced increase in CD86-expressing cells after activation with IAV. Interestingly, the presence of tBHQ decreased the percentage of CD80 expressing B cells, while it did not decrease the percentage of CD86 expressing B cells. The decrease CD80 is consistent with our previously published study, which showed that ATO decreased induction of the Th1 cytokine IFNγ by IAV-activated T cells. Likewise, this is consistent with the other observations in the current study, e.g. the expression of surface IgG, which was suppressed in the presence of ATO.

While there are many strengths to this in vitro infection model, which uses primary human immune cells, there are also some limitations. Influenza A virus, a respiratory pathogen, infects cells of the nasal, oropharyngeal, and bronchial epithelium *in vivo,* which is obviously not accounted for in this in vitro model. Another limitation is that local responses of cells and in the secondary lymphoid organs are not necessarily modeled well using PBMCs. However, this *in-vitro* infection model is scalable and thus suitable for medium throughput assays, which could be useful for both investigation of mechanisms and chemical screening—the data from which could potentially be used for predictive toxicity modeling.

The use of PBMC samples from unknown donors also likely introduces some level of variance into these studies. In particular, the unknown vaccination and previous exposure status could certainly introduce some variability in the results. It is important to note, however, that in PBMCs

from every individual we have tested so far, we have observed a rapid and robust T cell and B cell response to this viral strain, indicating that the PR8 influenza strain consistently produces a memory response from most healthy donors. Overall, this suggests broad community immunity against this particular virus strain (VEH group in Fig 8-11). However, it also suggests that this approach models a secondary response to IAV and a different peptide or virus would be needed to assess a primary response.

Overall, our results suggest that ATO has an inhibitory effect on the B cell response to IAV, which is consistent with other published studies. Other groups have shown that arsenic trioxide reduces T and B cell activation, numbers, and transplant rejection [343, 344]. There have also been reports that arsenic trioxide decreases glutathione (GSH) in T and B cells in vitro [345]. Decreasing GSH would predispose B cells potentially to oxidative stress, which they are prone to during activation and proliferation. A murine inhalation study of arsenic trioxide found no changes in LPS-mediated B cell activation, but major changes in the T cell-dependent humoral response to sheep red blood cell vaccination [346].

A potential confounding factor in these studies may be the presence of other immune cells, which themselves react to influenza virus. We measured the impact of ATO on T cell function [331] and on NK cell function (manuscript in preparation at the time of writing). To eliminate potential confounding factors, future studies may use magnetic or flow cytometric isolation or enrichment of B cells prior to the assay. This strategy could also be used to differentiate between B cell subtypes in peripheral blood, and subtype specific effects could be illuminated. Peripheral blood mononuclear cells contain both antigen-experienced B cells, such as memory B cells, and naïve B

cells. I hypothesize that enriching the assay for antigen-experienced B cells may amplify the effect of IAV challenge. It would be interesting to quantify the effect of ATO on these antigen-experienced B cells, as there may be a differential effect of ATO on more differentiated memory B cells compared to the less differentiated naïve B cells.

Exposure to arsenic trioxide occurs most commonly as an environmental contaminant through drinking water, however, exposure may also occur through other routes. Clinically, ATO is used to treat promyelocytic leukemia. The therapeutic IV dosage for a 70kg human with 5.5L blood volume is 0.15mg/kg, which is equivalent to ~9µM plasma concentration [347]. This is a much higher concentration than used in these studies, as we adjusted for a higher susceptibility of cells *in vitro*. Exposure of humans to arsenic trioxide or other arsenite compounds through occupation (as in pesticides, wood preservatives, iron working) or environmental (as in oral ingestion or inhalation) is much harder to quantify. Arsenite compounds, including arsenic trioxide, As(III), and As(V) are found commonly found in drinking water, residually in foods, and rarely airborne in wildly varying concentrations globally [348]. Studies estimate that humans in western society are exposed to up to 50µg of arsenic species per day through ingestion, most of which is of the less bioactive organic variant. About 10µg are expected to be inorganic, and up to 4µg of these are consumed through drinking water [348-350]. Inorganic arsenites are converted to organic, biomethylated arsenic compounds and excreted through the urine, though some amount of bioaccumulation occurs [348]. It is important to note that the amount of exposure can vary markedly depending on location, dietary choices, and other lifestyle factors. Certain regions of Taiwan, Bangladesh, India, and southern South America have groundwater arsenic

concentrations in excess of 50µg/L [351-354]. In the US, the United States Geological Survey estimates that about 10% of people that rely on well water are exposed to inorganic arsenic levels exceeding 10µg/L, with clustering in the Upper Northeast, Southern Michigan/Northern Ohio, and the Southwest [355]. While exact numbers are impossible to extrapolate from these findings, these data indicate a clear need to characterize the impact of low to moderate arsenic exposure on immunity.

In the present study, we have shown that arsenic trioxide impacts the activation and antibody response of B cells to influenza virus. Perhaps more importantly, however, we have developed a novel in vitro model that can be used to characterize the impact of toxicants on the response of primary human B cells to influenza virus, which prior to the Covid-19 pandemic, was the 8th leading cause of death in the U.S. Overall, this study demonstrates that arsenic trioxide strongly impairs the B cell response to influenza virus, while also establishing the use of a novel, scalable model for immunotoxicity screening.

Chapter 3: Nrf2 dependent and independent effects of T-cell dependent B cell activation ex vivo

Abstract

B cells provide humoral immunity via the production of antibodies. The activation of B cells and generation of high-specificity antibody is tightly regulated via an intricate interaction with T lymphocytes *in vivo*, both in mice and men. This process is crucial for immunity against intra-and extracellular pathogens, long-lasting immunological memory, and vaccination strategies. Conversely, exaggerated antibody responses can be equally impactful and lead to autoimmunity and allergy. Our group has previously shown that the synthetic food additive, tert-butylhydroquinone (tBHQ) alters the response of T cells to both allergic and infectious challenges and increases the production of IgE- antibodies *in vivo*. tBHQ is an activator of nuclear factor erythroid 2-related factor 2 (Nrf2), which is a master regulator of antioxidant and detoxification pathways. To measure a potential Nrf2-dependent effect of tBHQ on B cell activation, we designed an *ex vivo* polyclonal activation assay that mimics T cell signaling via the B cell receptor, CD40, and cytokine signaling. We isolated splenocytes from wild-type and Nrf2-deficient mice, which were then treated with increasing concentrations of tBHQ and then activated. After 48 hours, the cells were harvested and analyzed using high dimensional intracellular spectral flow cytometry. We saw a concentration-dependent increase in the expression of the costimulatory molecule CD80 by tBHQ in activated B cells while decreasing the expression of the closely related costimulatory molecule CD86. tBHQ caused a decrease in the expression of the early activation markers CD69 and CD25 in activated B cells. Interestingly, IgG1 surface expression was reduced in both wildtype and Nrf2-deficient cells, suggesting an Nrf2- independent effect of tBHQ. Collectively, the data suggest disparate effects of the food additive tBHQ on the expression of

cell surface proteins and antibody production by activated splenic B cells, which may play a role in the effects of tBHQ in allergy and host immunity.

Introduction

B cells are the main mediator of humoral immunity, producing a range of antibodies against foreign and autoantigens. In addition to their immunoglobulin (Ig) producing role, B cells are professional antigen-presenting cells (APC), able to phagocytose, trim and present antigens to both CD8 and CD4 T cells [356].

The production of antibodies is tightly regulated. Naïve, inactive B cells will express immunoglobulin D (IgD) receptor transiently when they exit the bone marrow [357]. IgD is mostly replaced by the most prevalent Ig on resting B cells, Immunoglobulin M (IgM). Upon activation of B cells by T follicular helper cells (TFH), the B cell begins to secrete IgM antibodies. Depending on the signals provided by TFH cells, B cells can change their isotype from IgM or IgD to one of the late-phase, high specificity immunoglobulins, such as Immunoglobulin A (IgA), Immunoglobulin E (IgE), or Immunoglobulin G (IgG). This process happens concurrently with somatic hypermutation, which greatly enhances the specificity of the antibodies. B cells then either undergo apoptosis upon cessation of TFH signaling or develop into memory B cells or plasma cells. During these processes, B cells are subject to considerable oxidative stress, and the transcription factor Nrf2 presumably plays a role in mitigating this stress [358].

Nrf2 is a cytoprotective transcription factor that primarily responds to nucleophilic and electrophilic stress. Structurally it is a cap'n'collar transcription factor with a prominent basic leucine zipper domain [165, 359]. Inactive Nrf2 is typically located in the cytosol, where it is anchored to its repressor protein, Keap1. Nrf2 bound by Keap1 is ubiquitinated and directed to

the proteasome for degradation [360]. Under oxidative or electrophilic stress conditions though, Nrf2 translocates to the nucleus and promotes an array of cytoprotective genes[361-363].

Nrf2 activators have been shown to be protective in many different toxicant models, however, the role of Nrf2 has not been verified in all of these (important due to the promiscuity of Nrf2 activators). Our group has published numerous studies in immune cells using Nrf2 activators, such as cadmium[364], CDDO-Im [365], arsenic trioxide [330], and tert-butylhydroquinone (tBHQ) [212, 288, 314, 316, 365]. The latter is a common phenolic food preservative that prevents the oxidation of fatty acids in hydrocarbons [232]. This is particularly important in shelved products containing vegetable oils, margarine, and lards. Exposure to tBHQ is increased in a western style diet.

Nrf2 has been shown to orchestrate several effects within the immune system. Nrf2 alters the cytokine response of activated T cells and alters T cell differentiation [212, 365]. NK cells have been shown to have a less pronounced effector function in vitro when exposed to tBHQ [288]. Exposure to tBHQ in murine LPS-stimulated B cells increases IgM secretion and decreases expression of surface activation markers in an Nrf2 dependent-and independent fashion [162]. Whereas the previous study of tBHQ in B cells focused on a T cell-independent stimulus (LPS), the present study was designed to understand the effects of tBHQ on T cell-dependent activation. Since our previous studies demonstrate that tBHQ strongly impacts T cell function, we used an activation cocktail that models T cell-dependent B cell activation. This allowed us to investigate the effect of tBHQ on B cells specifically.

In this study, we demonstrate that the food additive tBHQ has disparate effect on the antibody production by splenic B cells and their expression of surface markers, which may indicate a role for tBHQ in allergy and host immunity.

Methods

Materials

The sources of fluorescent antibodies are shown in table 4. tBHQ was purchased from Sigma Aldrich (St. Louis, MO) and kept in an airtight, desiccated container. Media for cell culture were purchased from Gibco (Thermo-Fisher, Waltham, MA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

Splenocyte isolation

All animal protocols are in compliance with the Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. Splenocytes were isolated using aseptic technique from C57BL/6 female mice. The mice used were either wild-type or Nrf2-deficient. Spleens were mechanically dissociated and single cell suspension of splenocytes was centrifuged at 300g/5min, washed, underwent ACK lysis, and were washed twice again. Cells were resuspended in complete Gibco Roswell Park Memorial Institute (cRPMI) 1640 Medium. Cells were then seeded out in a 96-well U-bottom plate (Greiner Bio-One) at 1x10^6 cells per well. Cells were treated with either 2µl cRPMI (Background), 0.005% Ethanol (Vehicle), or an escalating concentration of tBHQ ranging from 0.1µM to 5µM. Cells were incubated at 37C/5% CO_2 for 30 minutes and then treated with either 6µl cRPMI (Background) or 6µl of our activation cocktail (10µg/ml F(ab')2-Goat-Anti-Mouse IgM, 1µg/ml Anti-Mo CD40 and 10ng/ml recombinant mouse IL-4) (Vehicle and all tBHQ treated groups). Cells were incubated for 48 hours and then harvested for analysis. Background cells are defined as cells that received neither activation cocktail nor tBHQ treatment, but equal amounts

of cRPMI instead. Vehicle cells received activation cocktail and 0.005% ethanol in cRPMI, which is the same amount of ethanol as received by cells exposed to tBHQ. tBHQ groups received stated concentration of tBHQ, 0.005% ethanol in RPMI and activation cocktail.

Activation cocktail

To simulate a T cell-dependent B activation *ex vivo,* we designed an activation cocktail consisting of 10µg/ml F(ab')2-Goat-Anti-Mouse IgM (Invitrogen), 1µg/ml Anti-Mo CD40 (eBioscience, clone 1C10) and 10ng/ml recombinant mouse IL-4 (Peprotech, 214-14).

Fluorescent labeling

Cells were labeled both on the surface and intracellularly using the following protocol: Cells were centrifuged at 300g/5min, the supernatant was discarded, and cells were washed with Calcium and Magnesium deficient Phosphate Buffered Saline (PBS). This step was repeated twice, then 100µl of amine-reactive LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Invitrogen) was added. Cells were incubated for 30 min at room temperature in the dark, then washed/centrifuged at 300g/5min with PBS containing 1% fetal bovine serum (FACS Buffer).

Cells were then treated with 20µl of Fc Receptor Binding Inhibitor Polyclonal Antibody ("FC block", Invitrogen) and incubated at 4C for 10 minutes. Fluorescent surface antibodies (see Table 2) were centrifuged at 10^4G for 10 minutes then added to Brilliant Stain Buffer (BD Biosciences) and added to the cell/FC block suspension. Cells were incubated for 30min at 4C and then washed/centrifuged at 300g/5min. Cells were then permeabilized using Foxp3 Transcription Factor Staining Buffer Set (eBioscience) for 1 hour at 4C. Cells were then centrifuged/washed twice with Perm/Wash buffer (eBioscience) at 700g/5min. Intracellular antibodies (Table 4) were

centrifuged at 10^4G for 10 minutes and then added to Perm/Wash Buffer and cells. Cells were incubated for 30min at 4C and then centrifuged/washed twice with Perm/Wash buffer. 100µl of Cytofix (BD Biosciences) was added to the cells and incubated for 15min at 4C. Cells were centrifuged at 700g/5min, the supernatant discarded, and then resuspended in FACS buffer and subsequently analyzed on a 5 Laser Cytek Aurora (Cytek Biosciences, Fremont, CA).

Table 4. Fluorescent antibodies used in Chapter 3.

Target	Fluorophore	Clone	Manufacturer
CD 45	BUV 615	I3/2.3	BD
B220	BUV 805	RA3-6B2	BD
OX40L	BV421	RM134L	BD
CD19	BV570	6D5	Biolegend
CD40	SB600	1C10	Invitrogen
IgG1	BV650	RMG1-1	Biolegend
GATA-3	BV711	L50-823	BD
CD86	BV785	GL-1	Biolegend
I-A/I-E (MHC II)	FITC	2G9	Biolegend
CD80	PerCPCy5.5	16-10A1	BD
CD23	PerCP-eFluor 710	B3B4	Invitrogen
CD25	Pe-Cy7	3C7	Biolegend
CD138	AF647	281-2	Biolegend
CD69	APC-Fire 750	H1.2F3	Biolegend
CD267	PE	8F10	Biolegend
CD27	APC	LG.3A10	Biolegend

Statistical Analysis

A total of 3 experiments was conducted and one wildtype mouse and one Nrf2 -/- mouse was used in each experiment, for a total n=3 per group. 3 technical replicates were included in each experiment. Unmixed FCS files from Cytek Aurora were analyzed using FlowJo (BD Biosciences, Franklin Lakes, NJ). Calculations and graphical visualization of the results were done using PRISM Graphpad 9.20 software (La Jolla, Ca) using 2-Way ANOVA and Tukey's test for post hoc multiple comparisons. Statistical comparisons were made against the vehicle (VEH) treatment group and the wild-type genotype.

Results

tBHQ shows cytotoxicity at higher concentrations

At 48 hours, the percentage of living cells is generally greater in the activated groups compared to the unactivated background group, indicating that treatment cell activation increases survival (Fig. 12). At 1µM and 5µM tBHQ, there is a significant decrease in viability in both wildtype and Nrf2-deficient splenocytes, suggesting either an Nrf2 independent cytotoxic effect, or a significant percentage of cells that were not activate, which made them more susceptible to death. Decreased viability at these concentrations was unexpected, as we did not observe similar issues in other in vitro models using different cell types, e.g., T cell activation [365].

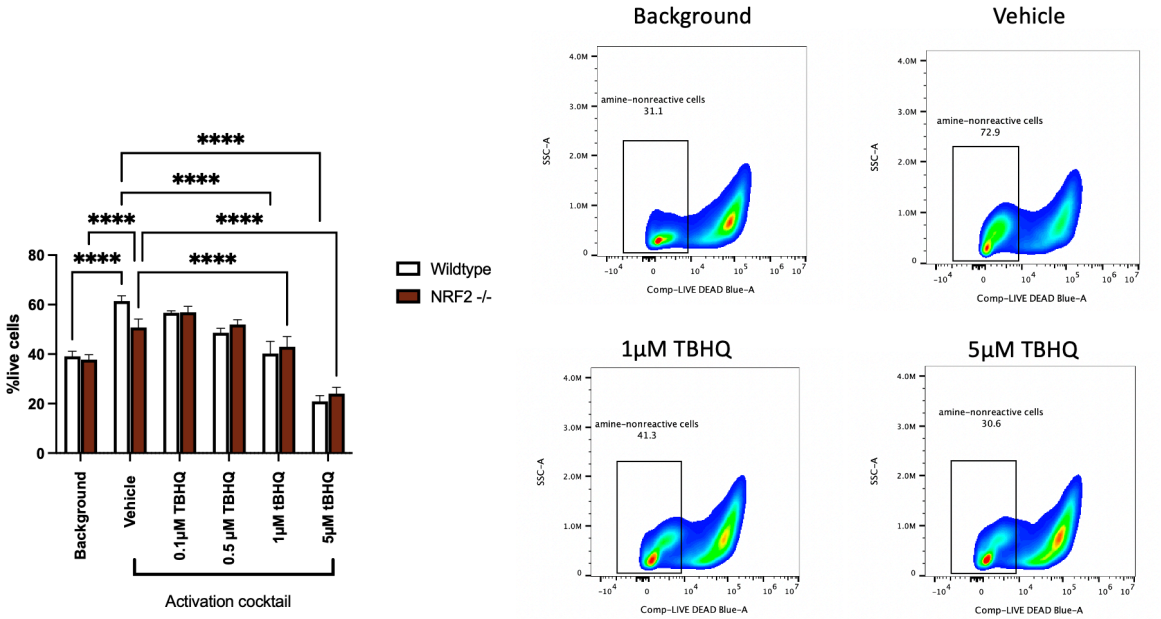


Figure 12. Viability changes are seen at 1µM and 5µM of tBHQ. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and fluorescent antibodies and analyzed using flow cytometry. Representative dot plots of Flow cytometric data are shown. Data are presented as the mean ± SE. N=3 per group (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

tBHQ decreases the percentage of CD19+ B cells within the splenocyte population

Excluding all amine-reactive (dead) cells, we next evaluated the percentage of CD19-expressing cells. CD19 is a surface marker expressed during all phases of B cell development, except for terminally differentiated antibody-producing plasma cells [366]. With the increase in viability of all cells observed in Fig.12, we expected to see a concurrent increase in CD19+ cells, hypothesizing that the increased viability was due to the expansion of the B cell population. We indeed observed an increase in the percentage of CD19+ cells in the activated VEH group as compared to the unactivated background group (Fig. 13). There was a marked decrease in CD19+ cells in the presence of tBHQ, staring at 0.1µM, which became more pronounced with increasing concentrations. At 1µM, the percentage of living CD19+ cells was comparable to the background group. Paradoxically, there was a reversal of that trend at 5µM, however the minor increase compared to 1µM is likely due to the small numbers of cells in that group, since the analysis quantifies the percentage of living CD19+ cells. Given that viability was measurably affected at 5µM, the cell populations became small, and the biological significance of this observation is

doubtful.

