The Ubiquitin Editing Enzyme A20 Maintains Immune Homeostasis and Prevents Autoimmunity

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Or

How A20 restricted most of my 20's

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Summary

The immune system is vital to ensure the surveillance of organisms against pathogens or malignant cells. However, the negative regulation of the immune system is equally essential, and defects in the termination of immune signals can result in autoimmunity and other pathologies.

The functioning of the immune system results from the integration of signals between and within cells. For some time, studies in immune signaling have focused on the molecular events playing a role in activating such cascades. However, little was known about how to turn off those signals. Just recently, new research started shedding light into mechanisms that ensure the negative regulation of immune signaling.

The enzyme A20 previously has been shown to be a fundamental intracellular negative regulator of immune signals. Mice deficient in the gene that codes for the A20 protein, *Tnfaip3*, have massive generalized inflammation and die prematurely. A20 deficient cells fail to terminate TNF and TLR induced responses.

In the first part of this work, we aimed to define the basal signals responsible for triggering the spontaneous phenotype of $A20^{-/-}$ mice. While genetically removing TNF signals by obtaining $A20^{-/-}TNF^{-/-}$ double-deficient mice does not rescue the disease observed in single A20-deficient mice, animals lacking the common TLR adaptor protein MyD88 ($A20^{-/-}MyD88^{-/-}$) have a clear amelioration of the $A20^{-/-}$ phenotype, indicating that there are tonic TLR signals that need to be constantly terminated by A20. We have shown that these signals are likely initiated in the gut, since treatment with broad-spectrum antibiotics that reduces commensal intestinal flora also ameliorates disease. Additionally, we have demonstrated that A20 also regulates MyD88-independent TLR signals.

In the second part of this work, we used a floxed allele of *Tnfaip3* to generate mice that lack A20 specifically in B-cells. While these mice develop normally and show no signs of the general inflammation observed in A20 globally deficient mice, *Tnfaip3*$^{fl/fl}$ and *Tnfaip3*$^{fl/+}$ CD19-Cre mice have disrupted lymphoid homeostasis and develop lupus-like autoimmunity. A20 deficient B-lymphocytes hyper-respond to signals initiated by the BCR, TLRs and CD40. We have demonstrated that A20 is necessary to restrict B-cell survival as A20-deficient B-cells are resistant to Fas-mediated cell death. This provides one mechanism by which A20 can prevent B-cell mediated autoimmunity.

All together, this work bestows several new insights on the mechanisms by which A20 plays a central role in maintaining the homeostasis of both the innate and the adaptive immune system. We show that there are tonic innate immune signals that need to be constantly terminated by A20. Furthermore, we show for the first time *in vivo* that A20 also plays a role in lymphocyte function. These findings relate to recent genetic associations between human A20, autoimmune diseases and cancer.

Sumário

O funcionamento do sistema imunitário é fundamental aos organismos desenvolvidos para garantir a prevenção contra microorganismos patogénicos ou células malignas. Ainda assim, a regulação negativa do sistema imunitário é igualmente essencial, e defeitos na terminação de sinais imunitários podem resultar em auto-imunidade e outras patologias.

O funcionamento do sistema immunitário resulta da integração de sinais entre e dentro das células. Na sua fase inicial, a investigação em sinalização imunitária dedicou-se principalmente aos processos moleculares responsáveis pela activação destas cascatas de sinalização. No entanto, pouco se sabia acerca de como terminar estes sinais de activação, de forma a que não se prolongassem indefinidamente. Só já mais recentemente, nova investigação começou a fornecer pistas acerca dos mecanismos que asseguram a regulação negativa da sinalização imunitária.

Alguma desta investigação recente demonstrou que a enzima A20 é um regulador negativo intracelular de sinais imunitários fundamental. Ratinhos deficientes no gene que codifica a proteína A20, *Tnfaip3*, desenvolvem níveis elevados de inflamação em vários órgãos e morrem prematuramente. Células que são deficientes em A20 não possibilitam a terminação de respostas induzidas por Tumor Necrosis Factor (TNF) ou Toll-Like-Receptors (TLRs).

Na primeira parte deste trabalho, o nosso objectivo foi definir quais os sinais existentes naturalmente, responsáveis por iniciar o fenótipo espontâneo de ratinhos $A20^{-/-}$. Eliminámos geneticamente sinais mediados por TNF, obtendo ratinhos $A20^{-/-}TNF^{-/-}$ e observámos o mesmo nível inflamação registada em ratinhos $A20^{-/-}$. No entanto, animais deficientes na proteína adaptadora comum à maioria dos TLRs, MyD88 ($A20^{-/-}MyD88^{-/-}$), mostraram uma clara diminuição da

inflamação comparativamente a $A20^{-/-}$. Estas observações indicam que há sinais inerentes mediados por TLRs que devem ser constantemente terminados através da acção de A20. Demonstrámos ainda que a origem mais provável destes sinais se encontra no intestino, já que o tratamento com antibióticos de largo espectro, que reduz a flora intestinal comensal, resulta num efeito semelhante. Demonstrámos ainda que A20 pode também regular sinais de TLRs que não dependem de MyD88.

Na segunda parte deste trabalho, usámos um alelo "floxed" de *Tnfaip3* e gerámos ratinhos sem A20 única e especificamente em células B. Estes ratinhos desenvolvem-se normalmente e não mostram sinais de inflamação generalizada como observado em ratinhos globalmente deficientes em A20. Ainda assim, ratinhos *Tnfaip3*$^{fl/fl}$ e *Tnfaip3*$^{fl/+}$ CD19-Cre apresentam alterações evidentes na homeostase dos seus linfócitos e desenvolvem uma doença com características semelhantes a lúpus. Linfócitos B deficientes em A20 respondem exageradamente a sinais iniciados pelo B-Cell-Receptor (BCR), TLRs e CD40. Demonstrámos que A20 é necessária para restringir a sobrevivência de células B, já que células B deficientes em A20 são resistentes à morte celular mediada por Fas. Esta descoberta sugere um mecanismo através do qual A20 pode prevenir a auto-imunidade mediada por células B.

Este trabalho fornece novos esclarecimentos sobre os mecanismos através dos quais a proteína A20 representa um papel central na manutenção da homeostase tanto do sistema imunitário inato como do adaptativo. Demonstrámos que há sinais imunitários inatos inerentes que devem ser constantemente descontinuados através da acção de A20. Mais ainda, demonstrámos pela primeira vez, *in vivo*, que A20 também têm um papel em linfócitos, o qual está relacionado com recentes associações genéticas entre A20 humano, doenças auto-imunes e cancro.

CHAPTER 1

Introduction

From Immunity to an Immune System

Throughout evolution, systems of defense against opportunistic pathogens arose in nearly all organisms. Such systems have been crucial for the fitness of progressively more complex species. Even though vertebrates continued to evolve more sophisticated immune processes, immunity can be found in invertebrates and plants, and remarkably, several of the same molecular pathways are shared among such different organisms (Howard and Jack, 2007; Litman and Cooper, 2007).

Jawed vertebrates as mammals evolved some of the most complex immune systems, and humans and mice have been at the center of much of the research in the field of Immunology. From the early days of vaccine discovery by Edward Jenner and Louis Pasteur, to the recent development of vaccinations and treatments against cancer, this research has had wide implications in medicine (Janeway, 2005; K. Abbas et al., 2007).

Vertebrate immune systems clear pathogenic infections as well as recognize and eliminate malignant cells, and as a result immunodeficiency is highly deleterious. Nonetheless, exaggerated immune responses can also harm the organism's own cells and cause equally severe pathologies. Therefore it is now extensively recognized that proper functioning of the immune system is fundamental and needs to be finely tuned (J. Kindt et al., 2007; Janeway, 2005).

Plants and invertebrates have several forms of immune defense but are jawed vertebrates that have evolved the most complex immune systems. Vertebrates like humans and mice have specialized immune cells, and have dedicated immune organs that allow the appropriate development of these cells as well as their efficient migration through the organism. Immune cells circulate through the blood and in dedicated lymphatic vessels, between sites of

surveillance and specialized lymphoid organs where they either develop (primary lymphoid tissues – bone marrow and thymus) or mount an immune response (secondary lymphoid tissues – spleen and lymph nodes). Immunity in vertebrates has been classified in two broad types: innate immunity and adaptive immunity (Janeway, 2005; K. Abbas et al., 2007).

Evolution of immunity: Innate Immunity and adaptive immunity

Innate immunity is common to vertebrates, invertebrates and plants. This kind of immunity has been characterized by being non-specific, and comprises anatomic barriers, physiological barriers, phagocytic and endocytic activities, production of anti-microbial proteins, and inflammatory barriers to foreign microbes (J. Kindt et al., 2007). Vertebrates like mice and humans also have professional innate immune cells (macrophages, neutrophils, dendritic cells (DCs)) that develop in the bone marrow and then circulate through the body or reside at particular locations. These cells are often the first set of cells to be recruited to a site of infection and mediate several of the aforementioned activities (K. Abbas et al., 2007).

The adaptive immune system is found only in jawed vertebrates and is characterized by being specific, enabling the development of memory for particular pathogens. Lymphocytes serve as the adaptive immune arm, and are comprised mainly of T and B cells. Conventional B and T lymphocytes can have their DNA somatically rearranged during their development, which allows each cell to express a different but specific receptor of its own. Each clone then goes through several steps of selection that guarantee its receptor can recognize an antigen – a small piece of a certain molecule - that, all working correctly, will recognize foreign components (non-self) but not self constituents. When a given clone is activated by the presence of its antigen, it rapidly proliferates and amplifies the immune response. After an infection is resolved, some of those cells

will be maintained as a pool of memory cells, eventually granting a faster and stronger secondary response (Janeway, 2005).

T-lymphocytes recognize antigens through the T cell receptor (TCR). To be activated, the TCR needs to bind antigens processed and presented by MHC (Major Histocompatibility Complex) molecules in Antigen Presenting cells (APCs) as DCs, illustrating the crosstalk between the innate and adaptive immune systems. Activated T-cells produce cytokines that aid the clearance of the infection (T helper cells) or directly kill infected or transformed cells (Cytotoxic T-cells), in what has been designated as the Cell-mediated Response (K. Abbas et al., 2007).

B-lymphocytes recognize antigens through the B cell Receptor (BCR) and are the cells responsible for producing antibodies (immunoglobulin), mediating the so-called Humoral Response. Binding of antibodies to antigenic particles activates molecular mechanisms that assist phagocytosis or killing of pathogens by other immune cells (Janeway, 2005).

Evolution of theories and models of immunity: from self-non-self recognition to the danger model

Once the existence of an immune system was recognized the central question in the field of immunology was (and still is to a certain extent) how does the system decide what it reacts to or not? How does it not attack the own organism and develop what has later been termed tolerance toward self? Or is it ignorance?

The immunity model that has prevailed the longest, and is still the theoretical framework for much of the research, is self-non-self recognition. In 1959, Frank Macfarlane Burnet suggested the Clonal Selection Theory of

Acquired Immunity (Burnet, 1959), in which he proposed that each lymphocyte expresses a single surface receptor for a foreign particle and that self-reactive lymphocytes are deleted early in development. In 1970, this model was further developed by Bretscher and Cohn (Bretscher and Cohn, 1970); after the discovery of hypermutation in activated B lymphocytes and thus the renewed chance for creation of autoreactive cells, these scientists proposed that there had to be a second signal for a cell to initiate a response, and that the second signal was coming from an helper cell (later found to be a T-cell). Soon after, Lafferty and Cunningham proposed a similar model for T-cell activation, which instead of "help" required "costimulation" (Lafferty and Cunningham, 1975).

For a while it was thought that lymphocytes were responsible for initiating the immune response. However, the need for a second signal posed a problem: if a second signal (like costimulation) was often not coming from a lymphocyte, and thus the adaptive immune harm of the response was not solely responsible for its initiation, how was self-reactivity avoided?

It is clear that the innate immune system recognizes foreign invaders (non-self) in a very different manner than cells of the adaptive immune system. However, the idea that innate immunity provides "non-specific" protection at mucosal barriers is an overly simplistic definition. In 1989, Charles Janeway Jr. proposed the revolutionary Pattern Recognition Theory (Janeway, 1989). He predicted that innate immunity encompassed the expression of Pattern Recognition Receptors (PRRs), which could recognize Pathogen Associated Molecular Patterns (PAMPs). Importantly, these PAMPs would consist of patterns not found in the host, and thus would be specific to the invading microbe or foreign body. PRRs would be responsible for directly sensing pathogens before intervention of the adaptive immune system. He called it discrimination between infectious non-self and noninfectious self, and evolutionarily this process of discriminating foreign bodies in an organism pre-dates the straight self / non-self discrimination done by the adaptive immune system (Janeway, 1992). His ideas

proved true and provided much of the framework that propelled a large amount of the Immunology research in the following decades. The discovery of Toll-Like-Receptors (TLRs) as the proposed PRRs was the first big step (Medzhitov, 2009).

Nevertheless, the infectious non-self model still has its limitations. Namely, if one thinks about the numerous instances in which there is the need to recognize self that might be harmful, like tumours, or the need to be tolerant of non-self that might be harmless, like commensals. Matzinger (from discussions with Fuchs) has proposed a Danger model (Matzinger, 1994). She adds another layer at the apex of the immune response, to include cells and signals from all tissues. Matzinger's viewpoint is that the immune system does not care as much about non-self discrimination as it does need to protect against danger, and that the initiation and polarization of an immune response depend on danger signals from the tissue affected by any sort of threat (Matzinger, 2002).

A number of Danger Associated Molecular Patterns (DAMPs) released by injured tissues have now been described. Remarkably, besides identifying new receptors, it was found that some PRRs also recognize DAMPs (Matzinger, 2002; Rubartelli and Lotze, 2007; Zhang et al., 2010). Furthermore, it has also been shown that several cell types in a plethora of tissues can respond to and produce cytokines, express PRRs, or have specialized, resident immune cells, contributing not only for the initiation but also the polarization of an immune response (Janeway, 2005; Matzinger, 2002). Hence, it seems clear that this dialog between tissues and the immune system is really happening, and playing a prominent role in the overall immune outcome.

Scope of this thesis: what breaks tolerance and do we really need a unifying model?

Nevertheless, is there really a need for a unifying model and de we have to decide what matters the most for an immune response? It seems to me that all

layers of immune recognition and alert have to be taken into account, with absolutely the same level of significance. If at times there have been trends to favor this or that aspect of immunity (sometimes just a single cell type), it might as well be about time to beware of such biased approaches. The barriers of what the immune system is have always been loose, after all. Notwithstanding a few defined immune organs, all the same immune cells can travel through numerous tissues (with a few exceptions, as the privileged brain) and in those instances it becomes harder to define what is the system itself versus what is an immune defense process that might as well not immediately involve an immune cell.

The scope of this work shines some light into just that. Perturbations in a single protein, in distinct aspects of the immune system (Toll-like-receptors in innate immune cells in chapter 2, B cells in chapter 3) depict how different cells and tissues play equally important yet distinct roles in maintaining the homeostasis of the immune system. Even if some outcomes do result more dramatic, altered intracellular regulation of a number of signals, in distinct cell types, appears each time to be sufficient to break immune tolerance at those various levels. In fact, conditional gene targeting will probably still be teaching us a great amount of new principles about the workings of these complex networks in many years to come. And just as selective pressures are diverse, there might as well not be a single model of immunity that fits it all. Or at least our knowledge will still have to evolve further to be able to create a broad enough theory. Ultimately, while interpretations might always be based on the wrong preconceptions, robust data might survive the test of time and speak louder than words.

Toll-like-receptors

TLRs are the broadest class of PRRs identified to date, first found in flies, and soon established to have an essential role in the immune systems of vertebrates like those of humans and mice. In mice and humans, TLRs can be expressed in both innate and adaptive immune cells, as well as in non-immune cells. However, their highest levels of expression are found in dedicated innate immune cells like macrophages, dendritic cells (DCs) or neutrophils (Beutler, 2009; Kawai and Akira, 2010; Medzhitov, 2009).

Demonstrating that innate immunity is more specific than previously thought, each TLR binds only a few specific ligands of its own. Confirming Janeway's prediction that PRRs would recognize patterns associated specifically with the invading pathogen and not the host itself, TLRs recognize motifs such as those of lypopolisacharyde (LPS), found in the wall of Gram negative bacteria (TLR4), double stranded RNA, which can be found in viruses (TLR3), or methylated DNA, from bacteria (TLR9). There have been 12 TLRs described in mice, and 10 in humans. TLRs 1-9 are conserved in both species (Kawai and Akira, 2010). Table 1.1 summarizes some basic features of TLRs.

TLR	Ligands	Adaptors
TLR1/2	Glycolipids, Triacyl lipoproteins	MyD88,Tirap
TLR2/6	Lipoteichoic acid, Zymosan, Diacyl lipoproteins	MyD88, Tirap
TLR3	polyI:C, dsRNA	TRIF
TLR4	LPS, Taxol, Heparan, Hyaluronate, F-prot, RSV, G-prot, VSV, Env prot, MMTV, others	MyD88,Tirap TRIF,TRAM
TLR5	Flagellin	MyD88
TLR7	ssRNA, imiquimod, loxoribine, other	MyD88
TLR9	CpG, methylated DNA	MyD88
TLR11	Profilin	MyD88
TLR12	?	?
TLR13	?	?

Table 1.1 Mouse TLRs, their principal ligands and downstream adaptor proteins. Adapted from Beutler, 2009.

TLR activation triggers signaling cascades that result in the expression of effector genes. Effector genes mediate direct functions in the activated cells, but also the expression of surface co-stimulatory molecules and a large number of cytokines and chemokines. Many of these are pro-inflammatory cytokines like IL-6 (Interleukin-6), IL-1β, TNF-α (Tumor Necrosis Factor α) (Beutler, 2009). Production of cytokines mediates the amplification and specification of the immune response and appropriate clearing of a particular infection. There has been ample evidence that innate immune system activation through TLRs is an essential component of effective immunity, and is critical for the efficient activation of the adaptive immune response (Beutler, 2009; Hou et al., 2008; Kawai and Akira, 2010).

Toll-like-receptor signaling

All TLRs except TLR3 bind the adaptor protein MyD88 (Myeloid differentiation primary response gene (88)). Upon TLR engagement, kinases and adaptor proteins are recruited by MyD88 and lead to the activation of downstream transcription factors. The main transcription factor responsible for the expression of pro-inflammatory cytokines downstream of MyD88 is NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) (Hayden and Ghosh, 2008).

Figure 1.1 summarizes some of the main players in the MyD88-dependent pathway of NF-κB activation.

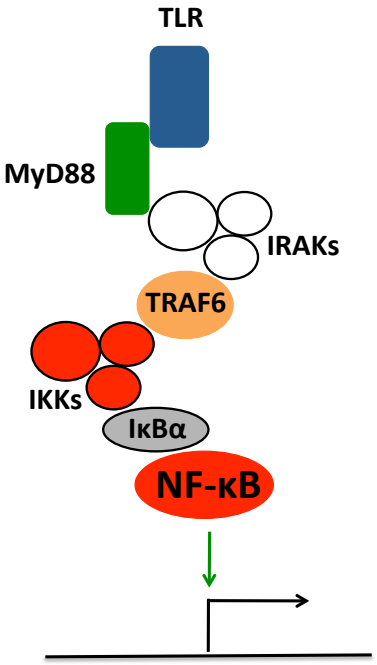

Figure 1.1 – TLR signaling through MyD88.

TRIF (TIR-domain-containing adapter-inducing interferon-β) is an alternate TLR adaptor. TLR3 signals exclusively through TRIF, and TLR4 signals through both MyD88 and TRIF. The TRIF-dependent pathway also activates NF-κB (and thus expression of pro-inflammatory cytokines), but it is best known for activating the transcription factor IRF3 (Interferon Regulatory Factor 3). IRF3 is the main factor responsible for the expression of type I interferons (IFN) alpha (IFN-α) and beta (IFN-β) (Kawai and Akira, 2010). IFNs are critical anti-viral cytokines, and thus the TRIF-dependent response is characterized by its anti-viral action. Even though IRF3 is the transcription factor leading to the most robust type I IFN transcription, IFNs have NF-κB binding sites in their promoters, such that both transcription factors can contribute to optimal cytokine production (Sun and Ley, 2008). There are two distinct pathway branches that activate either NF-κB or IRF3. These pathways were defined through the use of knockout cells from different TRAFs (TNF receptor associated factors), adaptor proteins downstream of TLRs (Häcker et al., 2006; Oganesyan et al., 2006). The pathway branch

leading to the activation of NF-κB depends on TRAF6, whereas the one that activates IRF3 depends on TRAF3. Figure 1.2 illustrates these findings.

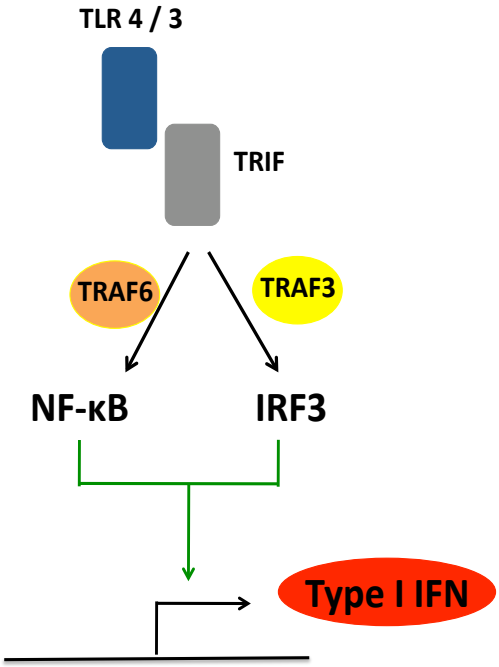

Figure 1.2 – TLR signaling through TRIF.

Nevertheless, this is only a simplified description of the prominent TLR signaling pathways, as they occur in innate immune cells (such as macrophages) and in response to most foreign agents. Thus, there are a number of variations in these signaling cascades, depending on cell types, ligands, or even cellular location of the receptors (Hayden and Ghosh, 2008; Kawai and Akira, 2010). The case of the specialized innate immune cells known as plasmacytoid DCs (pDCS, as opposed to conventional DCs, cDCs) illustrates this point well. These cells are known for being able to produce large amounts of type I interferons in response to TLR7 and TLR9 stimulation (Liu and Nussenzweig, 2010; Marshak-Rothstein, 2006). These are intracellular TLRs that respond to ssRNA and CpG DNA motifs, and thus detect viral and bacterial nucleic acids, respectively (Blasius and Beutler, 2010). Even though these TLRs signal through MyD88, and in other cell types

mediate only the production of pro-inflammatory cytokines through NF-κB, in pDCs the MyD88-dependent pathway can lead to the production of type I IFNs. It is thought this is due to high basal expression of another transcription factor in these cells, IRF7, that also transcribes IFNs α and β. IRF7 can be activated by the MyD88-dependent pathway. (Häcker et al., 2006; Kawai and Akira, 2010; Oganesyan et al., 2006).

Additionally, the activation state of the cells can also contribute to the differences in their response. For example, it is known that NF-κB can mediate the expression of other transcription factors, as IRFs (Hayden and Ghosh, 2008; Sun and Ley, 2008). Consequently, a secondary response, after the initial NF-kB activation, can vary from a primary one.

Toll-like-receptors in health and disease

Generation of knockout mice of different TLRs or their common adaptors results in impaired responses to pathogens and confirmed their essential role in immunity (Beutler, 2009). More recently it has become increasingly evident that appropriate TLR mediated responses are also critical in additional facets of the health of mice and humans (Kawai and Akira, 2010; Marshak-Rothstein, 2006). It was shown that TLR stimulation by commensal flora is essential for gut homeostasis (Rakoffnahoum et al., 2004). Furthermore, colonization by particular types of commensals can determine the kind of global response initiated by the immune system, and whether this is beneficial or harmful to the host (Ivanov et al., 2009). Finally, failure to regulate TLR responses can lead to chronic inflammation and autoimmune disease. In fact, besides the need for modulating the typical responses of TLRs to microbes, there has been increasing evidence that some TLRs, like intracellular TLRs 7 and 9, can recognize endogenous components, such as those of dying cells, and thus contribute to the triggering of a number of autoimmune diseases (Krieg and Vollmer, 2007; Marshak-Rothstein, 2006).

In conclusion, TLRs have emerged as fundamental components of the innate immune system. It is also recognized that they contribute to the orchestration of the whole immune response along with the adaptive immune system. Inappropriate TLR responses appear to be related to a number of pathologies. Hence, it seems imperative to understand the regulation of TLR signals.

B cells

B cells first develop in the bone marrow, from which they emigrate as immature B cells. They conclude most of their differentiation to mature B cells in the spleen, after which they can circulate in the blood stream or through other peripheral lymphoid organs (Allman and Pillai, 2008).

Figure 1.3 schematizes the life of a B cell.

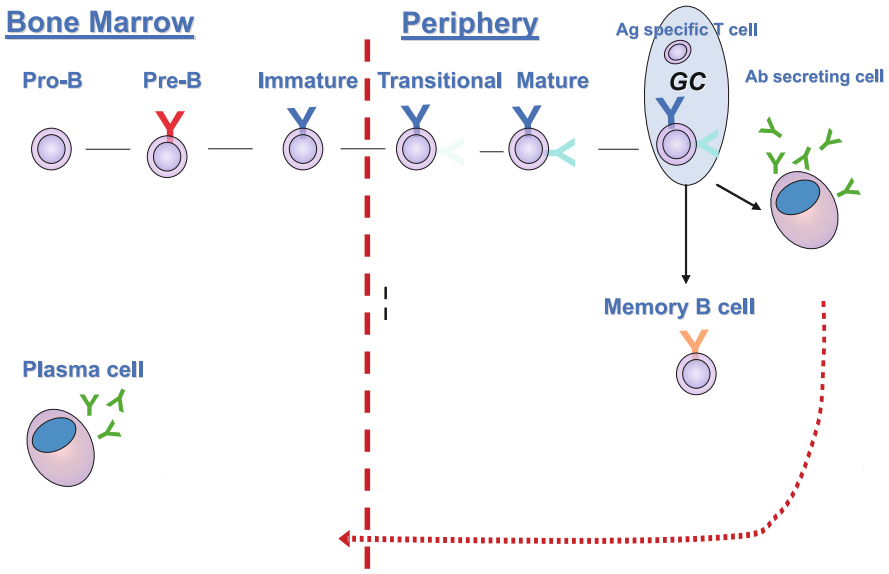

Figure 1.3 – Development and differentiation of B cells from the bone marrow to the periphery. Adapted from Dörner, 2009.

The antigen recognition component of the BCR is membrane-bound immunoglobulin (Ig). Two designated heavy chains and two light chains constitute immunoglobulin protein. Once a B-cell is activated, its ultimate goal is to generate and secrete large amounts of antigen-specific immunoglobulin. There are several isotypes of Ig, and the primary humoral response is initially characterized by IgM secretion. After encountering their antigen and becoming activated, B cells can

undergo a process called class-switch recombination: the Ig chains expressed are changed in order to produce other antibody isotypes, such as IgGs, IgA or IgD, each with particular characteristics appropriate for the type of response required (Goodnow et al., 2010; Janeway, 2005).

Immunoglobulin, including the BCR, binds unprocessed antigen, meaning that on the contrary of T lymphocytes, B cells do not recognize processed peptides presented by MHC molecules. Instead, they bind intact antigen, which they can access directly or extract from innate immune cells (Cyster, 2010; Qi et al., 2006). B cells can function as APCs themselves by expression of MHC class II molecules (K. Abbas et al., 2007).

Besides producing antibodies, B cells also produce cytokines and interact with other immune cells, contributing to the intricate conduction of the immune response. In fact, B lymphocytes express a diverse repertoire of different receptors besides the BCR, such as TLRs and TNFR family receptors, like CD40 and BAFFR (B-call Activating Factor Receptor). B cells integrate all these different stimuli in an effort to mount the most appropriate immunological outcome for the host (Dörner and Lipsky, 2006; Sen, 2006).

B cell selection

Like T cells, B cells go through steps of selection during their development that ensure their receptor binds a foreign element (positive selection) but not a self-antigen (negative selection). B cells are selected at several stages of development, allowing the diversification of checkpoints and decreasing the likelihood of failure (Goodnow et al., 2010; Shlomchik, 2008). The two main checkpoints of negative selection happen in the bone marrow and in structures called germinal centers (GCs), present in the spleen and lymph nodes. Both in the bone marrow (central tolerance) and in GCs (peripheral tolerance), negative

selection of autoreactive B cells can happen via three main mechanisms: clonal deletion (death of the autoreactive cell), anergy (the cell is turned unresponsive) or a process called receptor editing, in which the light chain of the BCR (at first κ) is replaced by an alternative one (type λ) by DNA rearrangement (Shlomchik, 2008; von Boehmer and Melchers, 2010; Yurasov and Nussenzweig, 2007).

It is accepted that selection takes in at least one basic principle: one signal alone is not enough to base an appropriate fate decision. The simplest version of this theory was first described as a two signal model, but today we understand that it might be more than a binary mechanism (Bretscher and Cohn, 1970; Goodnow et al., 2010; von Boehmer and Melchers, 2010). Regardless, it's probably safe to say that there should always be at least 2 signals, if not more, for a B cell to survive and be "given permission" to proliferate when encountering its antigen. Accordingly, much of this selection will involve growth versus death decisions (Shlomchik, 2009).

The first selection signal is usually from the BCR, and binding of antigen. To ensure that the B cell generates a productive BCR that recognizes an antigen (positive selection), weak BCR stimulation or no stimulation at all lead to B cell anergy or death, respectively. In fact, B cells die easily without any stimulation, in what can be designated as "death by neglect" (Sen, 2006). Nevertheless, excessively strong BCR stimulation without any other signal also leads to B cell death, likely ensuring the avoidance of what can be the signal of an abundant self-antigen (Dörner and Lipsky, 2006). Hence, strength of signal is also decisive. The second stimulus is usually co-stimulatory, and promotes B cell survival and even proliferation; it can be TLR stimulation, from antigenic moieties that most likely belongs to a pathogen, or TNFR family stimulation, like BAFFR in the bone marrow or CD40 in GCs (Shlomchik, 2009).

