Title
Roles of Ubiquitin Sensor ABIN-1 In The Immune System

Author
Callahan, Joseph Aaron

Publication Date
2013

Peer reviewed|Thesis/dissertation

Roles of Ubiquitin Sensor ABIN-1 In The Immune System

by

Joseph Aaron Callahan

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements:

This thesis represents several years of intense laboratory research and investments from many of my exceptional family members, friends, and colleagues who have taught me to think analytically and pursue scientific and personal goals.

I would like to first thank my thesis committee members Dr. Scott Oakes, Dr. Art Weiss, and Dr. Averil Ma. They have provided valuable insights about how to shape my thesis, select important, answerable research questions, and navigate the peer-reviewed research publication process. Averil, in particular, had a major influence through providing critical resources and funding throughout my thesis research. Averil has supervised these studies and provided many suggestions for experiments and projects. I appreciate his enthusiasm for excellent results. He has taught me to evaluate strengths and limitations of experiments, paths of investigation, and helped me publish first-author work.

I am grateful to the Tetrad graduate program for important assistance in navigating UCSF. Danny Dam, Rachel Mozesson, Billy Luh, Sue Adams, and Julia Molla worked together to provide excellent administrative assistance. It was always a pleasure talking with them, including when choosing potential rotation labs and shaping oral qualifying exam and thesis committees.

Among my labmates, I want to thank in particular, Dr. Shigeru Oshima, Dr. Bao Duong, and Dr. Gianna Hammer. For years, they have provided scientific advice, technical assistance, and counsel that helped me progress in my labwork. Dr. Shigeru Oshima is an excellent scientist and taught me how to do my first *ex vivo* leukocyte flow cytometry experiments. When I initially joined the lab, he spent dozens of late nights and weekends working on his experiments and advising me about how to shape questions and experiments. He encouraged me when results did not yield the hypothesized answers. Dr. Bao Duong has been an exceptional colleague and friend. I am thankful to Bao for being so generous with technical help and suggestions about how to revise project directions. Bao has a rarely paralleled level of curiosity and enthusiasm for scientific discovery. I am thankful to Dr. Gianna Hammer, who has taught me how to do numerous flow cytometry experiments. Gianna is very skilled at advocating for her work and for others'; she advised me about how to navigate the peer review process. She volunteered multiple times to jointly generate shared reagents. Shigeru, Bao, and Gianna have been excellent role models and mentors. They have taught me how to achieve major goals in the highly competitive scientific research field.

Several of my friends and colleagues in the Ma lab have contributed steadily to making lab a friendly and mutually supportive learning environment. I greatly appreciate Rommel Advincula, who taught me how to wean mice, create breeding plans, and manage my mouse colony. Rommel is an expert! I also want to thank Julio Barrera, Mihir Pendse, Alexander Agelidis, and Tammy Woo for

excellent technical support and lab management. They each contributed to making the lab an optimistic, mutually encouraging environment where we all cooperated to find solutions. I am grateful to Dr. Barbara Malynn, who has volunteered to help me revise scientific writing and data for grant proposals, manuscripts, and thesis drafts. I appreciate that Barbara repeatedly makes time to discuss potential experiments. Additionally, I am grateful for my friend, Dr. Ling Shao. Ling is an impressive experimentalist and has shown generosity and kindness to me more times than I can count. Ling looks for needs and leaps to meet them. He creates new lab jobs and does them himself! Ling is always happy to help, to listen, and to counsel about experiments or what to present for lab meetings. I also want to thank Dr. Leesun Kim, who is a formidable scientist, a deliberate worker, and is a model of perseverance. Leesun makes time to talk in lab and outside of lab about project strategies and possible future directions. She even brought us gifts and food for our late nights of experiments. It has been a pleasure building friendships and working daily with these experts.

I also want to acknowledge and thank several of my friends, colleagues, and professors who have helped by teaching me methods, generating mice, building collaborations, and aiding my growth as an investigator. They have modeled how to be very focused and achieve in the UCSF environment while training and building up others. I'm honored and grateful to have worked with: Dr. Michio Onizawa, Dr. Mike Whang, Dr. Celia Bovijn, Dr. Erwan Mortier, Dr. Rita Tavares, Dr. Albert Lee, Dr. Timothy Lu, Dr. Emre Turer, Dr. Min Wang, and Dr. Osamu

Hitotsumatsu. I am very grateful for support of several UCSF faculty, who have taught me how to think critically, navigate peer review, and make research presentations. It has been a consistent pleasure working on presentations, projects, and/ or qualifying exams with: Dr. Anita Sil, Dr. Dean Sheppard, Dr. Dave Morgan, Dr. Jason Cyster, Dr. Erin O'Shea, Dr. Lily Jan, Dr. Keith Mostov, and Dr. Michael Rosenblum. These experimentalists, medical scientists, and professors have all made major contributions to my scientific development.

Finally and most importantly, I thank my family and especially my wife, Elisabeth. Elisabeth, my parents, Darryl and Edith, and my older brother, Stephen have helped influence how I pursue education and work. All of my successes are our successes. My parents and my brother were my earliest teachers. They looked out for me and helped me focus on achieving academic excellence. I'm thankful to Steve for helping foster a curiosity and desire to ask well-crafted questions since childhood. My dad and mom taught me how to work hard even when discouraged. They encouraged me when I did my first research experiments as an intern working on vaccines adjuvants at Chiron. They supported me through many years of commuting to take classes and volunteer in the UC Berkeley cancer biology research lab. They taught me how to recover and learn from setbacks and failures. They taught me the importance of building a strong inner faith in God that helps me persevere and be courteous even when encountering unconstructive criticism. I'm thankful to God for giving me this opportunity to contribute to science through my thesis research. I am exceedingly thankful to

my compassionate and considerate wife, Elisabeth. Elisabeth has been very patient while we have been pursuing the successful completion of this thesis. Her kindness and exceptional devotion to making our goals reality made it possible for us to move to be 15 minutes walk away from the lab so I could do experiments at any time of day or night. She has helped me stay highly motivated and continue making time to do experiments, seek out collaborators for input, and build strong relationships that energize me to do my highest quality work. Elisabeth has been my closest ally and confidant during these years finishing this thesis.