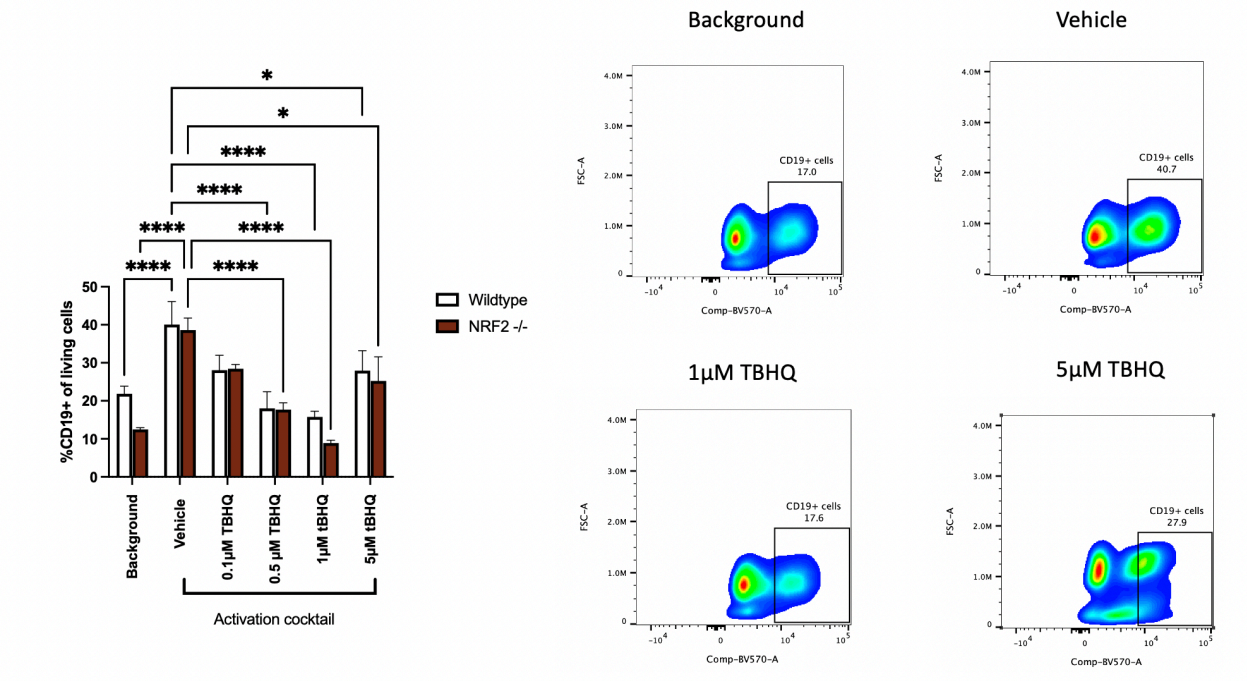


Figure 13. The number of CD19+ B cells is decreased by increasing concentrations of tBHQ. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and fluorescent antibodies and analyzed using flow cytometry. Representative dot plots of flow cytometric data are shown. Data are presented as the mean ± SE. N=3 per group. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

Changes in forward scatter (FCS) are seen at higher concentrations of tBHQ

FCS measures the dispersion of the blue laser along the laser beam's direction of travel, thereby measuring the size of cells. For lymphocytes, a modest increase in size and therefore median fluorescent intensity of FCS is common after activation. We observed a small, nonsignificant increase in FCS 48hrs after activation and a significant decrease at 1µM and 5µM (Fig 14). This decrease was robust in both wildtype and Nrf2 deficient cells, suggesting a Nrf2-independent effect on the size of B cells *ex vivo*.

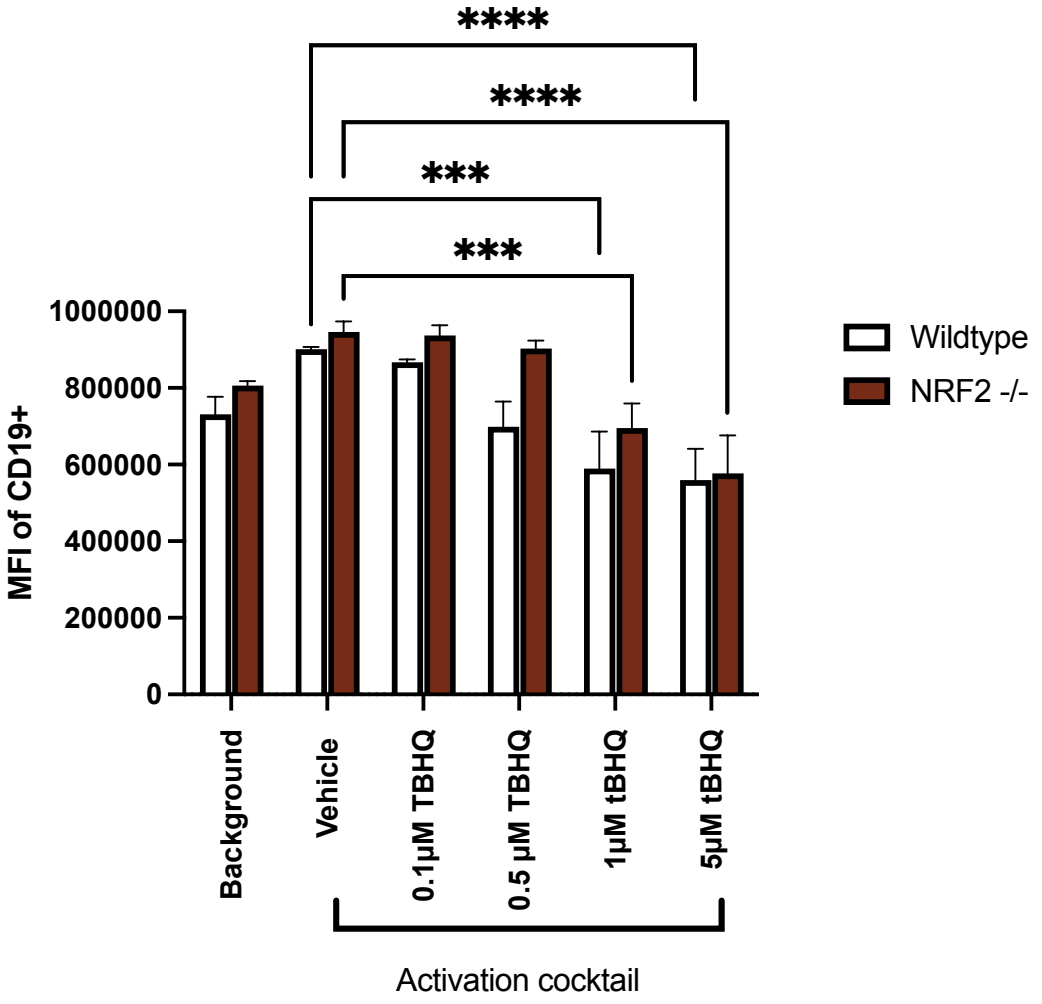


Figure 14. The median fluorescent intensity (MFI) of FSC of CD19+ B cells decreases at higher concentrations of tBHQ. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and fluorescent antibodies and analyzed using flow cytometry. Data represent the MFI of FSC channel and are presented as the mean ± SE. N=3 per group (ns indicates not significant, * indicates significance

Figure 14 (cont'd)

relative to the VEH group, p<0.05, ** indicates significance relative to the VEH group, p<0.01, *** indicates significance relative to the VEH group, p<0.001, **** indicates significance relative to the VEH group, p<0.0001)

The early activation markers CD69 and CD25 are decreased by tBHQ

CD69 is the one of the first activation markers upregulated on B lymphocytes following activation. CD25 is rapidly activated after CD69 induction. Our activation cocktail robustly induced expression of both markers (Fig 15 A and B.). CD69 expression trended downwards with escalating concentrations of tBHQ, reaching statistical significance at 1μM and a more pronounced effect at 5μM (Fig 15 A). There was no difference between genotypes, suggesting a tBHQ-independent effect. In contrast to CD69 which did not show any genotype-specific differences, CD25 was more highly expressed by Nrf2-deficient B cells in the background group (Fig 15B). After a robust induction, the expression of CD25 was decreased at 1μM of tBHQ. This effect was concentration dependent.

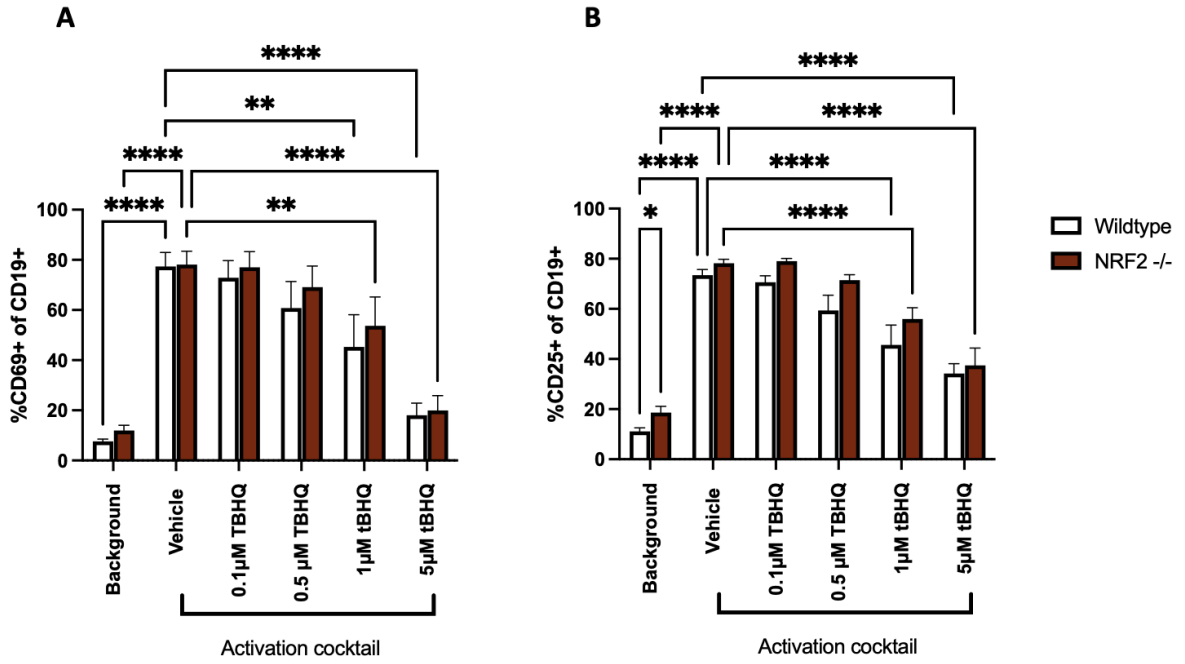


Figure 15. CD19+ cells show decreased expression of the surface markers CD69 and CD25 when exposed to high concentrations of tBHQ. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and fluorescent antibodies and analyzed using flow cytometry. Data are presented as the mean ± SE. N= 3 per group. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

CD80 expression increases while CD86 expression decreases following exposure to tBHQ

The costimulatory molecules CD80 and CD86 are implicated in the T-B cell interaction, where they bind to the T cell costimulatory molecules CD28 and CTLA-4 [342]. CD80 did not increase in response to activation by the cocktail, but showed an unexpected increase at 5µM, potentially due to low cell numbers (Fig. 16B). CD86 conversely showed the opposite effect: While markedly induced by activation, CD86 expression was diminished at 1µM and 5µM (Fig 16A). While there was a trend towards wildtype cells being more susceptible to inhibition of CD86 expression, this did not reach statistical significance.

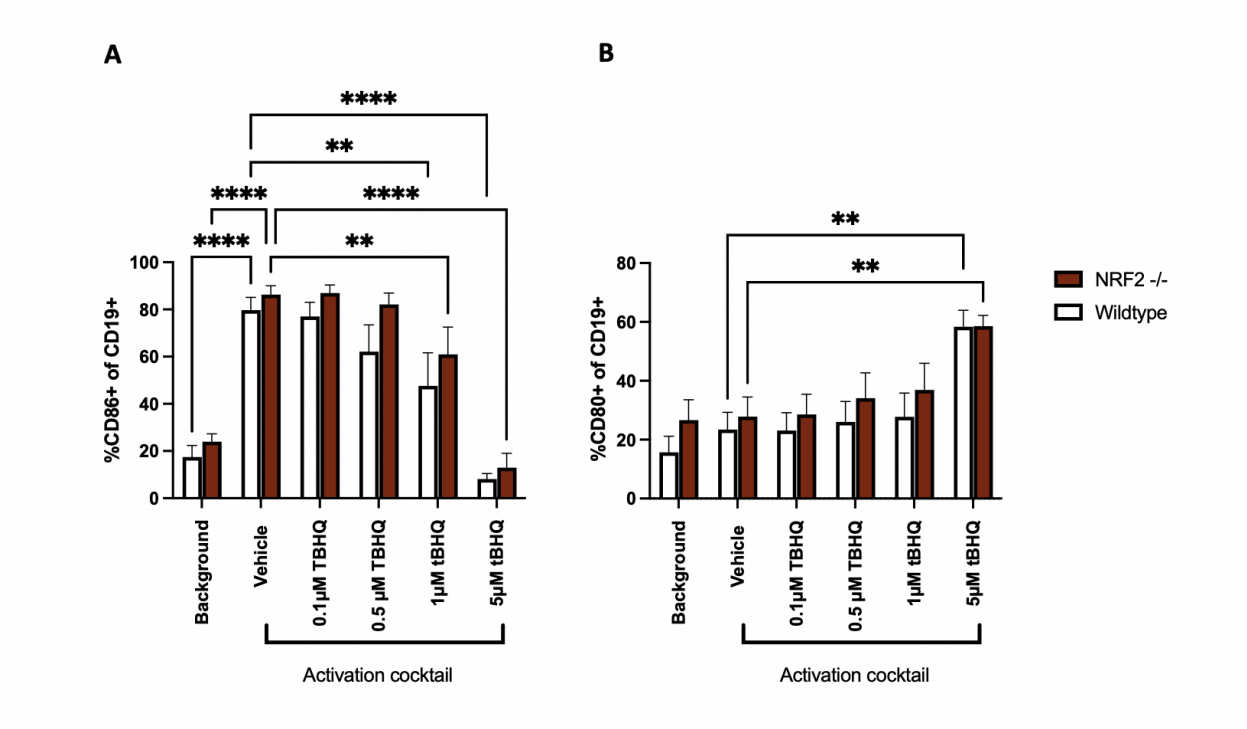


Figure 16. The expression of CD86 decreases in the presence of tBHQ while CD80 remains unchanged. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and fluorescent antibodies and analyzed using flow cytometry. Data are presented as the mean ± SE. N= 3 per group. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

The number of cells expressing IgG1 intracellularly is decreased in the presence of tBHQ

IgG1 is an intermediate immunoglobulin that B cells produce early in the process of isotype switching and thus can be a proxy measurement for B cells that are class switching. We hypothesized that the number of B cells expressing intracellular IgG1 would increase with activation and indeed we saw a robust increase in cell counts (Fig 17). This increase was diminished starting at 0.5µM tBHQ and decreased to baseline at 1µM and 5µM tBHQ. While there was a trend towards increased IgG1 expression in Nrf2-deficient cells, this did not reach statistical significance.

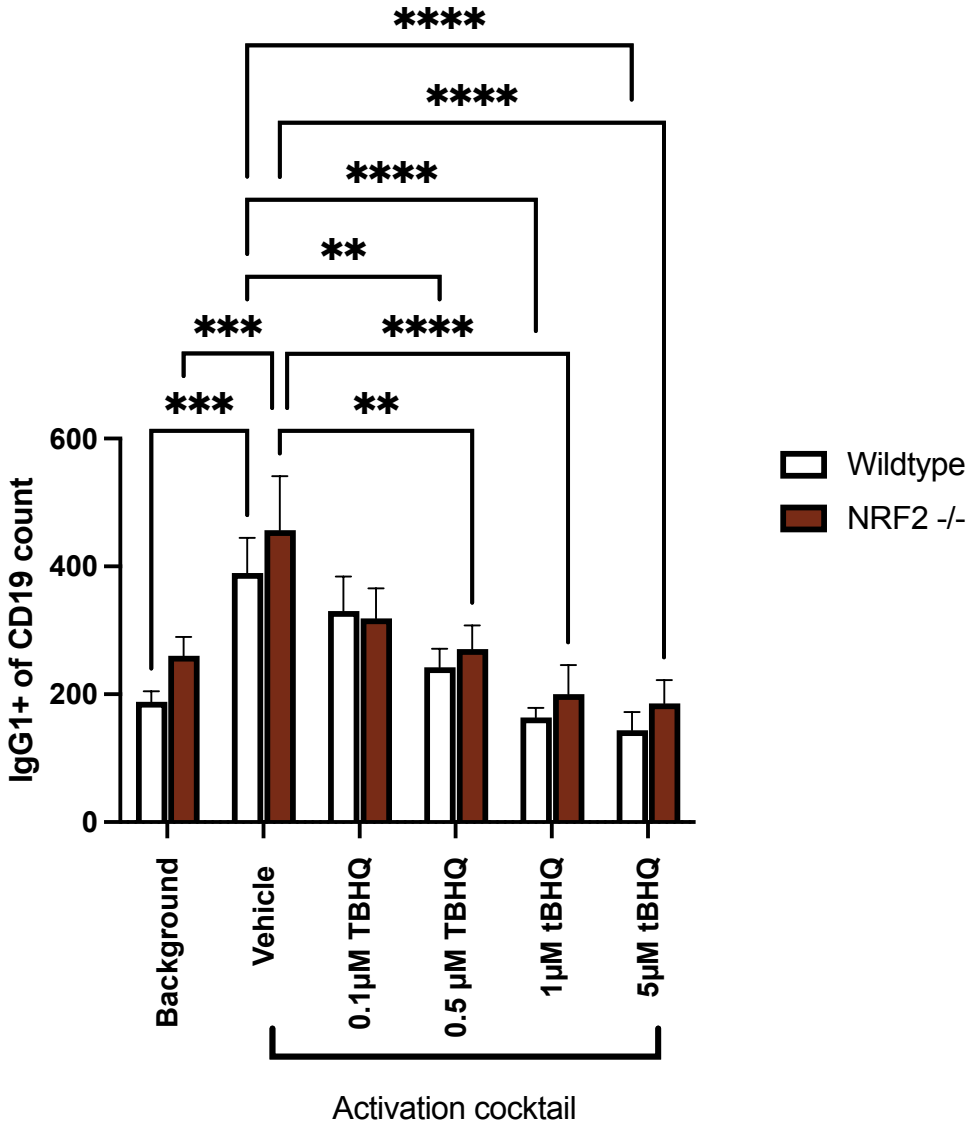


Figure 17. The number of IgG1-producing cells increases with activation but decreases in presence of tBHQ in a concentration-dependent manner. There is a non-significant trend towards a Nrf2 dependent, tBHQ mediated decrease in IgG1 counts. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and

Fig 17 (cont'd)

fluorescent antibodies and analyzed using flow cytometry. Graphs show the cell count of IgG1+ positive cells of all CD19+ cells. Data are presented as the mean ± SE. N= 3 per group (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

CD267 (TACI) expression in activated cells is inhibited by tBHQ at high concentrations

CD267 is a negative regulator of splenic B cell expansion and mediates maturation and survival [367]. We hypothesized that CD267 expression would increase with activation. Indeed, the percentage of CD267 expressing CD19+ cells increased with activation but was non-significantly decreased at 1μM tBHQ and ablated at 5μM (Fig 18). There was no statistically relevant difference between genotypes.