Autoreactive B cells can also be killed through direct stimulation of their death receptors. The most notable case is that of Fas (also known as CD95), which is expressed in B and T cells (Shlomchik, 2008). Fas deficient mice, and more strikingly, mice that lack Fas specifically on GC B cells, have profound autoimmunity, characterized by expansion of B an T cells and production of self-reactive antibodies (Hao et al., 2008; Rathmell et al., 1995). Activated T cells can also express FasL, and just as they give a survival and growth signal to B cells through CD40L, they will provide a death signal instead. Curiously, still, it was also observed that an activated B cell expressing B7.2 (also known as CD86) can bias a T cell to actually give a weak survival signal through FasL (Rathmell et al., 1998).

Germinal Centers

There is an extensive cross talk between B and T cells that modulates both sides of a response, i.e., the humoral and cell mediated arms of adaptive immunity (Dörner and Lipsky, 2006). The privileged meeting point for lymphocytes is generally in GCs, where B cells and T cells, along with a network of DCs (follicular DCs) are in tight communication. These structures enhance the likelihood of exposure to antigens (Goodnow et al., 2010).

After leaving the bone marrow (and thus having already passed central tolerance selection), naïve B cells will traffic until they are exposed to antigen and first get activated. An activated B cell can differentiate into either a plasma cell, a GC B cell or a memory B cell. Plasma cells are characterized by making large amounts of antibodies and memory B cells for being long lived. GC B cells divide rapidly and can themselves become memory cells or plasma cells. Even though we understand that these decisions also depend on the integration of several signals, the outcome cannot be easily predicted (Goodnow et al., 2010; Shlomchik, 2008).

In GCs, B cells divide rapidly, and this rate of division leads to expression of enzymes that allow both Class Switch Recombination and Somatic Hypermutation. Somatic hypermutation results in changes of about one nucleotide per cell division in the Ig complimentary determining regions of the B cell DNA. This nucleotide change results in either a silent mutation, where the same amino acid is encoded, or a replacement mutation, where a different amino acid is encoded. Ultimately the cell expresses a different amino acid and an altered BCR in the regions that determine antigen recognition. This can result in higher, lower or even inexistent affinity for the antigen that was bound by the BCR in the first place. High affinity clones are likely selected to survive and enriched in the resulting clonal population, whereas low affinity ones most likely die through an inability to compete for antigen and survival signals. This process is called Affinity Maturation (Yurasov et al., 2005).

During somatic hyper-mutation, however, some of these matured receptors can bind self-antigen creating another chance of escape of autoreactive cells. Therefore, there is the renewed need for negative selection. Once more, the outcome depends on the integration of several signals, to which the B cells are exposed during their stay at the GC. BCR strength is integrated with any TLR signals and the extensive crosstalk with the T cells. Besides expressing MHCII, activated B cells may also often express B7.1 (CD80), B7.2 (CD86), and ICOS-L (Inducible T cell Costimulator – Ligand), which are ligands for CD28, CTLA4 (Cytotoxic T-Lymphocyte Antigen 4), or ICOS on T cells, and act as co-stimulators. In turn, activated T cells will express CD40-L, Fas-L, and secrete IL-4. It is recognized that all of these factors play critical roles in the selection process. Still, mathematical modeling would probably be needed to try to predict the result of the integration of all the different signal combinations and their different intensities (Dörner and Lipsky, 2006; Goodnow et al., 2010; Rathmell et al., 1996; Shlomchik, 2009).

Figure 1.4 depicts some of these interactions.

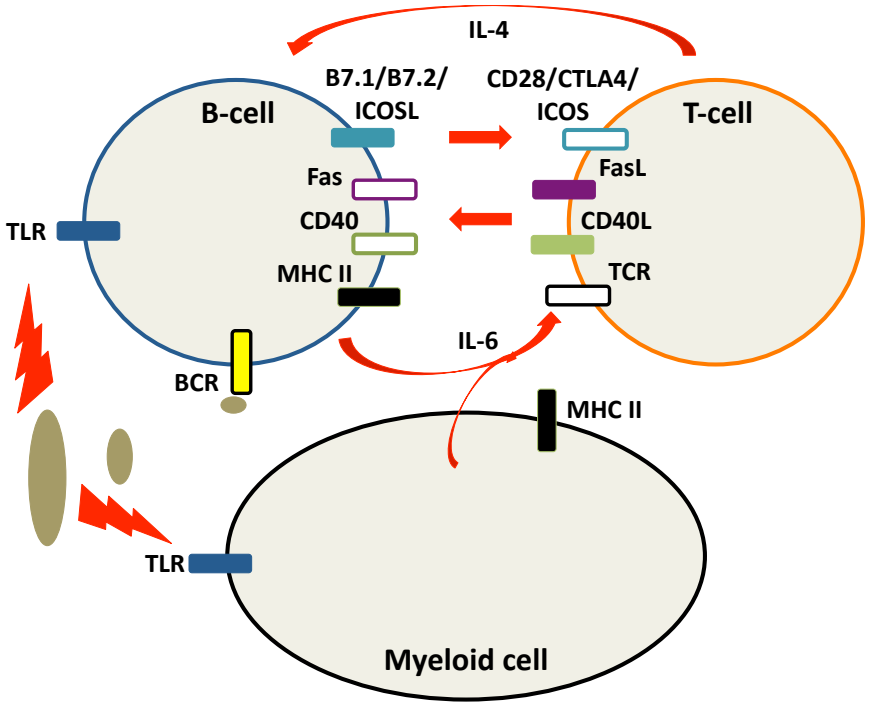

Figure 1.4 – B – T – myeloid cell interactions at Germinal Centers

B cells in autoimmunity and cancer

Failure to appropriately regulate each one of the processes described above can result in impaired selection, escape of autoreactive cells and ultimately systemic autoimmune disease. In fact, it is well documented that conditions of B cell mediated autoimmune disease as Systemic Lupus Erythematosus (SLE) are the consequence of defects in B cell selection, which result in a profound "break of tolerance" to self (Cappione et al., 2005; Fairhurst et al., 2006; Yurasov et al., 2005).

Besides the intricate control network required for maintaining tolerance, regulation of B cell function is also necessary to avoid cancer. B cell lymphomas are the result of uncontrolled expansion of a certain B cell population, and often show deregulated B cell activity (Küppers, 2009).

BCR stimulation leads to MAPK (Mitogen-activated protein kinases) and NF-kB activation. Similarly, TLRs, CD40 and the BAFFR also activate NF-κB. We could then hypothesize that much of the signal integration that mediates selection decisions and proper functioning of B cells depends on NF-kB signals strength and its regulation (Sen, 2006).

The transcription factor NF-κB

NF-κB was first indentified as a regulator of the *kappa* light chain of B cells. Soon it was discovered it functioned as the central transcription factor downstream of a diverse number of immune signaling networks. Over the past two decades, increasingly more functions of NF-kB have been described, many in immune related functions but some also in additional settings. Furthermore, the activation of NF-kB has turned into an excellence model of inducible gene expression, which principles of functioning go beyond the immune system (Hayden and Ghosh, 2008).

There are 5 different forms of NF-κB: p65 (RelA), p50 (NF-κB1), p52 (NF-κB2), cRel and RelB. All of these forms have in common a Rel homology domain (RHD) that accounts for DNA binding and homo- and heterodimerization, necessary for transcriptional activity. Inducible gene expression is achieved given that NF-κB is found in inactive states prior to a stimulating signal (Hayden and Ghosh, 2008).

In the predominant canonical pathway, IκBα (Inhibitor of NF-κB) binds p50/RelA dimers in the cytoplasm, keeping them from travelling to the nucleus and initiating transcription. Active IKKs (IκB kinases) mediate the phosphorylation and consequent degradation of IκBα, which allows initiation of transcription by NF-κB dimers. In the non-canonical pathways, instead of IκB proteins, unprocessed forms of NF-κB keep dimers inactive. In B cells, downstream of CD40, the BAFFR, or TNFRII, IKKα phosphorylates p100, resulting in its proteolysis into the active form p52 and release of p52/RelB dimers (Hayden and Ghosh, 2008; Sun and Ley, 2008).

The families of receptors discussed so far – TLRs, BCR, TNFR family receptors – all result in downstream induction of NF-κB activity, even receptor also

activates some additional transcription factors or other downstream responses of their own. Every one of these NF-κB activation pathways has its particular version of signaling intermediates, and in a given cell type results in the expression of a specific transcriptional program by NF-κB. Nonetheless, such routes seem to work by combining some of the same modules that in the end results in IKK activation (Kawai and Akira, 2010; Sen, 2006; Sun and Ley, 2008).

Regulation of NF-κB through ubiquitylation

While the first decade of research after the identification of NF-κB quickly provided a lot of knowledge about its activation mechanisms, less was understood about how to turn off these signals. Thus there remained a gap in our understanding in the regulation of inflammatory activity. Still today not much is known about what happens to stop NF-κB at the transcription level. But, in this past decade there has been remarkable progress on understanding the regulation of upstream cascades that signal to activate IKKs (Hayden and Ghosh, 2008; Sun and Ley, 2008).

Ubiquitylation is the post-translation addition of one or more (chains) ubiquitin molecules to proteins. Ubiquitin is a 76 amino acid protein that can be covalently affixed to other proteins. It requires E1 (activating), E2 (conjugating) and E3 (ligase) enzymes, as illustrated in Figure 1.5. Ubiquitylation was first identified as the addition of chains of ubiquitin with covalent bounds between lysines at position 48. These so-called K48 chains normally target proteins for degradation. However, it was found that mono-ubiquitylation and other types of ubiquitin chains can mediate various non-degrative functions. The most notable non-degrative ubiquitin chain type is K63 ubiquitin, which is necessary for diverse protein-protein interactions or protein activation (Sun, 2008)

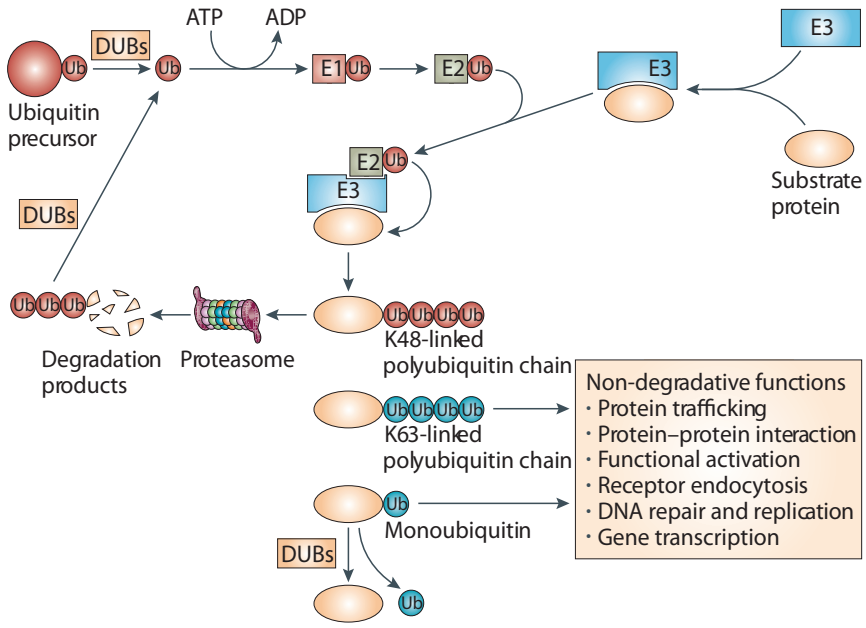

Figure 1.5 – Protein ubiquitylation. Sun, 2008.

More recently, ubiquitylation events have been identified in a number of signaling intermediates that lead to NF-kB activation. Notably, IKKγ is K63 ubiquitylated, as well as a number of TRAF molecules, namely TRAF6. Alongside with these discoveries, several proteins were described as negative regulators of immune signals. Some of the most significant NF-kB signaling regulators turned out to be ubiquitin-editing-enzymes, that can either remove ubiquitin chains (deubiquitylating enzymes, or DUBs) or mediate ubiquitin addition as E3 ligases. Importantly, a remarkable enzyme, A20, can perform both functions (Coornaert et al., 2009; Sun and Ley, 2008).

The ubiquitin editing enzyme A20

The A20 protein is encoded by the *Tnfaip3* gene. It was identified 20 years ago as an NF-κB inducible gene downstream of TNFα and initial observations suggested it could be part of a regulatory negative feedback mechanism (Krikos et al., 1992; Opipari et al., 1990). The generation of A20 knockout mice confirmed its fundamental role in terminating NF-κB signals. $A20^{-/-}$ mice present with multi-organ spontaneous inflammation and cachexia and die prematurely by 3-4 weeks of age. A20 deficient mouse fibroblasts (MEFs) and thymocytes are hyperresponsive to TNFα, showing prolonged NF-κB activation and TNF-induced apoptosis (Lee et al., 2000).

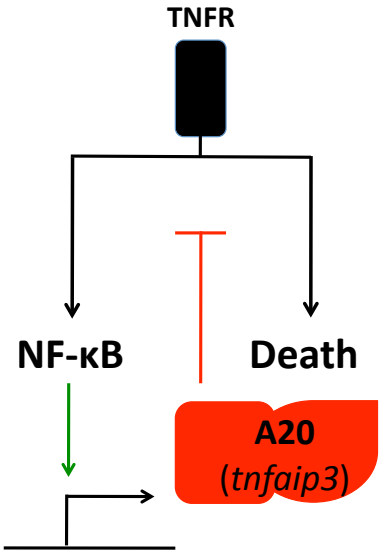

Figure 1.6 – Failure to terminate TNF-induced NF-κB and cell death responses in A20 deficient mice.

Analysis of the A20 sequence identified an N-terminal OTU (Ovarian Tumor) cysteíne protease domain and seven C-terminal Zinc Fingers. Further

research demonstrated that A20 is a dual function ubiquitin-modifying enzyme: It can remove K63 ubiquitin chains from RIP-1 (Receptor Interacting Protein 1), which needs them to operate as an essential mediator of TNFR induced NF-κB activity; Additionally, A20 can also use its zinc fingers to act as an E3 ligase and add K48 ubiquitin chains to RIP-1, targeting it for degradation (Wertz et al., 2004).

The latter observations uncovered a novel mechanism by which signals can be terminated through regulation of different ubiquitylation events. Nonetheless, there is still a lot to be understood about the many potential functions of this enzyme. Namely, it is remains unclear if there is ubiquitin type specificity for each one of A20's domains, whether that depends on each particular target, and finally, whether additional targets exist but remain unidentified. Furthermore, very recently, an additional mechanism for A20's action has been proposed, with evidence showing that A20 could antagonize TRAF E3 ligase activities by impeding them from binding E2 ligases (Shembade et al., 2010).

In spite of A20 being a potent inhibitor of TNF induced signals, $A20^{-/-}TNF^{-/-}$ mice also displayed spontaneous multi-organ inflammation and early lethality as $A20^{-/-}$ mice (Boone et al., 2004). This indicates signals other than TNF are responsible for initiating the spontaneous inflammation in the absence of A20. RAG (Recombination Activation Genes) deficiency (results in the absence of T an B cells) also does not rescue the $A20^{-/-}$ mice phenotype, demonstrating that B and T lymphocytes also are not responsible for initiating the generalized inflammation in these animals. On the other hand, A20 was shown to be required to terminate TLR and NOD (Nucleotide-binding oligomerization domain-containing) protein signals, and promote deubiquitylation of TRAF6 and RIP2, respectively (Boone et al., 2004; Hitotsumatsu et al., 2008).

More recently, some hints into the regulation of A20 itself have been discovered. Firstly, A20 binding proteins and some of their roles in collaborating

with A20 on signaling regulation have been described (Iha et al., 2008; Oshima et al., 2009; Papoutsopoulou et al., 2006; Shembade et al., 2007; Shembade et al., 2009; Wullaert et al., 2006). Secondly, post-translational modifications of A20 were also shown to have a role in signaling regulation. Downstream of TNF and LPS, IKKβ phosphorylates A20, which increases its inhibitory potential by and unknown mechanism (Hutti et al., 2007); In T an B cells, MALT1 can cleave A20 and disrupt its inhibitory activity (Coornaert et al., 2008).

A20 in autoimmunity and cancer

In the past 2 years there has been a striking number of reports of genetic associations between *TNFAIP3* polymorphisms and human autoimmune diseases: Inflammatory Bowel Disease (IBD), Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), Type I Diabetes (T1D), Coeliac Disease and Atherosclerosis (Bates et al., 2009; Duan et al., 2009; Graham et al., 2008; Kawasaki et al., 2010; Musone et al., 2008; Orozco et al., 2009; Plenge et al., 2007; Prahalad et al., 2009; Thomson et al., 2007; Trynka et al., 2009). Additionally, somatic mutations and deletions of A20 were found in various B cell lymphomas at remarkably high frequencies (Compagno et al., 2009; Honma et al., 2007; Honma et al., 2009; Kato et al., 2009; Malynn and Ma, 2009; Novak et al., 2009; Schmitz et al., 2009).

In conclusion, A20 has emerged as a fundamental regulator of immune signals. Its magnitude and versatility, suggest there is still a lot to be learned about its further roles in different cell types and pathways, and the variations of mechanism it uses to accomplish its tasks. Additionally, the recent associations with a large number of human diseases confirm its absolute importance.

THESIS AIMS AND RATIONALE

In the first part of this thesis work, described in chapter 2, we aimed to define which basal *in vivo* signals are responsible for initiating the systemic inflammation in the absence of A20. While TNF signals were excluded from accounting for triggering the multi-organ disease, it was found that A20 terminates TLR responses. Given that TLRs are essential and proximal regulators early during immune responses, and thus could trigger a widespread activation of the immune system, we asked whether TLR signals had to be constantly regulated by A20 in homeostatic conditions (i.e. without further external stimulation). Moreover, given that there is permanent TLR stimulation in the gut, we hypothesized this could be the site of initiation of such signals.

In chapter 3, our goal was to identify additional roles for A20 in the adaptive immune system. Precisely, we asked whether A20 regulates B cell function. Whereas in most cell types A20 expression is low before stimulation dependent induction, in B and T cells the baseline expression of A20 is higher, suggesting it could have a role in lymphocytes. Moreover, B cells are regulated by signals that lead to activation of NF-κB and which share some of the same intermediate molecules participating in A20 targeted pathways. Finally, the GWAS (Genome Wide Association Studies) that linked A20 with B cell mediated autoimmune disease (SLE) and the frequent somatic deletions and mutations of A20 in B cell lymphomas, further indicate a role for A20 in B lymphocytes.

CHAPTER 2

Homeostatic MyD88-dependent signals cause lethal inflammation in the absence of A20

Homeostatic MyD88-dependent signals cause lethal inflammation in the absence of A20

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SUMMARY

Toll-like receptors (TLRs) on host cells are chronically engaged by microbial ligands during homeostatic conditions. These signals do not cause inflammatory immune responses in unperturbed mice, even though they drive innate and adaptive immune responses when combating microbial infections. A20 is a ubiquitin-modifying enzyme that restricts exogenous TLR-induced signals. We show that MyD88-dependent TLR signals drive the spontaneous T cell and myeloid cell activation, cachexia, and premature lethality seen in A20-deficient mice. We have used broad spectrum antibiotics to demonstrate that these constitutive TLR signals are driven by commensal intestinal flora. A20 restricts TLR signals by restricting ubiquitylation of the E3 ligase tumor necrosis factor receptor – associated factor 6. These results reveal both the severe proinflammatory pathophysiology that can arise from homeostatic TLR signals as well as the critical role of A20 in restricting these signals in vivo. In addition, A20 restricts MyD88-independent TLR signals by inhibiting Toll/ interleukin 1 receptor domain – containing adaptor inducing interferon (IFN) β – dependent nuclear factor KB signals but not IFN response factor 3 signaling. These findings provide novel insights into how physiological TLR signals are regulated.

Abbreviations used: BMDM, bone marrow – derived macrophage; HSC, hematopoietic stem cell; IRAK-M, IL-1R – associated kinase M; IRF, IFN response factor; MCP, monocyte chemoattractant protein; mRNA, messenger RNA; PAMP, pathogen-associated molecular pattern; poly (I:C), poly-inosine:cytosine; RIP, receptor-interacting protein; R.U., relative units; SIGIRR, single Ig and TIR domain; SOCS, suppressor of cytokine signaling; TIR, Toll/ IL-1 receptor; TLR, Toll-like receptor; TRAF, TNF receptor – associated factor; TRIF, TIR domain – containing adaptor inducing IFN-β.

INTRODUCTION

Higher eukaryotes are colonized by microbial organisms, predominantly on interfaces with the environment such as the skin and gastrointestinal tract (1). Recent studies suggest that host cells, including professional antigen-presenting cells, " sense " the presence of microbial molecules under homeostatic conditions (2, 3). This sensing is performed by several families of cellular receptors, including Toll-like receptors (TLRs) and non-TLR proteins such as NOD/CATERPILLAR family proteins (4, 5). Collectively, these proteins are thought to bind to conserved pathogen-associated molecular patterns (PAMPs) and activate proinflammatory signals in host cells. The appreciation that microbial sensors such as TLRs may chronically or tonically engage PAMPs during homeostasis raises a major question: if TLRs trigger both adaptive and innate immune responses, why do homeostatic TLR signals not trigger inflammation? Despite the presence of up to 10^{11} bacteria per gram of luminal contents within the gastrointestinal tract, most humans tolerate this colonization without adverse effects. Indeed, host cell sensing of luminal microbes may provide beneficial signals under homeostatic conditions (2). How immune responses to commensal flora are restrained during homeostasis thus remains one of the central questions in immune regulation.

Inflammatory TLR signals might be distinguished from homeostatic TLR signals by the quantity or strength of TLR signals, by cell type – specific responses, by the cellular location of PAMP/TLR interactions (e.g., apical vs. basal surfaces or intracellular vs. membrane-bound receptors), or by additional extracellular cues (e.g., cellular stress response proteins) that modify the cellular interpretation of such signals. Among these general mechanisms, perhaps the most attention has been focused on proteins that regulate the intracellular strength, duration, and/or character of PAMP-triggered signaling.

TLR proteins contain leucine-rich repeat regions that mediate ligand

binding. TLRs also contain intracellular Toll/IL-1 receptor (TIR) domains that trigger signals via homotypic interactions with proximal adaptor proteins (6, 7). All TLRs signal via the adaptor MyD88, leading to activation of NF-KB and mitogen-activated protein (MAP) kinase pathways. NF-KB activation leads to a transcriptional program that up-regulates proinflammatory gene products such as IL-1β and TNF-α and, ultimately, to the activation and accumulation of monocytes. MyD88 is essential for most TLR-induced NF-KB signaling, as MyD88-deficient ($MyD88^{-/-}$) cells exhibit reduced and delayed NF-KB signaling activity (8, 9). TLR3 and TLR4 also use another adaptor, TIR domain – containing adaptor inducing IFN- β (TRIF). TRIF-dependent signals activate IFN response factor 3 (IRF3) in addition to NF-KB (10, 11). These transcription factors induce productive antiviral immune responses by binding to promoters of type I IFN and chemotactic genes (12).

Constitutive MyD88-dependent TLR signals provide beneficial, noninflammatory signals (2). These signals can also trigger spontaneous inflammation. Thus, a major issue arises as to how homeostatic TLR signals are distinguished from inflammatory TLR signals. One potential mechanism for distinguishing these TLR signals may be related to recent discoveries that endogenous proteins such as IL-1R – associated kinase M (IRAK-M), ST2, and single Ig and TIR domain (SIGIRR) can restrict TLR signals (13 – 18). These proteins can restrict the duration and/or intensity of TLR signals and modulate the cellular outcome of TLR signaling, thereby helping to determine whether TLR signals lead to homeostatic or inflammatory responses. Notably, mice lacking IRAK-M, ST2, and SIGIRR exhibit relatively modest inflammation and survive for longer than 9 – 12 mo (14, 16, 17). Suppressor of cytokine signaling (SOCS) 1 can also inhibit TLR signaling, and $SOCS\text{-}1^{-/-}$ mice exhibit severe spontaneous inflammation and lymphocyte-mediated perinatal lethality; however $SOCS\text{-}1^{-/-}$ mice are rescued by the absence of IFN-" signaling in $SOCS\text{-}1^{-/-}IFN\text{-}"^{-/-}$ double-mutant mice (18, 19). Thus, it is unclear whether these proteins restrict TLR signals in

unperturbed mice and whether restricting homeostatic TLR signals is important for immune homeostasis.

A20 is an inducible ubiquitin-editing enzyme that restricts both TLR- and TNF-induced responses by regulating the ubiquitylation of key signaling proteins (20, 21, 22, 23). A20 deficient ($A20^{-/-}$) mice spontaneously develop multiorgan inflammation, severe cachexia, and premature lethality that can start within 1 – 2 wk of age, demonstrating the potent antiinflammatory functions of this molecule (22). In addition, $A20^{-/-}$RAG-$1^{-/-}$ double-mutant mice spontaneously develop severe inflammation, cachexia, and premature death, indicating that A20 potently regulates innate immune homeostasis in the absence of adaptive lymphocytes (22). Although A20 is critical for restricting TNF-induced NF-KB signals, both $A20^{-/-}$ $TNF^{-/-}$and $A20^{-/-}$$TNFR1^{-/-}$ double-mutant mice spontaneously develop inflammation, cachexia, and premature death, indicating that A20 is critical for restricting inflammatory signals independently of TNF/TNFR1 signaling (22).

A20 expression is rapidly induced in macrophages upon TLR stimulation and is required for restricting the duration of TLR-induced NF-KB signaling and the quantity of TLR-induced proinflammatory cytokines (22). Hence, A20 functions as a negative feedback regulator of exogenous TLR-induced signals. As TLR ligands from commensal microorganisms may engage host cells during homeostatic conditions, we have investigated the potential roles A20 may play in regulating homeostatic TLR signals by interbreeding $A20^{-/-}$mice with $MyD88^{-/-}$mice. In addition, we have questioned the role of commensal bacteria in driving spontaneous inflammation in $A20^{-/-}$mice. Finally, we have used $A20^{-/-}$$MyD88^{-/-}$ double-mutant mice to examine the role of A20 in regulating MyD88-independent TLR signals.

RESULTS

MyD88 deficiency prevents premature lethality and cachexia in A20$^{-/-}$ mice

As commensal microbes or endogenous ligands may engage host TLRs under basal conditions, we investigated whether TLR signals contribute to the development of spontaneous inflammation in A20$^{-/-}$ mice by genetically removing MyD88-dependent TLR signals. A20$^{-/-}$mice were interbred with MyD88$^{-/-}$ mice and backcrossed at least five generations to C57BL/6. Remarkably, although only 30% of A20$^{-/-}$ mice survive to 8 wk of age, nearly 95% of A20$^{-/-}$MyD88$^{-/-}$ double-mutant mice survive past this age (Fig. 1 A). This improved survival rate is similar to that of MyD88$^{-/-}$mice. Additionally, surviving A20$^{-/-}$MyD88$^{-/-}$double-mutant animals do not exhibit the severe cachexia manifested by surviving A20$^{-/-}$mice (Fig. 1 B). A20$^{-/-}$ mice weigh only ≈60% of what wild-type controls weigh, whereas A20$^{-/-}$MyD88$^{-/-}$ mice weigh ≈90% of what MyD88$^{-/-}$ controls weigh (Fig. 1 C). These data suggest that MyD88 signals drive potent proinflammatory responses that lead to cachexia and premature death in the absence of A20.

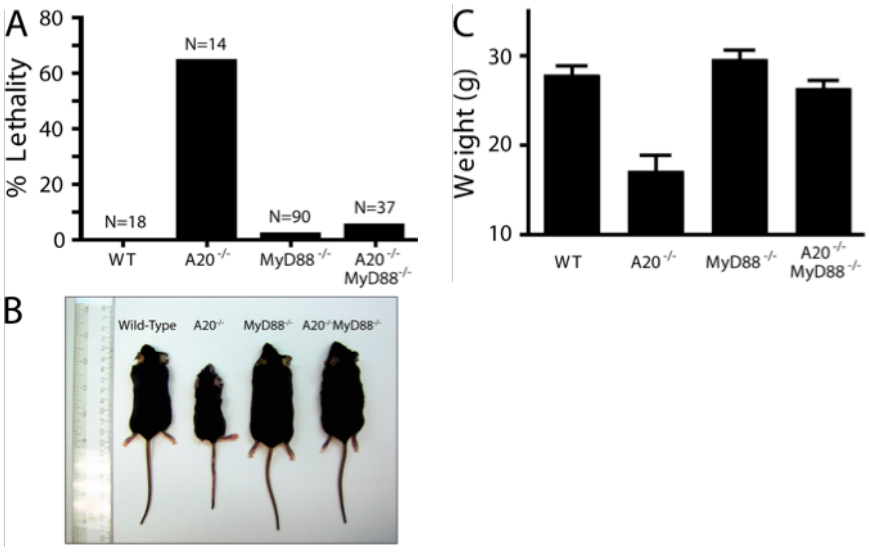

Figure 1. MyD88-dependent cachexia and lethality in A20$^{-/-}$ mice. (A) Survival of mice from the indicated genotypes at 8 wk of age (WT, A20 $^{+/-}$ MyD88$^{+/-}$; A20 $^{-/-}$, A20 $^{-/-}$MyD88$^{+/-}$; MyD88 $^{-/-}$, A20 $^{+/-}$MyD88$^{-/-}$; and A20$^{-/-}$MyD88$^{-/-}$). The percentage of mice in each cohort that dies before 8 wk of age is indicated by the column heights. Total numbers of mice in each cohort are indicated above each column.