Part of the data and text of this thesis is a reprint of the material as it appears in: Callahan, J.A., Hammer, G.E., Agelides, A., Duong, B.H., Oshima, S., North, J., Advincula, R., Shifrin, N., Truong, H.A., Paw, J.*, et al.* (2013). Cutting Edge: ABIN-1 Protects against Psoriasis by Restricting MyD88 Signals in Dendritic Cells. J Immunol *191*, 535-539.

Oshima, S., Turer, E.E., Callahan, J.A., Chai, S., Advincula, R., Barrera, J., Shifrin, N., Lee, B., Benedict Yen, T.S., Woo, T.*, et al.* (2009). ABIN-1 is a ubiquitin sensor that restricts cell death and sustains embryonic development. Nature *457*, 906-909.

The coauthor, Professor Averil Ma, M.D., listed in these publications directed and supervised the research that forms the basis for the thesis.

Abstract

Heterologous expression of ABIN-1 (A20 Binding and Inhibitor of NFκB-1, gene name *Tnip1*), like A20, was suggested to restrict TNF-induced inflammation and cell death. ABIN-1's physiological function was unknown. To interrogate ABIN-1's roles *in vivo*, we generated ABIN-1-deficient mice. ABIN-1 was required for successful embryonic development. TNF-deficiency rescued this lethality, demonstrating that ABIN-1 restricts potentially lethal TNF-induced signals. ABIN-$1^{-/-}$ $TNF^{-/-}$ mice develop a striking immune phenotype, suggesting that ABIN-1 restricts TNF-independent signals. ABIN-$1^{-/-}$ $TNF^{-/-}$ RAG-$1^{-/-}$ mice have splenomegaly, which supports that ABIN-1 expression in innate cells preserves immune quiescence. ABIN-$1^{-/-}$ fetal liver chimera develop partially cell-intrinsic widespread immune activation, suggesting that ABIN-$1^{-/-}$ hematopoietic cells are sufficient for exaggerated immune activation.

Aberrant immune activation contributes to the pathogenesis of multiple autoimmune and inflammation-exacerbated diseases. The *Tnip1* gene is strongly associated with susceptibility to psoriasis in humans. Psoriasis is a chronic, inflammatory skin disease caused by a combination of environmental and genetic factors. We demonstrated that mice lacking ABIN-1 specifically in dendritic cells (DCs), ABIN-1^{Flox} CD11c-Cre mice, exhibited perturbed immune homeostasis. ABIN-1 deficient DCs displayed exaggerated NF-κB and MAP kinase signaling and produced more IL-23 than normal cells in response to TLR ligands.

Challenge of ABIN-1^{Flox} CD11c-Cre mice with topical TLR7 ligand lead to greater numbers of TH17 and TCRγ/δ^+ T cells and exacerbated development of psoriaform lesions. These phenotypes were reversed by DC-specific deletion of the TLR adaptor MyD88. These studies link ABIN-1 with IL-23 and IL-17, and provide cellular and molecular mechanisms by which ABIN-1 regulates susceptibility to psoriasis. They support that ubiquitin sensor ABIN-1 regulates cell survival and restricts proinflammatory signals in DC to protect against psoriasis.

Table of Contents

List of Figures

Chapter 1

Introduction

Introduction

Immune activation combats infection and promotes inflammation.

Tightly regulated immune activation is critical for maintenance of host immunity. Immune activation is the process by which adaptive and innate immune cells adopt a phenotype specialized in combating infection. Temporary immune activation is part of the normal immune response and inflammation. Inflammation is the condition in which a part of the body develops swelling, reddening, and potentially pain and heat (Beutler and Cerami, 1988, 1989; Larsen and Henson, 1983). Cells in inflamed areas secrete inflammatory mediators to increase permeability of blood vessels and permit greater numbers of activated white blood cells to enter tissues. Myeloid cells at the site of injury consume pathogenic invaders, damaged host cells, and debris (Larsen and Henson, 1983). Myeloid cells then also secrete cytokines to activate additional cell types and coordinate the immune response. Transient inflammation during infection or injury promotes host immunity, sequestering the site of damage and recruiting cells to repair it. In contrast, prolonged and/ or excessively robust inflammation damages host cells and organ systems. As damaging inflammation contributes to disease pathogenesis, cellular and molecular mechanisms that restrict or terminate inflammation are critical for preventing inflammatory disease.

Immune homeostasis is a relatively quiescent status.

To maintain immune homeostasis, the relatively stable equilibrium between many components of the immune system in absence of infection, multiple specialized

hematopoietic white blood cell types (e.g., T cells, B cells, and innate immune cells) communicate with each other to upregulate and down-regulate immune responses. T cells employ membrane receptors, called T cell antigen receptors (TCRs), to recognize unique antigen epitopes presented on the surface of mammalian cells. Prior to antigen exposure in the periphery, T cells are naïve (producing few proinflammatory cytokines). T cells undergo terminal differentiation to adopt different cell fates, such as, TH17, memory phenotype T cells, or regulatory T cells (Reis e Sousa, 2006; Zhu et al., 2010). Terminally differentiated T cells are specialized in promoting and regulating adaptive immune cell-mediated responses. For example, TH17 produce cytokines (e.g., IL-17) to enhance inflammation by recruiting neutrophils and promoting subsequent production of additional growth factors and proinflammatory mediators (Huang et al., 2012). Memory phenotype T cells express high levels of activation markers suggesting antigen experience or prior immune activation.

Dendritic cells (DC) are innate immune cells that direct immune responses of both adaptive immune cells and other innate immune cells. DC activate naive T cells more effectively than other antigen presenting cells (Steinman, 1991, 2012). DC also provide activating and survival signals to B cells and myeloid cells. DC can also produce anti-inflammatory molecules to restore immune quiescence following resolution of infection. Restoring and preserving immune homeostasis requires maintaining multiple codependent immune cell types. DC have a unique

role in processing signals and determining when to rapidly shift the immune system from homeostasis to an activated state.

Dendritic cell types.