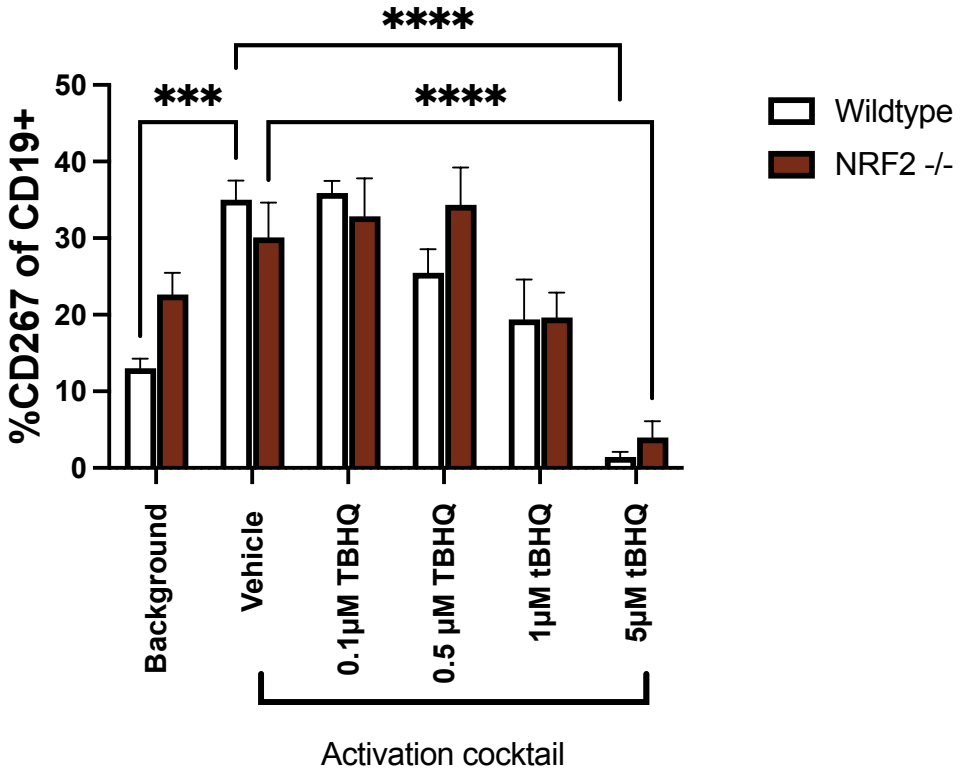


Figure 18. CD267 (TACI) expression is decreased at 5µM tBHQ. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and fluorescent antibodies and analyzed using flow cytometry. Data are presented as the mean ± SE. N=3 per group. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

The percentage of cells expressing high levels of MHCII (I-A/I-E) is decreased by exposure to tBHQ

MHCII I-A/I-E is expressed on the surface of antigen-presenting cells, such as dendritic cells, B cells, and macrophages. Antigen-derived peptides are presented on these molecules to CD4 T cells. Activated B cells typically express higher densities of MHC II, while virtually all B cells express this receptor to some degree. To assess changes in our assay, we gated on CD19+ cells with very high expression of MHCII (Fig. 19A). CD19+ cells with high expression of MHCII were indeed increased upon activation with our cocktail (Fig 19B). At 1µM and 5µM we saw a significant decrease in the MHCII high population, in both the wildtype and Nrf2 deficient cells, suggesting an Nrf2 independent effect.

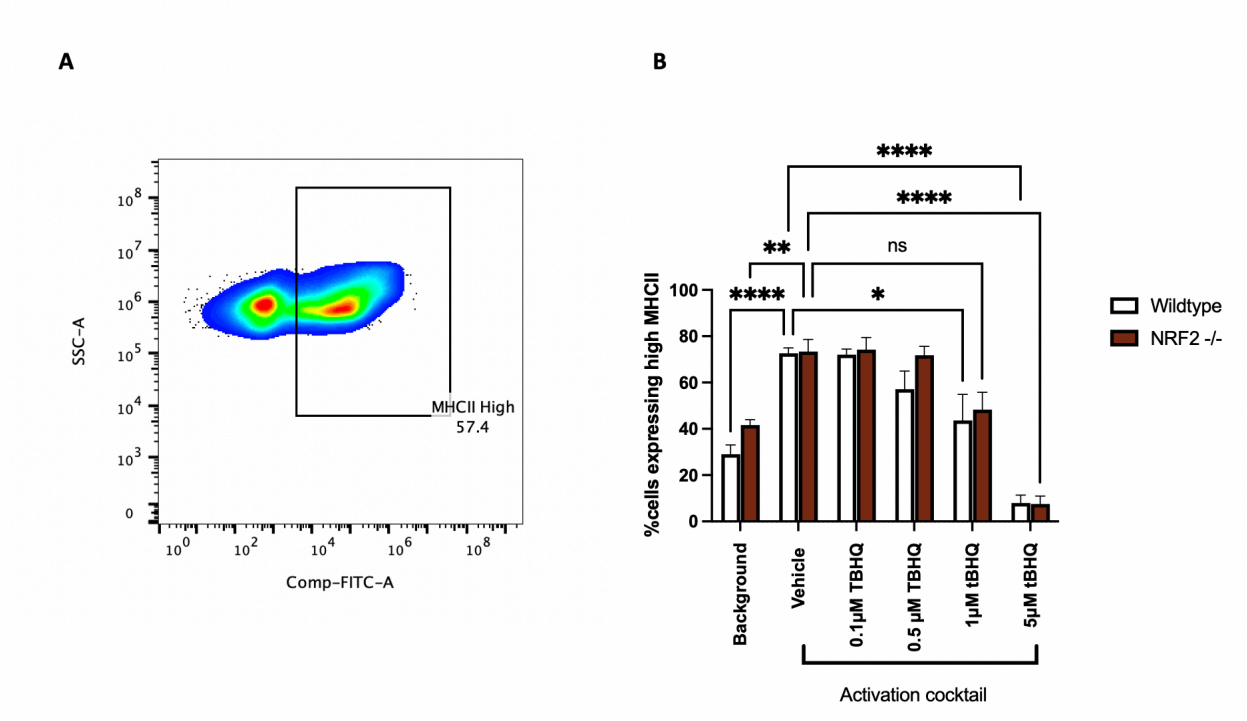


Figure 19. The number of cells highly expressing MHC II decreases at 5µM tBHQ. A shows representative gating strategy for MHC II High B cells on CD19+ living cells. B shows that activation significantly increased the percentage of MHCII high cells, which decrease at 1µM and 5µM. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and fluorescent antibodies and analyzed using flow cytometry. Data are presented as the mean ± SE. N=3 per group. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

Expression of CD23, an autoregulatory low-affinity IgE receptor, is decreased by tBHQ in a partially Nrf2-dependent manner

CD23 is an autoregulatory surface protein that binds to low-affinity IgE, supposedly to capture IgE-coated antigens in the lymphatic system or plasma and process them within the B cell [368]. CD23, in humans, can regulate the production of IgE antibody [369]. In mice, CD23 was found to be essential to enhance CD4 T cell responses and antibody production by B cells in a mechanism that also depends on CD11c cells [370]. CD23 is typically expressed in higher levels on activated B cells. In the background cells, we appreciated a genotype difference, with Nrf2-deficient cells having a higher baseline expression of CD23 (Fig 20). This Nrf-2 dependent trend was seen in all activated groups too, reaching statistical significance at 0.5µM. At 5µM, we observed an Nrf2 independent decrease in CD23 expression.

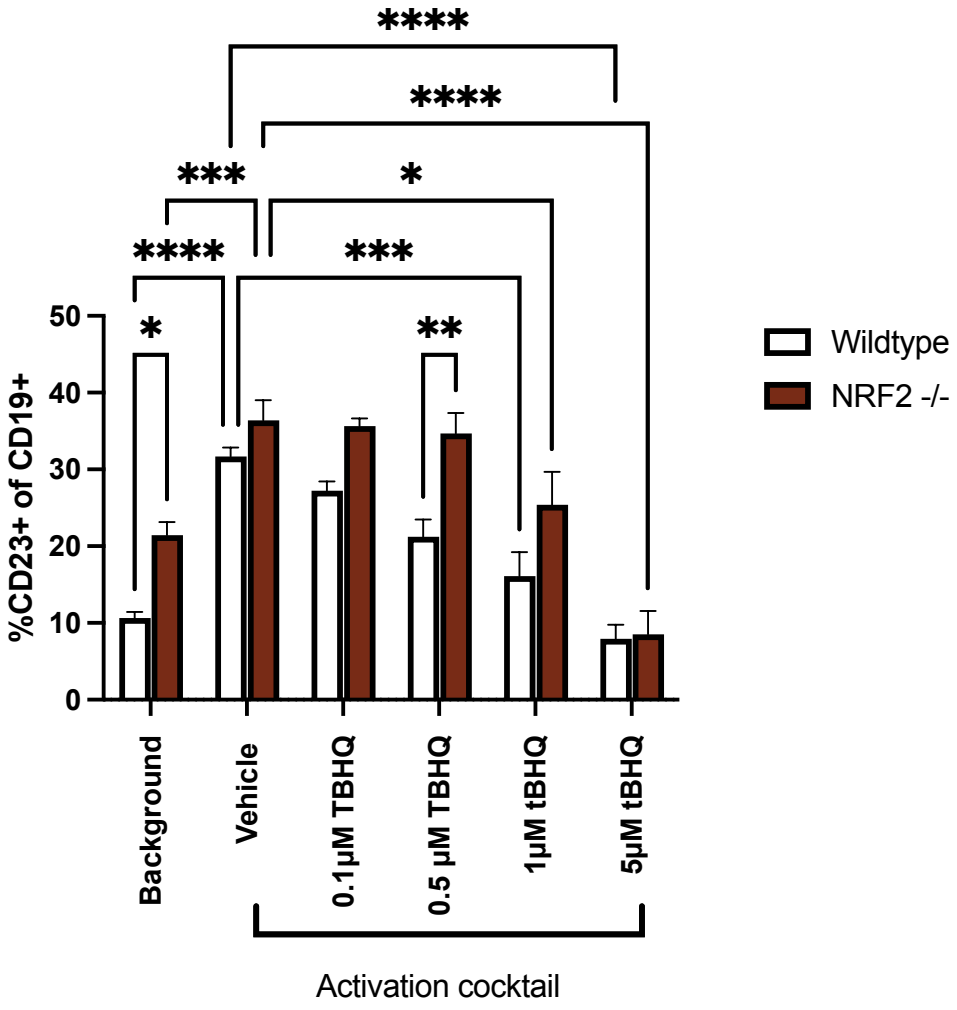


Figure 20. Genotypical difference in CD23 expression is mitigated by activation and restored at 0.5µM tBHQ. The effects are both Nrf2 dependent and at higher concentrations Nrf2 independent. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cells were harvested and labeled with amine-reactive live/dead discriminator and fluorescent antibodies and analyzed using flow cytometry. Data are presented as the mean ± SE. N=3 per group. (ns indicates not significant, * indicates significance relative to the VEH group,

Figure 20 (cont'd)

p<0.05, ** indicates significance relative to the VEH group, p<0.01, *** indicates significance relative to the VEH group, p<0.001, **** indicates significance relative to the VEH group, p<0.0001)

Induction of the B cell cytokine IL-6 induction is impaired in the presence of 1µM and 5µM tBHQ

IL-6 is a master regulator of cytokine networks and is secreted by activated B cells. IL-6 is canonically considered to be a pro-inflammatory cytokine [371] and can modulate the generation of new TFH cells [70]. In our *ex vivo* activation system, we expected a rise in IL-6 secretion after activation and a concentration-dependent decrease with tBHQ. Indeed, we saw an increase from ~10pg/ml to 60pg/ml when comparing the unactivated background to activated vehicle groups in Fig. 21. At 0.5µM and 0.1µM tBHQ, IL-6 secretion was stable, but we saw a decrease at 1µM and 5µM. At these concentrations, the suppression trended towards a greater decrease in the wild-type splenocytes, but this was not statistically significant.

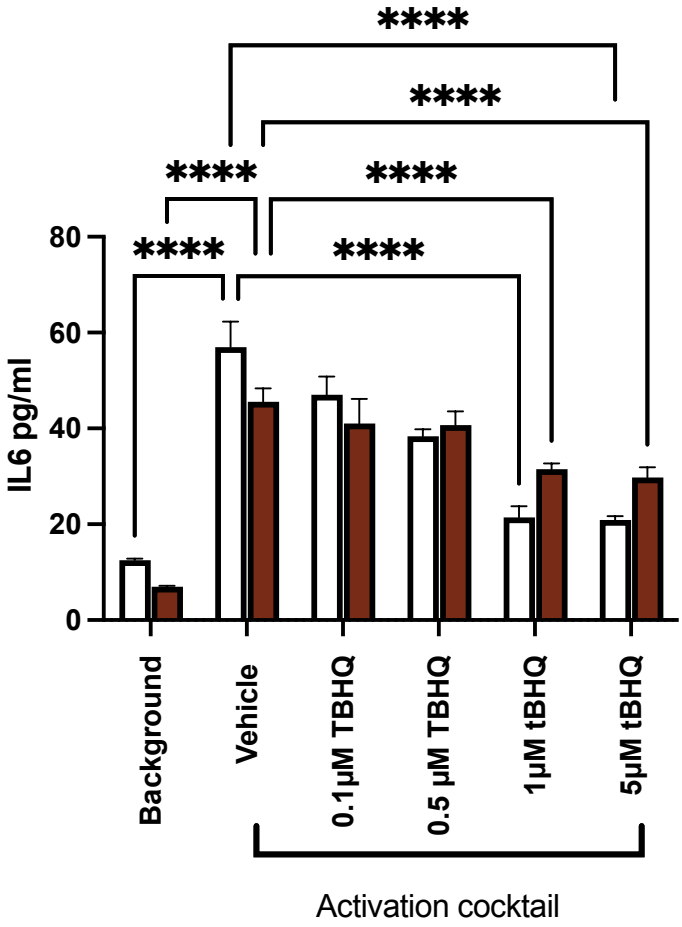


Figure 21. Production of the B cell cytokine IL-6 increases with activation but decreases at 1µM and 5µM tBHQ. Splenocytes were treated with either Vehicle (0.005% Ethanol) or an escalating concentration of tBHQ. After 30 min, Splenocytes were activated using our activation cocktail. After 48 hours, cell supernatant was harvested and IL-6 content was measured using IL-6 ELISA (Thermo Fisher, Waltham, MA). Data are presented as the mean ± SE. N=1 per group. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the

Figure 21 (cont'd)

VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

Discussion

This study demonstrates both Nrf2-dependent and -independent effects of tBHQ on B cell activation and surface marker expression. While the results are remarkable and reproducible, this *ex vivo* study simplifies the intricate T and B cell interaction, and it is impossible to predict if the tBHQ effects on T cells will have an additive, synergistic or inhibitory effect on B cells. tBHQ decreased the induction of the early activation marker CD69, which is rapidly expressed after activation of lymphocytes. Emerging studies indicate CD69 plays a metabolic role in T cells [372], though no work has been done on potential roles beyond a marker for early activation in B cells. A secondary activation marker, CD25, is a subunit of the high-affinity IL-2 receptor with a role in lymphocyte expansion. This is well characterized in T cells and there is emerging evidence that there is a similar role in B cells [340].

Both CD25 and CD69 are intended in our assay as markers of B cell activation, and we saw a robust increase with activation, and a subsequent decrease at 1μM tBHQ, indicating that B cells are less readily activated in presence of tBHQ. This could either work through an inhibition of activation pathways or a temporal delay in activation. To discriminate between these two possibilities, we could implement further mechanistic studies or repeat the study at different timepoints to establish a kinetic time course might be interesting.

We also compared the activation of the costimulatory molecules CD80 and CD86, which both interact with either CTLA-4 or CD28 expressed on the cell surface of T cells. CD80 and CD86 are structurally similar, but there have been indications that they convey different signals to a T cell, where CD80 promotes a Th1 fate and CD86 signaling promotes Th2 differentiation [336, 342].

In this chapter, we saw that our activation cocktail strongly increased the percentage of cells expressing CD86 but did not increase the percentage of cells expressing CD80. This may be due to limitations of this model.

While we included the major signals required for B cell stimulation, it is impossible to fully mimic the B cell niche required for T cell dependent activation, and it may be that there are additional signals, e.g. cytokines, required for robust induction of CD80 expression. Treatment with tBHQ significant decreased CD86 induction at 1µM. There was no significant change in CD80 expression when tBHQ was added. These observations contrast with the effect of ATO on CD80/CD86 expression in IAV-activated B cells in chapter 2, where the addition of IAV challenge induced both CD80 and CD86, but the Nrf2 activator ATO decreased only CD80 and did not change CD86. It is important to consider the substantial differences in experimental design between the two studies. The studies in chapter 2 were conducted on primary human immune cells derived from peripheral blood and a virus was the B cell activator, whereas in this chapter we used mouse splenocytes and challenged them with a B cell specific activation cocktail. Most importantly, different toxicants were used. Nonetheless, this observation is interesting because of the aforementioned possibility of the CD80/86 ratio modulating T cell polarization.

In addition, it is important not to draw early clinical implications out of those studies, as there is a significant difference between murine and human B cells. Unfortunately, sources for human B cells are somewhat limited. Peripheral blood-derived B cells are often antigen-experienced, which limits their susceptibility to an activation cocktail as proposed here. Ideal human tissue would be a lymph node or a spleen, but these are almost impossible to access without surgical

intervention. Some groups have used tonsil tissue discarded after elective tonsillectomy with great success, but that requires an otolaryngology clinic that performs non-emergent tonsillectomies as a collaborator to which we currently do not have access to. Furthermore, this study focused on splenic B cells, and while they are considered a good model, populations in the spleen differ from B cells in other compartments, as they are relatively enriched in marginal zone B cells (359). Marginal zone B cells are unlikely to be optimally activated by our activation cocktail, as it mimics the activation of follicular B cells by T cells. Some of the results described above were primarily seen at concentrations that coincided with alterations in cell viability. It is impossible to rule out that the changes might partially be due to enhanced cytotoxicity. The decreased viability may also be due to the signal we are providing with activation being very specific to FO B cells, and other B cell types as well as non- B cells may die due to the lack of survival signals. During analysis of flow cytometric data, we ensured that we only gated on cells that were neither amine reactive nor had FSC/SSC abnormalities that could be indicative of cell death. With these measures, we can be reasonably sure that the cell populations analyzed were only viable B cells. However, the effects of tBHQ were unexpected in magnitude, given the relatively low concentrations used, and are relevant to human exposure (226).

This raises an important question – does environmental tBHQ exposure alter the efficacy of antibody production in humans? At the time of writing, around 610.000.000 million doses of various vaccinations against COV-SARS-2 were administered in the United States (360), and around 93.000.000 cases were registered (361). Every single one of these immunological exposures to an antigen found in either the COV-SARS-2 virus or a vaccination triggers the

generation of neutralizing antibodies by B cells as outlined in the introduction. Considering the ubiquitous presence of tBHQ and other similar compounds in our environment, and the results of this *ex vivo* rodent study, it would be important to explore the magnitude of these effects in human populations.

Interestingly, the tBHQ effects observed here were independent of Nrf2. While we know that Nrf2 is strongly activated by tBHQ and that this specifically has implications on T cell activation [212, 314], we also described Nrf2-independent effects of tBHQ on immune cell function [162, 315]. This may be in part due to tBHQ's propensity inhibit NF-κB signaling [316]. Our lab has shown tBHQ effects on calcium signaling which may be Nrf2-independent [314], and potentially a calcium-dependent mechanism may be facilitating the effects seen here. Furthermore, tBHQ has been shown to activate the AHR [373], which could lead to Nrf2 independent effects.