(B) Representative 3-mo-old mice from the indicated genotypes. Note the cachexia of the A20$^{-/-}$but not the A20$^{-/-}$MyD88$^{-/-}$ mouse. (C) Weight of surviving mice. Graph represents mean body weights of 10 – 14-wk-old mice from the indicated genotypes. Error bars represent standard deviations. Data are representative of at least five mice from each genotype.

Homeostatic MyD88-dependent signals activate myeloid cells in A20$^{-/-}$ mice

MyD88-dependent TLR signals activate myeloid cells, leading to the production of cytokines that recruit and activate other innate immune cells. A20$^{-/-}$, A20$^{-/-}$RAG-1$^{-/-}$double-mutant, A20$^{-/-}$TNF$^{-/-}$double-mutant, and A20$^{-/-}$TNFR1$^{-/-}$ double-mutant mice all spontaneously accumulate myeloid cells in both lymphoid and nonlymphoid tissues (21, 22). To begin to examine the role of MyD88 in causing inflammation in A20 $^{-/-}$mice, we measured the spleen weights as a reflection of the cellularity of these organs. Spleen weights were dramatically increased in A20$^{-/-}$mice when compared with wild-type or MyD88$^{-/-}$mice (Fig. 2 A), and this splenomegaly was particularly impressive given that A20$^{-/-}$ mice were significantly smaller than the other genotypes of mice (Fig. 1 C). In contrast, the weight of spleens obtained from double-deficient A20 $^{-/-}$MyD88$^{-/-}$mice was significantly less than that obtained from A20 $^{-/-}$mice and more closely

approximated the weight of spleens obtained from wild-type and MyD88$^{-/-}$ mice (Fig. 2 A.)

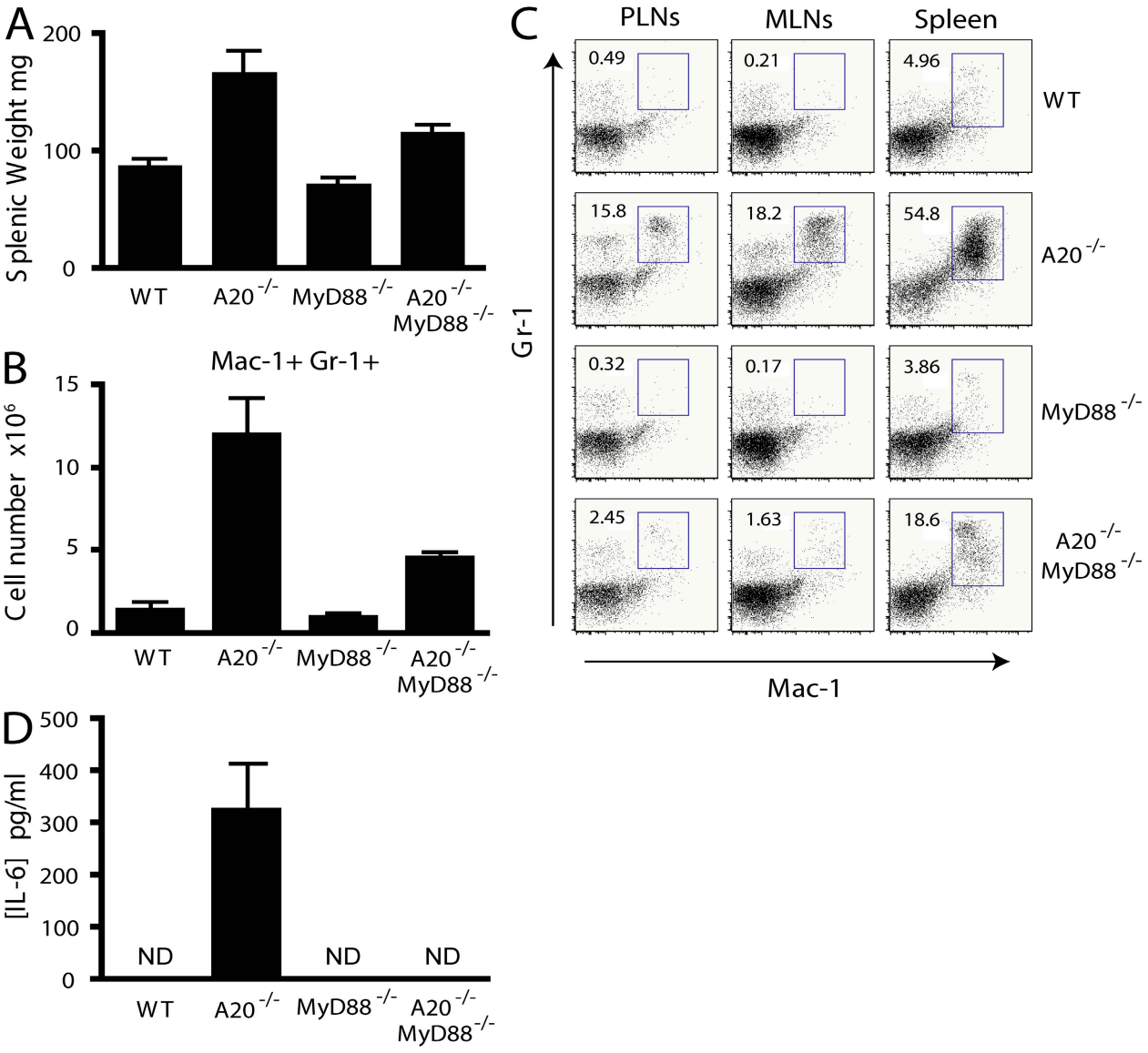

Figure 2. Reduced myeloid accumulation and splenomegaly in A20$^{-/-}$MyD88$^{-/-}$ mice. (A) Spleen weights from 6 – 8-wk-old mice of the indicated genotypes. (B) Total numbers of Mac1^{+} Gr1^{+} myeloid cells (activated macrophages and granulocytes) from spleens of mice of the indicated genotypes. (C) Representative flow cytometric analysis of Mac-1^{+} Gr-1^{+} myeloid cells from peripheral and mesenteric lymph nodes and spleens from mice of the indicated genotypes. Note

the decreased frequency and numbers of myeloid cells in lymphoid organs from A20-/-MyD88-/-mice compared with A20$^{-/-}$ mice. Numbers indicate the percentage of live Mac-1+ Gr-1+ splenocytes within the indicated gates. (D) ELISA analyses of IL-6 levels in serum from mice of the indicated genotypes. Error bars represent standard deviations. Data are representative of at least five mice from each genotype.

To further examine the cellular nature of MyD88-dependent inflammation in A20 $^{-/-}$ mice, we performed flow cytometric analyses of tissues from these wild-type, A20$^{-/-}$, MyD88$^{-/-}$, and A20$^{-/-}$MyD88$^{-/-}$ mice. These studies revealed that significantly greater numbers and percentages of myeloid (Mac1^{+} Gr1^{+}) cells accumulate spontaneously in spleens of A20$^{-/-}$ mice when compared with wild-type mice, whereas the numbers of these cells obtained from A20$^{-/-}$MyD88$^{-/-}$ mice were significantly less than those obtained from A20$^{-/-}$ mice and more closely approximated the numbers obtained from wild-type and MyD88$^{-/-}$ mice (Fig. 2, B and C).

Myeloid cells were selectively expanded in tissues of A20$^{-/-}$ mice at the cost of other cells, as the percentage of Mac1^{+} Gr1^{+} cells was increased when compared with wild-type or MyD88$^{-/-}$mice (Fig. 2 C). This expansion was also largely (but not completely) ameliorated in A20$^{-/-}$MyD88$^{-/-}$ mice. To assess the functional activation of myeloid cells such as macrophages, we measured serum levels of IL-6 in these mice. Serum IL-6 levels were dramatically elevated in A20$^{-/-}$ mice, as compared with MyD88$^{-/-}$ or wild-type mice, whereas A20$^{-/-}$ MyD88$^{-/-}$ mice had undetectable levels of IL-6 (Fig. 2 D). These findings indicate that A20 prevents spontaneous activation and recruitment of innate immune cells in mice by restricting homeostatic MyD88-dependent signals.

Homeostatic MyD88-dependent signals activate T cells in A20$^{-/-}$ mice

TLR-dependent signals play important roles in inducing adaptive immune response during microbial infections (24, 25). For example, MyD88-dependent signals on antigen-presenting cells support the activation and expansion of T cells. To determine whether basal MyD88-dependent signals cause spontaneous activation and expansion of T cells in unperturbed A20 $^{-/-}$mice, we examined the number and activation state of T cells from A20$^{-/-}$, A20$^{-/-}$MyD88$^{-/-}$, and control mice. Significantly greater percentages (and total numbers) of activated, memory phenotype (CD44^{Hi} CD62L^{Lo}) T cells were present in lymph nodes and spleens of A20$^{-/-}$mice when compared with wild-type mice (Fig. 3 A and not depicted). In contrast, T cells from A20$^{-/-}$MyD88$^{-/-}$ double-mutant mice were less activated than those from A20$^{-/-}$ mice and were more comparable to wild-type and MyD88$^{-/-}$ mice. This reduction in spontaneous T cell activation was observed in both CD4^{+} and CD8^{+} T cell populations (Fig. 3, B and C). This finding suggests that homeostatic MyD88-dependent signals cause T cell activation in the absence of A20.

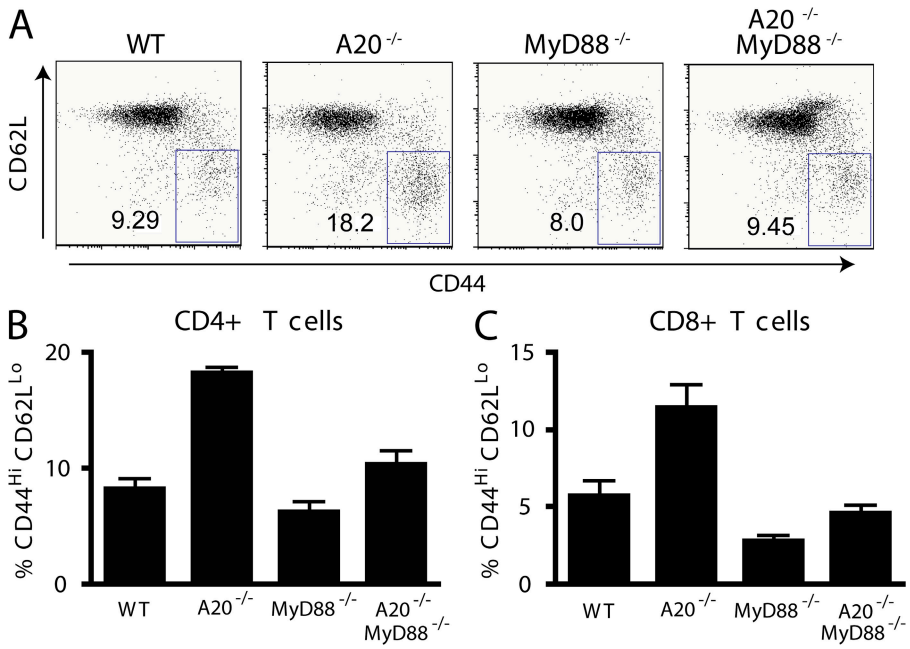

Figure 3. MyD88 dependence of T cell hyperactivation in A20$^{-/-}$ mice. (A) Flow cytometric analyses of T cell phenotypes from peripheral lymph nodes from 5 – 6-wk-old mice of the indicated genotypes. Representative FACS plots of CD4^{+} T cells. The numbers represent the percentages of CD4^{+} T cells that are CD44^{Hi} and CD62L^{Lo} (boxed gates), indicating an effector/memory T cell phenotype. Note the increased frequency of activated CD4^{+} T cells in A20$^{-/-}$ animals, and the reduced percentages of activated T cells in A20$^{-/-}$MyD88$^{-/-}$mice. (B) Quantitation of activated CD4^{+} (CD44^{Hi} CD62L^{Lo}) T cells. (C) Quantitation of activated CD8^{+} T cells. Error bars represent standard deviations. Data are representative of at least three mice per genotype.

Homeostatic MyD88-dependent signals in radiation sensitive hematopoietic cells drive myeloid cell activation in A20$^{-/-}$ mice

Hematopoietically derived cells such as macrophages and dendritic cells are able to sample surrounding microflora and transmit basal proinflammatory

signals. In addition, A20 and MyD88 are both expressed in epithelial cells and stromal cells, which can also respond to TLR ligands. Thus, A20's role in restricting MyD88-dependent signals could involve signals on either hematopoietic cells, stromal cells, or both. To isolate the potential role of A20 in regulating MyD88 signaling in hematopoietic cells, Ly5.2 C57BL/6 mice were lethally irradiated and reconstituted with bone marrow hematopoietic stem cells (HSCs) from either wild-type, $A20^{-/-}$, $MyD88^{+/+}$, $MyD88^{-/-}$, or $A20^{-/-}MyD88^{-/-}$ mice. Flow cytometric analyses of tissues from these chimera were then performed 6 wk after reconstitution. Similar to our findings with intact mice, spleens from radiation chimera reconstituted with $A20^{-/-}$ HSCs contained increased numbers of various myeloid cell types when compared with chimera reconstituted with wild-type HSCs (Fig. 4 A).

The myeloid cells that expand in $A20^{-/-}$ HSC-reconstituted chimera spleens include various myeloid populations such as macrophages (Mac-1^{+}, $F480^{+}$, and Siglec F^{-}), neutrophils (Mac-1^{+}, Gr-1^{+}, $F480^{-}$, and Siglec F^{-}), and eosinophils (Mac-1^{+}, Siglec F^{+}, and SSC^{Hi}) without any particular skewing toward particular subsets (Fig. 4 A). The absolute numbers of these populations were generally reduced in $A20^{-/-}MyD88^{-/-}$ HSC-reconstituted chimera compared with $A20^{-/-}$ HSC-reconstituted chimera, although some of these reductions were less dramatic than the differences observed in intact $A20^{-/-}MyD88^{+/+}$ and $A20^{-/-}MyD88^{-/-}$ mice (Figs. 2 and 4 A). To further characterize the nature of the spontaneous inflammation caused by $A20^{-/-}$hematopoietic cells in these chimera, we analyzed splenic production of cytokines. These studies revealed that spleens from $A20^{-/-}$ HSC-reconstituted chimera produced elevated levels of TNFα and IL-1β (Fig. 4 B).
These indicators of spontaneous inflammation were absent in $A20^{-/-}MyD88^{-/-}$ HSC-reconstituted chimera (Fig. 4 B). As IL-10 – deficient mice have recently been described to develop spontaneous colitis that resolves in the absence of MyD88 signals (3), we examined whether $A20^{-/-}$ HSC-reconstituted chimera were IL-10

deficient. A20$^{-/-}$ mice expressed elevated levels of IL-10, suggesting that IL-10 deficiency does not contribute to spontaneous inflammation in A20$^{-/-}$ mice (Fig. 4 B). A20$^{-/-}$ HSC-reconstituted chimera also expressed normal levels of IL-2 and possessed normal numbers of functional regulatory T cells (Fig. 4 B and not depicted). Thus, IL-2 deficiency is also unlikely to explain the spontaneous inflammation in A20$^{-/-}$ mice. Finally, A20$^{-/-}$ HSC-reconstituted chimera expressed elevated levels of IL-13 as well as the prototypical Th1 cytokines TNFα and IFN-γ (Fig. 4 B and not depicted). Hence, no gross evidence of Th1 or Th2 skewing is evident in these mice.

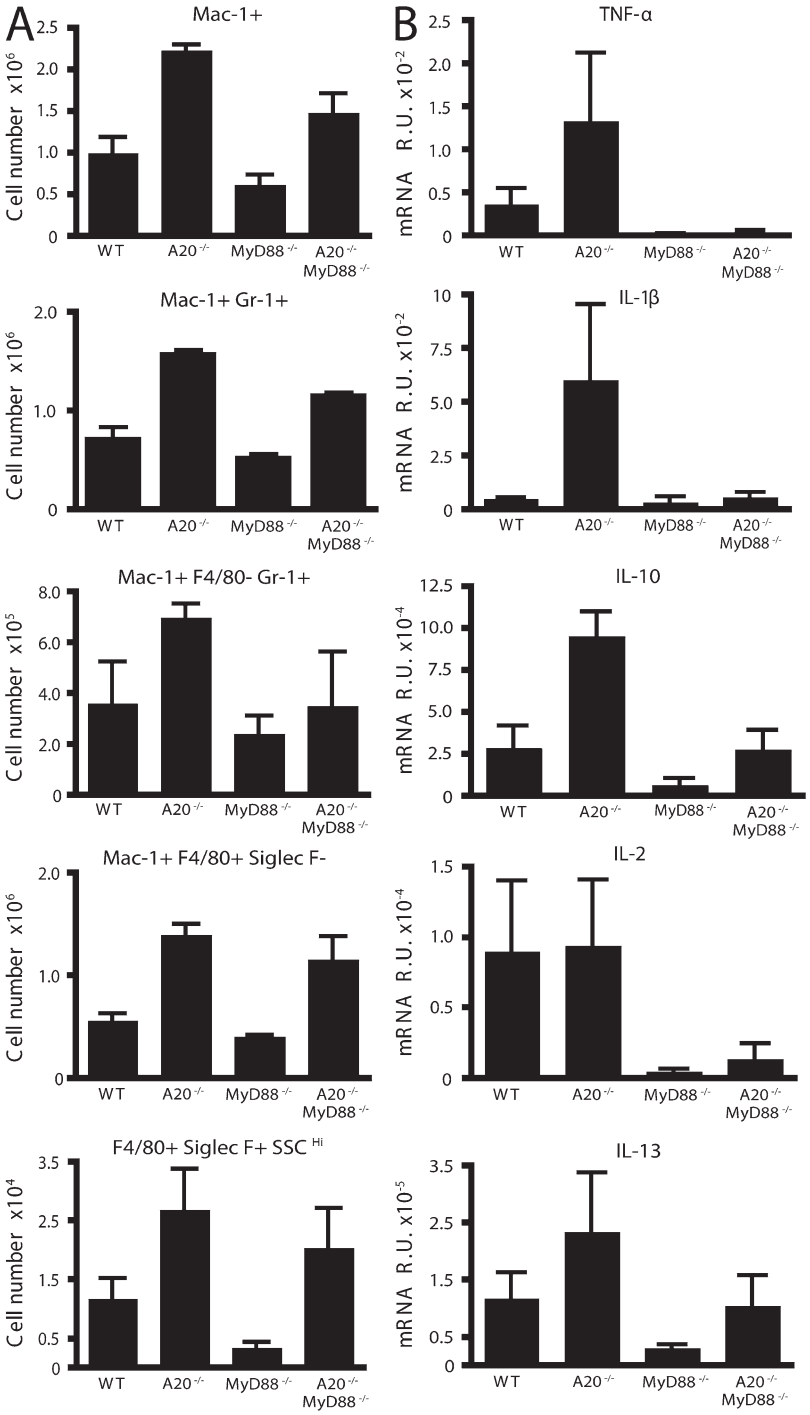

Figure 4. A20 expression in hematopoietic cells restricts MyD88-driven spontaneous inflammation in radiation chimera. (A) Flow cytometric analyses of chimeric mice generated by transfer of A20$^{+/-}$MyD88$^{+/-}$, A20 $^{-/-}$MyD88$^{+/+}$, A20 $^{+/+}$ MyD88$^{-/-}$, or A20$^{-/-}$MyD8$^{-/-}$ bone marrow HSCs into lethally irradiated C57BL/6J

mice. 6 wk after reconstitution, spleens from chimeric mice were analyzed by flow cytometry for the number of the indicated myeloid cell types ($Mac1^+$ $Gr1^+$, activated macrophages and neutrophils; $Mac1^+$ $F4/80^-Gr1^+$, neutrophils; $Mac1^+$ $F4/80^+$ Siglec F^-, macrophages; and $F4/80^+$ Siglec F^+ SSC^{Hi}, eosinophils). The numbers of Mac-1^+, Mac-1^+ $Gr\text{-}1^+$, and Mac-1^+ $F4/80^-Gr\text{-}1^+$ cells were statistically different ($P < 0.05$) between $A20^{-/-}$ and $A20^{-/-}MyD88^{-/-}$ mice. (B) Quantitative real-time PCR analyses of mRNA levels of the indicated cytokines in spleens from chimeric mice. Splenic expression levels of the indicated cytokines are shown in R.U. for the chimeric mice described in A. All cytokine mRNA levels were normalized to β-actin mRNA. The levels of cytokines were statistically different between $A20^{-/-}$ and $A20^{-/-}MyD88^{-/-}$ mice for all cytokines. Error bars indicate standard deviations. Graphs display results from at least three independent mice per genotype.

Collectively, these data suggest that homeostatic MyD88 signals in hematopoietic cells drive a broad spectrum of spontaneous proinflammatory pathways in the absence of A20.

Commensal bacteria stimulate homeostatic MyD88-dependent signals

Homeostatic MyD88-dependent signals may be derived from host molecules or from commensal microbes that express TLR ligands and are in constant contact with host cells. By far, the most abundant source of commensal microbes is the intestinal lumen. Thus, to determine whether commensal intestinal microbes stimulate the homeostatic MyD88-dependent signals that drive inflammation in $A20^{-/-}$ mice, we first generated radiation chimera using HSCs from $A20^{+/+}$ and $A20^{-/-}$ bone marrows. 2 wk after irradiation, we treated these chimera with oral broad spectrum antibiotics previously shown to markedly reduce the

number of intestinal bacteria (vancomycin, neomycin, metronidazole, ampicillin, and trimethoprim-sulfamethoxazole). After 3 wk of treatment with this antibiotic cocktail (or with trimethoprim-sulfamethoxazole alone as a control), chimeric mice were analyzed for the numbers of intestinal bacteria as well as for evidence of spontaneous inflammation. Chimera reconstituted with $A20^{-/-}$ HSCs developed spontaneous cachexia and lost weight when compared with chimera reconstituted with wild-type HSCs (Fig. 5 A). These results suggest that $A20^{-/-}$ hematopoietic cells recapitulate the spontaneous inflammation observed in intact $A20^{-/-}$ mice. Chimera treated with the cocktail of broad spectrum antibiotics possessed several orders of magnitude fewer intestinal bacteria (not depicted). In contrast with $A20^{-/-}$ HSC-reconstituted chimera treated with trimethprim-sulfamethoxazole alone, $A20^{-/-}$ HSC-reconstituted chimera treated with the cocktail of broad spectrum antibiotics maintained their weight (Fig. 5 A). Thus, antibiotic treatment prevents cachexia caused by A20 deficiency. Large numbers of $Mac1^{+}$ $Gr1^{+}$ myeloid cells spontaneously accumulated in the spleens of $A20^{-/-}$ HSC-reconstituted chimera but not in spleens from antibiotic-treated $A20^{-/-}$ HSC-reconstituted chimera (Fig. 5 B). Finally, spleens from $A20^{-/-}$ HSC-reconstituted chimera spontaneous produced elevated levels of IL-6 and TNF messenger RNAs (mRNAs) compared with spleens from wild-type HSC-reconstituted chimera (Fig. 5 C). These indicators of inflammation were ameliorated in $A20^{-/-}$ HSC-reconstituted chimera treated with broad spectrum antibiotics (Fig. 5 C). Collectively, these results indicate that commensal intestinal bacteria drive spontaneous cachexia and myeloid inflammation via MyD88 signals in the absence of A20.

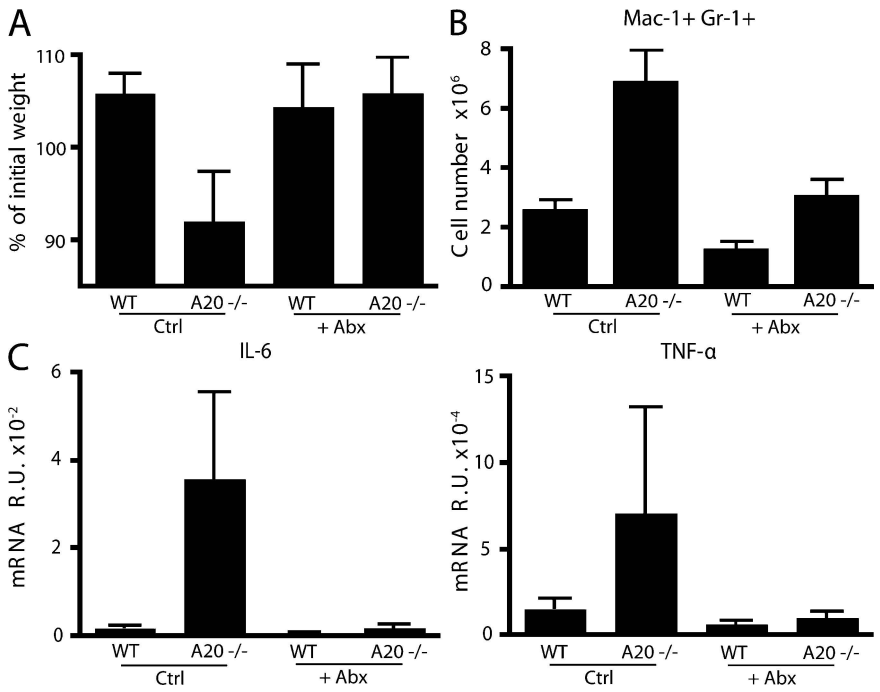

Figure 5. Depletion of commensals with broad-spectrum antibiotics diminishes inflammation driven by A20$^{-/-}$ hematopoietic cells. Chimeric mice were generated from transfer of A20$^{+/+}$ (WT) or A20$^{-/-}$ Ly5.1^{+} bone marrow HSCs into sublethally irradiated congenic Ly5.2^{+} C57BL/6J mice. 2 wk after irradiation, animals were given 0.5 gram per liter of vancomycin, 1 gram per liter of ampicillin, 1 gram per liter of neomycin, 1 gram per liter of metronidazole, and trimethoprim-sulfamethoxazole in drinking water for 3 – 4 wk (+Abx) or maintained on trimethoprim-sulfamethoxazole (Ctrl) for the same period. (A) The percent weight change of chimeric mice reconstituted with HSCs of the indicated genotypes and treated with either broad spectrum (+Abx) or control antibiotics (Ctrl). The percent change was calculated by dividing the weight of each mouse on the day of death/analysis by the weight on the day of irradiation and HSC reconstitution. (B) Flow cytometric analyses of Ly5.1^{+} Mac1^{+} Gr1^{+} cells from spleens of chimeric mice. (C) Quantitative real-time PCR analysis of mRNA levels of IL-6 and TNF in spleens of chimeric mice. Note that A20$^{-/-}$ HSC-reconstituted chimera spontaneously lose weight, accumulate myeloid cells, and express higher levels of splenic IL-6 and TNF than A20$^{+/+}$ HSC-reconstituted chimera, whereas treatment

of A20$^{-/-}$ HSC-reconstituted chimera with broad spectrum antibiotics prevents these signs of inflammation. Error bars represent standard deviations. Data are representative of five mice.

TRIF-dependent TLR signals are restricted by A20

In the absence of the TLR adaptor MyD88, TLR3 and TLR4 signal through an alternate adaptor protein, TRIF. TRIF-dependent responses lead to the production of type I IFNs (IFN-α and IFN-β) and are critical for productive antiviral immunity (10, 11). We thus asked whether A20 was physiologically required for restricting TRIF-dependent TLR responses. To examine this question, we injected LPS intraperitoneally into either A20$^{-/-}$MyD88$^{-/-}$ or MyD88$^{-/-}$ HSC-reconstituted chimeric mice and measured the resulting TRIF-dependent inflammatory response. We first measured the production of IFN-β and monocyte chemoattractant protein (MCP) 1, two TRIF-dependent proinflammatory cytokines that are produced by macrophages and dendritic cells. Quantitative real-time PCR analysis of splenocytes from these mice revealed that IFN-β and MCP-1 expression are induced by LPS to a greater degree in A20$^{-/-}$MyD88$^{-/-}$ HSC-reconstituted chimera than in MyD88$^{-/-}$ HSC-reconstituted chimera (Fig. 6 A). To assess the role of A20 in regulating MyD88-independent recruitment of granulocytes to LPS, we quantitated the number of Mac-1^{+} Gr-1^{+} F4/80^{-}cells (neutrophils) recruited to the peritoneal cavity 2 h after LPS injection. These experiments indicated that far greater numbers of these cells were obtained from the peritoneal lavage of LPS-stimulated A20$^{-/-}$MyD88$^{-/-}$ HSC-reconstituted chimera than from MyD88$^{-/-}$ HSC-reconstituted chimera (Fig. 6 B). We also measured the production of MCP-1 protein in peritoneal lavages from these mice by ELISA and found that MCP-1 protein was produced at higher levels in A20$^{-/-}$MyD88$^{-/-}$ HSC-reconstituted chimera than in MyD88$^{-/-}$ HSC-reconstituted chimera (Fig. 6 C).
Finally, IFN- β stimulates IL-15 and IL-15Rα production, leading to NK cell

activation in early antiviral immunity. Soluble IL-15Rα is released into the serum during this process (unpublished data). We thus measured serum IL-15Rα protein levels in LPS-stimulated chimeric mice by ELISA, and these assays showed that higher levels of soluble IL-15Rα were present in LPS-stimulated A20$^{-/-}$MyD88$^{-/-}$ chimera than in LPS-stimulated MyD88$^{-/-}$ chimera (Fig. 6 D). Collectively, these findings indicate that A20 is physiologically required for restricting acute, MyD88-independent, TRIF-dependent proinflammatory signals in vivo.