DC are highly specialized innate immune cells that activate adaptive and innate immune cells through cytokines and cell surface markers (Iwasaki and Medzhitov, 2010). Most DC in circulation are either conventional DC (cDC) or plasmacytoid DC (pDC). The majority of DC are cDC. cDC express constitutively high levels of CD11c integrin and MHC class II antigen presentation molecules. pDC are a far less abundant subset especially effective in producing Type I Interferons (IFNs) in response to viral and bacterial products (Kang et al., 2008). pDC express lower levels of CD11c than cDC and often express CD45 RABC (B220), making them separable by flow cytometry. In cooperation with thymic epithelial cells, DC present antigenic peptides on cell-surface expressed MHC class II molecules to shape the developing T cell repertoire in the thymus. DC express costimulatory molecules CD40, CD80, and CD86 to directly transmit activating signals to T cells. Activation marker expression increases when DC encounter damage or danger signals. DC maturation is associated with an increase in DC antigen presentation function and costimulatory molecule expression (Reis e Sousa, 2006). TLR stimulated DC produce proinflammatory cytokines (e.g., IL-6, TNF, IL-23, IL-12p40, IL-12p70) (Joffre et al., 2009). Well-regulated DC production of cytokines helps fight infections and promote host immunity. Poorly regulated cytokine production can generate cachexia and

systemic inflammation. DCs specialize in supporting T cells through antigen presentation and producing proinflammatory cytokines that enhance immune activation.

TLR signaling protects against infection and initiates inflammation.

DC recognize multiple molecular patterns and convert those activating signals into coordinated cellular responses. Toll like receptors (TLRs) are non-catalytic, transmembrane receptors in DC and other cells that induce responses to danger and damage signals (Kawai and Akira, 2006; Medzhitov et al., 1997). TLR4 and TLR7 are expressed on the cell's plasma membrane. Other TLRs are in intracellular vesicle membranes (Blasius and Beutler, 2010). TLRs are a subtype of pattern-recognition receptor (PRR) (Takeda et al., 2003). As sentinels for infection, DC use TLRs to detect pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). PAMPs are molecules produced by microbes, but not generated by the host. PAMP detection relies on self vs. non-self discrimination to indicate infection. DAMPs are molecules that can initiate and perpetuate an inflammatory response in absence of infection. DAMPs are usually released when host cells are damaged or lysed and release intracellular contents (Matzinger, 2002; Rubartelli and Lotze, 2007). For example, TLR4 binds lipopolysaccharide, a major structural component of the outer membrane of Gram-negative bacteria. Mouse TLR7 (mTLR7) and human TLR8 (hTLR8) bind single stranded RNA, as found in RNA viruses. Additionally, mTLR7/ hTLR8 bind the small molecule imiquimod, which mimics a

nucleic acid. Excessive signaling through mTLR7/ hTLR8 can induce psoriasis and/ or contribute to autoimmunity. TLR-mediated recognition of PAMPs and PAMP-like shapes allows DC to respond to infection and provides tools for inducing proinflammatory immune signaling.

Upon TLR ligand binding, DC can generate a potent, multipronged response to trigger immune activation. TLR4 and/ or TLR7 engagement leads to recruitment of adaptor MyD88, IL-1 Receptor associated kinases (IRAKs), and E3 ligase TRAF6. This TLR-proximate signaling complex can modulate additional downstream proteins to propagate signals through the cytoplasm to activate gene transcription factors (e.g. NF-κB). TLR engagement induces DC activation, DC maturation, and often stimulates production of cytokines and/ or chemokines (Beutler, 2009; Reis e Sousa, 2006). Chemokines are cytokines that attract immune cells to migrate directionally through a gradient. Cytokines can direct cells to apoptose, differentiate into alternate cell types, or inhibit protein production, among other functional activities. Antibody-mediated cytokine neutralization remedies symptoms of inflammatory autoimmune diseases. Controlling and restricting the abundance of TLR-induced cytokines protects the host from immunopathology, i.e., damage from the immune response. As inadequately restrained TLR signaling can lead to disease, proper restriction of TLR signals is crucial.

Negative regulators constantly restrict TLR signaling.

Negative regulation of TLR signaling occurs constantly at the levels of dissociation of adaptor complexes, degradation of signal proteins, and transcriptional regulation. Microbes (including commensal bacteria) on epithelial surfaces constantly express PAMPs, but the immune system does not induce rampant inflammation despite this steady-state exposure. Several molecules dissociate signaling adaptor complexes involved in TLR signaling or prevent formation of TLR-induced proinflammatory complexes. IRAK-M inhibits recruitment of IRAK1 and IRAK4 to adaptor MyD88 to restrict downstream MAPK and NFkB (Kobayashi et al., 2002). Similarly, β-arrestin-1 and β-arrestin-2 compete with TRAF6 in steady state to inhibit TRAF6 proinflammatory signaling (Wang et al., 2006). Additionally, ST2 acts as a signaling inhibitor, as ST2 has a Toll/ IL-1 Receptor (TIR) domain that binds to MyD88, but ST2 is unable to propagate signals downstream of MyD88 (Brint et al., 2004). Genetic knockout studies have demonstrated that many of these molecules are crucial for restricting MyD88-dependent signaling, but not for MyD88-independent signaling. DC and/ or macrophages lacking IRAK-M, β-arrestin-1, β-arrestin-2, or ST2 show exaggerated proinflammatory cytokine production, and mice lacking any of these targeted genes exhibit increased susceptibility to endotoxin-induced inflammation (Brint et al., 2004; Kobayashi et al., 2002; Wang et al., 2006). Deficiency of these key TLR signaling restricting genes unleashes potent pro-inflammatory cytokines (e.g. TNF) that activate multiple cell types and pathways.

TNF signaling promotes activation and/ or apoptosis.

TNF signaling activates immune cells and can induce apoptosis. TNF binding to TNFR initiates formation of a TNFR-proximate signaling complex. TNFR-proximate signaling complexes can mediate either proinflammatory, survival-enhancing NF-κB activating signaling or caspase-mediated apoptotic cell death signals (Chen and Goeddel, 2002; Hsu et al., 1996). The presence, absence, and/ or ubiquitination status of signaling proteins — such as receptor interacting serine/ threonine protein kinase 1 (RIP1), FADD (Fas associated protein with death domain) — and Caspase 8, can determine which of these outcomes is favored. Ubiquitination, or the attachment of the small peptide ubiquitin, marks some of these proteins for degradation or facilitates interaction with downstream binding partners. Both TLR and TNF signaling can induce NF-κB proinflammatory signals.