In future studies, we would like to determine the effect of tBHQ on B cells at different time points, e.g., 120 hours to be able to measure immunoglobulin secretion or 24 hours for the very early activation markers. It would also be feasible to adapt this system to a human *ex vivo* system, in which we could use human PBMC-derived B cells *ex vivo* and change the activation cocktail to human orthologues of our activators, e.g. anti-human IgM F(ab), human recombinant IL-4 and human recombinant CD40L. This would allow us to screen different toxicants quickly and efficiently for their effects on B cell activation. We would not be able to assess Nrf2 specificity initially for the lack of human Nrf2-deficient cells, however, we have explored the possibility of using the CRISPR-CAS9 system to generate Nrf2deficient PMBCs.

In this study, we have shown in a novel *ex vivo* model that the common food preservative tBHQ impacts T cell-dependent B cell activation in a partially Nrf2-dependent manner. B cell activation and antibody production are critical to humoral immunity and therefore essential to the well-being and host defense of mammals. We demonstrate the marked effects of tBHQ on this process in a rodent model, which warrants further studies. We also established a rapid and easy model to screen potential toxicants for their effect on B cell activation.

Chapter 4: The Food Additive tert-Butylhydroquinone Increases Plasma IgE during transdermal allergen sensitization in correlation with increased CD40 and CD138 expression on B cells

Abstract

Atopic diseases, such as allergies, are a global public health problem with concerning epidemiological dynamics. Both the severity and incidence of food allergies are increasing in the western world. Congruently, the use of synthetic food additives such as tert-butylhydroquinone (tBHQ) has also been increasing. Our lab has previously described a change in T cell polarization towards a T helper cell type 2 (TH2) phenotype when mammalian cells were exposed to tBHQ *in vitro*. These effects were mostly mediated by the stress-activated transcription factor Nuclear Factor erythroid-2 related factor 2 (Nrf2). Due to the close relationship of T and B cells in the production of high-affinity immunoglobulins, the question of the role of tBHQ in the generation of atopic disease arose. In this study, we demonstrate that exposure to low concentrations of tBHQ increases the concentration of plasma IgE and changes the differentiation status and phenotype of B cells isolated from the lymph nodes in a mouse allergy model of transdermal sensitization. Specifically, we found that dietary tBHQ increased expression of CD40, a molecule associated with T cell signaling and activation in B cells, and CD138, a marker of plasma cell differentiation. Furthermore, tBHQ increased the percentage of IgE+ CD138+ cells, which are known to be causative in allergic sensitization. In contrast, other molecules associated with B cell activation were not different between the tBHQ and control groups. Taken together, the data suggest that tBHQ promotes T cell signaling and plasma cell differentiation in B cells, which may contribute to the increased plasma concentrations of IgE during transdermal sensitization.

Introduction

Food allergies are a significant public health problem with rising concern. The incidence of allergies to common foods, such as peanuts, shellfish, eggs, and stone fruit has been steadily rising over the last decades [374]. Current estimations are that about 10% of children suffer from some form of food allergy, which results in 300,000 emergency room visits and 100-150 deaths annually [375, 376]. This phenomenon initially observed in the western world is also seen in developing countries and correlates with their rate of economic growth [377]. With asthma being the most prevalent atopic disease, food allergies are the second most common and have been called the "second wave of the allergic pandemic" [374].

Although food allergies often present as mild nonspecific discomfort, gastroenteric issues such as diarrhea or constipation and/or airway restriction, the most serious cases results in life-threatening acute anaphylactic shock [338]. Hospitalizations for anaphylactic shock have increased by 13.4% between 1994 and 2004 in Australia, which keeps emergency room admission statistics publically available at a centralized location, unlike other western countries [378].

The hygiene hypothesis has been postulated as an explanation for the rise in the incidence in allergies, arguing that the lack of exposure to certain pathogens hampers the development of the functional immune system [379, 380]. While these mechanisms of immune dysfunction may contribute to the rising prevalence of atopic disease, the epidemiological dynamics suggest that there may be other factors which remain unidentified. Notably, there has been an increase in the exposure to synthetic food additives congruent with the increased severity and incidence of food allergies in the same time frame. While it is important to note that this correlation does not in

itself mean that there is a causal relationship, I want to emphasize little work has been done to identify the effects of food additives on the pathology of food allergies.

Our lab has demonstrated that the synthetic food additive tBHQ activates the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and polarizes T helper cells to a type II phenotype, which is a key step in the development of atopic disease [212]. tBHQ is widely used as a preservative to stabilize vegetable oils in processed foods, is rapidly absorbed upon digestion and can reach blood concentrations exceeding 200μM [234]. The Food and Agriculture Organization/World Health Organization meeting in 1999 reported that in a typical western diet, folks may be exposed to 1100% of the allowed daily intake of tBHQ (0.7mg/kg body weight) [239]. Research from our lab suggests Nrf2 as a possible mediator of many of the immune effects of tBHQ. Nrf2 is a member of the cap'n'collar subfamily and has a highly conserved CNC domain [381]. Nrf2 can be activated by cell stress, including oxidative and electrophilic stressors [359]. tBHQ is also a robust Nrf2 activator through direct interaction with thiol groups of cysteine molecules on the Keap1 protein, which disrupt the binding of Keap1 to Nrf2 [382].Under homeostatic conditions, most Nrf2 is bound by its repressor protein Keap1, which polyubiquitinates Nrf2 and thus directs it to the proteasome forl degradation [360, 383].

Food allergy, by definition, is an IgE-dependent reaction to food antigen. The most severe manifestation of food allergies, anaphylactic shock, occurs when IgE bound on mast cell Fcε receptors binds to an antigen, crosslinks, and causes mast cell degranulation[384]. IgE is classified into two subforms, high-affinity IgE and low-affinity IgE. Low-affinity IgE is involved in the defense against helminths and large multicellular parasites[95, 105], while high-affinity IgE is the product

of the germinal center reaction and somatic hypermutation [108, 385]. In serum, IgE has a very short half-life of ~2 days [100], but once bound to Fc ε receptors on mast cells, IgE is extremely stable [59]. The decision as to whether a B cell produces IgE or another immunoglobulin subclass depends heavily on the interaction of the B cell with TFH cells in the germinal cente reaction and the signals provided to the B cell [385]. As our lab has previously described polarization changes in CD4 T cells upon exposure to tBHQ *in vitro* [212, 314, 316] and changes in B cell activation *in vitro* [162], the purpose of the present study is to determine the effects of low-dose oral tBHQ on B cell activation in an experimental mouse food allergy model.

Methods and Materials

Materials

The sources of fluorescent antibodies are shown in table 5. Rodent diets were purchased from Dyets (Bethlehem, PA) and stored at -20C. PBS for cell preparation was purchased from Lonza (Thermo-Fisher, Waltham, MA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

Sensitization

All animal protocols are in compliance with the Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. 4-week-old female BALB/CJ were acquired from the Jackson Laboratory and maintained with either a normal AIN-93G (0.0014% tBHQ) diet or modified tBHQ-free AIN-93G. Diets were procured from Dyets Inc, Bethlehem PA. After 1 week, mice were subjected to a thoracodorsal shave. One day later, 100µl of chicken ovalbumin (Sigma Aldrich, Burlington, MA) (100mg/ml) in sterile normal saline (allergy group, n=5) or sterile normal saline (control group, n=5) were applied to their exposed thoracodorsal skin and covered with a commercially available adhesive wound dressing (Meijer, Grand Rapids, MI). Sensitization was repeated 7 days and 14 days later and mice were sacrificed three days post last sensitization. The total number of mice was 5 per group.

OVA-specific IgE Quantification

Plasma was isolated from cardiac blood and analyzed for IgE using OVA-specific IgE ELISA (Biolegend, LaJolla, CA). ELISA was read using a Tecan Infinite M1000 (Tecan Trading AG, Hombrechtikon, Switzerland).

Cell preparation and flow cytometry labeling

Lymph nodes were placed between glass slides and mechanically dissociated by rubbing the slides together. Single cell suspension was washed twice and plated out in 96 well U bottom plates (Greiner, Kremsmuenster, Austria). The cells were then resuspended in FACS buffer (1%FBS in DPBS) and stained with amine-reactive live/dead blue (Invitrogen, Waltham MA). After 30 min, cells were washed twice with FACS buffer and anti-Fc-gamma receptor I, II, III (anti-CD16, anti-CD32, anti-CD64, Invitrogen, Waltham, MA) was added to diminish non-specific binding. After 15 minutes, cells were labeled with fluorescent surface antibodies (see Table 1) and incubated for 30 min. Cells were then permeabilized using the mouse FOXP3 intracellular staining kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions and labeled with intracellular antibodies (see Table 2). After 30 min, cells were washed twice with Perm/Wash Buffer and fixated using Cytofix (BD Pharmingen, San Diego, CA). Cells were then resuspended in FACS buffer and analyzed on a Cytek Aurora (Cytek Biosciences, Fremont, CA). The data presented herein were obtained using instrumentation in the MSU Flow Cytometry Core Facility. The facility is funded in part through financial support from Michigan State University's Office of Research & Innovation, College of Osteopathic Medicine, and College of Human Medicine. Flow

cytometric data were analyzed using FlowJo (BD Bioscience, Waltham, MA) and exported to Graph Pad Prism (GraphPad, LaJolla, CA).

Table 5. Fluorescent Antibodies used in Chapter 4.

Target	Fluorophore	Clone	Manufacturer
CD 4	BUV 496	GK1.5	BD
OX 40	BUV 563	OX-40	BD
CD 45	BUV 615	I3/2.3	BD
B220	BUV 805	RA3-6B2	BD
OX40L	BV421	RM134L	BD
IgE	BV480	R35-72	BD
CD19	BV570	6D5	Biolegend
CD40	SB600	1C10	Invitrogen
IgG1	BV650	RMG1-1	Biolegend
GATA-3	BV711	L50-823	BD
CD86	BV785	GL-1	Biolegend
I-A/I-E (MHC II)	FITC	2G9	Biolegend
PD-1	BB-700	J43	BD
CD80	PerCPCy5.5	16-10A1	BD
CD23	PerCP-eFluor 710	B3B4	Invitrogen
CXCR5	PE	L138D7	Biolegend

Table 5 (cont'd)

IL-4	PE-Dazzle 594	11B11	Biolegend
FOXP3	Pe-Cy5	FJK-16s	Invitrogen
CD25	Pe-Cy7	3C7	Biolegend
Bcl-6	APC	7D1	Biolegend
CD138	AF647	281-2	Biolegend
CD3	AF700	17A2	Biolegend
CD69	APC-Fire 750	H1.2F3	Biolegend
CD40L	SB 436	MR1	BD
CXCR5	BUV737	2G8	BD
IgM	BV480	R6-60.2	BD

Analysis

Flow cytometric data were analyzed using FlowJo (BD Bioscience, Waltham, MA) and exported to Graph Pad Prism (GraphPad, LaJolla, CA). ELISA was read using a Tecan Infinite M1000 (Tecan Trading AG, Hombrechtikon, Switzerland) and exported to GraphPad Prism (GraphPad, LaJolla, CA).

Statistical analysis was done using a two-way ANOVA and Tukey's post hoc test comparing average of the different diet groups and sensitization groups (row and column effect). Results show average ± SEM. ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$

Results

Serum IgE is increased upon exposure to tBHQ

An increase in plasma IgE is one of the hallmarks of food allergy. While being a very rare immunoglobulin isotype, IgE plasma concentrations rise considerably during allergic sensitization or parasitic infections. In this study, we measured serum IgE specific for OVA on day 17 after the first exposure to OVA. In Fig 22, we show that at baseline, both mice on the non-tBHQ diet and mice on the tBHQ diet have comparably low levels of OVA-specific IgE. Our sensitization protocol increases IgE about 7-fold in the non-tBHQ mice, indicating a robust response to the sensitization. In the non-tBHQ group, however, serum IgE is increased 16fold, indicating that dietary tBHQ markedly increases the plasma OVA-specific IgE levels in our transdermal sensitization model ($p<0.01$).

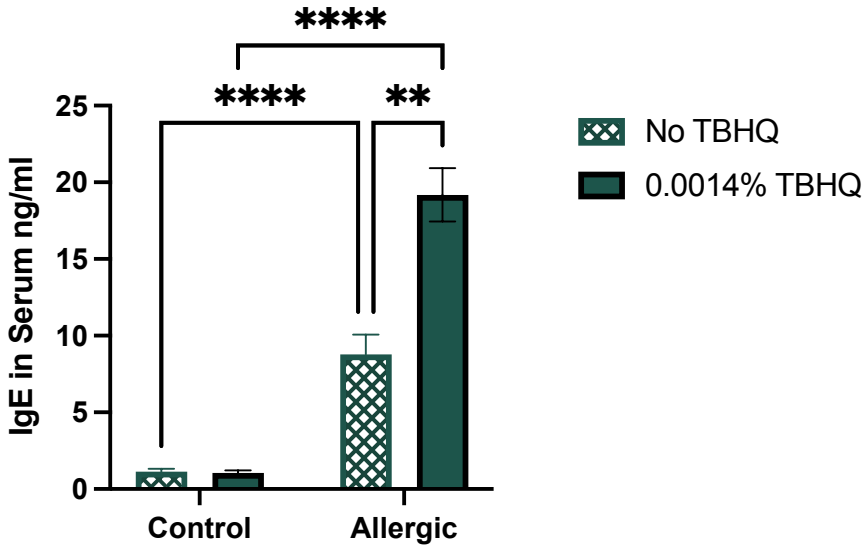


Figure 22. tBHQ diet increases Serum IgE after sensitization. 5-week-old mice where sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization. Blood was collected using cardiac puncture and serum isolated using heparinated vacuettes (Greiner, Kremsmünster, Austria). Serum was analyzed for OVA-specific IgE using ELISA (Biolegend, La Jolla, CA). Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

CD19-expressing cells increase with sensitization but show little difference in treatment groups

To further characterize the B cell response to tBHQ, we used high-dimensional spectral flow cytometry on lymph node cells to phenotype the B cell response in this model. In Fig 23, we show that the percentage of CD19-expressing cells within lymph nodes increased significantly with the

sensitization protocol (p<0.0001) with no statistically significant difference between the tBHQ and control groups.

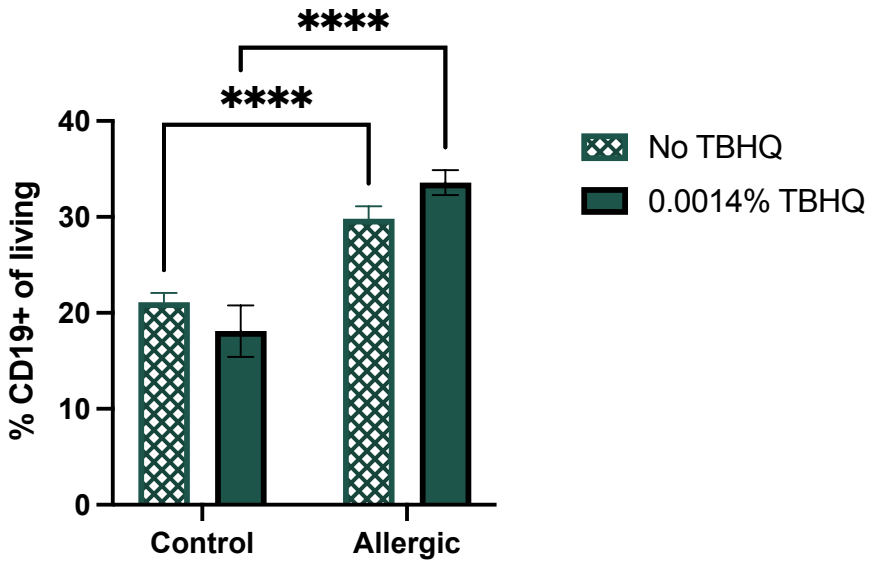


Figure 23. The percentage of CD19+ cells increases with sensitization. 5-week-old mice where sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization. Axillary and brachial lymph nodes were isolated, mechanically dissociated, and single cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. Gating strategy involved FCS/SSC selection, doublet exclusion, exclusion of dead cells and gating on CD19+ cells. DATA ARE PRESENTED AS THE MEAN ± SE. N=5 per group(ns indicates not significant, * indicates significance p<0.05, ** indicates significance p<0.01, *** indicates significance p<0.001, **** indicates significance p<0.0001)

Induction of the early activation marker CD69, but not CD25, after second sensitization

Expression of both CD69 and CD25 are induced shortly after B cell activation and thus these molecules are sometimes used as activation markers. Three days after the second sensitization,

we observed a robust increase in CD69 expression in the sensitized groups with a statistically non-significant increase in the tBHQ group (Fig 24A). Neither sensitization nor tBHQ had any effect on the expression of the early-activation marker CD25 (Fig 24B). Given that this time point is early in the development of allergy, we hypothesize that at later time points there may be an increase in the expression of CD25.

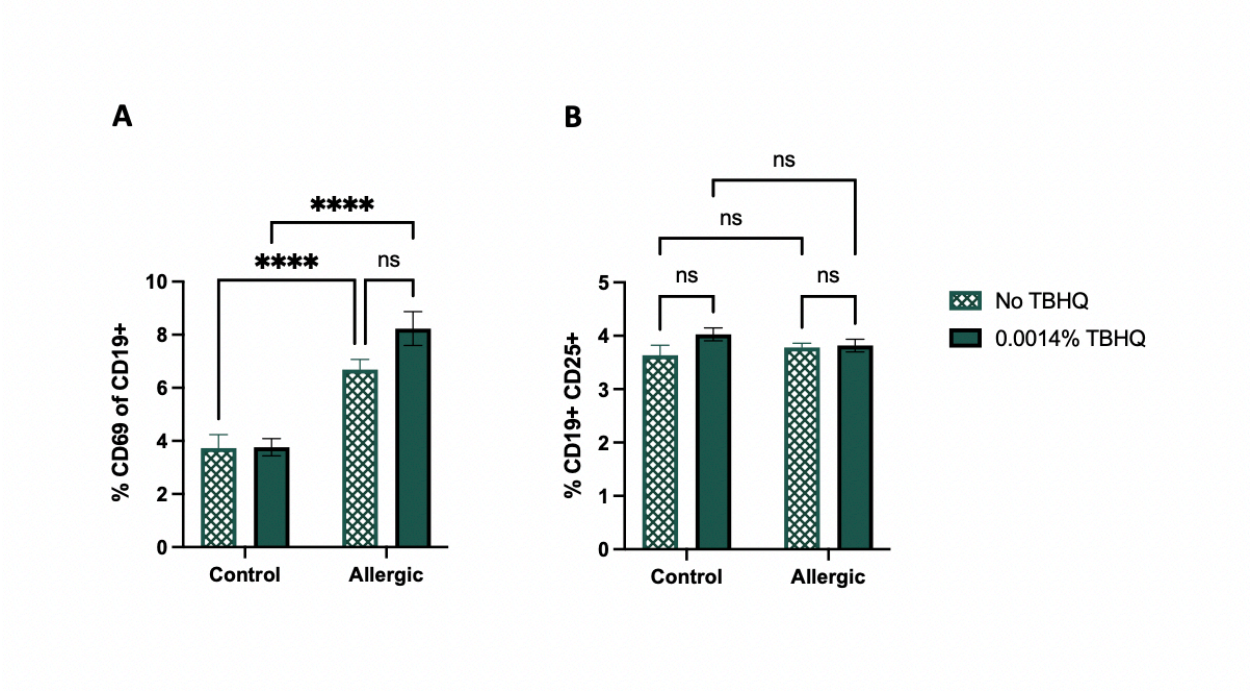


Figure 24. Sensitization increases CD69 but not CD25.5-week-old mice where sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization. Axillary and brachial lymph nodes were isolated, mechanically dissociated, and single cell suspension of lymphocytes was labeled with fluorescent antibodiesand analyzed on a Cytek Aurora spectral flow cytometer. Gating strategy involved FCS/SSC selection, doublet exclusion, exclusion of dead cells and gating on CD19+ cells. Data are presented as the mean ± SE. N=5 per group. (ns indicates

Figure 24 (cont'd)

not significant, * indicates significance p<0.05, ** indicates significance p<0.01, *** indicates significance p<0.001, **** indicates significance p<0.0001)

Increased expression of the costimulatory molecule CD86, but not CD80, following sensitization

CD80 and CD86 are co-stimulatory molecules that interact with CD28 on T cells during B cell activation [59]. CD80 and CD86 are considered immunologically similar with respect to T cell activation but have different signaling properties. CD80 has been shown to promote Th1 polarization in T cells, while CD86 has been associated with Th2 polarization [337]. Th2 polarization is particularly important for this study as allergies are traditionally associated with Th2 polarized T cells [108] and our previous studies showed tBHQ promotes Th2 polarization [212, 315]. In Fig 25, we show that our sensitization protocol does not alter the surface expression of CD80 (Fig 25A) but increases the expression of CD86 (Fig 25B), which is consistent with the hypothesis that CD86 promotes a Th2-like response. There was no difference between the control and tBHQ groups.