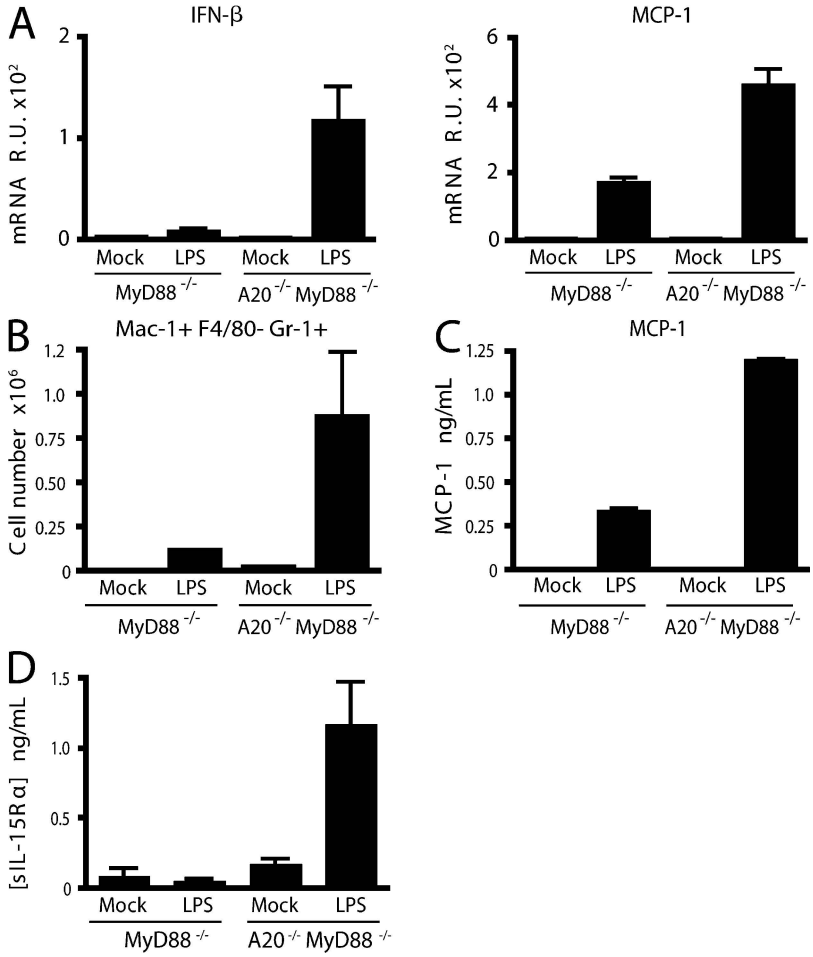

Figure 6. A20 expression in hematopoietic cells restricts TRIF-dependent LPS responses in vivo. Chimeric mice were generated using HSCs of the indicated genotypes and lethally irradiated C57BL/6J mice. 6 wk after reconstitution, chimeric mice were injected with 100 µ g LPS or PBS (mock) intraperitoneally and killed after 2 h. (A) Real-time PCR analyses of IFN-β and

MCP-1 mRNA levels in splenocytes from the indicated mice. mRNA levels were normalized to GAPDH mRNA and expressed as R.U. (B) Flow cytometric analyses of the number of $Mac1^{+}$ $Gr1^{+}$ $F4/80^{-}$ neutrophils recruited to the peritoneal cavities of the indicated mice. Absolute numbers of neutrophils obtained in 5 ml of peritoneal lavage are shown. (C) ELISA determination of levels of MCP-1 protein obtained in peritoneal lavages of mice of the indicated genotypes. (D) ELISA determination of soluble IL-15R a levels measured in the serum of the indicated mice. Error bars represent standard deviations. Data are representative of three mice in each treatment group.

A20 regulates TRIF-dependent TLR-induced NF-KB signaling but not IRF3 signaling

TRIF-dependent TLR signals involve both NF-KB and IRF3-dependent signals (26, 27). To determine how A20 regulates TRIF-dependent TLR responses, we first interbred A20 $^{-/}$MyD88$^{-/-}$ mice with TNF$^{-/-}$ mice. The resulting triple-mutant mice allowed us to evaluate TRIF-dependent TLR signals in the absence of secondary TNF signaling. We then prepared bone marrow – derived macrophages (BMDMs) from these TNF$^{-/-}$MyD88$^{-/-}$ and A20$^{-/-}$TNF$^{-/-}$MyD88$^{-/-}$ mice.

Comparable numbers and phenotypes of these two types of BMDMs were obtained after 6 d of culture in MCSF-supplemented media (not depicted). We then tested the responses of these cells to LPS or poly-inosine:cytosine (poly (I:C)), ligands for TLR4 and TLR3, respectively. After either of these stimuli, A20$^{-/-}$ TNF$^{-/-}$MyD88$^{-/-}$ BMDMs secreted greater amounts of IFN-β than TNF$^{-/-}$MyD88$^{-/-}$ BMDMs (Fig. 7 A and not depicted). Thus, A20 appears to be directly required for restricting TRIF-dependent TLR responses independently of TNF signaling.

The production of IFN-β is dependent on both NF-KB as well as IRF3 signaling, as the IFN-β promoter contains binding elements for both these transcription factors. To further understand the mechanism by which A20 restricts TRIF-

dependent TLR signaling, we stimulated A20$^{-/-}$TNF$^{-/-}$MyD88$^{-/-}$and TNF$^{-/-}$MyD88$^{-/-}$ BMDMs with LPS, prepared whole-cell lysates at various time points, and analyzed these lysates for the expression of IκBα and phospho-IκBα by immunoblotting. When compared with TNF$^{-/-}$MyD88$^{-/-}$BMDMs, A20$^{-/-}$TNF$^{-/-}$MyD88$^{-/-}$ cells exhibited prolonged expression of phospho-IκBα and delayed recovery of IκBα, indicating that A20 is required for restricting TRIF-dependent NF-κB signaling (Fig. 7 B).

In addition to activating NF-κB signaling, TLR3- and TLR4-induced activation of TRIF also causes IRF3 phosphorylation, which in turn leads to its dimerization, nuclear translocation, and stimulation of IFN- α/β gene transcription (12). TNF$^{-/-}$MyD88$^{-/-}$ and A20$^{-/-}$TNF$^{-/-}$MyD88$^{-/-}$ BMDMs were stimulated with LPS for various time periods. Phospho-IRF3 levels were then measured by immunoblotting of nuclear protein extracts. Surprisingly, TNF$^{-/-}$MyD88$^{-/-}$ and A20$^{-/-}$ TNF$^{-/-}$MyD88$^{-/-}$ BMDMs activated phospho-IRF3 with similar kinetics and intensity (Fig. 7 C). Thus, A20 is required for restricting TRIF-dependent NF-κB signaling but not TRIF-dependent IRF3 signaling. Moreover, these data suggest that increased IFN-β production from A20$^{-/-}$TNF$^{-/-}$MyD88$^{-/-}$ BMDMs, when compared with A20$^{+/+}$ TNF$^{-/-}$MyD88$^{-/-}$ BMDMs, is caused by increased NF-κB signaling and not increased IRF3 signaling.

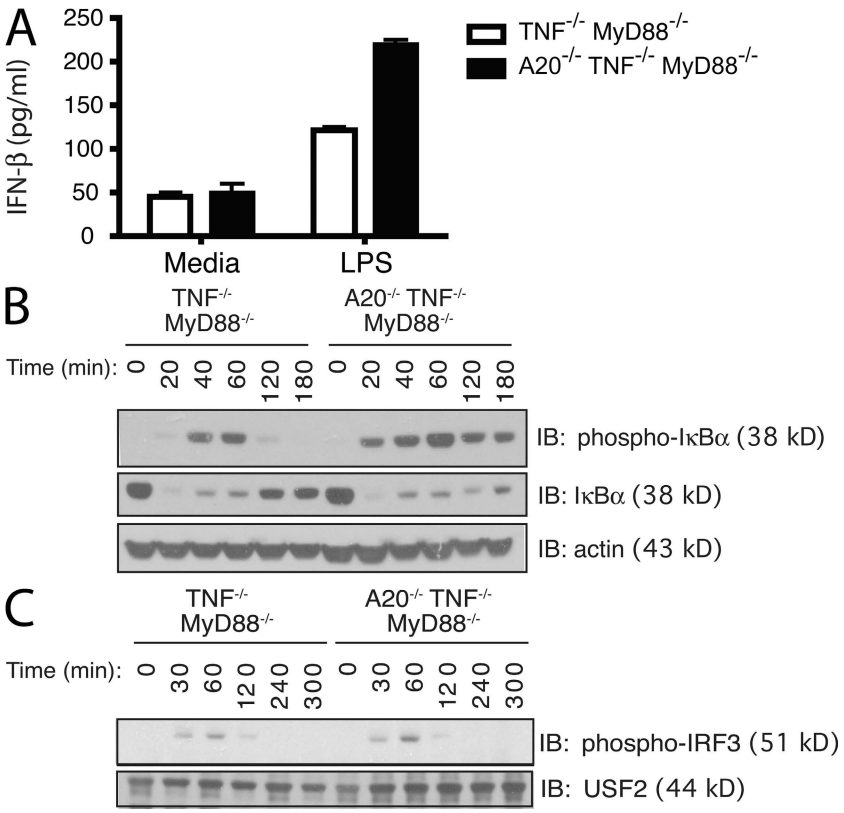

Figure 7. A20 is critical for directly restricting MyD88-independent LPS responses. (A) ELISA analysis of IFN-β secretion by LPS-stimulated BMDMs. TNF$^{-/-}$MyD88$^{-/-}$ and A20$^{-/-}$TNF$^{-/-}$MyD88$^{-/-}$ BMDMs were stimulated for 24 h with LPS, after which supernatants were harvested for ELISA. (B) Immunoblotting analyses of phospho-IκBα and IκB protein expression by LSP-stimulated BMDMs. TNF$^{-/-}$ MyD88$^{-/-}$ and A20$^{-/-}$TNF$^{-/-}$MyD88$^{-/-}$ BMDMs were stimulated with LPS for the indicated times, after which cells were lysed for immunoblotting analyses for the indicated proteins. Actin protein levels are shown as a loading control. (C) Immunoblotting analyses of nuclear lysates for phospho-IRF3 protein expression by LPS-stimulated BMDMs. Nuclear USF2 protein levels are shown as a control. The black line indicates that intervening lanes have been spliced out. Data are representative of at least three experiments.

A20 is essential for restricting TLR-induced TNF receptor – associated factor (TRAF) 6 ubiquitination

Our previous studies showed that A20 restricts TLR-induced NF-κB signaling (22). Our current studies indicate that A20 regulates TRIF-dependent NF-κB signaling but not IRF3 signaling. Collectively, these data suggest that the major function of A20 in TLR signaling is to directly regulate a signaling event that is common to MyD88- and TRIF-dependent NF-κB signaling and that is not shared by TRIF-dependent IRF3 signaling. TRAF6 is an E3 ligase that is involved in both MyD88- and TRIF-dependent NF-κB signaling (28 – 30). Upon stimulation with TLR ligands, TRAF6 is recruited to the TLR in both MyD88-dependent and -independent signaling pathways. TRAF6 autoligates lysine-63 ubiquitin chains upon TLR or IL-1R stimulation to activate NF-κB, and our previous studies showed that A20 is able to use its OTU protease domain to deubiquitylate TRAF6 (22, 30). Thus, TRAF6 may be a physiological target for A20 during TLR signaling. To directly determine if A20 is essential for regulating TRAF6 ubiquitylation during TLR stimulation, $A20^{+/+}$ and $A20^{-/-}$BMDMs were stimulated with LPS for various periods of time, after which cell lysates were analyzed for their expression of ubiquitylated TRAF6. Endogenous TRAF6 is ubiquitylated after 20 min of LPS stimulation (Fig. 8). Importantly, whereas TRAF6 ubiquitylation is transiently induced in $A20^{+/+}$ cells, declining after 40 min, TRAF6 ubiquitylation is prolonged to 40 and 60 min in $A20^{-/-}$ cells (Fig. 8). As these lysates were boiled in 1% SDS before immunoprecipitation of TRAF6, the ubiquitylated proteins we observe are likely to be predominantly ubiquitylated TRAF6 rather than noncovalently associated proteins. This finding indicates that A20 is essential for restricting endogenous TRAF6 ubiquitylation after TLR stimulation.

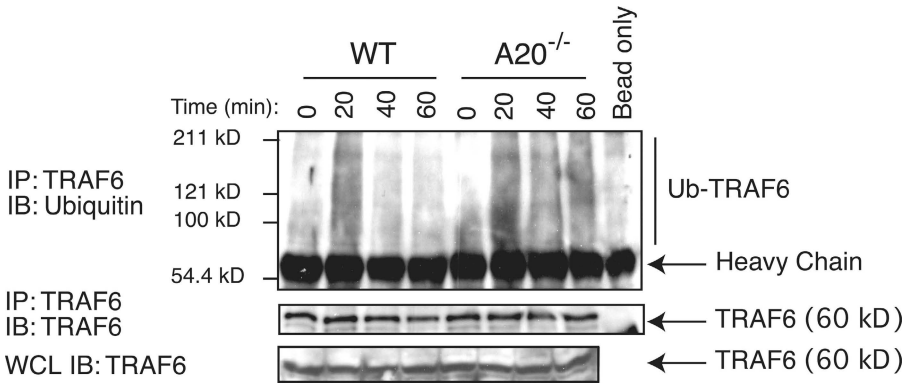

Figure 8. A20 is essential for restricting TRAF6 ubiquitylation. Immunoblotting analysis of endogenous TRAF6 ubiquitylation in LPS-stimulated BMDMs. $A20^{-/-}$and $A20^{+/+}$ BMDMs were stimulated with LPS, and cell lysates were generated at the indicated time points. Samples were boiled in 1% SDS to disassociate noncovalent protein – protein interactions. After dilution to 0.1% SDS, TRAF6 protein was immunoprecipitated from the lysates and immunoblotted for ubiquitin. Data are representative of at least three independent experiments.

DISCUSSION

Our studies have shed new light on the importance and regulation of homeostatic TLR signals. Recent studies have indicated that homeostatic TLR signals may be chronically triggered by PAMPs from microbes at mucosal surfaces such as the intestinal epithelium. MyD88-dependent TLR signals may be beneficial for intestinal homeostasis under normal circumstances (2, 31). The appreciation of such homeostatic TLR signals has raised several questions, including whether homeostatic TLR signals are potentially inflammatory. Our finding that unperturbed A20$^{-/-}$MyD88$^{-/-}$ mice are dramatically less inflamed and survive far longer than A20 $^{-/-}$MyD88$^{+/-}$mice suggests that homeostatic TLR signals can in fact be profoundly inflammatory and rapidly lethal. We have also elucidated a new function for A20 in regulating TRIF-dependent NF-κB signals but not IRF3 signals.

The idea that homeostatic MyD88 signals can cause spontaneous inflammation is broadly consistent with recent findings that constitutive MyD88 signals drive colitis and eventual mortality in IL-10$^{-/-}$ mice (3). However, although IL-10$^{-/-}$ mice develop progressive colitis during the first 4 – 6 mo of life and eventually die from this disease, the majority of A20$^{-/-}$mice die within the first 3 – 6 wk of life from widespread systemic inflammation and cachexia (3, 32). Thus, our findings indicate that homeostatic MyD88 signals can drive severe perinatal systemic inflammation in addition to intestinal inflammation. Moreover, although IL-10 is particularly important for restricting intestinal inflammation, A20 is critical for restricting systemic inflammation.

Although A20 prevents widespread inflammation, a major source of constitutive, proinflammatory TLR signals in A20$^{-/-}$ mice may nevertheless be

intestinal commensal bacteria. We have found that the removal of many of these bacteria with antibiotics reduced systemic inflammation in chimeric mice bearing A20$^{-/-}$ hematopoietic cells. Although previous studies have shown that intestinal bacteria can drive intestinal inflammation in several models (33), our current results link these bacteria and MyD88-dependent signals to A20-mediated regulation of innate immune cells and to systemic immune activation.

The different physiological roles of IL-10 and A20 in restricting MyD88-dependent signals is likely related to the distinct biochemical functions of these immunoregulatory proteins. IL-10 triggers STAT3-mediated transcriptional inhibition of proinflammatory genes, whereas our data indicate that A20 restricts MyD88-dependent TLR signals by regulating TRAF6 ubiquitylation. Thus, the critical physiological role that A20 plays in preventing constitutive homeostatic MyD88 signals from becoming inflammatory is likely caused by A20's biochemical role in directly regulating MyD88-dependent signals.

Proteins such as A20, IRAK-M, ST2, and SIGIRR that restrict TLR signals could regulate the duration and/or intensity of TLR signals and modulate the cellular outcome of TLR signaling, thereby helping to determine whether TLR signals lead to homeostatic or inflammatory responses (14 – 18). Notably, although unperturbed mice lacking IRAK-M, ST2, and SIGIRR exhibit relatively modest inflammation and survive for longer than 9 – 12 mo of age, A20$^{-/-}$ mice typically die within the first few weeks of life (13 – 17). Although SOCS-1 $^{-/-}$ mice exhibit perinatal lethality similar to A20$^{-/-}$ mice, SOCS-1$^{-/-}$ mice are rescued by the absence of IFN-γ signaling in SOCS-1$^{-/-}$IFN-γ$^{-/-}$ double-mutant mice (19 – 21). Thus, among proteins known to directly restrict TLR signaling, A20 appears to play a more critical role in regulating homeostatic TLR-driven inflammation in vivo. There are several potential (non-mutually exclusive) reasons why A20 has a greater physiological impact on homeostatic TLR signals. First, A20 enzymatically regulates ubiquitylation of signaling proteins, thereby regulating both the activity

and stability of such targets. Thus, A20 may not only deactivate signaling proteins but may also prevent further reactivation of these proteins. Second, A20 may regulate TLR signaling downstream of other known TLR regulating proteins, thus exerting a more definitive impact on the transcriptional outcome of TLR signals. Third, A20's physiological roles in restricting MyD88-dependent TLR signals may be amplified by A20's roles in regulating other non-TLR signals, e.g., TNF signals. However, marked survival differences between $A20^{-/-}MyD88^{-/-}TNF^{-/-}$ mice and $A20^{-/-}TNF^{-/-}$ mice suggest that restricting MyD88 signals may be the dominant physiological function of A20 during homeostatic conditions. Finally, it is possible that A20 may also regulate IL-1 or IL-18 signals, which also use MyD88 (9). Although we have not yet evaluated the contribution of aberrant IL-1 or IL-18 signaling to spontaneous inflammation in $A20^{-/-}$ mice, IL-1 receptor antagonist – deficient mice do not develop spontaneous inflammation within the first 4 mo of life (34). Thus, restricting basal IL-1 signals may not be critical for immune homeostasis in unperturbed mice. Collectively, A20 appears to be one of the most critical proteins for restricting homeostatic TLR signals in vivo.

Constitutive or basal MyD88-dependent signals could derive from microbial or host ligands. Our experiments with antibiotic-mediated depletion of commensal bacteria reinforce the notion that intestinal bacteria are a major source of MyD88-dependent signals triggering inflammation in unperturbed $A20^{-/-}$ mice. Collectively with previous findings that constitutive MyD88-dependent signals from luminal bacteria are required for maintaining intestinal health, the current results suggest that a moderate but not excessive magnitude of MyD88 signaling in the intestine is essential for intestinal homeostasis.

In the intestine, homeostatic TLR signals might also be distinguished from inflammatory TLR signals by microenvironment- and cell type – specific TLR signals. For example, apical TLR signals on epithelial cells might preferentially induce homeostatic signals, whereas TLR signals on dendritic cells trigger

inflammation. Although we have not yet directly addressed the roles of A20 in regulating epithelial cell function, our discoveries that radiation chimera bearing A20$^{-/-}$MyD88$^{-/-}$hematopoietic cells exhibit less spontaneous inflammation than chimeric mice bearing A20$^{-/-}$MyD88$^{+/+}$ cells indicates that A20-mediated restriction of homeostatic TLR signals specifically in hematopoietic cells is important for immune homeostasis. Although it is possible that TLRs on epithelial cells may also be constitutively engaged on mucosal surfaces, our results using radiation chimera indicate that engagement of TLRs on hematopoietic cells occurs in unperturbed mice and must be properly restricted to maintain immune homeostasis. Indeed, one example of how TLRs on hematopoietic cells might tonically bind PAMPs from luminal microbes is via the extrusion of dendrites by mucosal dendritic cells through epithelial tight junctions into the intestinal lumen (35 – 37). Future studies using gene-targeted mice bearing lineage-specific deletions of A20 should facilitate studies of A20's specific role in dendritic cells and other cell types.

The generation and characterization of A20$^{-/-}$MyD88$^{-/-}$mice also facilitates further studies of A20's roles in regulating additional signaling pathways. Milder but progressive spontaneous inflammation in older double-mutant A20 $^{-/-}$MyD88$^{-/-}$ mice and triple-mutant A20$^{-/-}$MyD88$^{-/-}$TNF$^{-/-}$ mice suggests that A20 regulates additional immune signals besides MyD88-dependent TLR and TNF signals. TRIF-dependent TLR signals may be one of these types of signals, and we have used A20$^{-/-}$MyD88$^{-/-}$mice to discover that A20 is also required for restricting TRIF-dependent TLR responses. We have also used A20$^{-/-}$MyD88$^{-/-}$ and A20$^{-/-}$MyD88$^{-/-}$ TNF$^{-/-}$ cells to decipher the biochemical mechanism by which A20 regulates TLR signaling. Specifically, both A20$^{-/-}$MyD88$^{-/-}$ mice and BMDMs derived from these mice produce more type I IFNs than A20$^{+/-}$MyD88$^{-/-}$mice and BMDMs after LPS stimulation. Previous work demonstrated that TRIF associates with TANK-binding kinase 1, TRAF3, and TRAF6 during the activation of NF-κB and IRF3 signaling

(10, 38). Both NF-κB and IRF3 are required for TRIF-dependent IFN transcription. Receptor-interacting protein (RIP) 1 ubiquitylation, possibly mediated by TRAF6, is also involved in TRIF-dependent NF-κB signaling (39, 40). Our data suggest that A20 restricts TRIF-dependent NF-KB signaling and not IRF3 phosphorylation. Hence, A20's nonredundant function in regulating TRIF-dependent signaling appears to be restricted to NF-κB signaling. A20 can inhibit TLR3 signals and can bind TRAF6, Nef-associated kinase/TANK-binding kinase 1, and IκB kinase e (41 – 43). Thus, it is likely that A20 restricts TRIF-dependent signaling by directly regulating proteins in this signaling complex. Our data do not support the previous finding that heterologous A20 expression inhibits IRF3 dimerization, and these differences could be caused by the distinct experimental systems used (43). Collectively, our data suggest that A20 restricts TRIF-dependent IFN responses by limiting TRIF-dependent NF-κB signaling.

Previous work has shown that conjugation of K63-linked ubiquitin chains to TRAF6 is an essential step in TLR-induced NF-KB activation (30). Our earlier studies also showed that A20 is a ubiquitin-modifying enzyme that can both deconjugate ubiquitin chains and ligate ubiquitin onto target proteins such as RIP and TRAF6 (18, 23). Our current data directly demonstrate that A20 is essential for restricting endogenous TRAF6 ubiquitylation after TLR stimulation, further reinforcing this biochemical mechanism. As TRAF6 binds to both MyD88 and TRIF after TLR stimulation, TRAF6 likely activates NF-κB signals in both of these pathways. RIP1 is also involved in TRIF-dependent TLR signaling, and A20 restricts RIP1 ubiquitylation in response to TNF (39, 40). Thus, it is possible that A20 also restricts TRIF-dependent NF-κB signaling by restricting RIP1 ubiquitylation. However, RIP1 is not involved in MyD88-dependent NF-κB signaling, so it is unlikely that A20's effects on RIP1 can explain all of A20's roles in TLR signaling. In contrast, A20's restriction of TRAF6 ubiquitylation may be a common mechanism by which A20 restricts both MyD88- and TRIF-dependent NF-κB signaling.

TRAF3 has recently been implicated in TRIF-dependent signaling (44, 45). TRAF3 appears to be more important for IRF signals, whereas TRAF6 is more important for NF-KB signaling. We and others have not been able to obtain evidence that A20 binds to or modifies TRAF3 (unpublished data), and our finding that IRF3 phosphorylation occurs normally in the absence of A20 suggests that A20 may preferentially regulate TRAF6- but not TRAF3-dependent signaling. These two proteins (along with TRAF2, TRAF4, and TRAF5) share structurally homologies, including RING and Zn finger motifs that may mediate E3 ligase activity. Selective regulation of TRAF6-but not TRAF3-dependent signaling by A20 might be explained if A20 preferentially binds TRAF6 rather than TRAF3. Alternatively, although TRAF6 is modified with K63-linked polyubiquitin chains during TLR initiated signal transduction, it is currently unclear if TRAF3 is similarly ubiquitylated. Thus, A20 may bind K63 polyubiquitylated TRAF6, whereas TRAF3 may not undergo this type of ubiquitylation modification. In either scenario, our findings indicate that A20 is a selective regulator of TRAF6- and not TRAF3-dependent signal transduction and provides new insights into how NF-κB and IRF signaling may be discriminated and differentially regulated.

Our finding that A20 $^{-/-}$MyD88$^{-/-}$ mice virtually all survive to adulthood with modest amounts of inflammation contrasts sharply with our previous findings that A20$^{-/-}$, A20$^{-/-}$RAG-1$^{-/-}$, A20$^{-/-}$TNF$^{-/-}$, and A20$^{-/-}$TNFR1$^{-/-}$ mice all spontaneously develop severe inflammation, cachexia. and premature death (18, 19). This result suggests that dysregulated homeostatic TLR signals stimulate downstream innate and adaptive immune signals in the absence of A20 (e.g., TNF, IL-12, IL-6, and chemokine production, T cell activation, B cell activation, etc). Although A20 may play multiple important roles in regulating immune cell signals, its role in restricting homeostatic TLR signals may be physiologically critical because TLR signals are situated at the apex of immune responses. Moreover, our results showing that antibiotics ameliorate inflammation in A20$^{-/-}$ chimeric mice suggest that commensal intestinal flora trigger these homeostatic MyD88-dependent TLR

signals. Finally, we have established a biochemical mechanism by which A20 restricts both MyD88- and TRIF-dependent TLR signals. In summary, our findings demonstrate the profoundly pro-inflammatory nature of homeostatic MyD88-dependent signals and identify A20 as a critical protein that prevents homeostatic signals from becoming inflammatory. Further studies elucidating how A20 restricts different types of TLR signals should yield important insights into the complex interplay between commensal microbes and host immune cells, as well as the biochemical mechanisms by which signals triggered by microbial molecules are regulated and interpreted.

MATERIALS AND METHODS

Mice and cell preparations. The generation and characterization of $A20^{-/-}$ mice have been previously described (20). $A20^{-/-}$ mice were back-crossed for eight generations onto a C57BL/6J background. $MyD88^{-/-}$ mice were provided by S. Akira (University of Osaka, Osaka, Japan) and R. Medzhitov (Yale University, New Haven, CT) and were backcrossed for five generations onto a C57BL/6J background before being interbred with $A20^{-/-}$ mice. Chimeric mice were generated by reconstitution of lethally irradiated congenic ($Ly5.2^{+}$) mice with bone marrow HSCs. Flow cytometric analyses of tissues from mice were performed with a flow cytometer (LSR2; Becton Dickinson) and FlowJo software (Tree Star, Inc.). For LPS-induced peritonitis experiments, mice were injected intraperitoneally with 100µg LPS from *Salmonella typhimurium* (Sigma-Aldrich) and sacrificed after 2 h for real-time PCR, ELISA, and flow cytometric analyses as described. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

BMDMs were generated in DMEM containing 10% FCS and in 10% CMG14-12 – conditioned media containing MCSF. After 3 d, nonadherent cells were aspirated and adherent cells were given fresh MCSF-containing media. After an additional 3 d, BMDMs were quantitated and phenotyped by flow cytometry with antibodies specific for F4/80 (Serotec) and Mac-1 (BD Biosciences). Macrophage purity was typically 90 – 95%. BMDMs were allowed to adhere to tissue culture plastic overnight before stimulation.

Depletion of commensal intestinal bacteria in chimeric mice. For antibiotic-mediated depletion of commensal bacteria, chimeric mice were generated by transfer of wild-type or $A20^{-/-}$ bone marrow HSCs into sublethally (700 rads)

congenic Ly5.2^{+} C57BL/6J recipient mice. All mice were given trimthoprim-sulfamethoxazole immediately after reconstitution. 2 wk after reconstitution, some mice were given 0.5 grams per liter of vancomycin hydrochloride (Novaplus), 1 gram per liter of ampicillin (Sandoz), 1 gram per liter of neomycin sulfate (Pharma-Tek), and 1 gram per liter of metronidazole (Baxter) in drinking water. After 3 – 4 wk, mice were sacrificed and analyzed by flow cytometry. To quantitate colonic microflora, fecal matter was removed from colons, weighed, and homogenized in luria broth at 0.1 g/ml before culturing on LB-agar plates.

TLR ligands and ELISAs. LPS from *S. typhimurium* and poly (I:C) (GE Healthcare) were used at concentrations of 1 μ g/ml and 50 ng/ml, respectively. BMDMs were stimulated with either LPS or poly (I:C) for 24 h, and culture supernatants were assayed by ELISA for IFN-j (PBL Biomedical Laboratories). Peritoneal lavages from LPS-stimulated mice were assayed by ELISA for MCP-1 secretion (BD Biosciences), and sera from these mice were assayed by ELISA for soluble IL-15Ra (Duoset; R & D Systems).