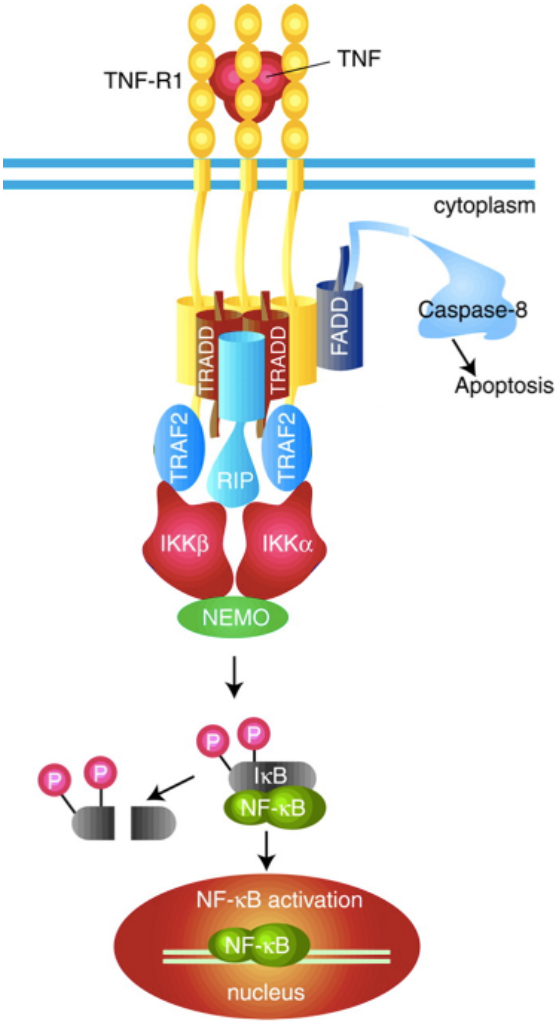


Figure 1. TNF-Induced Signaling.

TNF engagement of TNFR1 induces formation of signal-propagating complexes containing TRADD, TRAF2, and RIP. Recruitment of FADD and Caspase 8 can promote TNF-induced apoptosis. Alternately, interaction with IKK complex (containing IKKα, IKKβ, and NEMO) can promote IκBα degradation and NF-κB activation. Figure is adapted from (Chen and Goeddel, 2002).

NF-κB is a regulator of immunity, inflammation, and cell survival.

NF-κB signaling pathway is a regulator of immunity, inflammation, and cell survival. TNFR and TLR signaling rely on intracellular signaling cascades, such as NF-κB. NF-κB signaling relies critically on ubiquitination at multiple steps, including IKK complex-mediated phosphorylation, ubiquitination, and subsequent degradation of IkBa (inhibitor of NF-κB) (Ghosh and Karin, 2002; Karin and Ben-Neriah, 2000). Degradation of IκBα releases the NF-κB transcription factor. The prototypical NF-κB is composed of p65 (also known as RelA) and p50 subunits. NF-κB then enters the nucleus and binds to DNA sequences and promotes transcription of proinflammatory cytokines and survival-enhancing proteins. Selective ubiquitination regulates both TLR and TNF signaling axes.

Ubiquitination determines protein fates.

Ubiquitination comprises a series of posttranslational modifications that regulate protein function. Addition of the 76-amino acid globular ubiquitin peptide can direct proteins to multiple intracellular destinations. Ubiquitination can mediate signal transduction in proinflammatory signaling pathways (e.g. NF-kB) or target proteins for proteasomal degradation (Chen and Chen, 2013; Oudshoorn et al., 2012; Pickart and Fushman, 2004). Ubiquitination events mediate signaling in: DNA damage response, cell cycle, and endocytosis. Covalent ligation of a ubiquitin monomer to a target relies on a sequence of transient protein-protein interactions involving the cell's E1, E2, and E3 ubiquitination machinery. First, ubiquitin is bound to ubiquitin-activating enzyme (E1) in an ATP-consuming step.

Second, the activated ubiquitin is transferred from the E1 onto one of many potential ubiquitin-conjugating enzymes (E2). Third, in the presence of the ubiquitin ligase (E3), which binds both E2 and the target substrate protein, the ubiquitin is transferred from the E2 and onto the substrate protein. The interaction of E1, E2, and E3 allows for construction of chains with multiple different linkages.

Specific ubiquitin linkages designate the fates of substrate proteins (Kim et al., 2006; Li and Ye, 2008). Monomeric ubiquitination can direct endocytic removal of a receptor from the plasma membrane. Ligation of ubiquitin chains in which each ubiquitin is linked to the next through the lysine that is the 48^{th} amino acid residue (K48-linked) are typically targets substrates for proteasomal degradation. Ubiquitin chains linked through lysines other than K48 (e.g. K63) can target ubiquitinated proteins for non-degradative routes. K63 ubiquitination frequently occurs in receptor-mediated immune cell activation pathways, and propagates proinflammatory signals. As a tunable, reversible posttranslational modification, ubiquitination offers an effective way to rapidly activate, modulate, and terminate signaling. Regulation of ubiquitination requires interaction of multiple ubiquitin modifying enzymes.

Ubiquitin modifying enzymes modify signaling outcomes.

Ubiquitin modifying enzymes include E3 ubiquitin ligases and deubiquitinating enzymes (DUBs). Most ubiquitin ligases contain either a HECT (Homologous to

E6-AP Carboxyl Terminus) or RING (Really Interesting New Gene) finger domain that aids in transfer of ubiquitin to substrates (Pickart, 2001). RING-containing E3 ligases stabilize the interaction between substrate and E2 to mediate transfer of ubiquitin from E2 directly to substrate. HECT-containing E3 ligases form a thioester bond with the ubiquitin and then transfer ubiquitin to the substrate. In humans, ~600 E3 ligases exist. In addition, E3 ligases can interact with or act as ubiquitin-binding proteins/ ubiquitin sensors to aid in recruitment to substrates. Deubiquitinating enzymes modify ubiquitin-mediated signaling by facilitating removal of ubiquitin from substrates via their DUB domains (e.g. UBP, USP, JAMM, and OTU) (Wilkinson, 1997). Specific DUBs remove specific types of ubiquitin chain linkages to alter signaling pathways and immune homeostasis. For example, the DUB cylandromatosis protein (CYLD) restricts NF-κB and apoptotic signaling upstream of IKK activation. CYLD uses its UBP domain to cleave K63-linked, but not K48-linked, polyubiquitin chains (Chen and Sun, 2009). CYLD-deficient mice have multiple phenotypes consistent with increased IKK and NF-κB activity as well as loss of CYLD's tumor suppressing function (Sun, 2008). Ubiquitin ligases, ubiquitin sensors, and DUBs collaborate to shape the ubiquitin code and determine signaling outcomes.