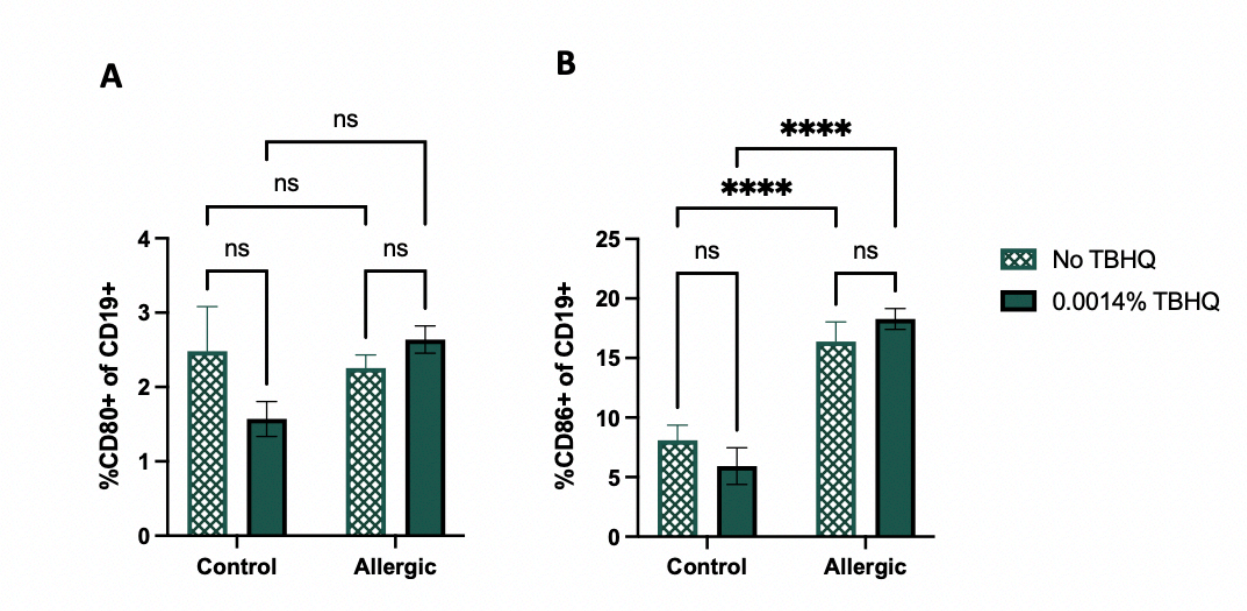


Figure 25. Sensitization does not increase the costimulatory molecule CD80 but does increase CD86. 5-week-old mice were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization. Axillary and brachial lymph nodes were isolated, mechanically dissociated, and single cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. Gating strategy involved FCS/SSC selection, doublet exclusion, exclusion of dead cells and gating on CD19+ cells. A shows the percentage of CD80+ cells of CD19+ cells. B shows the percentage of CD86+ of CD19+. Data are presented as the mean ± SE .N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

Expression of the costimulatory marker CD40 is increased by tBHQ at baseline and further increases with allergic sensitization

CD40 is a costimulatory molecule of the tumor necrosis factor receptor superfamily. It is expressed on APCs and interacts with CD154 (CD40-L) on T cells. This interaction is required for the germinal center reaction, isotype switching, and formation of plasma cells. FO B cells express CD40 constitutively; however, expression of CD40 is increased following FO B cell activation. We defined these cells as CD40 high. In our study, we found that even at baseline, mice on a tBHQ diet had a somewhat higher percentage of CD40-high cells (Fig 26). Sensitization increased the percentage of CD40+ high cells, but this increase was much more pronounced in mice in the tBHQ group compared to those on control diet ($p<0.01$).

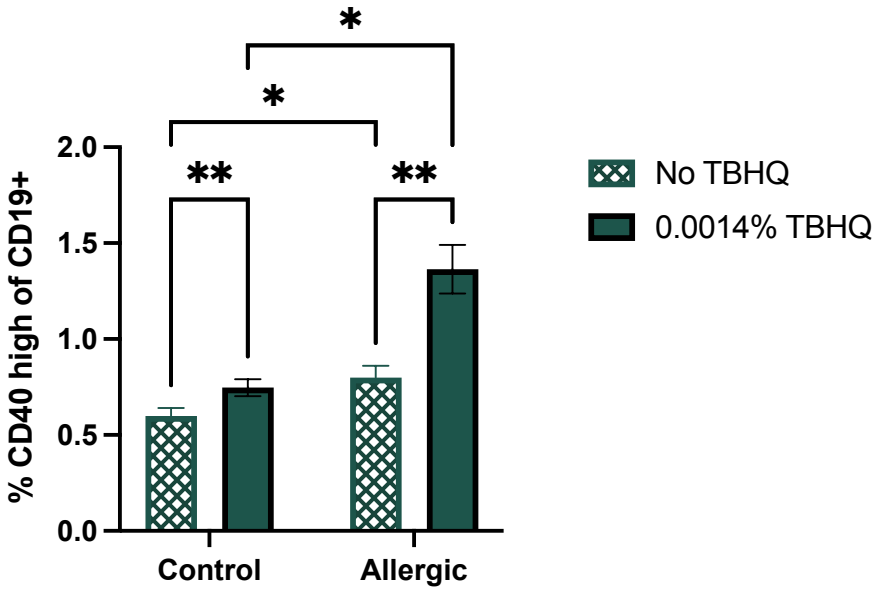


Figure 26. The percentage of B cells highly expressing the costimulatory molecule CD40 is increased with tBHQ consumption, even in unsensitized mice. 5-week-old mice where sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization. Axillary and brachial lymph nodes were isolated, mechanically dissociated, and single cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. Gating strategy involved FCS/SSC selection, doublet exclusion, exclusion of dead cells and gating on CD19+ cells. Shown is the percentage of CD40+ cells of CD19+ cells. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

Increase in IgE following sensitization was not different between tBHQ and control groups

IgG1 is an intermediate in the isotype switching process to IgE[105]. We used intracellular labeling to detect whether IgG1 or IgE were expressed by CD19+ cells . Unexpectedly, there was

no difference in intracellular IgG1 (Fig 27A) in our study, independent of sensitization status or tBHQ exposure. In contrast, IgE expression increased with allergic sensitization in both diet groups (Fig 27B). While we saw a sizeable 40% increase in IgE+ expressing cells (non-tBHQ mice= 2.47%, tBHQ mice = 3.54%) in sensitized mice on the tBHQ diet as compared to those on control diet, this effect was not statistically significant (p=0.21)

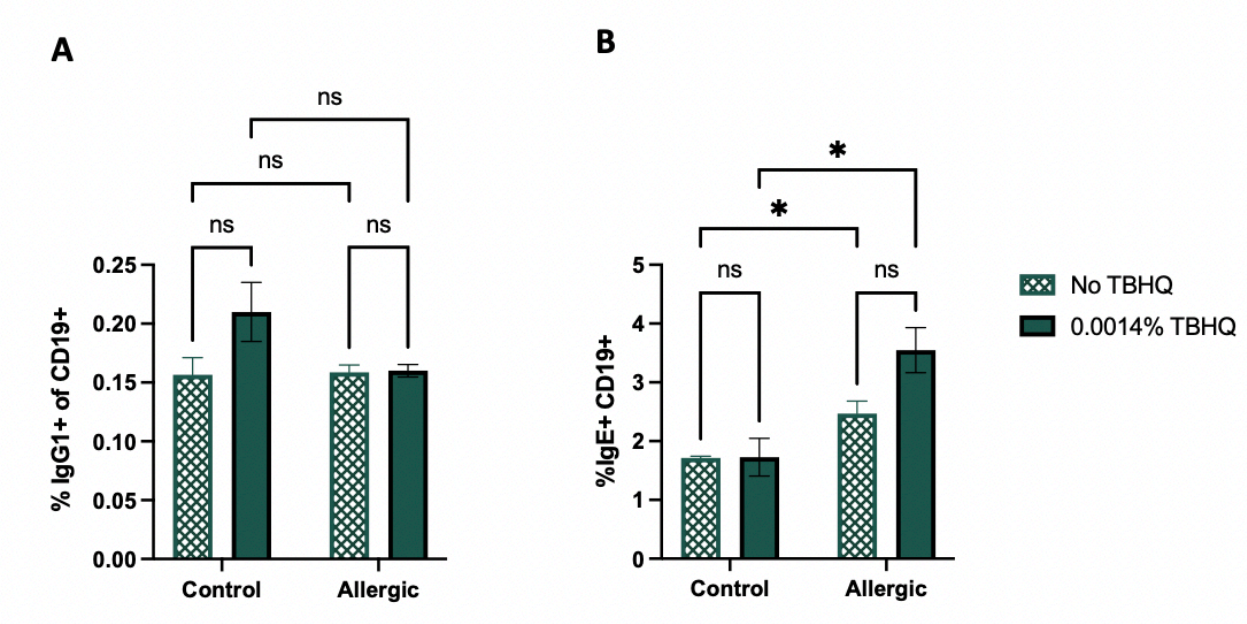


Figure 27. Sensitization has no effect on IgG1 but modestly increases IgE.4-week-old mice where sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization. Axillary and brachial lymph nodes were isolated, mechanically dissociated, and single cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. Gating strategy involved FCS/SSC selection, doublet exclusion, exclusion of dead cells and gating on CD19+ cells. A shows the percentage of CD19+ cells expressing intracellular IgG1. B shows the percentage of CD19+ cells expressing intracellular

Figure 27 (cont'd)

IgE. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

The percentage of CD138 expressing cells among all lymph node-derived cells is increased with both sensitization and tBHQ exposure

CD138 (Syndecan-1) is a marker of plasma cell differentiation and indicates that cells have started producing antibodies [386]. At this stage, B cells typically cease to express traditional B cell markers such as CD19 [387]. We evaluated the percentage of CD138-expressing cells in the lymph nodes and found that there was a baseline increase in CD138-expressing cells when mice were on the tBHQ diet (Fig 28A). Sensitization increased CD138 expression in both the non-tBHQ and the tBHQ groups, but the increase in the tBHQ group was more marked. This was somewhat unexpected, as typically cells exit the lymph node following isotype switching and home to either the bone marrow or peripheral tissues where they produce antibodies. We then measured the number of CD138+ cells that expressed IgE intracellularly, which represents a rare population among lymphocytes. Again, we saw a minuscule, but statistically significant increase in IgE+ plasma cells in unsensitized mice on the tBHQ diet (Fig 30B). Sensitization enhanced the difference in CD138 expression between treatment groups significantly (Fig 28B). This observation dovetails with Figure 22, the plasma IgE data. Overall, the data suggest that tBHQ may promote plasma cell differentiation, and specifically favor IgE-producing plasma cells.

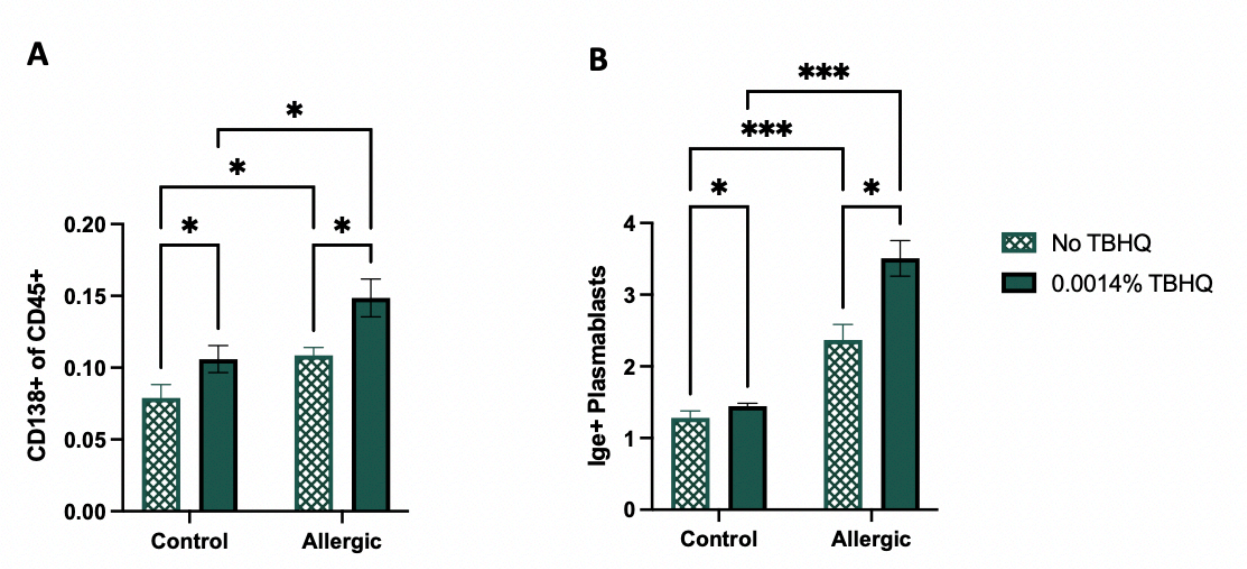


Figure 28. The overall percentage of plasmablasts is elevated in mice fed tBHQ, and the percentage of IgE producing plasma cells increases with both tBHQ and sensitization. 5-week-old mice where sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization. Axillary and brachial lymph nodes were isolated, mechanically dissociated, and single cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. Gating strategy involved FCS/SSC selection, doublet exclusion and exclusion of dead cells. A: In the remaining live lymphocyte population, CD138 surface expression was measured. B: Among CD138+ cells, presence intracellular IgE was measured. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

Discussion

The studies presented here show that exposure to tBHQ via ingestion increases serum IgE and alters B cell phenotypes in this murine model of allergic sensitization to ovalbumin. Allergic sensitization increased the expression of CD69, CD86, CD40 and intracellular IgE on CD19-expressing B cells and increased the percentage of plasma (CD138-positive) cells within the lymph node. IgE is the key molecule in allergic and anaphylactic reactions. In this study, we show a direct impact by the food additive tBHQ on IgE blood concentrations specific to the model antigen. It is important to acknowledge that the dosage of tBHQ used here in this study is very low – it is the standard dose used in rodent chow and less than what model diets estimate for the exposure of a typical western consumer. While this is a specific allergy model in mouse, it suggests the need to consider the impact of tBHQ in humans and raises the question whether tBHQ may alter or impair humoral immunity.

Similarly to the chapters before, we measured the percentage of cells expressing CD80 and CD86, as they are implicated in modulating T cell-B cell interactions and in potentially influence Th1 or Th2 polarization [336, 342]. There is evidence to suggest that CD86 signaling promotes polarization of Th2 cells, a cell type that is known to be causative in allergy, and thus highly relevant to this chapter. Indeed, we saw that mice that were sensitized had a higher percentage of CD86-expressing cells. The percentage of CD80-expressing cells showed a large amount of variation and neither sensitization nor oral tBHQ had an impact. These observations are consistent with the idea that increased CD86 signaling is associated with a Th2 response, as

allergies are typically considered to be Th2-mediated. The hypothesis following this, namely that oral tBHQ increases CD86 expressing B cells, is not supported by the data shown in this chapter. This study focused on 4-week old, female Balb/cj mice. This reductionist study was designed to exclude confounding factors such as age (which we assess in chapter 5), sex, and inbred mouse strain. There are important sex differences in mice, specifically with respect to mast cell function, a downstream effector cell type in food allergies [388]. In general, sex differences in the immune response are well described in mouse models [389] Clinically, in humans, there is a slight skew towards females having a higher incidence of atopic disease and allergies [390].

A discrepancy we observed in this study was the very robust increase in plasma OVA-specific IgE, while the number of IgE-producing B cells was not significantly altered by tBHQ (Fig27B). One of the key things to consider here is that this is a very dynamic process in which B cells develop into either memory cells, plasmablasts or plasma cells. Fig 28B shows that among CD138+ cells of lymphoid lineage, IgE expression is significantly increased when tBHQ is present. CD138+ CD45+ cells signify either plasmablasts or plasma cells, which are committed to the antibody-producing role. The ELISA results shown in Fig 22 show a more marked increase in OVA-IgE plasma concentrations; however, this may be reflective of differences in the parameters as OVA-IgE was quantified in Fig. 22, whereas total IgE+ 138+ cells were quantified in Fig. 27. In addition, protein quantification by ELISA is more sensitive than intracellular labeling and flow cytometry.

The ELISA shown In Fig 22 shows a more marked increase, but we must remember that not only are ELISA typically more sensitive than flow cytometric data, but also that this ELISA measured

OVA-specific IgE compared to general IgE, which is a more precise tool to quantify the response to sensitization.

This study is limited in that it only evaluates cells in the lymph node and therefore in only one compartment of the body. We selected the lymph node because it is the site of the germinal center reaction and somatic hypermutation, which arguably have the largest impact on antibody production and humoral immunity. Future studies into other compartments, such as peripheral blood and bone marrow, would likely also be informative to include—particularly with respect to characterization of the effect of tBHQ on plasma cell differentiation. These findings are important as they indicate that tBHQ promotes allergic sensitization in this model and suggest the need to investigate the effect of tBHQ on allergy in humans. The data also suggest tBHQ may also impact humoral immunity and could potentially alter the efficacy of vaccinations and the generation of high affinity antibodies as a response to seasonal, epidemic, or pandemic infections. Further, the role of tBHQ in the global rise of atopic diseases should be considered. The increasing exposure of the average western consumer to synthetic food additives such as tBHQ may contribute to the prevalence and increasing incidence of food allergies and related diseases, such as asthma, eczema, and allergic rhinitis, in industrialized countries. The significant morbidity and burden of allergic disease on patients warrants further investigation into the toxicological implications of these findings.

Taken together, in this study, we have shown that a very low dose of the synthetic food additive tBHQ, as found in standard mouse chow and standard western diets, promotes the development of allergy in a mouse model closely mimicking human allergy pathogenesis.

Chapter 5: The impact of age on murine B cell response to transdermal sensitization

Abstract

Food allergies are a common and sometimes life-threatening problem encountered in up to 8% of the pediatric population. The incidence of food allergies is increasing in the U.S. and globally. To study food allergies, robust and physiologically relevant animal sensitization models are required. Many models use adjuvants such as cholera toxin and physiologically-irrelevant delivery methods of the potential allergen, such as intraperitoneal injection.