RNA isolation and quantitative PCR analyses. RNA was isolated from spleens using extraction of homogenized tissue (RNeasy Mini Kit; QIAGEN). RNA was treated with Rnase-free DNase (QIAGEN) to eliminate contaminating genomic DNA and quantified using a spectrophotometer (ND-1000; Nanodrop). Complementary DNA was generated using an RT kit (Quanti-Tect; QIAGEN), and real-time quantitative PCR was performed using an SYBR green PCR kit (QuantiTect; QIAGEN). The following gene-specific primers were used for quantitating

TNF-α (sense, 5'-TGGCCTCCCTCTCATCAGTT-3'; antisense, 5'-TCCTCCACTTGGTGGTTTGC-3'),

IL-1β (sense, 5'-CCGTGGACCTTCCAGGATGA-3'; antisense, 5'-GGGAACGTCACACACCAGCA-3'),

IL-10 (sense, 5'-TGAATTCCCTGGGTGAGAAG-3'; antisense, 5'-

CTCTTCACCTGCTCCACTGC-3'),
IL-2 (sense, 5'-AAAAGCTTTCAATTGGAAGATGCTG-3'; antisense, 5'-TTGAGGGCTTGTTGAGATGA-3'),
IL-13 (sense, 5'-GGAGCTGAGCAACATCACACA-3'; antisense, 5'-TTGAGGGCTTGTTGAGATGA-3'),
β-actin (sense, 5'-AAGTGTGACGTTGACATCCGTAA-3'; antisense, 5'-TGCCTGGGTACATGGTGGTA-3 '),
MCP-1 (sense, 5'-CCCAATGAGTAGG CTGGAGA-3 '; antisense, 5'-TCTGGACCCATTCCTTCTTG-3'),
IFN-β (sense, 5'-CAGCTCCAAGAAAGGACGAAC-3'; antisense, 5'-GGCAGTGTAACTCTTCTGCAT-3 '),
and GAPDH (sense, 5'-AACGGGAAGCCCATCACCATCTT-3 '; antisense, 5'-GCCCTTCCACAATGCCAAAGTT-3 ').

Real-time PCR was performed and analyzed (ABI 7300; Applied Biosystems). mRNA relative units (R.U.) were calculated as $2^{n-(Ct[gene\ of\ interest]-Ct[control])}$, as previously described (46).

Immunoblotting and immunoprecipitations. For in vitro TLR signaling experiments, BMDMs were stimulated for the periods of time indicated in the figures with 1 μg/ml LPS, washed in PBS, and lysed for 20 min at 4 ° C in RIPA lysis buffer (1 × PBS, 20 mM β-glycerophosphate, 1 mM Naorthovanadate, and complete protease inhibitor cocktail; Roche) and sonicated. Lysates were cleared by centrifugation at 14,000 *g* for 20 min at 4 ° C, and supernatants were removed and boiled in Laemmli buffer for immunoblot analysis of IκBα, phospho-IκBα, or phospho-IRF3 (Cell Signaling Technology). For endogenous TRAF6 immunoprecipitations, 10% SDS was added to cleared lysates for a final concentration of 1% SDS and boiled for 5 min. Boiled lysates were diluted 10-fold in PBS, and anti-TRAF6 – coupled (Santa Cruz Biotechnology, Inc.) protein A beads (Thermo Fisher Scientific) were added. Samples were washed three times

with 1 × PBS and boiled in Laemmli buffer for immunoblot analysis with either antiubiquitin (P4D1; Santa Cruz Biotechnology, Inc.) or anti-TRAF6 (EMD) antibodies.

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AUTHOR CONTRIBUTIONS

R. M. Tavares planned and performed experiments in Figures 4, 5, 6 and 7, while the remaining experiments (Figures 1, 2, 3 and 8) were undertaken by E. E. Turer.

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CHAPTER 3

The Ubiquitin Modifying Enzyme A20 Restricts B-cell Survival and Prevents Autoimmunity

The Ubiquitin Modifying Enzyme A20 Restricts B Cell Survival and Prevents Autoimmunity

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SUMMARY

A20 is a ubiquitin modifying enzyme that restricts NF-κB signals and protects cells against tumor necrosis factor (TNF)-induced programmed cell death. Given recent data linking A20 (*TNFAIP3*) with human B cell lymphomas and systemic lupus erythematosus (SLE), we have generated mice bearing a floxed allele of *Tnfaip3* to interrogate A20's roles in regulating B cell functions. A20-deficient B cells are hyperresponsive to multiple stimuli and display exaggerated NF-kB responses to CD40-induced signals. Mice expressing absent or hypomorphic amounts of A20 in B cells possess elevated numbers of germinal center B cells, autoantibodies, and glomerular immunoglobulin deposits. A20-deficient B cells are resistant to Fas-mediated cell death, probably due to increased expression of NF-kB-dependent antiapoptotic proteins such as Bcl-x. These findings show that A20 can restrict B cell survival, whereas A20 protects other cells from TNF-induced cell death. Our studies demonstrate how reduced A20 expression predisposes to autoimmunity.

INTRODUCTION

Maintenance of B-cell homeostasis requires proper intracellular integration of signals delivered from multiple surface receptors such as the B cell antigen receptor, Toll-like receptors (TLRs), B cell-activating factor (BAFF) receptor, and CD40, as well as intracellular cues. Failure to integrate pathways such as NF-κB signaling can lead to B cell deficiency, aberrant B cell activity, or even lymphoma. Aberrant B cell tolerance and selection can cause production of autoantibodies, formation of immune complexes (IC), and ultimately tissue damage and autoimmune disease (Fairhurst et al., 2006).

Tnfaip3 encodes the A20 protein, a ubiquitin-modifying enzyme (Wertz et al., 2004; Boone et al., 2004). A20 was initially identified as a TNF-induced molecule that restricts TNF induced signaling (Opipari et al., 1990). Targeting of *Tnfaip3* in mice revealed A20s critical anti-inflammatory functions, given that A20-deficient (*Tnfaip3*$^{-/-}$) mice exhibit severe spontaneous multiorgan inflammation, cachexia, and perinatal death (Lee et al., 2000). Epistasis experiments revealed that A20 restricts TLR and nucleosome-binding oligomerization domain (NOD) triggered NF-κB signaling, in addition to TNF-induced NF-κB and programmed cell death (PCD) signaling (Lee et al., 2000; Boone et al., 2004; Hitotsumatsu et al., 2008). Thus, A20 restricts a number of innate immune signaling pathways in macrophages and fibroblasts. The severe systemic inflammation and cachexia caused by A20 deficiency is ameliorated in mice that also lack the TLR adaptor protein MyD88 (Turer et al., 2008). Radiation chimeras bearing *Tnfaip3*$^{-/-}$ hematopoietic cells also develop spontaneous systemic inflammation, which is alleviated by depletion of commensal intestinal bacteria with antibiotics (Turer et al., 2008). Thus, A20 maintains immune homeostasis and restricts the potentially proinflammatory nature of basal MyD88-dependent signals.

In addition to the innate immune functions described above in macrophages and fibroblasts, A20 is also expressed in T and B cells (Sarma et al., 1995; Lee et al., 2000). During T cell activation, A20 is recruited to the MALT-1-Bcl-10 scaffold complex, and is cleaved by the paracaspase MALT-1 (Coornaert et al., 2008). A20 has also been reported to de-ubiquitinate MALT-1 to restrict TCR signals (Duwel et al., 2009). A20 cleavage is also observed in B lymphoma cell lines in response to BCR stimulation (Coornaert et al., 2008). Other clues that A20 may play important roles in adaptive lymphocytes derives from human genetic studies that implicate A20 (or *TNFAIP3*) as a susceptibility gene for systemic lupus erythematosus (SLE) – an autoimmune disease associated with aberrant B cell function--as well as studies showing that A20 is a tumor suppressor gene in B cell lymphomas (Graham et al., 2008; Musone et al., 2008; Compagno et al, 2009; Kato et al, 2009; Novak et al, 2009; Schmitz et al, 2009). Nevertheless, the physiological roles of A20 in T and B cells are largely undefined.

B cells are regulated by BCR, TLR, BAFF and CD40 signals. These signaling cascades share some of the same ubiquitin-dependent signaling molecules utilized by TNF and TLR ligands (e.g., TRAF2, TRAF6, IKKγ) (Hayden and Ghosh, 2008). Given A20's role in preventing inflammation, its genetic linkage to human B cell lymphomas and SLE, and the central role B cells play in SLE pathogenesis, we hypothesized that A20 may regulate B cell homeostasis and prevent autoimmunity. To determine the cell intrinsic functions of A20 in regulating B cells, we have generated mice lacking A20 specifically in these cells.

RESULTS

B-lineage deletion of A20 perturbs lymphoid homeostasis

In order to analyze the cell intrinsic functions of A20 in B cells, we generated a targeting construct in which exon 2 of the *Tnfaip3* gene was flanked by loxP sites, a "floxed" allele. The targeting construct was transfected into C57BL/6 ES cells and neomycin resistant clones were screened for the targeted allele (Figures 1A and B). Transient transfection of Cre recombinase resulted in removal of the neomycin cassette to obtain the floxed *Tnfaip3* allele (Figures 1A and B). ES clones were injected into albino C57BL/6 blastocysts, and the resultant chimeras were bred with albino C57BL/6 mice. Non-albino C57BL/6 progeny were screened for the presence of the floxed allele, $Tnfaip3^{fl}$ (Figure 1B).

Mice carrying the $Tnfaip3^{fl}$ allele were bred with CD19-Cre knock-in mice to generate $Tnfaip3^{+/+}$, $Tnfaip3^{fl/+}$ and $Tnfaip3^{fl/fl}$ mice bearing a single copy of the Cd19-Cre allele (Rickert et al., 1997). All mice described in this study were heterozygous for the CD19-Cre targeted allele (Cd19-$Cre^{+/-}$) to control for potential nonspecific effects of Cre expression while maintaining CD19 expression. For simplicity, Cd19-$Cre^{+/-}$ mice will subsequently be referred to as Cd19-Cre mice. As has been found for other "floxed" alleles, $Tnfaip3^{fl/fl}$ CD19-Cre mice had efficient and B cell specific deletion of *Tnfaip3* exon 2, as assessed by genomic polymerase chain reaction (PCR) and Southern blot (Figure 1C and data not shown). Flow cytometry sorted immature and germinal center (GC) B cells, subsets represented in smaller proportions, were also nearly 100% deleted as measured by quantitative genomic PCR (Figure 1D). A20 protein is constitutively expressed in B cells and T cells (Figure 1E). Deletion of *Tnfaip3* exon 2 on both alleles ($Tnfaip3^{fl/fl}$ CD19-Cre) led to complete loss of A20 protein in splenic B cells

(Figure 1E). Note that deletion of one allele of *Tnfaip3* in *Tnfaip3*$^{fl/+}$ CD19-Cre mice causes hypomorphic (~50%) expression of A20 protein in B cells (Figure 1E).

Figure 1

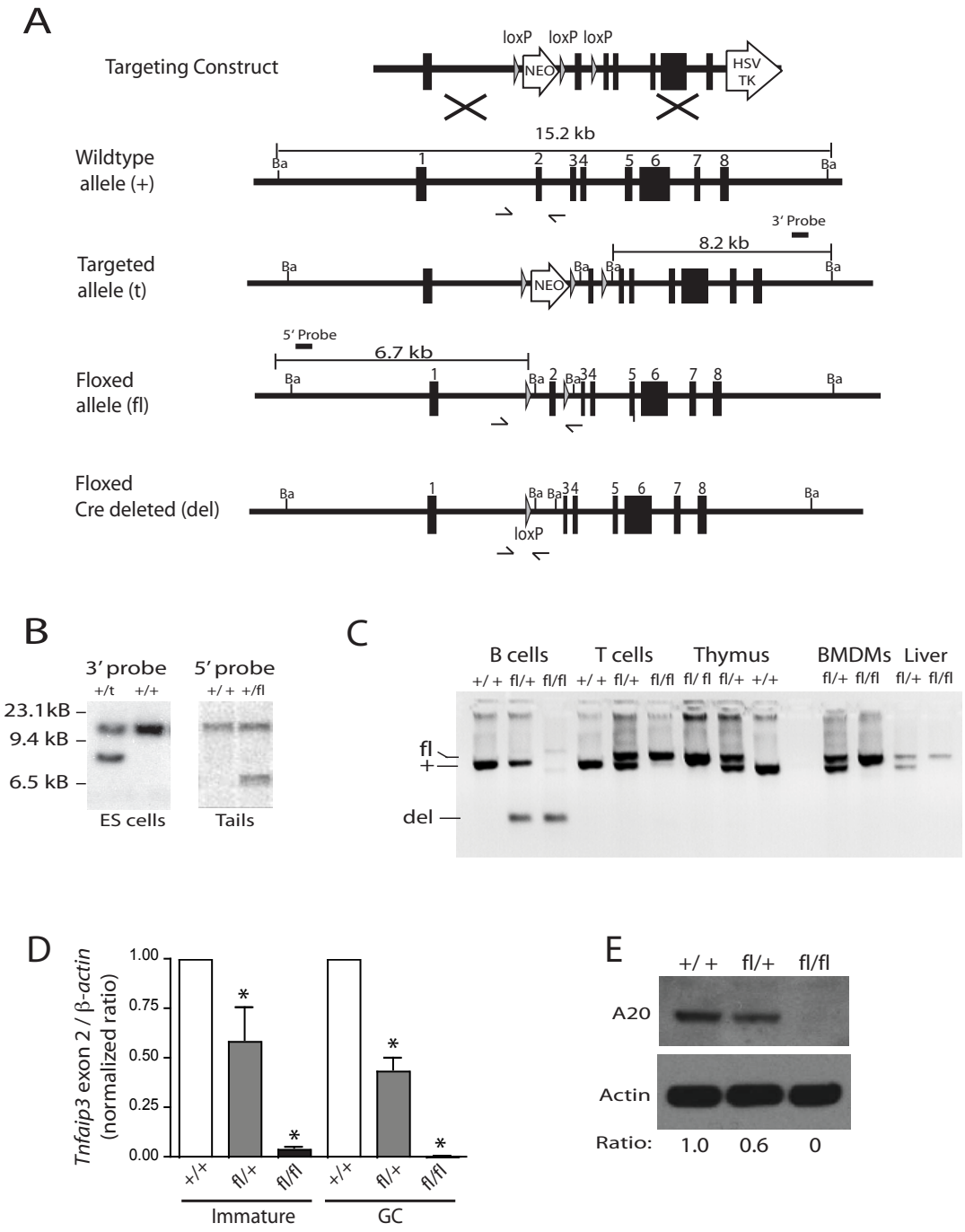

Figure 1. Gene targeting strategy to generate mice lacking *Tnfaip3* in B cells.

(A) Schematic representation of the gene targeting construct and screening strategy for obtaining the *Tnfaip3* floxed (fl) allele. (B) Southern blots of BamHI digested genomic DNA from ES cells showing the targeted allele (left blot) and from tails from mice with germline inheritance of the fl allele (right blot). (C) Genomic DNA PCR analysis of deletion of *Tnfaip3* exon 2 in the indicated cell types from mice of the indicated genotypes. BMDMs are bone marrow derived macrophages. All mice are CD19-Cre$^{+/-}$. Sizes of PCR products for floxed (fl), wild type (WT) and deleted (Del) alleles are indicated. Data are representative of 5 mice per genotype. (D) Quantitative genomic DNA PCR of flow cyomtery-sorted populations from immature (CD19^{+} CD93[AA4.1]$^{+}$) and GC (CD19^{+} GL7^{+} CD95^{+}) B cells from mice of the indicated genotypes. Error bars show S.E.M. of 3 mice per genotype. (E) Immunoblot analysis of A20 expression in B cells from the indicated genotypes of mice. The A20/actin protein ratio relative to *Tnfaip3*$^{+/+}$ CD19-Cre cells is shown below the blots.

Tnfaip3$^{fl/fl}$ CD19-Cre mice were obtained in Mendelian numbers and developed normally. Hence, these mice differed dramatically from mice lacking A20 in all cells or in all hematopoietic cells, both of which develop severe spontaneous inflammation and early lethality (Lee et al., 2000; Boone et al., 2004; Turer et al., 2008). To begin to assess the roles of A20 in regulating B cells, we quantitated lymphoid populations from 5-7 week old *Tnfaip3*$^{fl/fl}$ CD19-Cre, *Tnfaip3*$^{fl/+}$ CD19-Cre and *Tnfaip3*$^{+/+}$ CD19-Cre littermates by flow cytometry (Table 1, top panel). *Tnfaip3*$^{fl/fl}$ CD19-Cre mice contained moderately increased numbers of B cells (CD19^{+}), particularly immature B cells (CD19^{+}IgM^{hi}) and germinal center (GC) B cells, when compared to *Tnfaip3*$^{+/+}$ CD19-Cre control mice (Table 1, Figure 2A, B, C). Although the percentage of B1a (IgM^{+}, CD5^{+}) cells in the

peritoneal cavity of *Tnfaip3*$^{fl/fl}$ CD19-Cre mice was lower than *Tnfaip3*$^{+/+}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre mice, the absolute number was not significantly different (Figure 2C, S1A, Table 1). Although A20 deletion in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice occurs in B cells and not T cells (Figure 1C), both B cells (CD19^{+}) and T cells (TCRβ+) were modestly expanded in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice (Figure 2A and Table 1). The relative percentages of T cell subpopulations (CD4^{+}, CD8^{+}, and Tregulatory) were normal (data not shown). Taken together, these findings suggest that A20 restricts the numbers of B cells, particularly immature and GC B cells.

Heterozygous *Tnfaip3*$^{fl/+}$ CD19-Cre mice possess largely normal numbers of lymphoid populations, even though *Tnfaip3*$^{fl/+}$ CD19-Cre B cells express half the amount of *Tnfaip3* protein as wild type *Tnfaip3*$^{+/+}$ CD19-Cre B cells (Figure 1E). A notable exception is that the numbers of germinal center (GC) (CD95^{+}GL7^{+}) B cells in *Tnfaip3*$^{fl/+}$ CD19-Cre mice approximates the number present in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice (Figure 2C and Table 1). Thus, proper regulation of GC B cell homeostasis requires more A20 protein than other B cell populations.

Bone marrow from *Tnfaip3*$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre mice contained normal numbers of B lineage cells, with normal proportions of pro-B (CD43^{+}, IgM-) and pre-B (CD43^{-}, IgM^{-}) cells (Figure S1B). There was a small decrease in the percentage of IgM^{+} B cells in *Tnfaip3*$^{fl/fl}$ CD19-Cre bone marrow, which reflected reductions in mature or re-circulating (IgM^{+}, IgD^{+}) B cells (Figure S1B). As CD19 is expressed throughout B cell development, these results suggest that A20 is not required for early B cell differentiation.

The differences in peripheral lymphocyte populations described above persisted but were not further exaggerated in 6 month old mice (Figure S1C and data not shown). In addition, six month old *Tnfaip3*$^{fl/+}$ CD19-Cre and *Tnfaip3*$^{fl/fl}$ CD19-Cre mice contained increased percentages of splenic plasma cells when

compared to *Tnfaip3*$^{+/+}$ CD19-Cre mice (Figure 2D). While markers of B cell activation were expressed normally in 5-7 week old mice, spontaneous B cell activation became apparent in 6 month old *Tnfaip3*$^{fl/fl}$ CD19-Cre but not *Tnfaip3*$^{fl/+}$ CD19-Cre mice (Figure 2E, S1D). Spontaneous T cell activation was not observed (data not shown). These findings suggest that A20 expression in B cells prevents spontaneous activation and differentiation of B cells over time.

Overall, elevated numbers of T and B cells and spontaneous B cell activation were observed in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice by six months of age. In addition, elevated numbers of GC B cells were observed in both heterozygous *Tnfaip3*$^{fl/+}$ CD19-Cre mice and homozygous *Tnfaip3*$^{fl/fl}$ CD19-Cre mice.

Figure 2

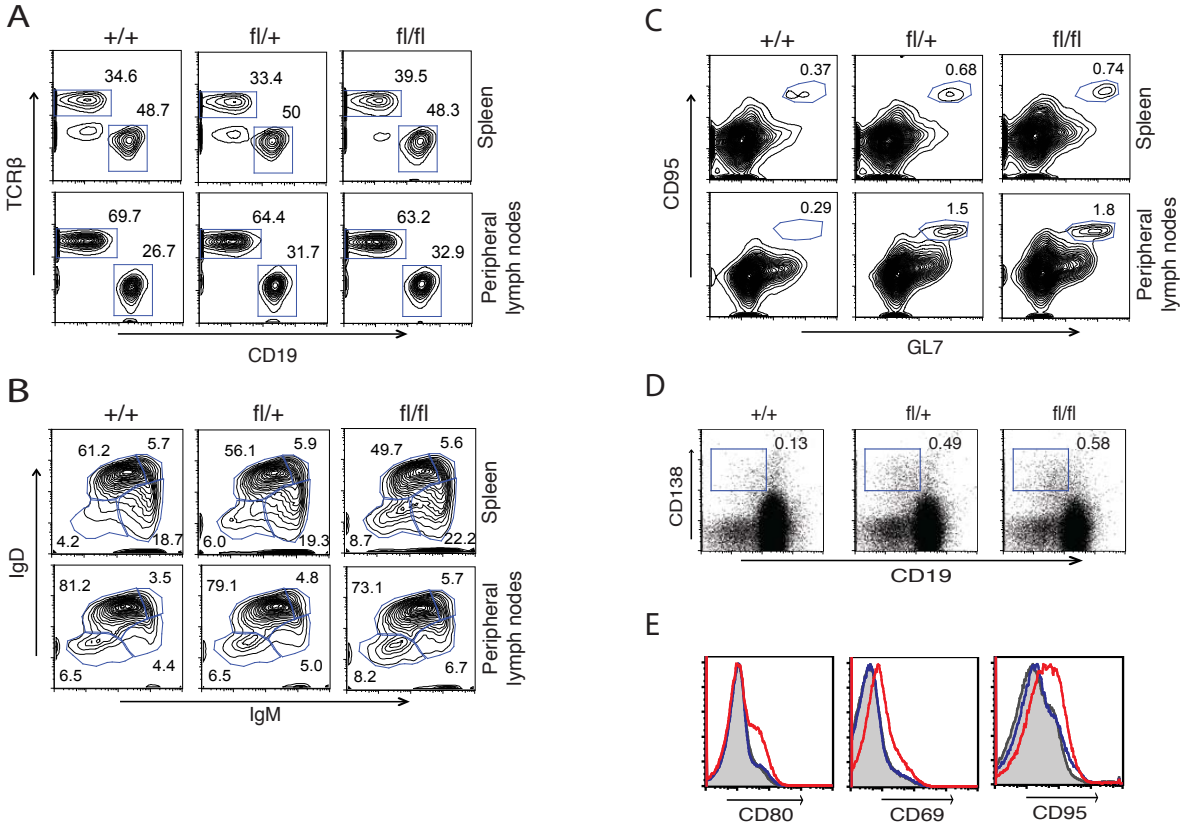

Figure 2. Flow cytometric analyses of B lymphocyte populations in

***Tnfaip3*$^{fl/fl}$ CD19-Cre mice.**

Flow cytometric analyses of lymphoid tissues. (A) Analyses of lymphoid populations in spleens and peripheral lymph nodes from 5 - 7 week-old mice. (B) Analysis of CD19^{+} gated cells showing B-cell maturation (IgM, IgD) from 5 - 7 week-old mice.. (C) Analysis of CD19^{+} gated cells showing GC B cells (GL7^{+} CD95^{+}) from 5 - 7 week-old mice. (D) Plasma cells (CD19^{+} low CD138^{+}) within TCRβ- CD19^{+} gated splenic B cells from 6 month old mice are shown. Percentages of cells within the indicated gates are shown on plots. E) Histograms comparing expression of B cell activation markers (CD80, CD69, and CD95) on CD19^{+} gated cells from *Tnfaip3*$^{+/+}$ cells (shaded histogram) *Tnfaip3*$^{fl/+}$ cells (blue line) and *Tnfaip3*$^{fl/fl}$ B cells (red line). All data compare littermates of the indicated genotypes and are representative of 3-5 mice per genotype.

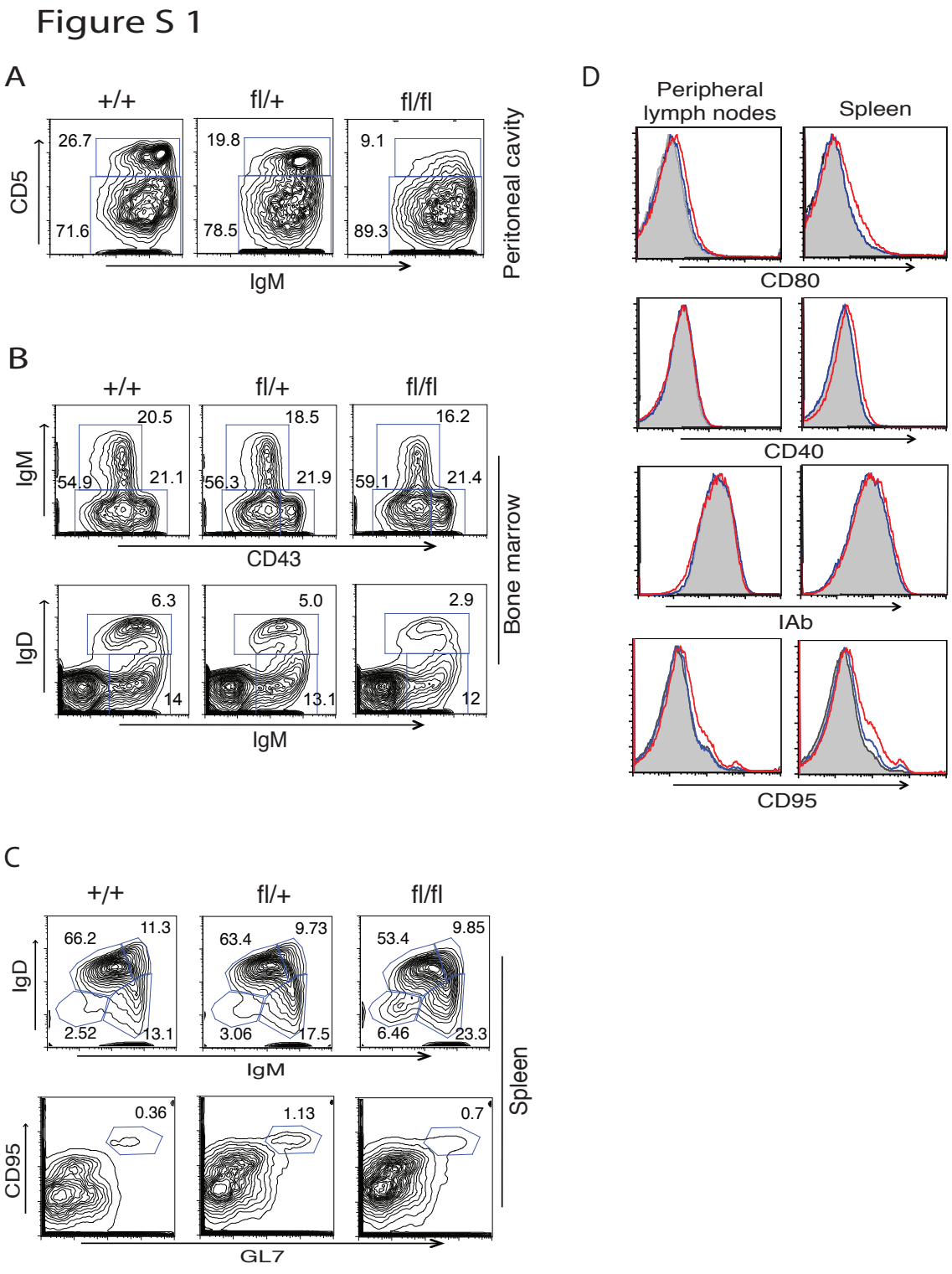

Figure S1.

Flow cytometric analyses of B cells from lymphoid organs of *Tnfaip3*$^{+/+}$,

***Tnfaip3*$^{fl/+}$, and *Tnfaip3*$^{fl/fl}$ mice.** (A) Peritoneal cells from 5 to 7 week old mice were lymphocyte gated and analyzed for the percentages of B1a (IgM^+ $CD5^+$) and B2 (IgM+ CD5-) cells. (B) Bone marrow cells from 5 to 7 week old mice were lymphocyte gated and analyzed for the percentages of B cells at various differentiation stages. (C) Spleen cells from 6 month old mice were gated on $CD19^+$ cells and analyzed for B cell maturation stages (IgM, IgD) and GC B cells ($GL7^+$ $CD95^+$). Numbers in the plots represent the percentages of B cells of the demarcated subsets (A-C). (D) Spleens and lymph nodes from 5 to7 week old mice were gated on $CD19^+$ and analyzed for expression of the indicated surface activation markers: *Tnfaip3*$^{+/+}$ (shaded histogram), *Tnfaip3*$^{fl/+}$ (blue line), and *Tnfaip3*$^{fl/fl}$ (red line).