A20 is a multifunctional editing enzyme that restricts proinflammatory signaling.

A20 is an unusual ubiquitin-modifying enzyme harboring both E3 ligase and DUB activities. A20 (gene name *Tnfaip3*) is a TNF-inducible gene (Krikos et al., 1992;

Opipari et al., 1990). Using its N-terminal ovarian tumor (OTU) cysteine protease domain A20 removes K63-polyubiquitin chains to restrict receptor-induced proinflammatory signaling (Wertz et al., 2004). A20 uses its fourth zinc finger domain to mark proteins for proteasomal degradation by ligating K48-polyubiquitin chains to them (Wertz et al., 2004). A20-deficiency severely compromises immune homeostasis. A20-deficient mice develop severe multi-organ inflammation and die prematurely. Challenge of $A20^{-/-}$ mice with TNF or LPS causes lethality within 2 hours of injection (Lee et al., 2000). These and other experiments suggest that A20 may restrict signaling downstream of TNFR and TLRs. A20 may selectively ubiquitinate or deubiquitinate multiple targets (e.g., TRAF6, RIP1) to restrict alternate signaling pathways. Additionally, A20 may interact with binding partners that cooperate with A20 to perform its functions.

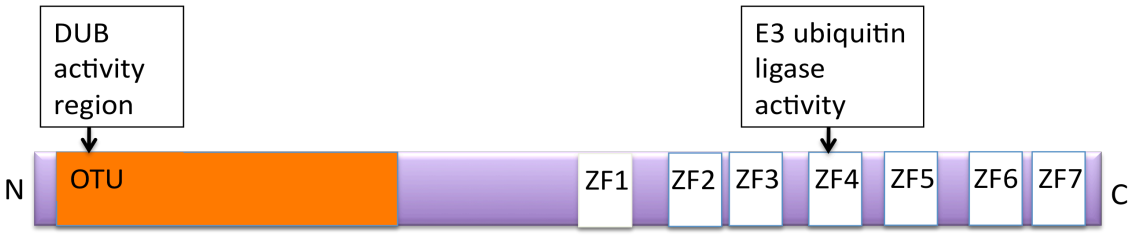


Figure 2. A20 schematic.

A20 contains an OTU domain that deubiquitinates K63-polyubiquitinated targets. A20 has seven zinc fingers. Zinc finger 4 can act as an E3 ligase by adding K48-polyubiquitin chains to targets.

ABIN-1 protein binds A20, restricts proinflammatory signals, and is linked to disease.

An unbiased search for A20 binding partners yielded ABIN-1. ABIN-1 (A20 Binding and Inhibitor of NF-κB-1, gene name *TNIP1*) may collaborate with A20 in restriction of NF-κB signaling. ABIN-1 has an NF-κB binding site in its promoter and is inducible by TNFα (Tian et al., 2005). Suggestive of negative feedback capacity, heterologously expressed ABIN-1 restricts proinflammatory signaling downstream of TNF, LPS, and IL-1 (Heyninck et al., 1999). ABIN-1 can bind to NEMO (NF-κB Essential Modulator, the regulatory subunit of the IKK complex). To test which interactions were critical for NF-κB restriction, ABIN-1 truncation mutants lacking the A20-binding region, NEMO-binding region, or both were made. Deletion of either the A20-binding region, or NEMO-binding region of ABIN-1 led to no reduction of ABIN-1's NFkB inhibitory effect (Mauro et al., 2006). Deletion of both disabled ABIN-1's inhibition of TNF-induced NF-κB signaling. ABIN-1 shares partially overlapping regulation and activity with A20. ABIN-1 might help recruit A20 to specific targets. Studies of A20 and other negative regulators of proinflammatory signaling demonstrated that tightly regulated proinflammatory signaling is critical to maintain host immunity and immune quiescence in absence of infection.

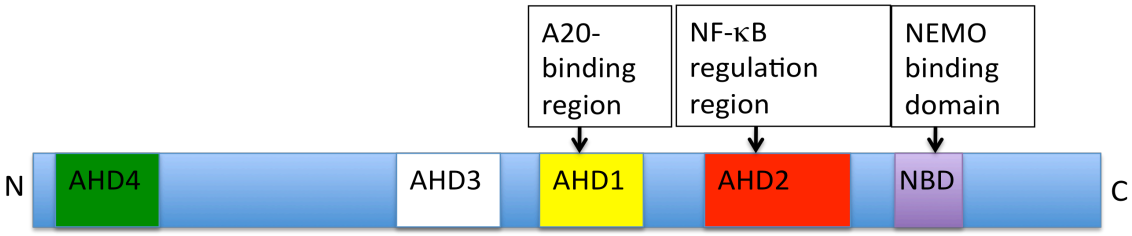


Figure 3. ABIN-1 schematic.

ABIN-1 has four ABIN homology domains (AHD) and a NEMO binding domain. AHD1 has been suggested to interact with A20, and AHD2 regulates NF-kB signaling intensity upon heterologous expression.

Both A20 and ABIN-1 are linked to multiple human diseases. Identification of factors that may support or direct A20 activity could help determine how critical negative regulators of inflammation function at a molecular level. GWAS linked *TNFAIP3* SNPs to multiple diseases, including: psoriasis, SSc (Systemic Sclerosis), SLE (Systemic lupus erythematosus), rheumatoid arthritis, type 1 diabetes, coeliac disease, Crohn's disease, coronary artery disease in type 2 diabetes (Adrianto et al., 2011; Boonyasrisawat et al., 2007; Cai et al., 2010; Consortium, 2007; Dieude et al., 2010; Elsby et al., 2010; Eyre et al., 2010; Fung et al., 2009; Graham et al., 2008; Hammer et al., 2011; Han et al., 2009; Hughes et al., 2010; Koumakis et al., 2012; Musone et al., 2008; Musone et al., 2011; Nair et al., 2009; Plenge et al., 2007; Shimane et al., 2010; Strange et al., 2010; Thomson et al., 2007; Trynka et al., 2009). Reflective of human pathologies, model organism reports demonstrated that reducing A20 expression levels in B cells or in gut commensal-sensing cells could unleash exaggerated proinflammatory signals that mediate SLE-like autoimmune disease and compromise immune homeostasis in the gut, respectively (Tavares et al., 2010; Turer et al., 2008). *TNIP1* SNPs also correlated with several of these A20-linked diseases. ABIN-1 is linked to: psoriasis, SSc, SLE, psoriatic arthritis, myasthenia gravis (Adrianto et al., 2012; Allanore et al., 2011; Bossini-Castillo et al., 2013; Bowes et al., 2011; Gateva et al., 2009; Gregersen et al., 2012; Han et al., 2009; Kawasaki et al., 2010; Nair et al., 2009; Sun et al., 2010). Interrogating ABIN-1's function in cells and in intact organisms may help demonstrate why ABIN-1 mutations predispose to disease.