In contrast, transdermal sensitization without an adjuvant closely mimics dermal absorption of food-derived molecules in humans. We have previously shown that dietary exposure of 5-week-old mice to the synthetic food additive tert-butylhydroquinone promotes allergic sensitization resulting in increased plasma concentrations of IgE and increased numbers of plasmablasts. In this study, we investigated the impact of age on the increased allergic sensitization in mice on a tBHQ diet. We found that 5-week-old mice on a tBHQ diet did not show a statistically significant difference in the expression of the activation markers, CD69, CD25, CD80 or CD86, in comparison to those on control diet. Nor did tBHQ impact the number of IgE-producing B cells in 5-week-old mice. However, tBHQ increased the number of plasmablasts in 5-week-old mice, but this effect was diminished with age. Furthermore, while transdermal sensitization to OVA increased the number of IgE+ plasmablasts in 5-week-old mice, this increase was diminished in 8-week-old and 16-week-old mice, suggesting that older animals may be less susceptible to allergic sensitization in this model. Overall, this study shows that age significantly blunts transdermal sensitization in this model and diminishes the enhancing effect of tBHQ on allergic sensitization.

Introduction

Food allergies are a significant public health concern [391]. About 8% of children and 4% of adults have some form of allergic reaction to food-derived antigens during their lifetime [392, 393] and the incidence has been rising over the past few years [392]. In some western states, the rate of the most severe outcome of food allergies, anaphylactic shock, increases by 10% every 10 years [374, 377]. In the previous study we have shown that the synthetic food additive tBHQ can increase the production of IgE and the number of plasma cells following sensitization. tBHQ is an activator of Nrf2 and has been shown to promote Th2, while inhibiting Th1, polarization [212]. Nrf2 belongs to the subfamily of Cap'n'collar proteins and has a well-conserved CNC domain [381]. As stated above, Nrf2 can be activated by tBHQ, but also by a host of cellular stressors, such as heavy metals, food additives, and nucleophilic compounds [359]. Under non-stress conditions, Nrf2 is bound to its repressor protein Keap-1. Upon activation by the aforementioned compounds, Nrf2 translocates to the nucleus and activates the antioxidant response element, triggering the expression of cytoprotective genes [360, 383, 394]. Under non-stress conditions, Keap-1 polyubiquitinates Nrf2 and directs it thus to proteasomal degradation [360]. In this study, we explore the impact of age at the start of sensitization by comparing mice that started sensitization at 5 weeks of age, 9 weeks of age and 16 weeks of age. We hypothesize that similar to epidemiological observations in humans [391], older mice are less susceptible to the development of food allergies.

Methods

Materials

The sources of fluorescent antibodies are shown in table 5. Rodent diets were purchased from Dyets (Bethlehem, PA) and stored at -20C. PBS for cell preparation was purchased from Lonza (Thermo-Fisher, Waltham, MA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

Sensitization

All animal protocols are in compliance with the Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. BALB/cj mice were acquired from Jackson Laboratories and housed in a specified germ-free environment. Mice were fed either AIN-93G rodent chow (containing 0.014% tBHQ) or tBHQ-free AIN-93G. At 4, 8, or 16 weeks of age, mice were shaved thoracodorsically to expose the epidermis. One day later, 100µl of 100mg/ml chicken ovalbumin (Merck, Darmstadt, Germany) in 0.9% saline or just 0.9% saline were applied to the backs of the mice and subsequently covered with a commercially available flexible fabric bandage (Johnson & Johnson, New Brunswick, NJ). The procedure was repeated every 7 days for 3 weeks for mice in the 5-week age group and for 4 weeks in the 9 and 16-week age group. Lymph nodes and blood were harvested three days after their last sensitization. Lymph nodes were mechanically dissociated to collect lymphocytes. Brachial and axial lymph nodes of each mouse were processed together and pooled, to account for anatomic variation in lymph node size and grouping. Each sample represents lymphocytes from a single mouse, pooled from axillary and brachial lymph nodes,

which we have previously determined to be the primary draining lymph nodes. Lymphocyte single cell suspension in PBS was washed twice prior to culturing the cells in 96 well U bottom microplates (Greiner, Kremsmünster, Austria).

Flow cytometry

Cells were washed twice in Phosphate buffered saline and stained with amine-reactive live/dead blue (Invitrogen, Waltham MA). After 30 min, cells were washed twice with FACS buffer, and anti-Fc-gamma receptor I, II, III (anti-CD16, anti-CD32, anti-CD64, Invitrogen, Waltham, MA) was added to diminish non-specific binding. After 15 minutes, fluorescent surface antibodies (see Table 1) were added and incubated for 30 min. After a FACS wash, cells were subjected to permeabilization using the mouse FOXP3 intracellular staining kit (BD Pharmingen, San Diego, CA). After 1 hour, cells were washed twice using Perm/Wash Buffer (BD Pharmingen, San Diego, CA) and incubated with intracellular antibodies (see Table 2). After 30 minutes, cells were washed twice with Perm/Wash Buffer and fixated using Cytofix (BD Pharmingen, San Diego, CA). Cells were then resuspended in FACS buffer and analyzed on a Cytek Aurora (Cytek Biosciences, Fremont, CA). The data presented herein were obtained using instrumentation in the MSU Flow Cytometry Core Facility. The facility is funded in part through the financial support of Michigan State University's Office of Research & Innovation, College of Osteopathic Medicine, and College of Human Medicine. FCS files were analyzed using FlowJo (BD Bioscience, Waltham, MA).

Table 6. Fluorescent antibodies used in Chapter 5.

Target	Fluorophore	Clone	Manufacturer
CD 4	BUV 496	GK1.5	BD
OX 40	BUV 563	OX-40	BD
CD 45	BUV 615	I3/2.3	BD
B220	BUV 805	RA3-6B2	BD
OX40L	BV421	RM134L	BD
IgE	BV480	R35-72	BD
CD19	BV570	6D5	Biolegend
CD40	SB600	1C10	Invitrogen
IgG1	BV650	RMG1-1	Biolegend
GATA-3	BV711	L50-823	BD
CD86	BV785	GL-1	Biolegend
I-A/I-E (MHC II)	FITC	2G9	Biolegend
PD-1	BB-700	J43	BD
CD80	PerCPCy5.5	16-10A1	BD
CD23	PerCP-eFluor 710	B3B4	Invitrogen
CXCR5	PE	L138D7	Biolegend
IL-4	PE-Dazzle 594	11B11	Biolegend
FOXP3	Pe-Cy5	FJK-16s	Invitrogen

Table 6 (cont'd)

CD25	Pe-Cy7	3C7	Biolegend
Bcl-6	APC	7D1	Biolegend
CD138	AF647	281-2	Biolegend
CD3	AF700	17A2	Biolegend
CD69	APC-Fire 750	H1.2F3	Biolegend
CD40L	SB 436	MR1	BD
CXCR5	BUV737	2G8	BD
IgM	BV480	R6-60.2	BD

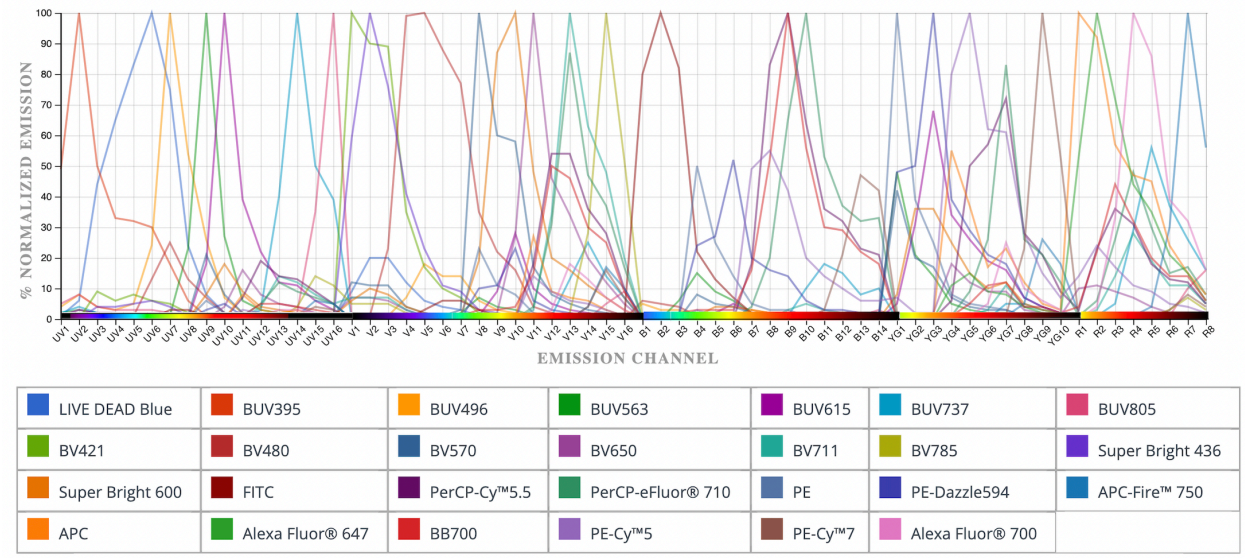


Figure 29. Spectral unmixing sample of the *in-vivo* panel. Exemplary spectral analysis of a model sample in which all fluorophores were present.

Analysis

The data were compiled from 5 mice per group and are presented as the mean ± standard error of the mean. Each data sample represents measurement from one individual mouse. Two-way ANOVA was used to determine statistical differences between treatment groups, and Šídák's post hoc test was used to compare the diet groups and sensitization groups (row and column effects). Calculations and graphical visualization of the results were done using PRISM Graphpad 9.20 software (La Jolla, Ca). ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$

Results

Transdermal sensitization results in CD19+ expansion in 5-week-old and 9-week-old mice

After sensitization, we expect an increase in the B cell population due to clonal expansion and the germinal center reaction. The number of B cells increases during this process. In this study, we defined B cells as CD19+ cells, and CD3- cells. Indeed, we saw a statistically significant increase in the 5-week-old mice and the 9-week-old mice, and a diminished, non-significant trend in the 16-week-old mice (Fig. 30). The increase in the 5-week-old mice was much more robust than in the older subjects.

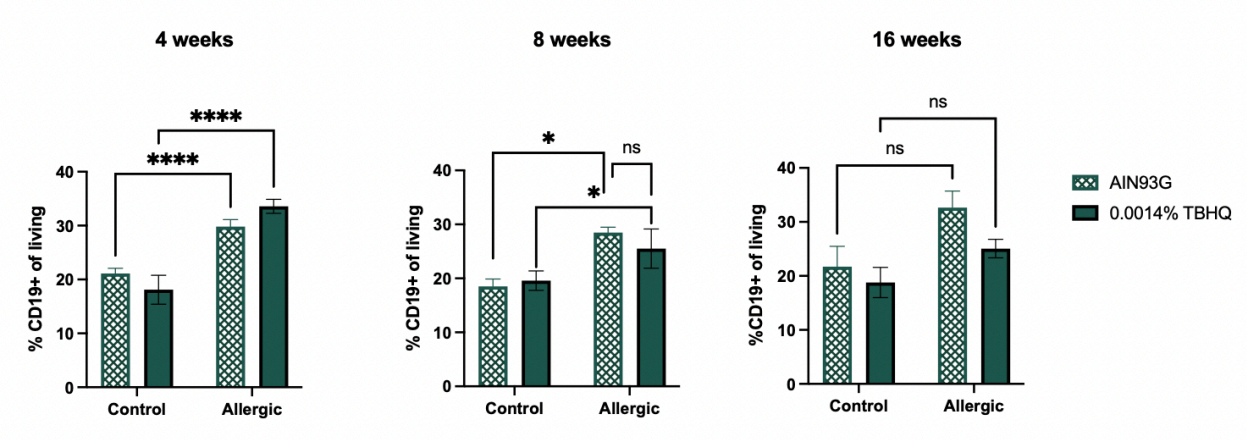


Figure 30. The percentage of CD19+ cells increases more strongly after sensitization in young mice. Mice at different ages were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization for the 5-week-old mice and 3 days after the 4th sensitization for older mice. Axillary and brachial lymph nodes were isolated, and mechanically dissociated, and single-cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. The gating strategy involved FCS/SSC selection, doublet exclusion, and exclusion of dead cells. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

CD69 is a C-type lectin protein that is expressed early in lymphocyte activation.

We hypothesized that sensitization would increase the percentage of CD69-expressing B cells. Approximately 4% of B cells express CD69 in naïve 5-week-old mice, and sensitization significantly increases this percentage (Fig 31A). In 9-week-old mice, we see a comparably higher baseline of CD69 expression, but no increase after sensitization. In mice aged 16 weeks at the start of the treatment, the baseline of CD69 expression is very low with considerable variability, and there is no significant increase in expression following allergic sensitization. tBHQ does not show any effect in either naïve nor sensitized mice of any age group. CD25 was not affected by allergic sensitization or tBHQ treatment (Fig 31B). Of note, there was an odd and unexpected high percentage of CD25 expressing cells in the unsensitized, tBHQ-fed 16-week-old mice that was statistically significant. Currently, it is not clear at this time why this spike occurred and whether it is biologically significant.

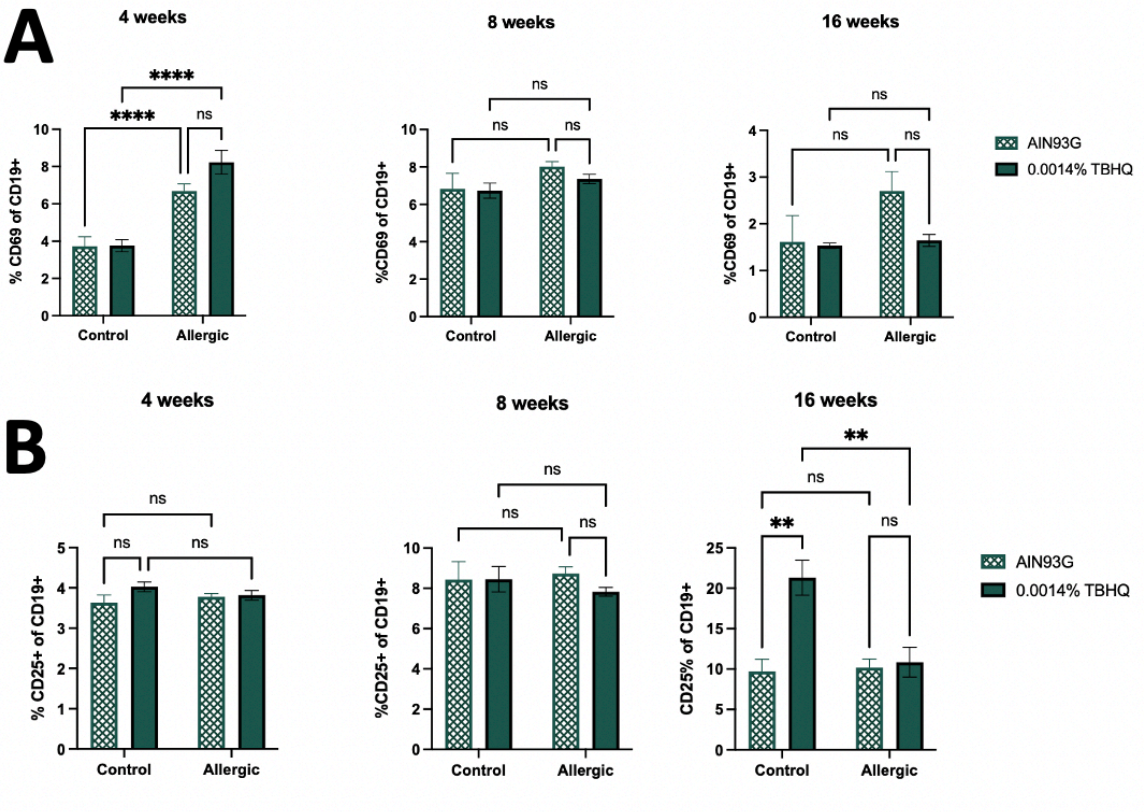


Figure 31. Contrasting patterns of induction for CD69 and CD25 after sensitization. Mice at different ages were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization for the 5-week-old mice and 3 days after the 4th sensitization for older mice. Axillary and brachial lymph nodes were isolated, and mechanically dissociated, and single-cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. The gating strategy involved FCS/SSC selection, doublet exclusion, and exclusion of dead cells. A shows the percentage of CD19+ cells expressing CD69. B shows the percentage of CD19+ cells expressing CD25. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, **

Fig 31 (cont'd)

indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

Expression of CD86, but not CD80, is induced on CD19+ B cells following allergic sensitization

The costimulatory molecules CD80 and CD86 interact with receptors at the T cell surface and are considered robust activation markers of B cell activation. There is some evidence that the CD80:CD86 ratio can influence T cell cytokine secretion and therefore serve as a signaling pathway in the adaptive immune response [395, 396]. In this study, we observed some variability, but no effect of either tBHQ nor sensitization status on CD80 expression. Interestingly, the baseline expression of CD80 increased in the 9-week-old mice compared to 5-week-old mice and was greatest in the 16-week-old mice (Fig 32). In contrast, CD86 expression increased with sensitization in all age groups, though the overall induction was somewhat blunted in the 16-week-old animals. The induction of CD86 in 16-week-old mice following sensitization was further dampened in mice exposed to tBHQ (Fig 33).