Table 1. Cellulariry of B lymphocytes populations in *Tnfaip3*$^{+/+}$, *Tnfaip3*$^{fl/+}$ and *Tnfaip3*$^{fl/fl}$ *CD19-Cre* mice

Total cellularity (x10^6)	+/+	fl/+	fl/fl
Lymph Nodes	14.3 ± 0.7	15.8 ± 1.2	25.7 ± 3.7 *
Spleen	44.4 ± 11	55.4 ± 9.0	74.2 ± 11
Bone Marrow	31.4 ± 3.2	30.7 ± 5.6	31.2 ± 4.1
Peritoneum	4.7 ± 0.7	3.8 ± 1.1	8.1 ± 1.6
Lymph Nodes (x10^6)			
B-cells	3.9 ± 0.2	4.7 ± 0.5	8.8 ± 1.1 *
T-cell	8.9 ± 0.7	8.4 ± 0.8	12.9 ± 1.6 *
Myeloid Cells	0.15 ± 0.03	0.15 ± 0.03	0.24 ± 0.03
Mature B-cells	2.5 ± 0.1	2.9 ± 0.3	3.3 ± 0.5
Immature B-cells	1.2 ± 0.1	1.1 ± 0.2	3.9 ± 0.3 *
Germinal Center B-cells	0.01 ± 0.003	0.08 ± 0.02 *	0.05 ± 0.02 *
Spleen (x10^6)			
B-cells	23.7 ± 6.0	26.5 ± 4.5	37.8 ± 6.4
T-cell	16.5 ± 0.8	16.1 ± 1.7	24.7 ± 2.5 *
Myeloid Cells	0.7 ± 0.03	1.0 ± 0.2	1.4 ± 0.3
Mature B-cells	11.2 ± 1.4	15.1 ± 3.6	17.9 ± 3.1
Immature B-cells	9.8 ± 0.6	9.2 ± 0.7	14.8 ± 2.6 *
Germinal Center B-cells	0.17 ± 0.03	0.6 ± 0.15 *	0.45 ± 0.08 *
Marginal Zone B-cells	1.6 ± 0.08	1.1 ± 0.3	0.9 ± 0.2
Peritoneum (x10^6)			
B-cells	1.6 ± 0.34	2.7 ± 0.25	4.1 ± 0.37
B1a	0.45 ± 0.04	0.69 ± 0.16	0.6 ± 0.24
B2	0.55 ± 0.16	0.93 ± 0.11	1.6 ± 0.12

Table 1. Cellularity of lymphoid organs and respective subpopulations in

***Tnfaip3*^{fl} CD19-Cre mice.**

Quantitation of lymphoid populations in the indicated tissues from the indicated genotypes of 5-7 week old mice. The total cellularity of lymphoid organs is shown in the top panel. Subpopulations were identified by flow cytometry using the indicated markers: B cells ($CD19^+$); T-cells ($TCR\beta^+$); myeloid cells ($Mac\text{-}1^+$); mature B-cells ($CD19^+$, IgM^{Lo}, IgD^+); immature B-cells ($CD19^+$ IgM^{hi}); marginal zone B-cells ($CD21/35^{Hi}$ $CD23^{Lo}$); GC B cells ($CD19^+$, $GL7^+$, $CD95^+$); B1a (IgM^+, $CD5^+$). * indicates significant difference relative to $^{+/+}$ ($p < 0.05$, using one-way Anova). For GC B-cells, means are from 5 mice per genotype; for all other subpopulations, means of 5 *Tnfaip3*$^{+/+}$ CD19-Cre, 11 *Tnfaip3*$^{fl/+}$ CD19-Cre and 11 *Tnfaip3*$^{fl/fl}$ CD19-Cre mice are shown.

Exaggerated responses of *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells in vitro.

Our prior work indicated that A20 restricts TNF, TLR, and NOD induced NF-kB signals as well as TNF induced PCD in fibroblasts and macrophages (Lee et al., 2000; Boone et al., 2004; Hitotsumatsu et al., 2008; Turer et al., 2008). B cells receive activation, proliferation and survival signals from BCR, CD40 receptor, and other receptors (Skaug et al., 2009). To test if A20 directly regulates B cell responses, we assayed responses of *Tnfaip3*$^{fl/fl}$ CD19-Cre, *Tnfaip3*$^{fl/+}$ CD19-Cre and *Tnfaip3*$^{+/+}$ CD19-Cre splenic B cells to LPS, cytosine phosphoguanine oligodeoxynucleotide (CpG) and to agonist anti-IgM and anti-CD40. As noted above, unstimulated splenic B cells from young (5-7 week old) *Tnfaip3*$^{fl/fl}$ CD19-Cre mice resembled B cells from *Tnfaip3*$^{fl/+}$ CD19-Cre and *Tnfaip3*$^{+/+}$ CD19-Cre mice (Supplementary Figure 1). *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells expressed higher levels of CD80 (B7.1) and CD69 after stimulation with several B cell agonists (Figure 3A and data not shown). *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells also proliferated to a greater extent than control cells (Figure 3B). Thirdly, *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells produced more IL-6 than *Tnfaip3*$^{fl/+}$ CD19-Cre and *Tnfaip3*$^{+/+}$ CD19-Cre cells after

treatment with LPS and CpG (Figure 3C).

To control for potential differences in B cell populations and to avoid potential caveats associated with developmental abnormalities, we sought to eliminate A20 expression in mature B cells after B cell development. Accordingly, we interbred *Tnfaip3*^{fl} mice with estrogen receptor (ER)-Cre [Gt(ROSA)26ER-Cre] mice to obtain mice in which A20 (*Tnfaip3*) deletion would not occur until cells were exposed to 4-hydroxytamoxifen (4-OH-T). Splenic B cells enriched from *Tnfaip3*$^{fl/fl}$ ER-Cre mice and treated with 4-OH-T in vitro effectively ablated A20 protein expression (Figure 3D). We then tested responses of these B cells. Consistent with our findings with *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells, mature splenic *Tnfaip3*$^{fl/fl}$ ER-Cre B cells rendered acutely A20 deficient with 4-OH-T exhibited exaggerated responses to all receptor stimuli when compared to control *Tnfaip3*$^{fl/+}$ ER-Cre cells (Figure 3E). Thus, A20 expression in mature B cells restricts B cell responses independently of any potential roles of A20 in regulating B cell development.

Figure 3

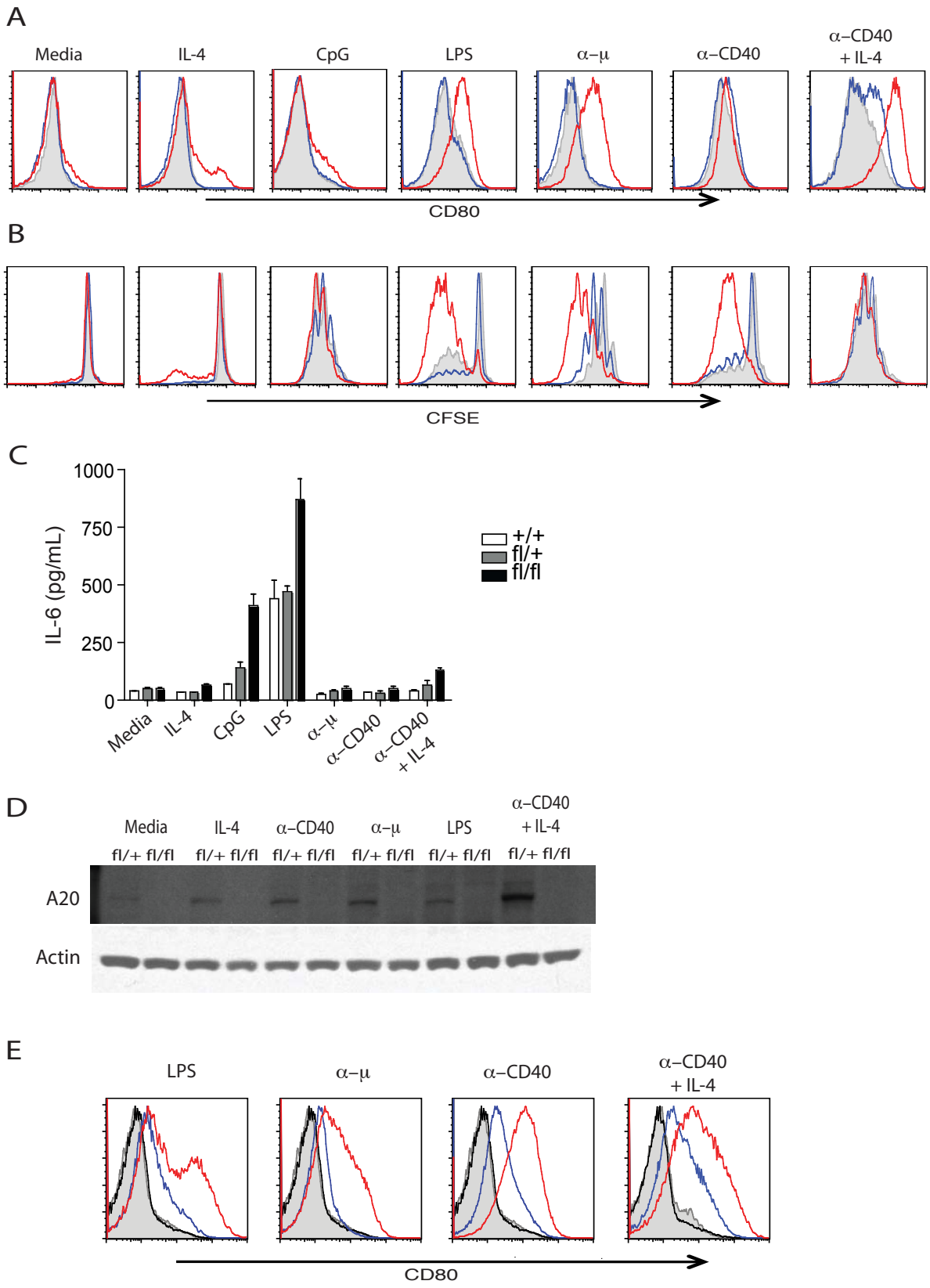
A
Media
IL-4
CpG
LPS
α–μ
α–CD40
α–CD40
+ IL-4
CD80
B
CFSE
C
IL-6 (pg/mL)
1000
750
500
250
0
+/+
fl/+
fl/fl
Media
IL-4
CpG
LPS
α–μ
α–CD40
α–CD40
+ IL-4
D
Media
IL-4
α–CD40
α–μ
LPS
α–CD40
+ IL-4
fl/+ fl/fl
A20
Actin
E
LPS
α–μ
α–CD40
α–CD40
+ IL-4
CD80

Figure 3. Hyper-responsiveness of A20-deficient B cells.

In vitro responses of purified B cells after stimulation with the indicated stimuli for 72h. Flow cytometric analyses of (A) surface expression of the activation marker CD80 (B7.1) and (B) dilution of CFSE-labeled cells. *Tnfaip3*$^{fl/fl}$ CD19-Cre cells are shown in red lines; *Tnfaip3*$^{fl/+}$ CD19-Cre cells shown in blue lines; and *Tnfaip3*$^{+/+}$ CD19-Cre cells shown in shaded gray histograms. (C) ELISA determination of IL-6 secretion in the supernatants of 72 hr B cell cultures. Means and standard deviations of triplicate wells are shown. (D) Immunoblots of A20 and actin protein expression in purified Rosa-ER-Cre+ B cells of the indicated genotypes. Lysates were isolated after 72 hr stimulation with the indicated ligands in the presence of 4-OH-T. (E) Expression of CD80 in purified B cells after treatment with 4-OH-T and the indicated ligands for 72hr. *Tnfaip3*$^{fl/fl}$ GT-Rosa-Cre+ B cells treated with IL-4 or media (black lines) or the indicated ligands (red lines); *Tnfaip3*$^{fl/+}$ GT-Rosa-Cre+ B cells treated with IL-4 or media (gray shaded histograms) or the indicated ligands (blue lines). All data are representative of 3 independent experiments.

A20 restricts NF-kB activation signals downstream of CD40.

A20 restricts cellular responses to TNF, TLR, and NOD2 ligands by restricting NF-kB signaling (Lee et al., 2000; Boone et al., 2004; Hitotsumatsu et al., 2008; Turer et al., 2008). CD40 is a tumor necrosis factor receptor (TNFR) family member that triggers NF-κB signals and supports B cell activation and survival (Elgueta et al., 2009). To test whether A20 directly restricts CD40 induced NF-kB signals, splenic B cells from *Tnfaip3*$^{+/+}$ CD19-Cre and *Tnfaip3*$^{fl/fl}$ CD19-Cre mice were stimulated with agonist anti-CD40 antibody. A20 protein was dramatically induced by CD40 engagement in *Tnfaip3*$^{+/+}$ CD19-Cre B cells, whereas no A20 expression was observed in *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells (Figure 4A). *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells displayed increased and prolonged canonical NF-kB signaling as measured by IkBα phosphorylation in response to anti-CD40

(Figure 4B). Agonist anti-CD40 also induced greater levels of phospho-p100, an indicator of non-canonical NF-κB signaling, in *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells when compared to *Tnfaip3*$^{+/+}$ CD19-Cre B cells (Figure 4C). This increased non-canonical NF-κB signaling correlates with increased basal p100 protein levels in *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells (Figure 4C). By contrast, Erk phosphorylation was similarly induced in *Tnfaip3*$^{fl/fl}$ CD19-Cre and control B cells (Figure 4D).

Figure 4

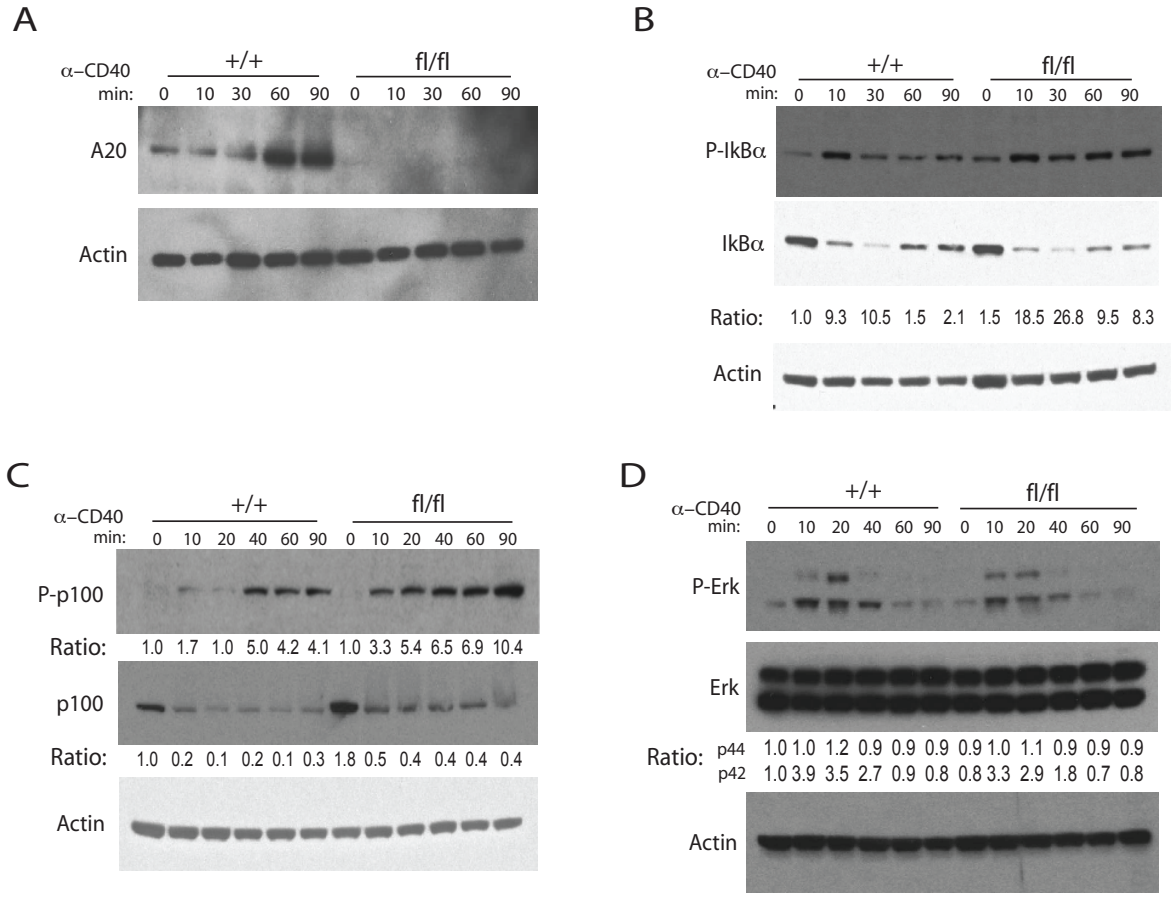

Figure 4. A20 restricts NF-κB signaling downstream of CD40 signals.

(A) Immunoblot analysis of A20 protein induction by agonist anti-CD40. (B) Immunoblot analyses of phospho-IκBα and IκBα after CD40 stimulation. Ratios of pIkBα/IkBα were normalized to time 0 of *Tnfaip3*$^{+/+}$ CD19-Cre cells and are shown

below. (C) Immunoblot analyses of NF-κB P-p100 (upper panel) and p100 (middle panel) protein levels in response to agonist anti-CD40 antibody. Ratios of P-p100/actin, normalized to time 0 sample in *Tnfaip3*$^{+/+}$ CD19-Cre B cells, are shown below P-p100 immunoblot. Ratios of p100/actin, normalized to time 0 sample in *Tnfaip3*$^{+/+}$ CD19-Cre B cells, are shown below p100 immunoblot. (D) Immunoblot analysis of Erk signaling. Ratios of pErk/Erk were normalized to time 0 of *Tnfaip3*$^{+/+}$ CD19-Cre cells and are shown below. Actin protein levels shown below all panels as loading controls. Data are representative of 3 independent experiments.

Normal antigen specific B cell responses in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice.

A20's capacity to restrict B cell activation in vitro suggests that *Tnfaip3*$^{fl/fl}$ CD19-Cre mice might exhibit exaggerated antigen specific B cell responses in vivo. We thus tested antigen specific B cell responses to NP-KLH in these mice. At baseline, *Tnfaip3*$^{fl/fl}$ CD19-Cre mice have more IgM and modestly elevated amounts of IgGs (Figure S2A). *Tnfaip3*$^{fl/fl}$ CD19-Cre mice produced proportionately higher amount of anti-NP IgM antibodies before and after immunization with NP-KLH (Figure S2B). Anti-NP IgG levels were similar in the various genotypes of mice (Figure S2C). Thus, T cell dependent B cell responses occur largely normally in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice. Anti-NP-Ficoll IgM responses paralleled T cell dependent NP-KLH responses in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice, indicating that T cell independent B cell responses also occur normally in these mice (data not shown).

Figure S 2

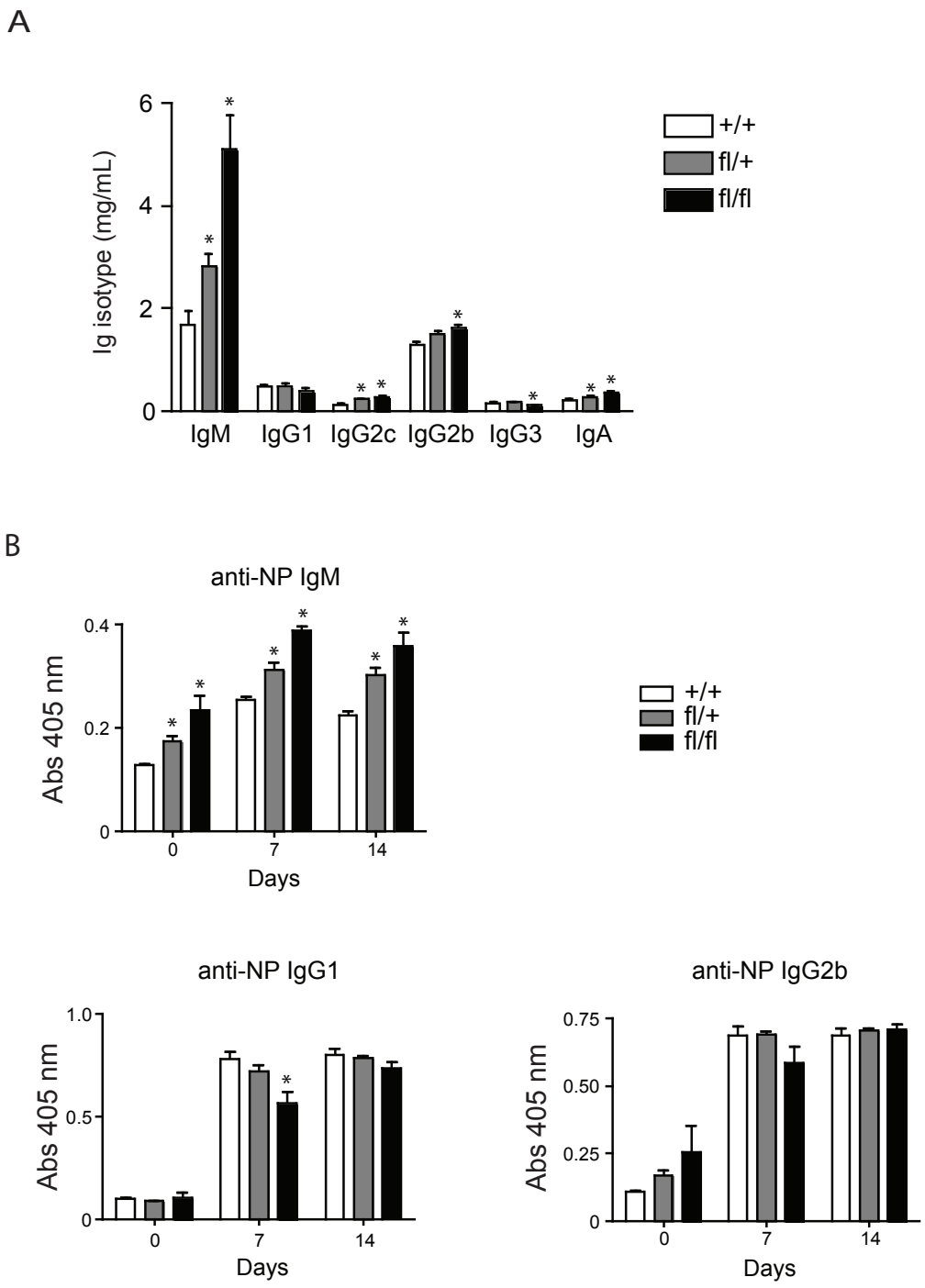

Figure S2.

Normal antigen specific B cell responses in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice.

ELISA analyses of serum immunoglobulin amounts from *Tnfaip3*$^{+/+}$, *Tnfaip3*$^{fl/+}$,

and *Tnfaip3*$^{fl/fl}$ mice. (A) Ig isotypes levels in unimmunized 5 to 7 week-old mice (n=6) plotted as means ± SEM. * indicates $p < 0.05$ by one-way ANOVA. (B) T-cell dependent, antigen specific B cell responses to NP-KLH. NP-specific antibody amounts before and after immunization at the indicated time points were plotted as the geometric mean ± SEM of 5 mice. Serum dilutions of 1:200 for IgM and 1:100 for IgG1 and IgG2b are shown. * indicates $p < 0.05$ by two-way ANOVA.

Tnfaip3$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ Cd19-Cre mice develop autoimmunity.

Increased numbers of immature and germinal center B cells suggest that auto-reactive B cells may accumulate and produce autoantibodies in *Tnfaip3*$^{fl/fl}$ CD19-cre mice. In addition, several SNPs near the human A20 (*Tnfaip3*) gene are independently associated with susceptibility to SLE (Graham et al., 2008; Musone et al., 2008). To characterize the autoantibody profile of *Tnfaip3*$^{fl/+}$ CD19-Cre mice and *Tnfaip3*$^{fl/fl}$ CD19-Cre mice as compared to *Tnfaip3*$^{+/+}$ CD19-Cre mice, we used large-scale 1152-feature protein and peptide microarrays to detect autoantibodies directed against over 140 antigens (Robinson et al., 2002). Antibodies to over 46 self antigens were detected, including antibodies to nuclear antigens (e.g., single stranded DNA, small ribonuclear proteins A and C, Ku protein), glomerular antigens (e.g., vimentin, collagen X, proteogylcan, and aggrecan), and heat shock proteins (Figure 5A). Importantly, the serum autoantibody profiles from *Tnfaip3*$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre mice clustered well with each other and away from *Tnfaip3*$^{+/+}$ CD19-Cre mice (Figure 5A). These autoantibodies were observed in both male and female mice and were observed in C57BL/6 inbred mice, a strain that is relatively resistant to SLE-like disease. Elispot analysis for anti-DNA indicate both an increase in the number and size of anti-DNA producing B cells in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice (Figure 5B and data not shown). These findings indicate that A20 expression in B

cells prevents spontaneous production of autoantibodies.

Autoantibodies can be deposited in glomeruli of kidneys of SLE patients and ultimately cause glomerulonephritis. To determine whether serum autoantibodies in *Tnfaip3*$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre mice lead to glomerular Ig deposits, we examined kidneys from 6 month old mice by histology and immunofluorescence. Although kidney sections appeared histologically normal, IgM deposits were observed in the kidneys of both *Tnfaip3*$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre mice (Figure 5C). IgG deposits were more prominent in *Tnfaip3*$^{fl/+}$ CD19-Cre mice (Figure 5C).

In addition to autoantibody producing B cells "escaping" from negative selection, activation of innate immune cells and type I interferon (IFN I) secretion may be key factors contributing to autoantibody production and the pathogenesis of SLE (Fairhurst et al., 2006; Shlomchik, 2008). CpG triggers B cell activation and production of type I interferons (IFN), and increases class switching to pathogenic autoantibody isotypes (Ehlers et al., 2006). We thus asked whether *Tnfaip3*$^{fl/+}$ CD19-Cre and *Tnfaip3*$^{fl/fl}$ CD19-Cre mice develop more autoimmune disease after stimulation with CpG. CpG treatment of intact mice enhanced production of IgG anti-dsDNA antibodies in serum as well as pathogenic deposition of IgG in renal glomeruli of both *Tnfaip3*$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre mice but not *Tnfaip3*$^{+/+}$ CD19-Cre mice (Figure 5D and data not shown). Taken together, these findings indicate that A20 expression in B cells prevents autoimmunity.

Figure 5

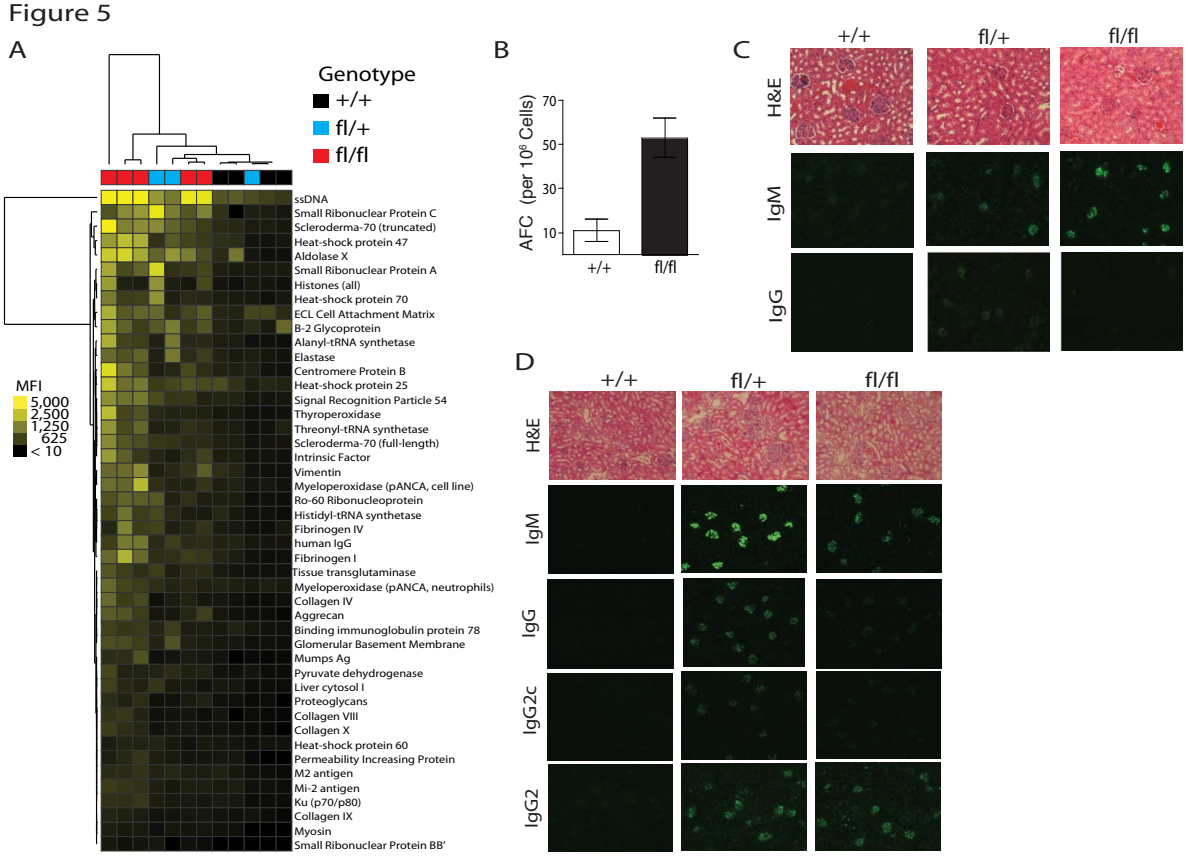

Figure 5. Spontaneous autoantibody production in *Tnfaip3*$^{fl/+}$ CD19-Cre and *Tnfaip3*$^{fl/fl}$ CD19-Cre mice.

(A) Protein array analyses of autoantibodies in sera from 3 month old *Tnfaip3*$^{fl/fl}$ CD19-Cre (n=5), *Tnfaip3*$^{fl/+}$ CD19-Cre (n=3), and *Tnfaip3*$^{+/+}$ CD19-Cre (n=4) mice. Heat map shows relative reactivity to the respective antigens on the arrays, hierarchically clustered in both axes by Euclidean Distance. The reactivity intensities (MFI) are depicted on a relative color scale. Statistically different antigens were identified using 2-class SAM with an unpaired t-test. (B) Antibody Forming Cells (AFC) measured on an Elispot for anti-dsDNA Ig producing B-cells. Counts were plotted as the mean of triplicate wells and S.D. is shown. Data are representative of 3 independent experiments. (C) Immunofluorescent analyses of glomerular Ig deposition in 6 month old mice of indicated genotypes. Analyses of IgM and IgG deposits shown in upper and middle panels, respectively. Data are representative of at 3 mice per genotype. Sections stained with hematoxylin and

eosin are shown above. (D) Immunofluorescent analyses of glomerular deposition of Igs of the indicated isotypes after CpG treatment. 8-10 week old mice (n=4) of the indicated genotypes were treated with 40 μg of CpG intraperitoneally every other day for 2 weeks. Mice were analyzed 6 weeks after start of treatment. H&E sections shown above. All sections 100X magnification.