This thesis explores the roles of ABIN-1 in the immune system. In chapter 2, we present studies, which demonstrate that ABIN-1 restricts TNF-induced apoptosis. We show that ABIN-1 expression in innate cells preserves immune homeostasis. These experiments support that ABIN-1 restricts TNF-dependent and TNF-independent signals. In chapter 3, we present studies, which demonstrate that ABIN-1 expression in DC is required to restrict MyD88-dependent TLR signals and protect against inflammatory autoimmune disease psoriasis.

Chapter 2

ABIN-1 Protects Against TNF-Induced Embryonic Lethality And Supports Immune Quiescence

Summary

Heterologous expression of ABIN-1 (A20 Binding and Inhibitor of NFκB-1), like A20, was suggested to restrict TNF-induced inflammation and cell death. ABIN-1's physiological function was unknown. To interrogate ABIN-1's roles *in vivo*, we generated ABIN-1-deficient mice. Endogenous ABIN-1 was required for successful TNF-competent mouse embryonic development, as ABIN-$1^{-/-}$ embryos died with fetal liver apoptosis, anemia and hypoplasia. TNF-deficiency rescued this lethality, demonstrating that ABIN-1 restricts potentially lethal TNF-induced signals during embryogenesis.

ABIN-$1^{-/-}$ $TNF^{-/-}$ mice had a striking immune phenotype. ABIN-$1^{-/-}$ $TNF^{-/-}$ RAG-$1^{-/-}$ mice showed that ABIN-1-deficient mature T cells and B cells were not required to generate splenomegaly. Using ABIN-$1^{-/-}$ fetal livers for stem cell transfer into chimera, we showed that ABIN-$1^{-/-}$ hematopoietic lineage-specific deletion of ABIN-1 was sufficient to generate this partially cell-intrinsic widespread immune activation. These studies provide new insights into how ubiquitination and ubiquitin sensing proteins regulate cellular and organism survival and immune quiescence.

Introduction

Human GWAS studies implicate regulators of innate and adaptive immune signaling in disease susceptibility. Negative regulators of proinflammatory signaling protect against destructive immune activation. Identification and investigation of functions of regulatory genes can help in design of targeted therapies. Disease susceptibility SNPs identified in both A20 and ABIN link these genes to a partially overlapping list of autoimmune diseases and diseases that are exacerbated by damaging inflammation (Ma and Malynn, 2012). These connections support that biochemical and cell-biological dissection of ABIN-1 physiological functions could elucidate mechanisms by which ABIN-1 protects against aberrant inflammation and disease.

In this study, we investigated the roles of ABIN-1 in maintaining cell survival and immune quiescence. We have demonstrated a critical role of ABIN-1 in restricting TNF-induced apoptosis. Additionally, we demonstrated that ABIN-1 expression in innate cells preserves immune homeostasis through regulating TNF-independent signals.

Results

ABIN-1 is required for successful embryonic development.

To generate ABIN-1 deficient mice, we generated a gene-targeting construct in which mouse *Tnip1* exons 12 - 15 were flanked with LoxP sites. We introduced this construct into C57BL/6 inbred mouse embryonic stem cells and bred individuals with germline transmission of the targeted allele to mice bearing E2A-Cre recombinase. Mice bearing the null allele lacking exon 12-15 were then bred back to B6 to make ABIN-$1^{+/-}$ mice.

We found that only 2-3% of live-born pups were ABIN-$1^{-/-}$. ABIN-$1^{-/-}$ embryos were found in Mendelian ratios at post coital days E12.5 through E18.5 (Table 1). The majority of ABIN-1-deficient mice die during embryonic development with lower than normal body mass, pale color, and anemia (Figure 1A-C). ABIN-$1^{-/-}$ embyos develop excessive cell death in the liver, as characterized by hypocellularity and immunohistochemical staining of cleaved Caspase 3 (Figure 1D). This indicates that ABIN-1 has a role in supporting the development of viable embryos. TNF induces RelA and many NF-κB survival-enhancing genes (Gallagher et al., 2003). RelA-deficient mice die of late stage TNF-dependent embryonic lethality (Doi et al., 1999). Adenoviral expression of ABIN-1 blocked TNF-induced apoptosis in hepatocytes (Wullaert et al., 2005). We hypothesized that ABIN-1 may also restrict TNF-induced apoptosis during embryogenesis. Accordingly, we tested whether TNF-deficiency could rescue ABIN-1-deficient

embryos. ABIN-1$^{-/-}$ embryonic lethality is largely TNF-dependent, as ABIN-1$^{-/-}$ TNF$^{-/-}$ embryos are born at near Mendelian ratios. This demonstration of TNF-dependent death *in vivo* argues strongly that endogenous ABIN-1 protects against TNF-induced cell death during embryonic development.

		ABIN-1 genotype				
TNF genotype	Age	+/+	+/-	-/-	-/- (%)	Total #
	E15.5 pc	12	32	17	(27.9%)	61
	E16.5 pc	6	29	11	(23.9%)	46
TNF +/+	E18.5 pc	11	16	11	(28.9%)	38
	newborn	12	28	1	(2.4%)	41
	3-4 week	37	92	4	(3.0%)	133
TNF +/-	live born	19	28	4	(7.8%)	51
TNF -/-	live born	27	61	22	(20%)	110

Table 1. ABIN-1 is required for regulating TNF signals in utero.

Numbers of embryos or live born pups of the indicated genotypes at various stages of development obtained from ABIN-$1^{+/-}$ heterozygote intercrosses are shown. Total embryos/pups genotyped at each stage of development for each TNF genotype is indicated at right (total). The percentages of embryos that were homozygous deficient (ABIN-$1^{-/-}$) among specific TNF genotypes and at specific stages of development are indicated in parentheses.