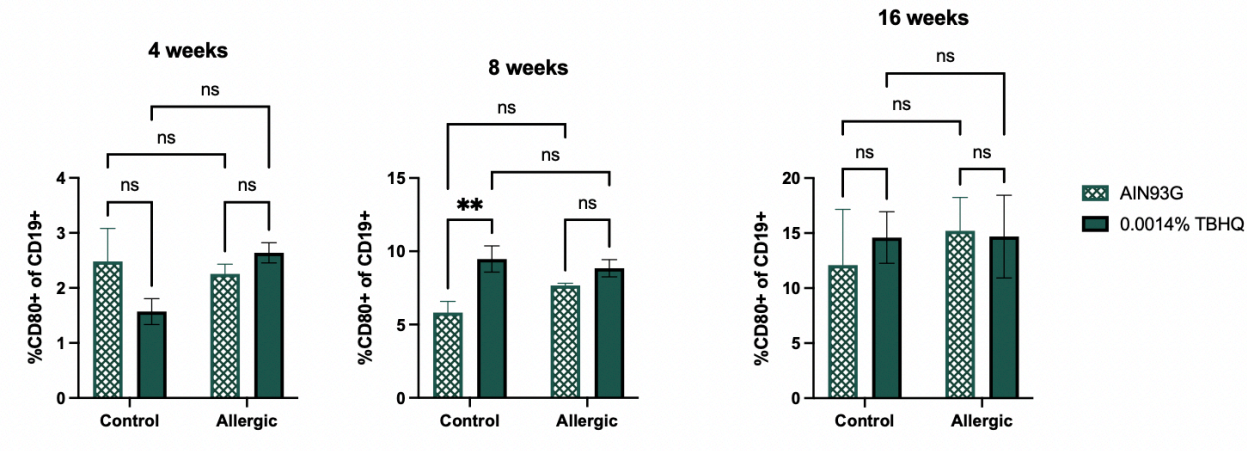


Figure 32. CD80 expression does not change with sensitization independent of age. Mice at different ages were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization for the 5-week-old mice and 3 days after the 4th sensitization for older mice. Axillary and brachial lymph nodes were isolated, and mechanically dissociated, and single-cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. The gating strategy involved FCS/SSC selection, doublet exclusion, and exclusion of dead cells. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance p<0.05, ** indicates significance p<0.01, *** indicates significance p<0.001, **** indicates significance p<0.0001)

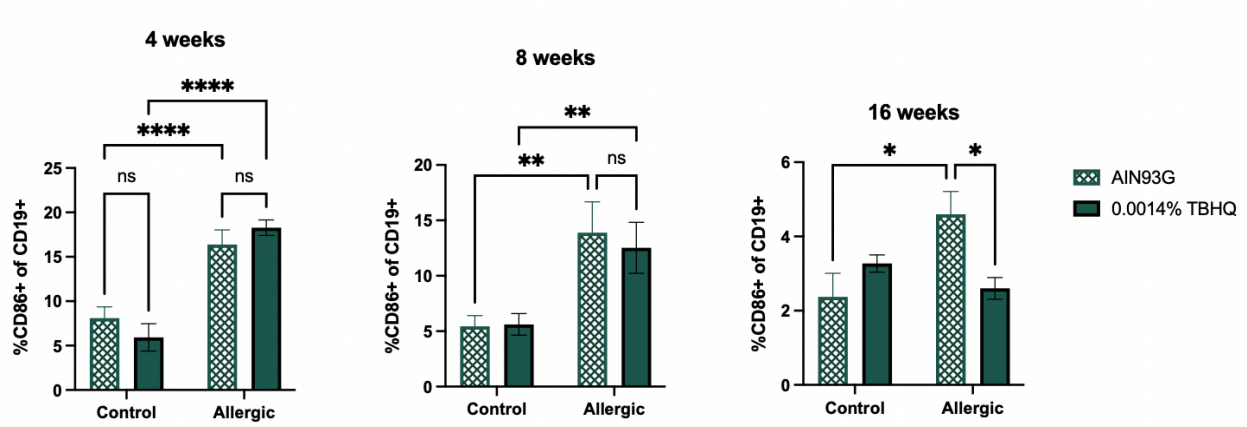


Figure 33. CD86 is increased with sensitization and the amplitude of this effect is inversely correlated with age. Mice at different ages were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization for the 5-week-old mice and 3 days after the 4th sensitization for older mice. Axillary and brachial lymph nodes were isolated, and mechanically dissociated, and single-cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. The gating strategy involved FCS/SSC selection, doublet exclusion, and exclusion of dead cells. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

Exposure to tBHQ does not impact the percentage of IgE+ or IgG1+ B cells following sensitization in any age group

Following activation and in certain cellular microenvironments, B cells may undergo antibody class switching in which they transition away from production of IgM to producing one of the other classes of antibody. IgE and IgG1 are classes of antibodies that are associated with type 2 immune responses and allergies. Thus, we measured whether tBHQ and allergic sensitization

increased the percentage of B cells expressing IgE and IgG1. In Fig 34, we found that the percentage of IgE-expressing B cells increased robustly with sensitization in 5-week-old mice and 9-week-old mice, but that tBHQ did not have a significant effect. In 16-week-old mice, there was greater variability with a trend towards an increase due to sensitization in the control diet group, but not in the tBHQ group. IgG1, an immunoglobulin often seen as an intermediate precursor to IgE, is also sometimes associated with allergy. In contrast to IgE, there was no effect of sensitization on IgG1 in the 5-week-old mice at this time point, and generally a low baseline expression. In the 9-week-old group, however, there was a robust increase in sensitization in both the control diet and tBHQ groups. In 16-week-old mice, the baseline expression was very low again, and considerable variability was observed (Fig. 35)

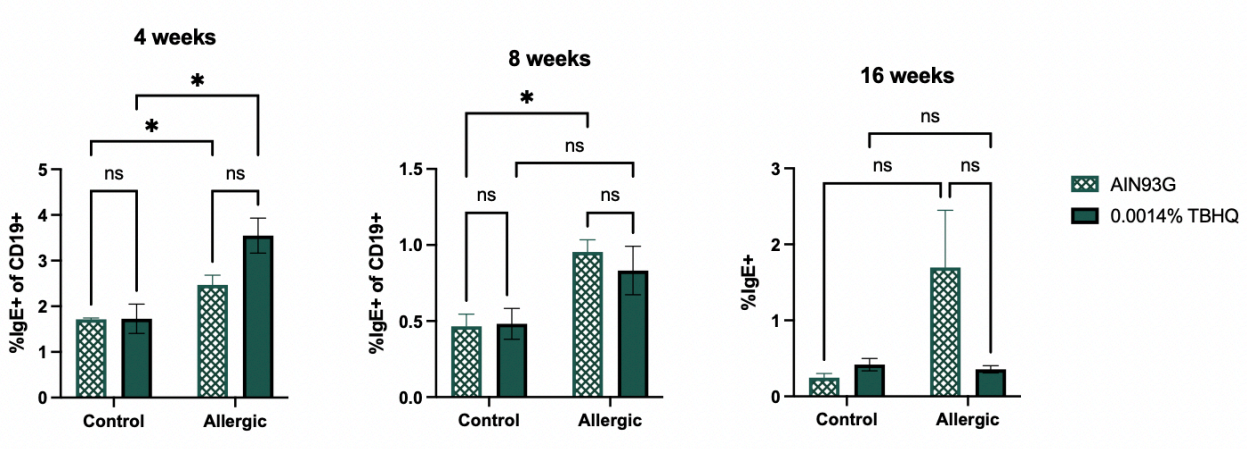


Figure 34. The percentage of IgE-producing CD19+ cells increases with sensitization in the younger groups but not in mice who start sensitization at 16 weeks. Mice at different ages were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization for the 5-week-old mice and 3 days after the 4th sensitization for older mice. Axillary and brachial lymph nodes were isolated, and mechanically dissociated, and single-cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. The gating strategy involved FCS/SSC selection, doublet exclusion, and exclusion of dead cells. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance relative to the VEH group, $p<0.05$, ** indicates significance relative to the VEH group, $p<0.01$, *** indicates significance relative to the VEH group, $p<0.001$, **** indicates significance relative to the VEH group, $p<0.0001$)

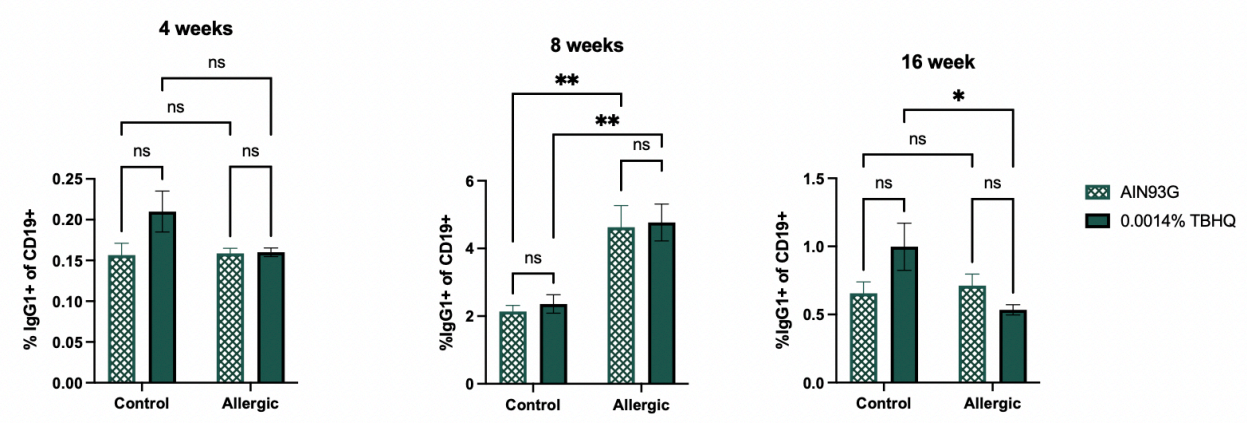


Figure 35. IgG1-producing CD19+ cells increase in mice that start sensitization at 8 weeks, but not in younger or older mice. Mice at different ages were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization for the 5-week-old mice and 3 days after the 4th sensitization for older mice. Axillary and brachial lymph nodes were isolated, and mechanically dissociated, and single-cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. The gating strategy involved FCS/SSC selection, doublet exclusion, and exclusion of dead cells. Data are presented as the mean ± SE. N=5 per group (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

Dietary exposure to tBHQ promotes plasmablast differentiation in 4-week-old, but not older, mice

In addition to class-switching, B cells are also able to differentiate into plasma cells, which produce large amounts of antibodies and are thought to be the largest producers of IgE in allergy. The expression of CD138 can be used to identify plasma cells. Accordingly, we assessed the

percentage of CD138+ plasma cells among all living cells (Fig 36). In the 5-week-old mice, both the presence of tBHQ and sensitization status significantly increased the number of plasma cells (defined as CD138+ CD45+ cells). 9-week-old mice showed a similar trend, but much less pronounced and not statistically significant. Among 16-week-old mice, there was considerable variability, but no observed effect of either sensitization or tBHQ.

Taking a closer look at these plasmablasts, we measured the percentage of CD138+ cells that expressed intracellular IgE (Fig 37). Interestingly, in mice aged 5 weeks at the start of sensitization, both dietary tBHQ and allergic sensitization increased the percentage of IgE-producing plasmablasts. In mice 9 weeks at the start of sensitization, only sensitization increased the percentage of plasma cells, whereas tBHQ had no effect. In mice 16 weeks at the start of sensitization, no effect was seen with either tBHQ or sensitization. Overall, concerning IgE-producing plasma cells, the data suggest that younger mice are more sensitive to both transdermal sensitization and dietary tBHQ exposure.

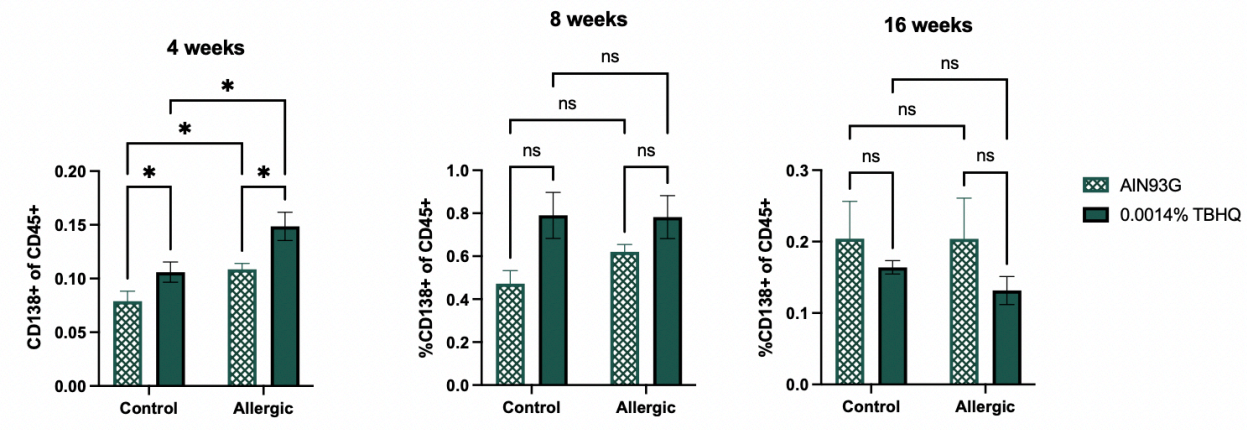


Figure 36. The percentage of plasmablasts increases with sensitization and with dietary tBHQ independently in younger mice, but not in older mice. Mice at different ages were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization for the 5-week-old mice and 3 days after the 4th sensitization for older mice. Axillary and brachial lymph nodes were isolated, and mechanically dissociated, and single-cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. The gating strategy involved FCS/SSC selection, doublet exclusion, and exclusion of dead cells. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance p<0.05, ** indicates significance p<0.01, *** indicates significance p<0.001, **** indicates significance p<0.0001)

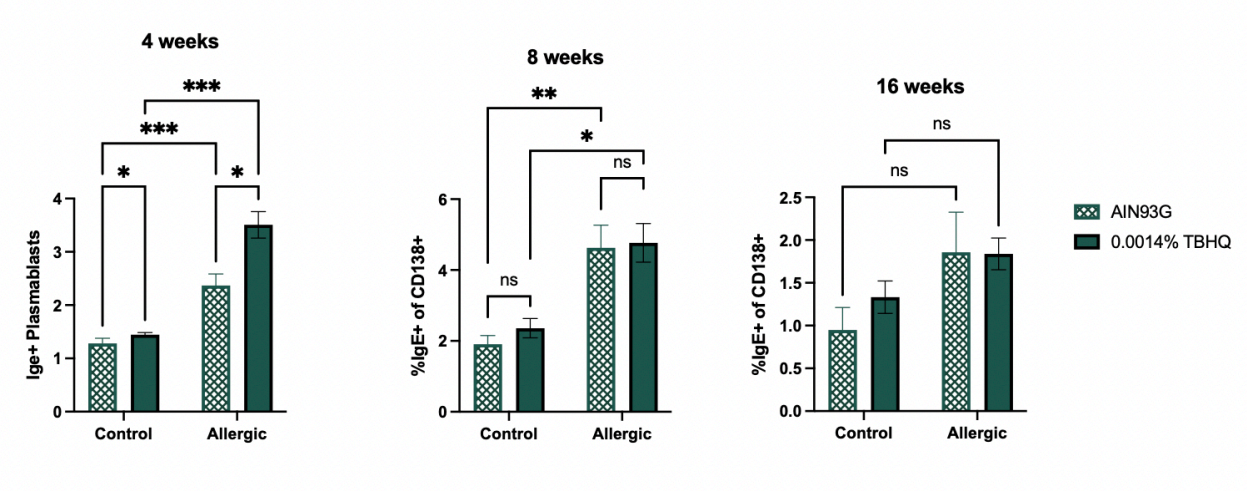


Figure 37. The percentage of plasmablasts producing IgE is increased with both sensitization and dietary tBHQ in younger mice, and only with sensitization in mice 8 weeks old at first sensitization. Mice at different ages were sensitized via their thoracodorsal skin against OVA 3 times and harvested 3 days after the third sensitization for the 5-week-old mice and 3 days after the 4th sensitization for older mice. Axillary and brachial lymph nodes were isolated, and mechanically dissociated, and single-cell suspension of lymphocytes was labeled with fluorescent antibodies and analyzed on a Cytek Aurora spectral flow cytometer. The gating strategy involved FCS/SSC selection, doublet exclusion, and exclusion of dead cells. Data are presented as the mean ± SE. N=5 per group. (ns indicates not significant, * indicates significance $p<0.05$, ** indicates significance $p<0.01$, *** indicates significance $p<0.001$, **** indicates significance $p<0.0001$)

Discussion

In this study, we demonstrate that there is a considerable effect of age at the start of sensitization in our transdermal sensitization model. Mice that are 5 weeks at the time of first sensitization have a much stronger response than 9-week-old mice, which in turn have a stronger response than 16-week-old mice. This tracks with human epidemiological data, where we see that young children are much more likely to develop new food allergies compared to adolescents, adults, or geriatric populations, in that order [391].

From previous, unpublished data and based on the clinical data in humans, we were concerned that older animals, such as in the 9-week-old and 16-week-old groups, would have diminished immune response to the allergen. Thus, we used a more robust sensitization regimen that included 4 sensitizations. The younger mice, which we knew had a robust IgE response, were only sensitized 3 times, as previous studies indicated that this was the optimal timepoint to detect both B cells and TFH cells in the lymph node. On the one hand, this slightly diminishes the meaningfulness of the comparisons made in this chapter, but on the other hand, 4 weeks of sensitization should, in theory, enhance the effect of sensitization allowing for better comparisons of the impact of tBHQ on allergic sensitization in animals of different ages. Interestingly, only the 9-week-old mice showed an increase in IgG1, an immunoglobulin considered an intermediate in IgE class switching, compared to the 5-week-old, which showed an increase in IgE. There are multiple possible explanations for this observation. In this study, we only observe one immunological compartment, the lymph node. B cells that differentiate into plasma cells will eventually leave the lymph node and either infiltrate antigen-rich tissues or take

up residence in the bone marrow, where they perpetually secrete immunoglobulins into the bloodstream [338]. There is a possibility that the timepoint of migration is different depending on the age of the mouse. Evaluating other compartments such as peripheral blood and bone marrow might reveal more about the plasma cell population. A murine study where all these immunological compartments are harvested simultaneously might shed some light on this. Also of interest would be a time-course study, evaluating the increase in plasma IgE levels throughout the course of sensitization at different ages. Interestingly, different allergens seem to affect different age groups in humans – toddlers and infants suffer more often from allergy to milk and egg, while adolescents react more often to nuts and shellfish [390, 397]. Considering this, a mouse study evaluating the induction of food allergies to different antigens in different age groups would further optimize this model. Once an antigen-specific optimal age is determined, we would be able to determine the impact of tBHQ on food allergy in the context of different, common food antigens.

Compared to adults, B cell subsets in peripheral blood, bone marrow and secondary lymphoid organs are not well characterized. Despite a dearth of information in this area; however, it is clear significant changes occur in the B cell population during infancy and childhood. Newborns have relatively fewer circulating lymphocytes overall, including B cells, in blood as compared to older infants and children [398]. Human infants have the greatest percentage of naïve B cells as compared to older children and adults where young infants have the highest percentage of naïve B cells which gradually decrease to adult levels by around 5 - 10 years of age. There is a concordant increase in the memory B cell population that is inversely proportional to the naïve

B cell population in humans. It seems likely there may be an activated B cell state that occurs during the transition of naïve B cells to memory B cells, however this has not been characterized. Our study shows a gradual increase in markers of B cell activation (CD25 and CD80) in mice with age. While it is difficult to directly correlate the ages of mice with those of humans, there are some developmental milestones that can be compared. Mice begin eating solid food at approximately 12 days of age, which occurs in humans at around 6 months of age. Mice begin to become reproductively mature at around 6 weeks of age, which could roughly approximate puberty. Thus, the mice in our youngest group (5 weeks at first sensitization) could be considered somewhat equivalent to pre-pubescent humans at the beginning of sensitization and adolescents at the end of sensitization. Although we did not directly assess B cell memory in this study, the increase in B cell activation markers over time suggests that mice, like humans, may have an increase in B cell memory as juveniles develop into adults.

In this present study, we demonstrated that there is a significant effect of age on transdermal sensitization in a rodent model. To our knowledge, we are the first group to evaluate this parameter in this very physiologically applicable model of food allergy. This informs the design of subsequent studies and makes a valuable argument for the enrollment of younger mice into allergy studies.

Chapter 6: Conclusions

Discussion of findings

In the chapters above, we investigated the impact of Nrf2 activators on various aspects of B cell function, including polyclonal in vitro activation, antiviral immunity, and allergic sensitization. Chapter 2 demonstrated that in an *in vivo* infectious disease model, the heavy metal arsenic trioxide impairs B cell activation and function. In chapter 3, an *ex vivo* rodent model modeling T cell-dependent B cell activation showed that tBHQ impacts this activation in a partially Nrf2-dependent fashion. Chapter 4 took this finding *in vivo* and showed that in a murine model of allergic sensitization, tBHQ exacerbates the allergic phenotype, including higher serum IgE, a higher percentage of IgE-producing B cells, and higher activation status of B cells. In Chapter 5, we evaluated the impact of age on the efficacy of the allergic sensitization model, finding that younger mice are much more susceptible to the induction of allergy. This tracks with human epidemiological studies. Taken together, these findings suggest that common environmental toxicants that stimulate Nrf2 signaling can impact B cell activation and function as well as the ratios of Immunoglobulins produced.

A common thread in these studies was that the activation markers CD69 and CD25 were impaired in the presence of ATO and tBHQ, respectively. In chapter 2, I found that CD25 expressing cells were increased after IAV challenge and decreased with ATO. In chapter 3, I showed that both CD25 and CD69 increase after activation *ex vivo* and decrease with 1µM of tBHQ. In chapter 4 and 5, I observed that during allergic sensitization *in vivo,* CD69 increased, but not CD25 in mice that are 5 weeks of age at the start of sensitization, and that effect disappeared when mice were older at the beginning of sensitization. The common thread in between different species and

toxicants is that there is a decrease in activation markers when Nrf2-activating compounds are present. With the data collected thus far; however, I cannot distinguish between an overall inhibition versus a delay in B cell activation or some combination of these two possibilities.