A20 restricts B cell survival to Fas-mediated PCD.

Tnfaip3$^{fl/+}$ CD19-Cre mice share two key features with *Tnfaip3*$^{fl/fl}$ CD19-Cre mice: increased numbers of GC B cells and susceptibility to autoimmunity. These observations suggest that A20 expression levels may regulate B cell selection in germinal centers. One possible mechanism for negative selection of autoreactive B cells in GCs is Fas-induced PCD (Hao et al., 2008). We thus tested the susceptibility of *Tnfaip3*$^{+/+}$ CD19-Cre, *Tnfaip3*$^{fl/+}$ CD19-Cre, and *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells to Fas-induced PCD. Splenic B cells were activated with agonist anti-CD40 to induce Fas sensitivity. As expected, activation of *Tnfaip3*$^{+/+}$ CD19-Cre B cells caused increased expression of Fas and dose dependent sensitivity to Fas mediated PCD (Figure 6A, B). *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells expressed higher levels of Fas than *Tnfaip3*$^{+/+}$ CD19-Cre B cells after CD40 stimulation, while *Tnfaip3*$^{fl/+}$ CD19-Cre B cells expressed intermediate levels (Figure 6A). Remarkably, *Tnfaip3*$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre B cells were resistant to Fas-mediated PCD when compared to control *Tnfaip3*$^{+/+}$ CD19-Cre cells--even though *Tnfaip3*$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre B cells expressed greater amounts of Fas (Figure 6B). This finding is particularly surprising given our prior observation that *Tnfaip3*$^{-/-}$ fibroblasts are more susceptible to TNF induced PCD than *Tnfaip3*$^{+/+}$ cells (Lee et al, 2000). Thus, A20 supports Fas mediated PCD in B cells while inhibiting TNFR induced PCD in fibroblasts.

To understand why A20 deficient B cells are resistant to Fas mediated

PCD, we hypothesized that increased NF-kB signaling in these cells might lead to increased expression of anti-apoptotic proteins such as Bcl-x. After stimulation with agonist anti-CD40 in vitro, Bcl-x mRNA levels increased within one hour, and rose to a higher level in *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells compared with *Tnfaip3*$^{fl/+}$ CD19-Cre or *Tnfaip3*$^{+/+}$ CD19-Cre B cells (Figure 6C). CD40 triggered induction of Bcl-x mRNA was blocked by the NF-kB inhibitor, NEMO-binding domain (NBD) peptide, but not by control peptide (Figure 6C). Hence, NF-kB signaling appears directly required for induced Bcl-x mRNA transcription. Consistent with enhanced Bcl-x mRNA expression, markedly higher levels of Bcl-x protein appear in *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells compared with *Tnfaip3*$^{fl/+}$ CD19-Cre or *Tnfaip3*$^{+/+}$ CD19-Cre B cells (Figure 6D). Taken together, these findings indicate that A20 restricts the survival of activated B cells by limiting the NF-kB dependent transcription of Bcl-x mRNA and the subsequent production of Bcl-x protein. They also provide a potential mechanism by which A20 deficient B cells are resistant to negative selection in germinal centers.

Figure 6

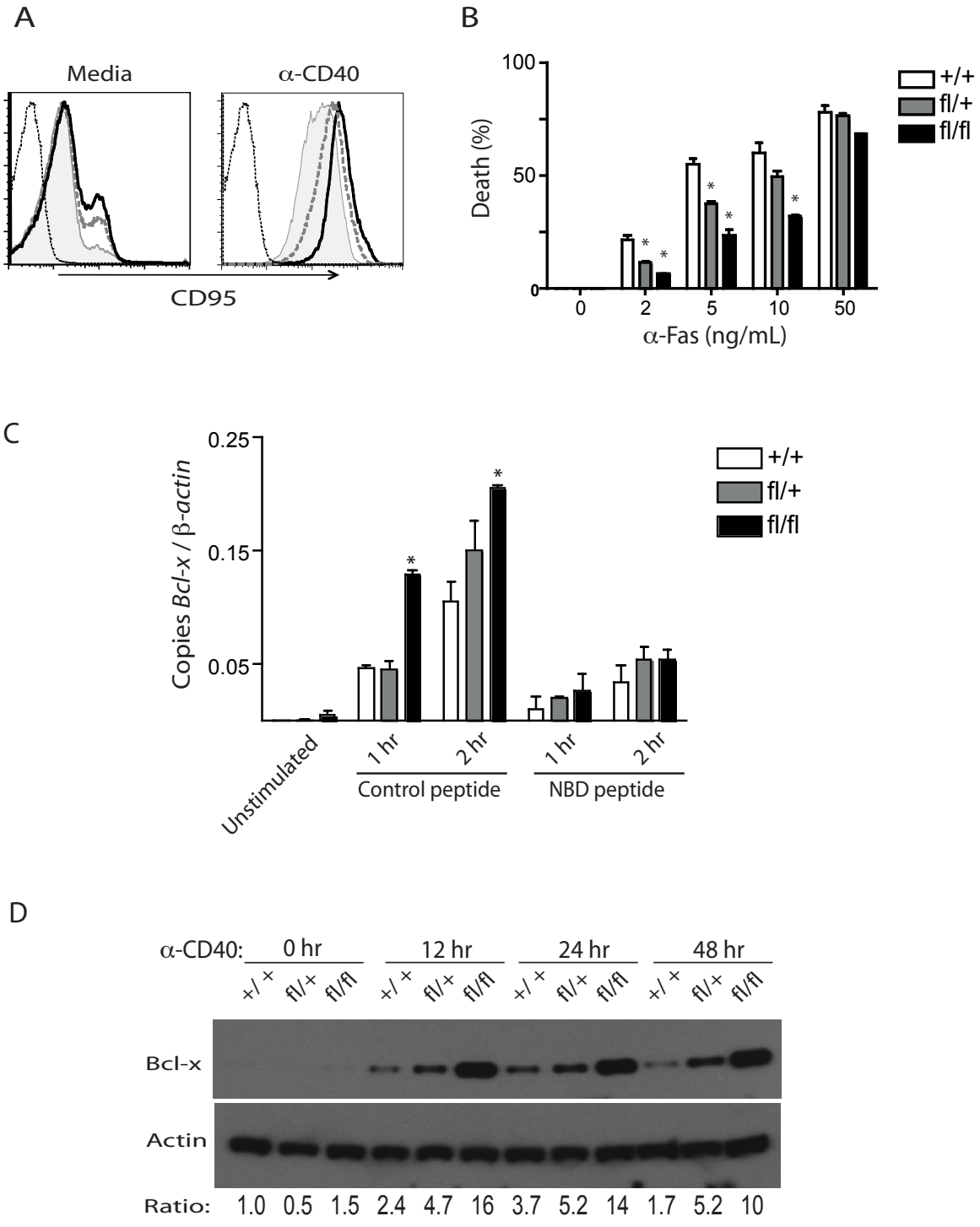

Figure 6. A20 deficient and hypomorphic B-cells are resistant to

programmed cell death.

(A) Flow cytometric analysis of CD95 (Fas) expression in enriched B cells after agonist anti-CD40 stimulation. Overlays of CD95 histograms on gated $CD19^+$ cells from *Tnfaip3*$^{+/+}$ CD19-Cre (shaded), *Tnfaip3*$^{fl/+}$ CD19-Cre (dashed line), *Tnfaip3*$^{fl/fl}$ CD19-Cre (black line), and unstained control (dotted line) are shown. (B) Enriched B-cells stimulated for 48 hours with agonist anti-CD40 and treated with the indicated concentrations of agonist anti-CD95 for 12 hours were analyzed for survival by measuring the percentage of dead (Annexin-V^+ $DAPI^+$) cells by flow cytometry. Percent death was calculated as [% Fas induced dead - % control dead / 100% - % control dead]. Data are plotted as mean ± S.D of triplicate wells. * indicates $p < 0.001$ using two way Anova. (C) Bcl-x mRNA levels measured by real time quantitative (RT qPCR) in enriched B-cells. Cells of the indicated genotypes were stimulated with agonist anti-CD40 for the indicated times in the presence of an inhibitor or a control peptide, or left unstimulated. (D) Immunoblot analysis for the expression of Bcl-x protein in B-cells of the indicated genotypes stimulated with agonist anti-CD40 for the indicated times. Actin protein levels are shown below as control. Ratios of Bcl-x to actin protein, normalized to the *Tnfaip3*$^{+/+}$ B cells at time 0, are shown below each sample. All data are representative of 3 independent experiments.

DISCUSSION

The generation and characterization of mice lacking A20 specifically in B cells, *Tnfaip3*$^{fl/fl}$ CD19-Cre mice, has allowed us to unveil several novel functions for A20. We have discovered that B cell specific expression of A20 restricts CD40 and BCR responses, terminates CD40 triggered NF-kB signals, restricts B cell survival, and prevents autoimmunity. These studies provide unique molecular insights into B cell homeostasis, human SLE and B cell lymphomas.

Tnfaip3$^{fl/fl}$ CD19-Cre mice are largely healthy, in marked contrast to mice lacking A20 expression in all cells or in all hematopoietic cells. This observation is consistent with our prior suggestion that the cachexia, myeloid dysregulation and perinatal lethality observed in globally deficient A20 mice is primarily due to myeloid cell dysfunction. Like other "floxed" alleles bred to CD19-Cre mice, *Tnfaip3*$^{fl/fl}$ CD19-Cre mice delete A20 in a highly B cell-specific fashion. While these mice contained mild expansion of T cell numbers, these perturbations are probably not due to aberrant A20 deletion as judged by our molecular analyses and by the published literature for this Cre strain (Rickert et al., 1997; Schmidt-Supprian and Rajewsky, 2007). Although *Tnfaip3*$^{fl/fl}$ CD19-Cre mice did not express measurable serum levels of IL-6 or IL-4 by ELISA, they did express increased levels of splenic IL-4 mRNA (data not shown); and *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells produced more IL-6 and expressed higher levels of co-stimulatory molecules upon stimulation. Thus, T cell expansion may be due to antigen-independent bystander effects induced by B cell derived cytokines and/or co-stimulatory molecules, as has previously been observed in other settings of B cell hyper-responsiveness (Homig-Holzel et al., 2008; Hao et al., 2008).

Our findings demonstrate that A20 performs important functions in adaptive immune cells in addition to previously described functions in innate immune cells

(Lee et al, 2000; Boone et al, 2004; Hitotsumatsu et al, 2008). A20's roles in restricting CD40 and BCR triggered NF-kB signals add to the spectrum of signaling cascades regulated by this ubiquitin modifying enzyme. NF-kB signaling is known to be important for regulating B cell homeostasis (Sen 2006; Siebenlist et al, 2005). The phenotypes of our mice lacking A20 expression in B cells reveal the importance of tightly regulating basal NF-kB signals in these cells.

B cell specific loss of A20 expression leads to increased numbers of autoantibody producing cells. B cells undergo several stages of negative selection to eliminate autoreactive cells both in the bone marrow and in peripheral lymphoid organs (Jacobi and Diamond, 2005; Shlomchik, 2008; Yurasov and Nussenzweig, 2007; von Boehmer and Melchers, 2010). In the bone marrow, immature B cells are selected as a consequence of BCR and BAFF signals, and intracellular cell survival factors. In the periphery, selection occurs during GC maturation. Germinal centers are sites where B cells undergo expansion, immunoglobulin class switching, somatic hypermutation, and affinity maturation (Klein and Dalla-Favera, 2008). B cells with low affinity for antigen or reactivity for self antigens are negatively selected within GCs (Shlomchik, 2008). Deletion of autoreactive B cells helps prevent autoimmunity, and defective GC selection of autoreactive B cells has been observed in human SLE patients (Cappione et al., 2005). Fas (CD95) is highly expressed on GC B cells (Watanabe et al, 1995). While the role of Fas mediated PCD in GC selection has been controversial (Smith et al., 1995; Takahashi et al., 2001; Mizuno et al., 2003; Hoa et al., 2008), Fas mediated PCD likely plays an important role in eliminating autoreactive B cells (Rathmell et al., 1995; Fukuyama et alk., 2002; William et al., 2002). Thus, the accumulation of GC B cells in *Tnfaip3*$^{fl/+}$ CD19-Cre and *Tnfaip3*$^{fl/fl}$ CD19-Cre mice may be due to the increased resistance of A20 deficient B cells to physiological PCD, leading to the escape of autoreactive B cells.

How might A20 deficiency in B cells render them resistant to PCD? One

possibility stems from the observation that NF-kB dependent proteins protect B cells against PCD. Indeed, both canonical and non-canonical NF-kB signaling downstream of BCR, CD40, BAFF, and TLR receptors are thought to promote B cell survival as well as proliferation and activation (Siebenlist et al., 2005; Sen, 2006; Homig-Holzel et al., 2008). NF-kB has been suggested to be necessary for mediating BCR induced resistance to Fas mediated PCD (Mizuno and Rothstein, 2003; Schram and Rothstein, 2003). Our studies indicate that A20 directly restricts canonical NF-kB signals and suggest that these signals may lead to elevated non-canonical NF-kB signals. These increased NF-kB signals lead to increased expression of anti-apoptotic proteins such as Bcl-2 and Bcl-x. Deregulated expression of these proteins has been shown to cause altered GC B cell selection (Grillot et al., 1996; Takahashi et al., 1999). Hence, increased expression of Bcl-x and/or other NF-kB dependent proteins may provide a molecular underpinning for increased numbers of GC B cells in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice.

Heterozygous *Tnfaip3*$^{fl/+}$ CD19-Cre mice contain similarly increased numbers of GC B cells and autoantibodies as homozygous *Tnfaip3*$^{fl/fl}$ CD19-Cre mice at young ages (i.e., 5-7 weeks old), suggesting that a high threshold level of A20 expression must be maintained for properly selecting (or deleting) these cells. Reduced A20 expression in other cell types leads to increased production of NF-kB dependent gene products, so endogenous levels of A20 expression appear to be limiting (O. Hitotsumatsu , S. Oshima, G. Hammer, unpublished data). Reduced (rather than absent) levels of A20 expression or hypomorphic A20 proteins may also link A20 (*Tnfaip3*) susceptibility SNPs with SLE in human patients (Musone et al, 2008). Thus, mice expressing reduced levels of A20 may prove to be highly relevant models of human autoimmune diseases.

While reduced A20 expression in B cells leads to accumulation of GC B cells and IgG autoantibodies, absent A20 expression also causes accumulation of

immature B cells and IgM in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice and progressive activation of B cells with age. Hence, a lower amount of A20 is necessary to preserve selection of immature B cells and to restrict spontaneous B cell activation than the amount required for proper GC selection. As IgM autoantibodies may be protective against IgG mediated autoimmune disease, higher IgM levels in homozygous *Tnfaip3*$^{fl/fl}$ CD19-Cre mice may reduce the degree of autoimmune disease observed in these mice (Witte, 2008). A20 levels are dynamically regulated, largely in response to NF-kB signals (Krikos et al, 1992). Thus, A20 expression levels appear to be finely tuned to regulate NF-kB signaling and survival of distinct subsets of B cells.

Tnfaip3$^{fl/fl}$ CD19-Cre mice exhibit largely normal antigen specific B cell responses in vivo, despite the fact that mature *Tnfaip3*$^{fl/fl}$ CD19-Cre B cells exhibit increased responses to BCR, CD40, and TLR ligands in vitro. These findings suggest that B cell independent factors such as T cells and myeloid cells can properly restrict antigen specific B cell responses, even if they allow progressive accumulation of autoreactive B cells in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice.

Our experiments indicate that A20 expression in B cells regulates GC B cell selection as well as B cell activation, thereby regulating key aspects of B cell tolerance. It is remarkable that B cell specific deletion of A20 alone is sufficient for autoimmunity in C57BL/6 mice. The appearance of IgG deposits suggests that abnormal B cells are sufficient for at least the initial stages of the autoimmunity and are part of the continuum to full blown disease. Hence, lupus prone *Tnfaip3*$^{fl/fl}$ CD19-Cre and *Tnfaip3*$^{fl/+}$ CD19-Cre mice as well as genetic derivatives of these mice should be useful models for understanding human SLE. Heterozygous *Tnfaip3*$^{fl/+}$ CD19-Cre mice may be particularly relevant, as reduced, rather than absent A20 expression may characterize this human condition.

Recent studies have shown that somatic loss of A20 in B cells causes

several types of Hodgkin, non-Hodgkin, and marginal zone B cell lymphomas in humans (Kato et al., 2009; Compagno et al., 2009; Schmitz et al., 2009; Novak et al., 2009). Our findings that A20 deficient B cells express high levels of Bcl-x and are resistant to Fas mediated PCD provide molecular insights into how A20 functions as a tumor suppressor in B cells. Remarkably, A20 is a pro-apoptotic protein in B cells even though it restricts TNF induced apoptosis in fibroblasts and hepatocytes (Lee et al., 2000; Arvelo et al., 2002). Hence, it is critical to analyze A20's physiological functions in cell type-specific contexts. Future studies testing the potential of A20 deficiency to collaborate with other B cell oncogenes may reveal the spectrum of A20's tumor suppressor functions in B cells.

In conclusion, we have demonstrated functions for A20 in regulating B cell responses, including the restriction of CD40 induced NF-kB signals. These cell autonomous functions are critical for B cell homeostasis and the prevention of autoreactive B cells and autoimmunity. In addition to unveiling new molecular mechanisms of B cell homeostasis, these studies provide critical insights into the pathogenesis of human SLE and B cell lymphomas.

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Author Contributions

R.M.T. planned and performed the experiments, analyzed the data, and assisted in writing the manuscript. E.E.T. made the conditional gene targeting construct, performed gene targeting in ES cells, and characterized the original $A20^{fl}$ germline mice. R.A. performed mice breeding and genotyping, and assisted with mouse injections and bleeding. C.L.L. performed the antibody array experiments and performed statistical analysis, under the supervision of P.J.U. C.A.L. and P.J.U. edited the manuscript. P.S. assisted in the analysis of the autoantibody data and mouse histology and immunohistochemistry, under the supervision of C.A.L. L.R. performed some of the initial experiments with R.M.T. J.B. provided excellent technical assistance. B.A.M. and A.M. helped plan and supervise the experiments and data analysis, and wrote the manuscript.

METHODS

Generation of A20 conditionally targeted (*Tnfaip3*$^{fl/fl}$) mice.

Recombineering was used to generate a gene targeting construct from a bacterial artificial chromosome (BAC) from the C57BL/6J strain containing the *Tnfaip3* gene. C57BL/6 ES cells (PRX-B6T, Primogenix) were transfected with this construct, and successfully targeted ES cells were identified by Southern blot analysis. Correctly targeted clones were then transfected with recombinant Cre and screened for removal of the neomycin cassette to obtain the *Tnfaip3* "floxed" allele. Blastocyst injections of targeted ES cells were performed by the UCSF Transgenic Core. Chimeras were bred to albino C57BL/6J mice and non-albino progeny were screened for the presence of the floxed allele (*Tnfaip3*$^{fl/+}$). Mice bearing the targeted allele in the germline were interbred with CD19-Cre mice on the C57BL/6 background (B6.129P2(C)-CD19tm1(cre)Cgn/J) (Rickert et al, 1997). *Tnfaip3*$^{fl/fl}$ or *Tnfaip3*$^{fl/+}$ mice homozygous for CD19-Cre were bred with *Tnfaip3*$^{fl/fl}$ or *Tnfaip3*$^{fl/+}$ mice without the CD19-Cre allele to generate experimental mice of various A20 genotypes (*Tnfaip3*$^{+/+}$, *Tnfaip3*$^{fl/+}$ and *Tnfaip3*$^{fl/fl}$) that have one copy of Cre and are heterozygous for CD19. Genotypes were initially confirmed by Southern blot analysis and subsequently identified by PCR using the following primers: 5'-AACTTTACAGTCCCCAGCAATGG-3' (sense); and 5' – GAGGAGGTTGGAAGACATAGAATCG-3' (antisense).

Cell preparation and analyses.

Single cell suspensions were prepared and incubated with the designated conjugated antibodies (all from BD Biosciences, except anti-CD5 and anti-CD93

(AA4.1), eBioscience), and live cells (DAPI-) were analyzed by flow cytometry (LSRII, BD Biosciences) using FlowJo software (Tree Star Inc.). BMDMs were prepared as previously described (Boone et al., 2004). For in vitro assays, B-cells were isolated by negative depletion with TCRβ, Mac-1, NK1.1 and Ter119 biotinylated antibodies (BD Biosciences) bound to streptavidin coated magnetic beads (M-280 Dynabeads, Invitrogen). Cells were stimulated with anti-CD40 (HM40-3, BD Biosciences) at 1 μg/ml, anti-μ chain (Jackson Immunoresearch) at 2 μg/ml, IL-4 (Peprotech) at 10 ng/ml and LPS (Sigma) at 1μg/ml. For in vitro deletion of *Tnfaip3* exon 2 from GT-Rosa Cre B cells, cells were treated with 4-OH-T (2.5 nM) for the first 12 hours of stimulation. For NF-kB inhibition, control peptide or NBD Peptide (Calbiochem) were added at 0.5 μM one hour prior to stimulation with agonist anti-CD40 antibody.

Mouse immunizations

For in vivo antigen responses, 6-8 week old mice were injected intraperitoneally (ip) and bled on the indicated days. Mice were injected with 50 μg NP-KLH (Biosearch Technologies) mixed 1:1 with Imject Alum (Thermo Scientific). For CpG treatment, 8 week old mice were injected ip, every other day for 2 weeks, with 40 μg CpG ODN 2395 (tcgtcgttttcggcgcgcgccg) with phosphorothioate bases (Invitrogen). Serum was collected before treatment, and 6 weeks post-treatment. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Immunoglobulin and AFC determination

Serum and supernatant immunoglobulin levels (total Ig, IgM, IgG1, IgG2c, IgG2b, IgG3 and IgA) were determined by isotype specific ELISA (Southern

Biotechnology, Birmingham, Alabama). NP (4-Hydroxy-3-nitrophenylacetyl) -specific antibody titers were determined by ELISA with plates coated with NP23-BSA (Biosearch Technologies). dsDNA-specific antibody titers were determined by ELISA with plates coated with Hind III (New England Biolabs) digested pUC19 in 0.1M Tris, over-night at room temperature. Cytokines levels were measured by ELISA as recommended by the manufacturer (BD Biosciences). Elispot was performed by incubating enriched B cells on plates coated with dsDNA as above. After overnight incubation, plates were washed and incubated with alkaline phosphatase (AP) conjugated anti-IgM (Southern Biotech), and subsequently developed with BCIP substrate (Sigma) dissolved into alkaline phosphatase buffer (0.1M Tris, 0.1 M NaCl, 5 mM MgCl2) and 0.6 % LMP Agarose (Sigma).

Autoantigen arrays were printed and processed as previously described. Arrays were probed with goat antibody specific for mouse Ig (Jackson Immunoresearch). Detailed protocols and lists of antigens have been published and are available online at http://utzlab.stanford.edu/protocols (Robinson et al., 2002). Significance Analysis of Microarrays (SAM) was applied to the data to identify antigens with statistically significant differences in array reactivity between mutant and wild type mice (Tusher et al., 2001).

Histology and immunohistochemistry

Kidneys and spleens were fixed in 10% formalin. Sections and H&E stain were performed by the UCSF VAMC Pathology Core. For immunohistochemistry, kidneys were embedded in Tissue-Tek OCTTM compound and snap frozen in Methyl-butane with dry ice. Tissue sections were then stained with IgM-FITC or IgG-FITC (Jackson Immunoresearch), IgG2c-FITC or IgG2b-FITC (Bethyl Laborotories Inc.).

Cell signaling assays

Enriched B cells were stimulated as indicated in the figures, lysed in 0.1% NP-40 (Calbiochem) lysis buffer, and nuclei spun down to yield cytoplasmic lysates. Lysates were cleared by centrifugation at 14,000 g for 20 min at 4 ° C, supernatants were removed, heated in Laemmli buffer and run on SDS-PAGE (Novex System, Invitrogen). Immunobots were probed for A20 (Boone et al, 2004), actin (JLA20, Calbiochem), phospho-IκBα, IκBα, phospho-Erk, Erk, phospho-NF-kB2 p100 and NF-kB2 p100/p52 (Cell Signaling), Bcl-x (Transduction Biotechnologies).

Fas-induced cell death assay

Fas-induced cell death assays were performed as described (Watanabe et al, 1995; Wang et al., 1996). Briefly, enriched B-cells were plated with agonist anti-CD40 (1 μg/ml) for 60 hours. Agonist anti-CD95 (Fas) was added to the cultures at the indicated doses for the last 12 hours. Cells were harvested and stained for flow cytometry with Annexin-V (BD Biosciences) and DAPI.

Real-time PCR assays

For quantitation of genomic A20 exon 2, DNA was prepared using DNeasy Kit (Qiagen), after which qPCR was performed using SYBR Green (Qiagen). Primers for exon 2 of A20 were the following: 5'-CTGACCTGGTCCTGAGGAAG-3' (sense); and 5'-GCAAAGTCCTGTTTCCACAA-3' (anti-sense). This qPCR assay was shown to detect less than 1% of exon 2 DNA in titrated mixtures of *Tnfaip3*$^{-/-}$ and *Tnfaip3*$^{+/+}$ cells (data not shown). For quantitation of Bcl-x mRNA

expression, mRNA was prepared using RNeasy Kit (Qiagen) and cDNA was obtained with Quantitect Reverse Transcription Kit (Qiagen). Primers for Bcl-x cDNA were the following: 5'-GCAGACCCAGTAAGTGAGCA-3' (sense) and 5'-AGAAAGTCGACCACCAGCTC-3' (antisense). In both types of assays, β-actin primers used as a reference were: 5'- AAGTGTGACGTTGACATCCGTAA -3' (sense) and 5'- TGCCTGGGTACATGGTGG TA -3' (antisense). Assays were performed using an ABI 7300 real-time PCR machine (Applied Biosystems).

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CHAPTER 4

Final Discussion

This thesis work covered two general but distinctly different aspects of A20's function in the immune system. While the first part focused on innate immune responses, the second turned the scope of our attention on A20's roles in adaptive immunity. In the end, just like both sides of the immune system are fundamental, these two stories might have complemented each other to build the route of my learning journey and hopefully still open doors that will lay the foundation for future research.

In chapter 2 of this work, we have shown that MyD88 deficiency largely reverses the $A20^{-/-}$ phenotype, demonstrating that MyD88-dependent signals need to be constantly regulated by A20 under homeostatic conditions, or otherwise cause systemic inflammation. Moreover, these MyD88-dependent signals appear to be initiated by gut commensals, as treatment with broad-spectrum antibiotics can also decrease spontaneous inflammation driven by A20 deficient cells. $A20^{-/-}$ hematopoietic cells seem to recognize microbes in the intestine and be sufficient to set off generalized inflammation. We have also demonstrated that A20 terminates MyD88-independent, TRIF mediated TLR responses. Nevertheless, A20 seems to specifically regulate NF-κB signals rather than IRF signals downstream of TRIF. This suggests that rather than having an indiscriminate inhibitory role, A20 may have preferences for certain targets, namely those upstream of NF-κB.

Very recently, a conditional allele of A20 was used in conjunction with Villin-Cre to obtain specific deletion of A20 in IECs (intestinal epithelial cells) (Vereecke et al., 2010). In contrast with our findings with hematopoietic cells, these mice do not develop any sort of spontaneous inflammation, even though they are more

sensitive to experimentally induced colitis than controls. Additionally, MyD88 deficiency in these mice renders them more sensitive to induced colitis, rather than being protective. In contrast, TNF deficiency is protective, and these signals in IECs seem to be the most deleterious in the absence of A20.

These diverging results highlight how A20 can have cell type specific regulatory functions. Furthermore, these findings are consistent with previous reports that TLR stimulation in the intestinal epithelia is required for gut homeostasis (Rakoffnahoum et al., 2004). TLR stimulation in the intestinal epithelia is mostly beneficial, and IECs might be less sensitive to increased TLR signals. On the other hand, exaggerated TLR responses in immune cells that might survey the intestinal basal layer are more proinflammatory and need to be constantly terminated by A20. Cytokines such as TNF, which is produced by these cells, can then in turn be harmful to IECs.

Generation of lineage specific deletions of A20 will certainly provide additional insights into such specificities. In particular, generation of DC and macrophage lineage specific deletions of A20 can further confirm whether TLR signals in these cells alone are indeed responsible for driving systemic inflammation in the absence of A20. That being the case, it reinforces the role of A20 in terminating innate immune signals under homeostatic conditions.

In chapter 3 of this work, we have shown that A20 regulates B cell functions and homeostasis. A20 terminates BCR, CD40 and TLR induced responses in B cells. Mice with absent or hypomorphic A20 expression in B cells develop spontaneous autoimmunity. A20 deficient and hypomorphic B cells have increased survival to PCD (programmed cell death) and this might be a mechanism by which autoreactive cells escape selection in the case of aberrant

function or expression of A20.

We have identified additional pathways that are regulated by A20. Future studies might use B cells to define novel targets and mechanisms of this enzyme. Particularly, prolonged NF-κB signals downstream of CD40 could owe to prolonged TRAF2 ubiquitylation downstream of this receptor. In fact, TRAF2 has already been reported to interact with A20 in other contexts (Shembade et al., 2010). Finally, just as A20 interacting proteins have been found to function in previously known A20 regulated pathways, it will be interesting to look at their roles in B cell signaling.