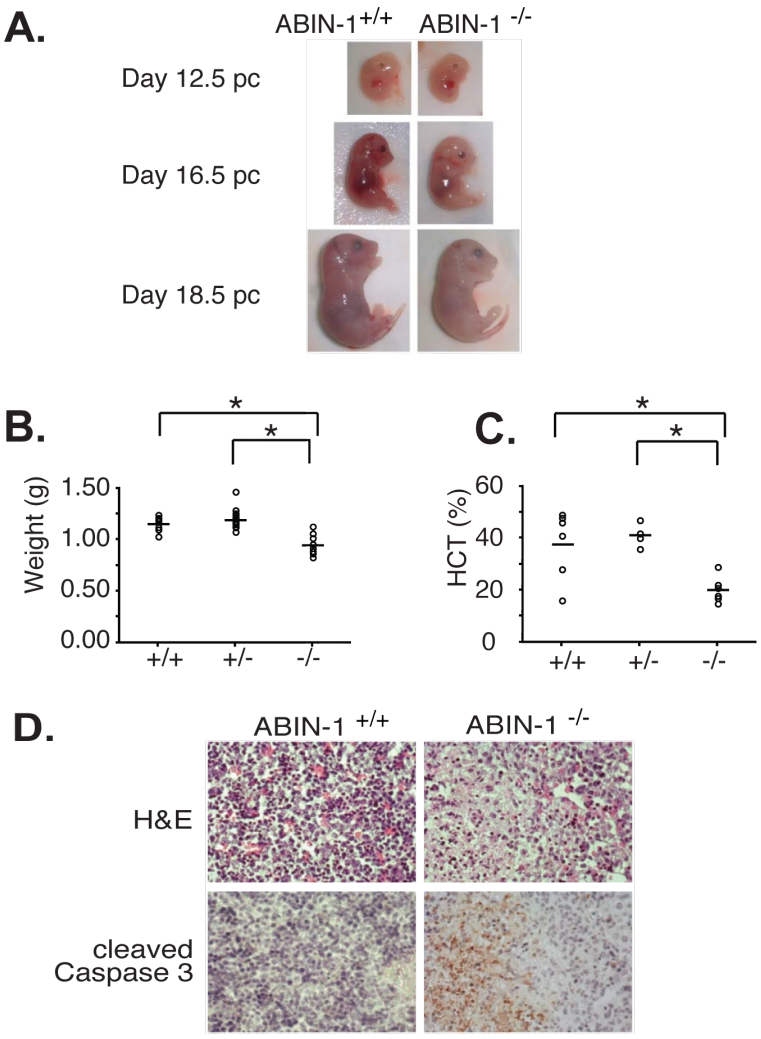


Figure 1. ABIN-1 is required for embryonic development.

(A) Gross appearance of ABIN-$1^{+/+}$ and ABIN-$1^{-/-}$ embryos. (B) Hypoplasia of E18.5 ABIN-$1^{-/-}$ embryos. Weights of individual embryos are shown as circles; horizontal bars indicate mean weights for each genotype. ABIN-$1^{-/-}$ embryos weigh less than ABIN-$1^{+/+}$ and ABIN-$1^{+/-}$ control embryos ($p < 0.01$ between ABIN-$1^{+/+}$ and ABIN-$1^{-/-}$ embryos; and $p < 0.01$ between ABIN-$1^{+/-}$ and ABIN-$1^{-/-}$ embryos, n=35). (C) Anemia of E18.5 ABIN$1^{-/-}$ embryos. ABIN-$1^{-/-}$ embryos are markedly anemic ($p < 0.02$ between ABIN-$1^{+/+}$ and ABIN-$1^{-/-}$ embryos; $p < 0.01$ between ABIN-$1^{+/-}$ and ABIN-$1^{-/-}$ embryos, n=18). (D) Hematoxilin and eosin

(H&E) histology (upper panels) and cleaved caspase 3 immunohistochemistry (lower panels) of sequential sections from ABIN-$1^{+/+}$ and ABIN-$1^{-/-}$ fetal livers. Apoptotic patches were found in 5 of 7 ABIN-$1^{-/-}$ fetal livers analyzed and in none of 10 ABIN-$1^{+/-}$ and ABIN-$1^{+/+}$ fetal livers analyzed ($p < 0.01$).

ABIN-1 mildly restricts TNF-induced NF-κB signaling *in vitro.*

TNF-induces proinflammatory signals as well as proapoptotic signals, and testing efficiency of endogenous ABIN-1 in restricting NF-κB signaling was a necessary follow-up experiment. Heterologously expressed ABIN-1 can restrict NF-|B (Mauro 2006). To test whether physiologically expressed levels of ABIN-1 are required to inhibit NF-κB signaling, we stimulated ABIN-$1^{-/-}$ MEFs with TNF and assayed NF-κB signaling by immunoblot. Comparing densitometry of p-IκBα: IκBα immunoblots, wildtype MEFs and ABIN-$1^{-/-}$ MEFs have very similar acute NF-κB responses. Accumulated NF-κB-driven proinflammatory cytokine IL-6 is produced in slightly higher quantity by ABIN-$1^{-/-}$ MEFs (compared to ABIN-$1^{+/+}$ controls). These experiments suggest that endogenous ABIN-1 modestly restricts TNF-induced NF-κB upon MEF stimulation. By comparison, $A20^{-/-}$ MEFs showed a clear exaggeration of NF-κB both in acute signaling and downstream IL-6 secretion (Figure 2A-B). This experiment suggested that A20 is far more efficient in restricting NF-κB compared to ABIN-1.