Similarly, we saw disparate results regarding the expression of the costimulatory molecules CD80 and CD86. As mentioned above, CD80 and CD86 bind to their ligands CD28 and CTLA-4 on T cells to convey stimulatory and inhibitory signals, respectively. CD86 signaling through CD28 can stimulate T cells towards a Th2 phenotype, while CD80 signaling via CD28 can stimulate towards a Th1 phenotype [336, 342]. In chapter 2, we saw that CD80 expression was decreased by ATO, while CD86 was not. In chapter 3, we saw that there was no increase in CD80-expressing cells in the activated groups, and tBHQ did not change the percentage of CD80-expressing cells. CD86 on the other hand was increased with activation and decreased at 1μM tBHQ. In Chapter 4 and 5, we saw that allergic sensitization in vivo did not impact levels of CD80-expressing cells, but increased CD86-expressing cells in sensitized mice independent of their diet. Considering the immunology of B cells in these models, this makes sense, as CD86 is the Th2-associated costimulatory molecule. In characterizing the role of Nrf2, the image is less clear – ATO decreases CD80 but not CD86 in a human *in vitro* model, while CD86 is decreased by tBHQ in a murine *ex vivo* model. With the data accumulated in this thesis, it is not possible to reconcile these disparate findings in a meaningful way. While the models and toxicants differ too much to draw conclusions, it is important to note that ATO and tBHQ activate Nrf2 via different molecular mechanisms, which may have functionally distinct outcomes. These findings, disparate as they may be, are highly interesting though, as the role of CD80 and CD86 in stimulating T cells is

underexplored in the context of infectious disease and allergy. The results presented here warrant more research into the role and impact of costimulation and how different signals change the ratio in which those costimulatory molecules are expressed. For toxicologists, a potential impact of environmental toxins on the CD80/86 expression could be a promising lead to explain immunological alterations.

Overall, the main function of B cells is the production of immunoglobulins. Antibodies need to have the right antigen affinity and the right isotype for optimal function. These studies have, with the exception of chapter 4, not directly measured antibody production, antibody specificity and antibody isotypes. Instead, I took proxy measurements, such as Ig subclasses expressed on the surface of B cells as part of the B cell receptor or intracellular Ig subclasses. In Chapter 2, I measured surface IgG as a response to IAV challenge. IgG is a later immunoglobulin class and only expressed on cells that have undergone isotype switching. Measuring the percentage of cells with IgG on their surface, therefore, served as a proxy for cells that have undergone isotype switching, or their daughter cells as this population expands. The measured increase of IgG+ cells in response to IAV is likely due to the expansion of a population of antigen-experienced B cells that recognized IAV antigen.

Chapter 3 used a much earlier time point to measure cell status, and labeled IgG1 specifically, one of the earliest immunoglobulins produced during isoclass switching in mice. In this assay, we used intracellular labeling, and were able to detect IgG1 inside the cell, which is a more sensitive assay than surface labeling. IgG1 was of particular interest because it can be a precursor to both IgG and IgE. There was a tBHQ concentration-dependent decrease in the counts of IgG1-

producing cells, which may signify either inhibition or delay of immunoglobulin production. An extension of this assay, with later collection timepoints and an added supernatant analysis for immunoglobulin content may shed light on this question. In chapters 4 and 5, I used an allergic model, and IgE was of particular interest. I used both intracellular staining to assess the percentage of B cells and plasmablasts actively producing IgE and OVA-specific IgE ELISA on plasma, which measures IgE that specifically binds to the model allergen. Taken together, the experimental data suggests that in the context of influenza in humans, ATO suppresses the percentage of IgG+ B cells, which likely would lead to lower overall levels of IgG specific to IAV. In our generic T cell-dependent B cell activation model, tBHQ lowered the number of IgG1-producing cells among mouse splenocytes. In contrast to those findings, dietary tBHQ *in vivo* increased the percentage of IgE-producing B cells, plasma cells and plasma IgE in the allergy model. Does this mean that tBHQ decreases IgG production and favors IgE production? It would be premature at this point to draw this conclusion; however, future studies could be designed to test this hypothesis.

There are several limitations to these studies. In chapter 2, we used human PBMCs, which are a specific subtype of B cells and differ from B cells found in the spleen and lymph nodes, where the germinal center reaction is located and where isotype switching and clonal expansion occur. This study also only used H1N1 influenza, a single antigenic target, to which only a subset of PBMCs responds. This limitation was addressed in Chapter 3, where we used a polyclonal activation cocktail that is not limited to a specific antigen but activates B cells in a way that closely mimics T cell-dependent activation without the need for an antigen. As we were able to use cells from

both wild-type mice and mice genetically ablated for Nrf2, we could demonstrate that these effects are at least partially Nrf2-dependent. In Chapter 4, we evaluated the impact of dietary tBHQ on the development of food allergy in young mice by inducing atopy using a transdermal sensitization model. This study did not use Nrf2-deficient mice and thus, the role of Nrf2 in the effects of tBHQ could not be evaluated. Chapter 5 evaluated the impact of age in the same allergic sensitization model.

Relative to other immune cell types, comparably less work has been done on the toxicology of B cells, and most of this work has focused on AHR signaling [149-151, 153, 155, 157]. The studies described in this dissertation add to this understudied research area and add a class of ubiquitous toxicants. The generation of diverse antibodies is of utmost importance as deficiency leads to severe clinical outcomes, such as hyper-IgM syndrome, which is an immunodeficiency defined by a lack of CD40-CD40L signaling. This is a key process to the B cell activation model used in chapters 3 and 4 [58].

In general, alterations in these signaling and activation pathways have the potential to greatly impact humoral immunity. It is important to note that for the average, immunocompetent western subject, humoral immunity operates on a high level, and standard vaccinations routinely elicit high titers of high-affinity antibodies, sometimes lasting for a lifetime, while xenobiotics like tBHQ are ubiquitous in everyday life. In that context, it seems unlikely that tBHQ has a major impact on B cell function related to humoral immunity. More likely is a small, insidious effect that subtly changes outcomes in immunoglobulin production, which is what I hypothesize based on the data shown here. However, small changes may be more dramatic and consequential if a

person is, due to genetic or pharmaceutical reasons, immunosuppressed or deficient. Furthermore, small, insidious processes are hard to measure in human populations due to the inherent variability between subjects, both genetically and behaviorally. B cell activation happens in secondary lymphoid organs, such as the spleen and the lymph node, and access to these tissues is almost impossible in human subjects. If they are excised, it is usually in the context of malignancy or other severe pathological processes, which change the dynamic in these organs, and pathology usually requires these organs for grading of disease processes. Biopsies like fine needle aspirations are theoretically possible but must be done under local or general anesthesia and are major procedures and thus unlikely to be available to basic scientists. PBMCs, even with the limitations stated above, are our best tool for immunotoxicity testing in human subjects. Our rodent models come with their limitations too, as rodent immune systems are only an approximation of human immune systems. The applicability of observations in mouse models to humans has been hotly debated in the scientific community [399]. Nonetheless, the results reported in chapters 2,3, and 4 are reproducible and indicate that these compounds alter B cell activation and effector function.

Allergy and atopic disease can be described, in a very simplified way, as overactivation of the immune system resulting in an inappropriate response to innocuous antigens. The discussion in the paragraphs above focused mainly on extrapolating the effects presented in this thesis in the context of humoral immunity. Looking at the results, specifically in chapter 4, through the lens of allergy, paints a different picture: the aberrant activation of the immune system is further exacerbated, and the immunoglobulin IgE is increased when tBHQ is present. Studies in our lab

that are yet to be published have shown that more advanced endpoints, such as anaphylactic shock, are also more severe in tBHQ-fed mice compared to non-tBHQ-fed mice. As mentioned in the introduction, the incidences of food allergies and anaphylactic shock are rising in the western world [378, 393, 400]. The data presented herein is not sufficient to establish a causal relationship between the increased prevalence of food allergy in humans and tBHQ exposure but is congruent with the hypothesis that dietary tBHQ promotes IgE sensitization in mice. B cells of sensitized mice that were fed tBHQ show higher expression of CD40, a higher percentage of IgE-expressing CD138 cells, and an increase in plasma IgE concentrations. The data suggest the need for further investigation into the impact of tBHQ on allergy in humans.

Significance of findings

Further characterization of these impairments is necessary, and these and further studies could be used to inform regulatory decision-making. For the public, these results may help personal decision-making regarding the consumption of highly processed foods that contain tBHQ. As mentioned above, it is important to strike a healthy balance in interpreting these results. On the one hand, the results are real, significant, and reproducible, on the other hand, we can estimate that most humans in the western world are exposed to Nrf2 activating compounds such as tBHQ daily, and the vast majority of humans in the western world have normal, effective humoral immunity. It is reasonable to recommend the avoidance of processed foods if possible and regular testing of well water for heavy metals, but we must be careful not to slip into alarmism and over-exaggeration of results. On the other hand, the number of people perishing from infectious diseases such as influenza, SARS-COV-2, and others, is still staggering. Infectious disease was the third leading cause of death in 2020, with over 400.000 deaths [401]. If there was a way to mitigate human exposure to ATO and tBHQ, and if this hypothetical change would cause a small but noticeable change in B cell function, would there be lives saved by this intervention? The answer to this is, of course, impossible to formulate without straying into speculation and sensationalism, but the data presented herein raises this very insurmountable question.

Chapter 5 answers a question we asked ourselves when we designed these studies, namely if age was a factor in this allergy model, and publication of our results may help other groups working on similar models fine-tune their methodology. We show that younger mice are more susceptible

to immunization, which correlates with clinical observations in humans. In Chapter 4, we show that the number of CD40 expressing cells and the number of IgE-producing plasma cells increases when tBHQ is present during the development of allergy, and that the concentration of plasma IgE is increased. These observations are limited to the mouse model but could be used as a basis to inform current food allergy patients and parents that try to avoid the development of food allergies in their infants through their dietary choices. Avoiding foods that contain tBHQ is likely neither curative nor ultimately preventative in the development of human allergy but could potentially mitigate the severity of food allergies and empower parents and patients. A possible caveat to this is socioeconomic – often foods that are rich in tBHQ are cheaper and more easily accessible, making the above recommendation selective in nature, as some families simply would not be able to afford a diet that stringently avoids tBHQ-containing food products. Adding a potential stigma in telling patients' families that they may be to blame for the development of their loved one's condition because they were not able to financially provide a diet would be an unfortunate outcome of this recommendation, which itself is based on evidence from a mouse model.

Future directions

In chapter 2, we described a novel human PBMC-based assay for immunotoxicity testing using influenza A as a challenge. This model is easily adapted to other toxicants and other challenges. We have started gathering preliminary data on other related compounds like sodium arsenite, which could be another way to model human exposure to As(III), which is a common exposure that is particularly of concern with rice-heavy diets [402]. It would be easy to use the same model to evaluate the effects of other heavy metals with relevant environmental or occupational exposure, such as cadmium, mercury, or lead compounds. In 2022, Washtenaw county in Michigan had a spill in hexavalent chromium (Cr (VI)), which is another metal that could be evaluated using this assay. Cr (VI) is also a known Nrf2 activator [403, 404], similarly to cadmium [214, 364, 405], lead [215, 406], and mercury [407]. To determine whether the ATO-dependent changes we have documented in Chapter 2 or any potential effects in follow-up experiments are Nrf2-dependent, we may explore the use of CRISPR/CAS9 to ablate Nrf2 in PBMC-derived human B cells [408]. Our lab has previous, yet unpublished experience in ablating Nrf2 in human PBMC-derived CD4 T cells, and protocols could be adapted to facilitate B cell-specific Nrf2 ablation. This experiment, with isolated and Nrf2-deficient B cells, would give us greater resolution and more precise data about the involvement of Nrf2.

precise data about the involvement of Nrf2.

The studies in chapter 2 exclusively used H1/N1 A/PR/8/34. Influenza is a very important model but considering the ongoing SARS-COV-2 pandemic, we are actively exploring the expansion of our model to include SARS-CoV-2 and other coronaviruses. Given that there is a significant

portion of the population that has received immunizations against SARS-COV-2, and a high number of subjects that recovered from active disease, I would love to pursue a bigger, IRB-requiring study that enrolls subjects with information regarding their vaccination status, previous disease history, age, and sex. Blood draws from this populace would be analyzed for the presence of anti-SARS-COV-2 antibodies and recombinant SARS-COV-2 spike protein, which has been used by others in *in-vitro* assays [409], as an infectious challenge. This modified assay could be used to assess the effect of different toxicants (such as the ones described above) on B cell function following a challenge by SARS-COV-2 protein, and we could differentiate between naïve and antigen-experienced B cells based on vaccination/previous disease status of the subject. This study would be highly relevant to the current global pandemic and has the potential to be immensely impactful.

In a similar vein, Chapter 3 is limited in its scope by using mouse splenocytes *ex vivo* for the studies. The scope could be expanded by using mouse cells from other compartments, such as lymph nodes or peripheral blood, thus changing the ratio of B cell subtypes in the preparation. This could also be achieved by using flow cytometry-assisted cell sorting or magnetic isolation of a particular B cell subtype, e.g. FO B cells, which in theory should respond more strongly to our activation cocktail. Similar to the studies in chapter 2, this study is easily scalable and could be performed with most toxicants. One possibility is even to use Nur77-GFP reporter mice, which exhibit a bright GFP signal when turning into a GC-reaction-like phenotype [410]. Hypothesizing that our activation cocktail activates this Nur77-GFP signaling, this model could even be used in high-throughput toxicology screening, where the GFP signal is the primary readout and a

reduction in GFP signaling would signify a reduction in GC-reaction-like phenotype in B cells. This would be an excellent starting point to screen toxicants on a large scale for inhibition of T cell-dependent B cell activation. Having a simple fluorescent read-out would enable that study to be used in a high throughput screen.

Chapter 3 used splenocytes from mice and a mouse-specific antibody cocktail. A very similar cocktail would be feasible to activate human PBMC-derived B cells. After verification of the activation by an anti-human IgM antibody fragment, anti-human CD40 or recombinant human CD40L, and recombinant IL-4, this could be a useful tool for further screening of the impact of toxicants on T cell-dependent B cell activation, though it would converge somewhat with the future directions outlined for chapter 2.

Regarding the *in vivo* allergy studies we describe in Chapters 4 & 5, the use of large-scale unbiased approaches, such as scRNAseq, could elucidate pathways that are differentially activated during sensitization when tBHQ is present, and thus direct further studies. It would be of particular interest to see whether there is differential activity of BLIMP-1 controlled genes, which control both B cell and plasma cell fate [63, 68]. The role of tBHQ on BLIMP-1 activity overall would be interesting to investigate as there is a mutually-antagonistic relationship between BLIMP-1 and Nrf2. Furthermore, BLIMP-1 is involved in many different immune processes, many of which are relevant to the present studies.

In all our previous allergy studies and the ones presented in this thesis, a tetramer detecting T or B cells specific for OVA would have been a great addition. MHC II tetramers that can detect OVA-specific T cells are readily available but were considered unreliable, though new, improved

tetramers have recently become available. Specifically for B cells, new protocols emerged in the last few years that effectively use tetramers to study antigen-specific B cells [411]. Measuring T and B cells that are specific to OVA would be an excellent addition to the studies in chapters 4 and 5.

Taking a step back from toxicology, a knowledge gap that my literature review revealed was the role of Nrf2 in B cells. B cells, once activated, have high metabolic turnover and are subject to significant oxidative stress [412]. Further investigation of the role of Nrf2 in B cells is an unexplored field with the potential to reveal important therapeutic targets. This is underscored by the findings in chapter 4, where we saw that even without sensitization, dietary tBHQ increased the percentage of CD40-expressing B cells and the number of plasmablasts in mice. Nrf2-deficient mice develop a lupus-like autoimmune disease with aberrant IgM, IgG, and C3 complexes leading to a nephritic syndrome, which hints that there is some B cell involvement [413]. Conversely, the data presented in chapter 3 suggest that many (though not all) of the effects observed in these studies may be Nrf2-independent. Nonetheless, studies confirming the effect (or absence of an effect!) of Nrf2 activation on B cell function would add to our understanding of B cell biology.

One question that arose in the discussion of limitations of chapters 4 and 5 was the location of IgE-secreting cells. While we were able to capture some in the lymph node at the timepoint chosen, plasma cells and memory B cells often do not dwell in secondary lymphoid organs [414]. While the consensus is that plasma cells relocate to the bone marrow and use its relatively high circulation as a base for antibody secretion, infiltrating B cells and plasma cells are often observed

at sites of infection, inflammation, or malignancy [127, 415-417]. The use of mice expressing IgE with a fluorescent VeriGem reporter construct [418] on an *in-vivo* imaging system like the Perkin-Elmer IVIS Spectrum, located in Michigan State's advanced molecular imaging facility, would allow us to measure multiple independent relevant data. Primarily, we would be able to track IgE+ cells and their movement after leaving the lymph node, potentially even after another sensitization or an oral or inhalatory antigen challenge. Secondly, using frequent imaging would us to better understand the kinetics of the B cell response and plasma cell differentiation following allergic sensitization in our model.

This remarkable mouse data would best be followed up by something that is more clinically applicable. Humanized mouse models for allergy research are possible, but complex. This is part due to the nature of allergy itself, which is a complicated mechanism involving many cell types and the recognition of an innocuous antigen [419]. XenoGraft vs Host disease is also a problem in these models, though newer protocols mitigate these issues [420]. Given these limitations, studies on humans are an attractive option. One idea is to use a large academic center with a dedicated allergy unit, such as Mott's Children's Hospital (Mary H Weiser Center for Food Allergies) in Ann Arbor or Detroit Children's Hospital and enroll patients for a correlative tBHQ study. While they visit the clinic for things like specific antigen testing, desensitization treatments, or educational events, urine samples could be collected and analyzed for tBHQ. At the same time, patients and loved ones may be asked to fill out a questionnaire regarding allergy symptom severity. Additionally, blood work, such as total IgE titers or antigen specific IgE titers, may be assessed. The analysis could compare plasma tBHQ levels, questionnaire results, and

potential lab values for correlation. Food allergy centers, such as Mary Weiser in Ann Arbor, already provide uncountable educational events, and another study could assess whether counseling and education towards a tBHQ-free diet have a measurable impact on urine tBHQ levels and food allergy symptoms.

Access to blood samples from food allergy patients could also provide an avenue for allergen-specific *ex vivo* assays. A prospective study may enroll patients with a specific allergy and collect PBMCs from patients via phlebotomy, and culture those cells as described in chapter 2. Instead of using influenza as a challenge model, we could expose the cells to the specific allergen (e.g., OVA for patients with egg allergies or crude peanut extract for patients with peanut allergy) to which the patient is allergic, and thus specifically screen circulating B and T cells for their activity. As described in Chapter 2, we could use different environmental toxicants to measure whether they have an impact on this allergen-specific activation. This would take the model described in chapter 2 directly from bench to bedside and provide a very clinically applicable measurement.

Ultimately, the studies presented here give us a starting point in assessing the impact of environmental toxicants and Nrf2 activators on B cell function, highlighting both Nrf2-dependent and -independent effects. The future directions suggested here would allow us on one hand to further quantify other toxicants for their effect on B cell activation, but also expand our understanding on whether xenobiotics present in our everyday life influence the activity and function of B cells. Finally, the human studies could determine whether or not there is a correlation between dietary tBHQ exposure and allergy severity.

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