B cells integrate stimuli from various receptors in order to provide an appropriate outcome. A20 deficient B cells are hyperresponsive to most of these stimuli *in vitro*, and these defects probably account for failure at several levels of B cell function *in vivo*. Interestingly, selection seems to be particularly affected, whereas antigen-specific responses are mostly normal. Likewise, while B cell mediated autoimmunity and increased GC B cell numbers were observed with both absent or hypomorphic expression of A20, B cell hyperactivation is only obvious with total A20 absence. Differences probably stem from complex combinations of B cell intrinsic and extrinsic factors, like different NF-κB intensities and B – T cell interactions, respectively. While it is hard to predict all the resulting outcomes, perhaps mathematically modeling some of these variables could shine new light into these problems.

Meanwhile, it might be useful to explore the role of B – T cell interactions, which occur both at the T cell / B cell border of the follicle and later in the GCs. As reported in B cell specific deletion of Fas (Hao et al., 2008), we have observed that altered B cell function can drive T cell expansion, and each one of these cell subsets might feed into each other to further disrupt homeostasis. From this perspective, mice double deficient for A20 in B and T cells might be of particular

interest to explore such dynamics.

We have found that A20 restricts B cell survival to Fas mediated PCD upon CD40 stimulation and this might explain defective negative selection in GCs in the face of A20 deficiency. Nonetheless, this does not exclude additional points of escape of autoreactive cells in the absence of A20. In fact, it is likely that there is also impaired negative selection in the bone marrow, given the abundance of IgM (non-switched) autoantibodies. In this context, it will be of special interest to test different types of cell death in B cells isolated from both spleen and bone marrow. Furthermore, examining *Tnfaip3*^{fl} mice for the additional mechanisms of B cell selection, receptor editing and anergy induction, can also be elucidating.

Finally, although we have not observed obvious signs of B cell lymphomas in mice aged up to one year old, given the many associations between A20 and B cell lymphomas it might be worth further exploration into the signs of malignancy in *Tnfaip3*$^{fl/fl}$ mice. Specifically, increased survival at GCs can result in increased AID (activation-induced cytidine deaminase) expression and result in increased oncogene translocations, related with malignancy (Robbiani et al., 2008; Takizawa et al., 2008). Because GC formation and retention is intimately tied to CD4 T cells, it remains possible that A20 deficiency in T cells as well as B cells may also lead to altered T cell help and ultimately the survival of malignant cells.

In conclusion, we have unveiled several novel functions of A20 that significantly contribute to the appropriate functioning of the immune system, innate and adaptive. Absent or altered expression of A20 in different cell types results in a number of defects, highlighting the increasingly accepted position of A20 as a central regulator of immune function.

Importantly, our observations emphasize how immune tolerance can be broken through different immune cell types, by manipulating a single gene. Complete ablation of expression in all immune lineages resulted in exaggerated inflammation in a broad, pathogen or danger associated signal. Concurrently, reduced expression in B lymphocytes alone affected the fine workings of self-non-self discrimination of acquired immunity.

In Chapter 1 I discussed how the operation of the entire immune system results from the incorporation of signals from different cells, belonging to both the innate and the adaptive immune systems, but also to the tissues with which immune cells interact. Each immune cell, in turn, integrates those different external inputs into the cell. While we continue to discover how A20 functions at the intracellular level of many cells, it is equally evident that these intracellular processes can result in system wide consequences. And even if some levels seem to be more of a tipping point than others, it seems evident that there have to be checking points at all of them, and that maintenance of immune homeostasis and tolerance depends on the efficient intracellular control by molecules as A20.

For all this, I think there are many reasons to predict that additional lineage specific deletions of A20 will continue to reveal exciting and hitherto unknown roles for A20 in immunity and beyond.

APPENDIX

Unpublished preliminary data and future directions

BAFF mediated survival and death by neglect of A20 deficient B cells

In order to assess A20's role in restricting B cell survival in additional contexts, we isolated B cells from spleen and bone marrow and tested them for cell death in further conditions. Specifically, BAFF is known to promote B cell survival and be determinant during B cell development and selection (Sen, 2006). Additionally, we observed that *Tnfaip3*$^{fl/fl}$ B cells have higher basal levels of p100, enabling these cells to hyperrespond to BAFF signals, which activate this non-canonical NF-κB pathway.

We have isolated B cells from bone marrow and spleens from *Tnfaip3*$^{+/+}$, $^{fl/+}$ and $^{fl/fl}$ CD19-Cre mice and cultured them with increasing doses of BAFF at different time points. In this primary data (Figure 2.1), we found no consistent differences between genotypes in BAFF mediated survival. On the other hand, we tested B cell survival in the absence of any stimulation in culture ("death by neglect") and found that there was a consistent difference between genotypes, with decreasing doses of A20 corresponding to increased survival to lack of stimulation.

These preliminary observations suggest that A20 might restrict B cell survival in particular contexts, rather than indiscriminately. A20 does not seem to limit BAFF mediate B cell survival, whereas its deficiency or lower expression result in increased survival to lack of stimulation. It still remains to be tested whether BCR induced death is affected by A20, in the absence or presence of different doses of counteracting survival cytokines IL-4 and BAFF. These results should contribute to further tease out the mechanisms by which A20 can regulate B cell survival and eventually negative selection.

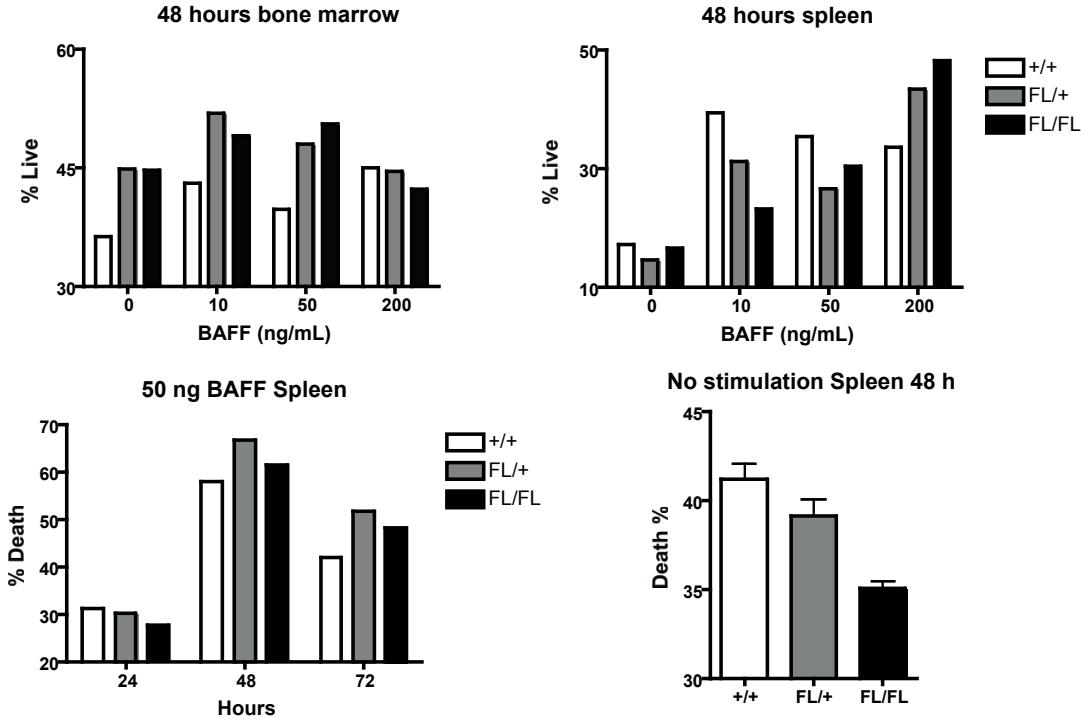

Figure 2.1 – BAFF mediated B cell survival. Data is representative of two independent experiments.

Kappa and lambda chains expression in *Tnfaip3*^{fl} mice

So as to assess other mechanisms of negative selection that could be affected by A20, we planned on measuring receptor editing in *Tnfaip3*^{fl} CD19-Cre mice. Induction of receptor editing might also depend on NF-κB activation levels during B cell development and differentiation (Derudder et al., 2009). We have not looked directly at receptor editing, but we have measured *kappa* (κ) and *lambda* (λ) chains in the serum of *Tnfaip3*^{fl} CD19-Cre mice, as well as the relative abundances of κ and λ positive B cells by flow cytometry. In serum, we have found progressively increased levels of both chains with lower or absent

expression of A20, probably due to corresponding increased levels of total Ig. There was a bias towards more Igλ in the absence of A20, which curiously was more apparent in anti-dsDNA autoreactive Ig (Figure 2.2). Flow cytometry analysis of spleen and bone marrow B cells showed no significant alterations in the percentages of each κ and λ individual populations between the 3 genotypes. However, again there seems to be a bias towards higher overall λ expression, with an increase in double positive cells corresponding to decreasing A20 (Figure 2.3)

Since receptor editing promotes replacement of Igκ light chains by Igλ ones, these observations suggest that there is no defect in receptor editing. On the contrary, there could be increased induction of expression of λ chains that could be a consequence of increased NF-κB in the absence of A20. Definitive conclusions would require direct assessment of receptor editing.

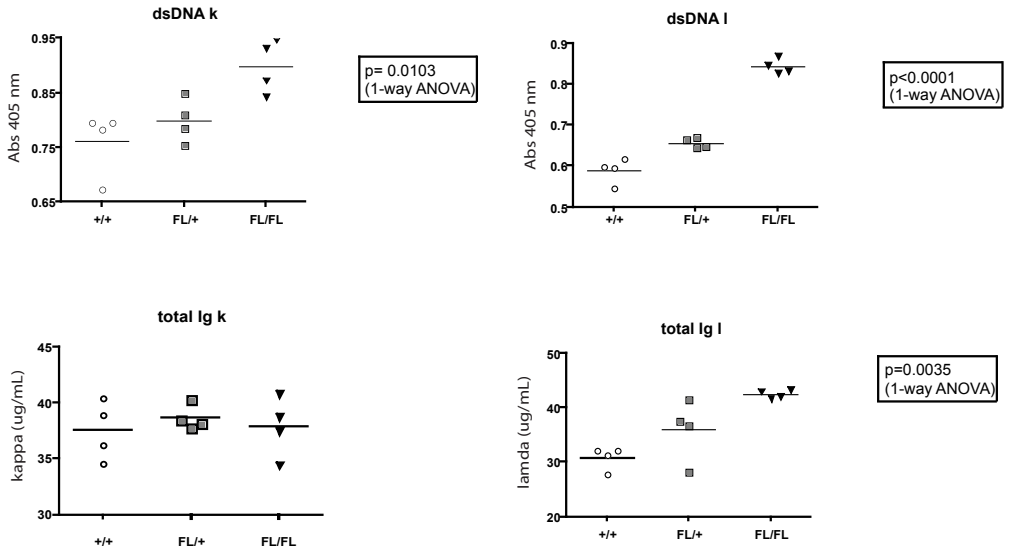

Figure 2.2 – Elisas of serum from the indicated mice genotypes at 3 months of age. (Top Graphs) Total Igκ and Igλ and (Bottom Graphs) anti-dsDNA specific Igκ and Igλ.

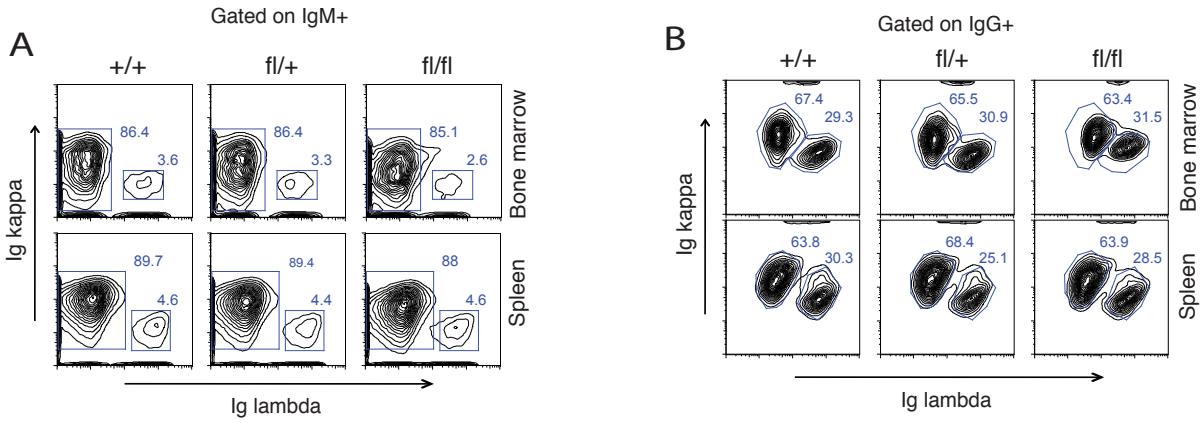

Figure 2.3 – Flow cytometry analysis of κ and λ B cell populations in spleen and bone marrow of *Tnfaip3*^{fl} CD19-Cre mice of the indicated genotypes. All samples were gated on CD19^{+} cells and (A) IgM^{+} (B) IgG^{+}. Data are representative of 3 sets of mice.

Decreased high affinity Ig in T-dependent responses in the absence of A20

We asked whether affinity maturation was altered in the absence of A20. Even though we have not made most direct evaluations, we measured antibodies in T-dependent antigen specific responses by NP_3 versus NP_{23} Elisas, which can give a good indication of the higher versus lower affinity nature, respectively, of the antibodies produced. Strikingly, even though *Tnfaip3*$^{fl/fl}$ CD19-Cre mice make some more IgM, IgG antibodies seems to be of considerably lower affinity. This might be explained by the increased survival of A20 deficient B cells. While in wild-type mice lower affinity B cells do not survive due to weaker stimulation, *Tnfaip3*$^{fl/fl}$ cells might live as a consequence of stronger signals in the absence of A20. Overall, this decreases the affinity of the Ig pool produced.

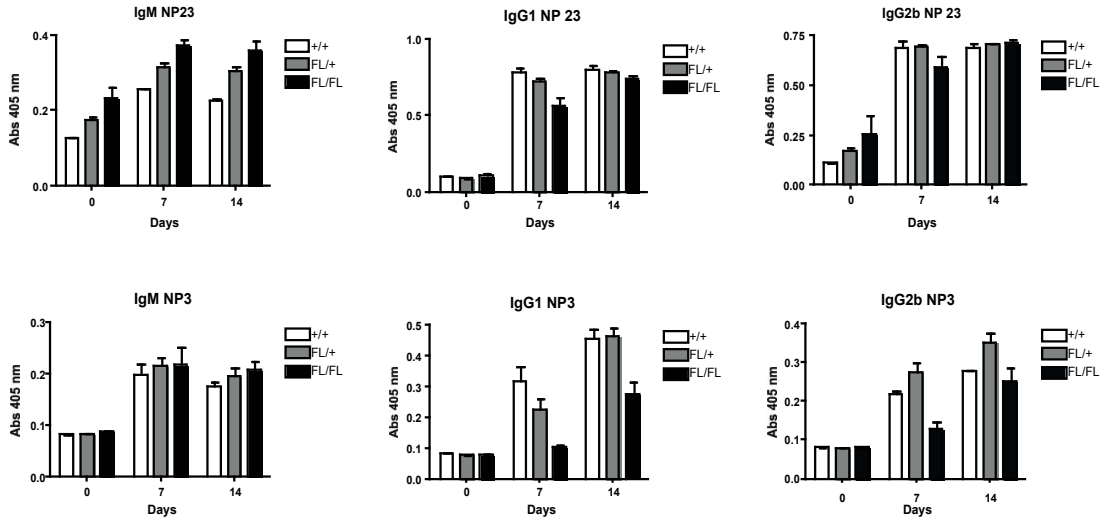

Figure 2.4 – Elisa plates coated with NP_3 (top) versus NP_{23}-BSA (bottom) were used to analyze the sera from mice of the indicated genotypes, immunized with NP-KLH, at the indicated days post-immunization. Means ± SEM of five mice per genotype.

Disrupted spleen architecture in *Tnfaip3*$^{fl/fl}$ CD19-Cre mice

We have examined the structure of spleens of *Tnfaip3*^{fl} CD19-Cre mice. 6 month-old mice have a marked loss of splenic architecture observable by H&E staining (Figure 2.5 top panel). We performed immunohistochemistry stains to look at cell populations in these spleens. In Figure 2.5, bottom panel, T cells are in green, B cells in blue, and Marginal Zone (MZ) in red. *Tnfaip3*$^{fl/fl}$ mice and to a lesser extent *Tnfaip3*$^{fl/+}$ show increased numbers of T cells outside the central T cell area normally observable in *Tnfaip3*$^{+/+}$ mice.

Some of the spleens with noticeable loss of splenic architecture had the appearance of pre-malignant lesions. Such observation could be a good pointer that is worth further aging these mice and pursue other signs of malignancy that

might not be grossly obvious. On another note, the observation of T cells moving to the B cell zone in *Tnfaip3*^{flfl} spleens reinforces the notion that B cells are influencing T cell behavior, driving its expansion and perhaps its positioning in follicles. Further analysis of this kind, in spleens and lymph nodes, might also reveal interesting. From the same perspective, analysis of *Tnfaip3*^{fl} CD19-Cre CD4-Cre double mice with A20 double in B an T cells might teach us more about these interactions.

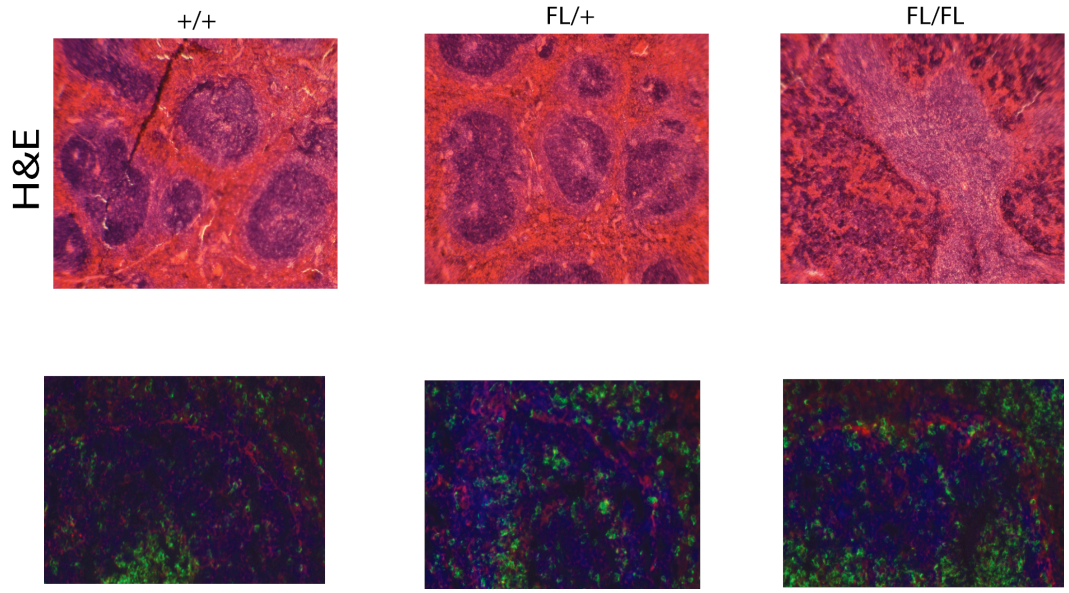

Figure 2.5 – Spleens from 6 month old mice of the indicated genotypes. Top panel H&E staining, 100X magnification. Bottom panel, immunohistochemistry in frozen sections, 500x magnification shows part of one follicle. Green Thy1.1, Blue B220, Red MadCam. Data are representative of 5 mice per genotype.

B – T cell interactions in *Tnfaip3*^{fl} CD19-Cre CD4-Cre double deleted mice

Tnfaip3$^{fl/fl}$ CD19-Cre B cells alone drive expansion of T cells, but no consistent T cell activation was ever observed in these mice. In light of the role of B –T cell interactions for lymphoid homeostasis and negative selection of B cells,

we have crossed *Tnfaip3*^{fl} CD19-Cre mice with CD4-Cre mice to obtain double B and T cell A20 deficient mice. T cell lineage specific deletion of A20 results in spontaneous T cell activation, but T cell numbers decrease, likely due to activation induced cell death (S. Oshima, unpublished data). We thus asked whether cooperation between hyperresponsive B and T cells could result in exacerbation of the two individual phenotypes.

Surprisingly, B cell percentages are markedly reduced in double Cre *Tnfaip3*$^{fl/fl}$ mice, at the expense of an increase in T cell percentages (Figure 2.6 A). As total cellularity is not lower but even increased in the absence of A20 (data not shown), the net result is moderately lower total B cell numbers and strikingly expanded T cell numbers. While no obvious differences were found in myeloid populations, GC B cell percentages are dramatically increased, similarly to what is observed in *Tnfaip3*^{fl} single CD19-Cre mice (Figure 2.6 A, B). So in spite of lower B cell numbers, there are still significantly higher GC B cell numbers. As in single CD19-Cre mice, there are increased total levels of Fas expressed in B cells in the absence of A20 (Figure 2.6 C). Additionally, we have measure ICOS-L (which binds ICOS on T cells) on the surface of B cells, and found similarly increased levels of ICOS-L in the absence of A20 (Figure 2.6 C).

$CD4^+$ T follicular cells (TFH) are known for interacting with B cells by moving to the B cell zone due to upregulation of CXCR5. These cells also express higher ICOS levels than non-follicular T cells. *Tnfaip3*$^{fl/fl}$ double Cre $CD4^+$ T cells did not express higher CXCR5, suggesting T cells might migrate to the B cell zone (Figure 2.5) independently of CXCR5 upregulation. Conversely, ICOS levels were elevated in the absence of A20, which along with higher ICOSL levels on B cells suggests crosstalk mediated by this receptor.

Finally, we measured T-cell activation in CD4 and CD8 T cells in spleens and lymph nodes of the double CD19 / CD-4 Cre mice. Remarkably, we found a

very significant increase in T cell activation, (2-4 fold) in both *Tnfaip3*$^{fl/fl}$ and *Tnfaip3*$^{fl/+}$ mice.

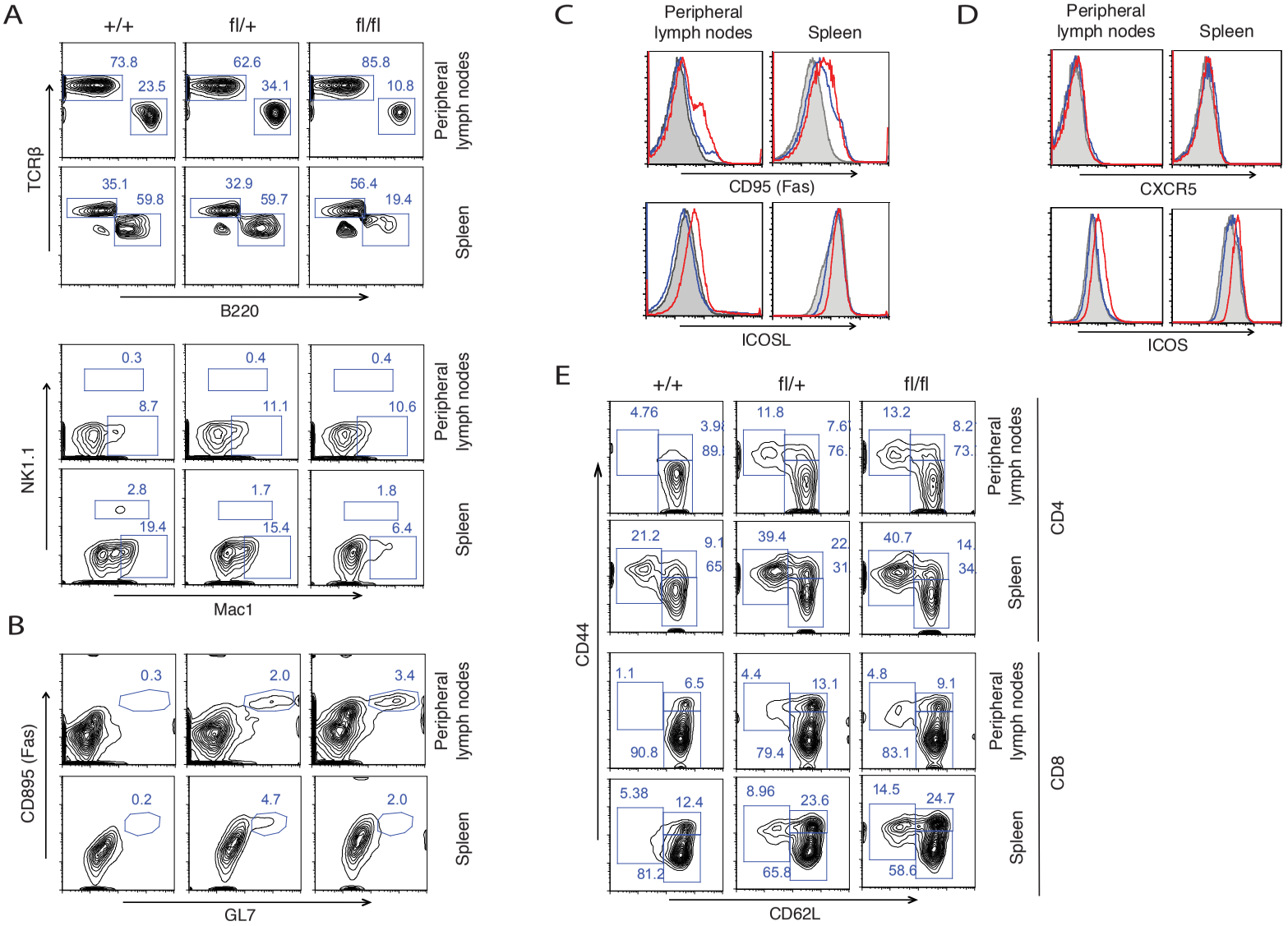
A
+/+
fl/+
fl/fl
73.8
23.5
62.6
34.1
85.8
10.8
Peripheral lymph nodes
TCRβ
35.1
59.8
32.9
59.7
56.4
19.4
Spleen
B220
0.3
8.7
0.4
11.1
0.4
10.6
Peripheral lymph nodes
NK1.1
2.8
19.4
1.7
15.4
1.8
6.4
Spleen
Mac1
B
0.3
2.0
3.4
Peripheral lymph nodes
CD895 (Fas)
0.2
4.7
2.0
Spleen
GL7
C
Peripheral lymph nodes
Spleen
CD95 (Fas)
ICOSL
D
Peripheral lymph nodes
Spleen
CXCR5
ICOS
E
+/+
fl/+
fl/fl
4.76
3.9
89.
11.8
7.6
76.
13.2
8.2
73.
Peripheral lymph nodes
CD4
21.2
9.1
65
39.4
22
31
40.7
14
34
Spleen
CD44
1.1
6.5
90.8
4.4
13.1
79.4
4.8
9.1
83.1
Peripheral lymph nodes
CD8
5.38
12.4
81.2
8.96
23.6
65.8
14.5
24.7
58.6
Spleen
CD62L

Figure 2.6 – Flow cytometry analysis of spleens and lymph nodes from *Tnfaip3*$^{+/+}$, $^{fl/+}$ and $^{fl/fl}$ CD19-Cre$^{+/-}$ CD4-Cre^{+} 4 month-old mice. A) Lineage stains for T-cells (TCRβ^{+}), B-cells (B220^{+}), Myeloid cells (Mac1^{+}) and NK cells (NK1.1^{+}). B) Stains for GC B cells (GL7^{Hi}, CD95^{Hi}) gated on B220^{+}. Histograms showing C) CD95 / Fas and ICOSL expression on B220+ gated cells and D) CXCR5 and ICOS expression in CD4^{+} gated cells. E) Stains for CD62L and CD44 in CD4^{+} or CD8^{+} gated cells as indicated. Activated T cells are CD44^{Hi} CD62L^{Lo} and naïve T cells are CD62L^{Hi} CD44^{Lo}. Data are representative of 3 mice per genotype

This data suggests that simultaneous A20 absent or hypomorphic expression in B an T cells may perhaps reveal additional details on T – B cell interactions, and how A20 contributes to regulate each arm of the adaptive immunity. In this context, it will be worth testing whether activated T cells are killing B cells through expression of Fas-L. Additionally, it is possible that activated A20 deficient B cells give survival signals to activated A20 deficient T cells that otherwise could die. Moreover, measuring autoantibodies and its deposits in these mice possibly will clarify whether this setting exacerbates autoimmunity, or on the contrary, myeloid cells or others, in the absence of A20, contribute to disease progression. Finally, investigating signs of malignancy in these mice can also clarify whether this could be a lymphoma prone setting.

In conclusion, although all these results are preliminary, they stress the many possibilities of carrying on exploring some of the novel functions of A20 first described in this thesis work.

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Provas de doutoramento em Ramo de Biologia e Especialidade de Imunologia Estrutural
Candidata: Licenciada Rita Margarida Morais Tavares
Data: 10 de Dezembro de 2010

Voto

Aprovado ☑

Recusado ☐

Justificação:

A candidata deve ver aprovada a concessão do título de Doutor, em vista da qualidade do seu trabalho e da forma inteligente como respondeu às questões que lhe foram colocadas. A candidata revelou extensos conhecimentos na sua área de trabalho e autonomia na execução de um projecto científico.
A candidata poderia no entanto ter prestado mais atenção à revisão da literatura, quer do ponto de vista histórico, quer em termos da profundidade do tratamento do tema.

Doutor Luís Paulo da Silva Nieto M. Rebelo

Doutor Paulo Júlio Miranda Vieira

Doutor Averil Ma

Doutor Sukalyan Chatterjee

Doutor Bruno M. de Carvalho e Silva Santos

Doutor Jorge Albino C. Araújo Carneiro