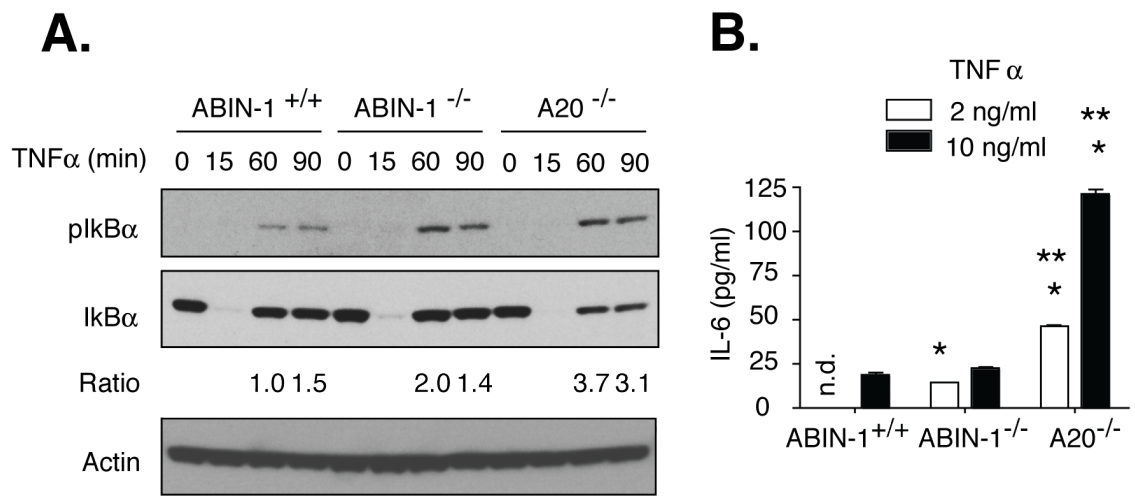


Figure 2. Endogenously expressed ABIN-1 mildly restricts TNF-induced NF-κB signals

(A) Immunoblot determination of IkBa and phospho-IkBa in wild-type, ABIN-$1^{-/-}$, or $A20^{-/-}$ MEFs. Actin is shown as loading control. Densitometry-determined ratios of phospho-IκBα: IκBα relative intensities are noted. Cells were incubated with 10 ng/mL TNF for indicated time intervals. (B) ELISA quantitation of IL-6 secreted by indicated genotype MEFs during overnight incubation with 2 or 10 ng/mL TNF. "n.d." indicates cytokine was not detected. Data are representative of 3 independent experiments.

ABIN-1 restricts TNF-induced apoptosis.

A20 restricts TNF-induced death, and ABIN-1$^{-/-}$ embryonic lethality is TNF-dependent. To test whether ABIN-1-deficient MEFs showed heightened TNF sensitivity, we stimulated them with apoptotic stimuli TNF and cycloheximide (CHX). ABIN-1-deficient MEFs were highly susceptible to TNF-induced death (Figure 3A). Consistent with programmed cell death, ABIN-1$^{-/-}$ MEFs showed abnormally high Caspase 3 cleavage and Bid cleavage (Figure 3B).

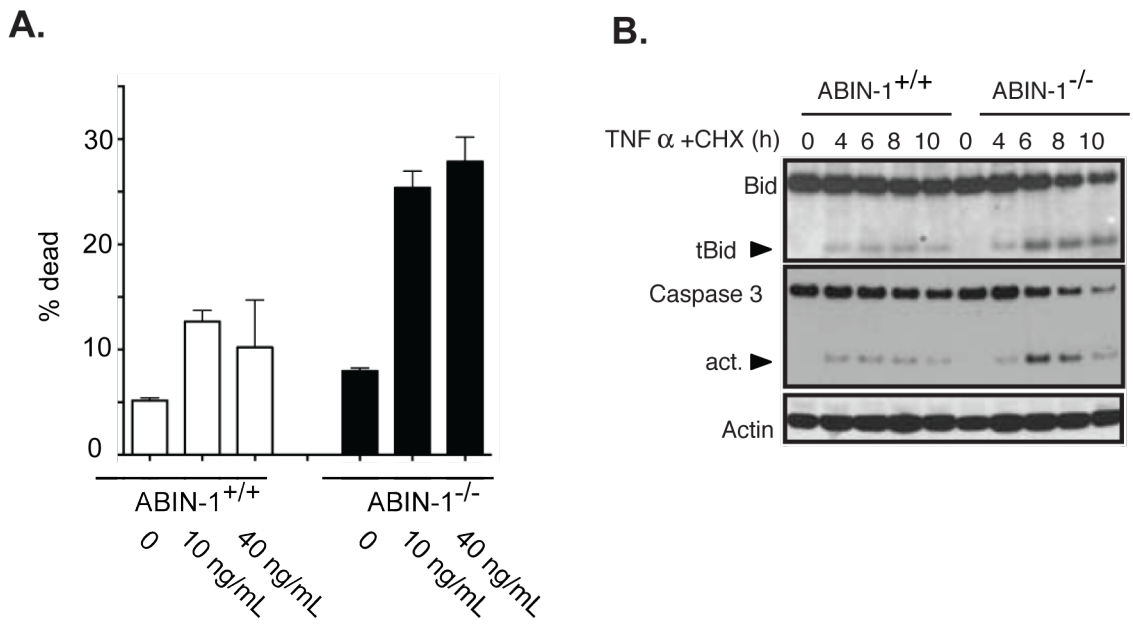


Figure 3. ABIN-1 restricts TNF-induced cell death *in vitro.*

(A) TNF-induced death measured by flow cytometry. ABIN-$1^{+/+}$ or ABIN-$1^{-/-}$ MEFs were treated with TNF and cycloheximide for 4 hours and stained with annexin. (B) TNF-induced cleavage of Bid and Caspase 3. Data are representative of 3 - 10 independent experiments.

ABIN-1 restricts TNF-independent proinflammatory signals in the immune system.

The survival of TNF-deficient ABIN-1$^{-/-}$ mice allowed us to analyze the effects of ABIN deficiency on the immune system. We hypothesized that if ABIN-1 had critical roles in addition to restricting TNF-induced death and TNF-induced NF-|B, ABIN-1$^{-/-}$ TNF$^{-/-}$ mice would develop an immune phenotype. We used flow cytometric analysis to detect T cell, B cell, and myeloid cells in the spleens and peripheral lymph nodes. ABIN-1$^{-/-}$ TNF$^{-/-}$ mice indeed developed a striking phenotype consisting of splenomegaly, accumulation of exaggerated percentages of memory phenotype T cells and granulocytes (Figure 4A-C). Total numbers of B cells remained roughly constant. Splenomegaly was a consistent feature of the ABIN-1$^{-/-}$ TNF$^{-/-}$ mice, however the size of the enlarged spleens varied dramatically (2-10 fold). This large range made total numbers of cells recovered from each ABIN-1$^{-/-}$ TNF$^{-/-}$ individual vary widely. A trend of increased memory phenotype T cells was evident (Figure 4B). Global deletion of ABIN-1 and TNF produced mice with multiple immune system aberrations. These findings support the conclusion that ABIN-1 is critical for restricting TNF-independent signals and maintaining immune quiescence in the adult mouse.

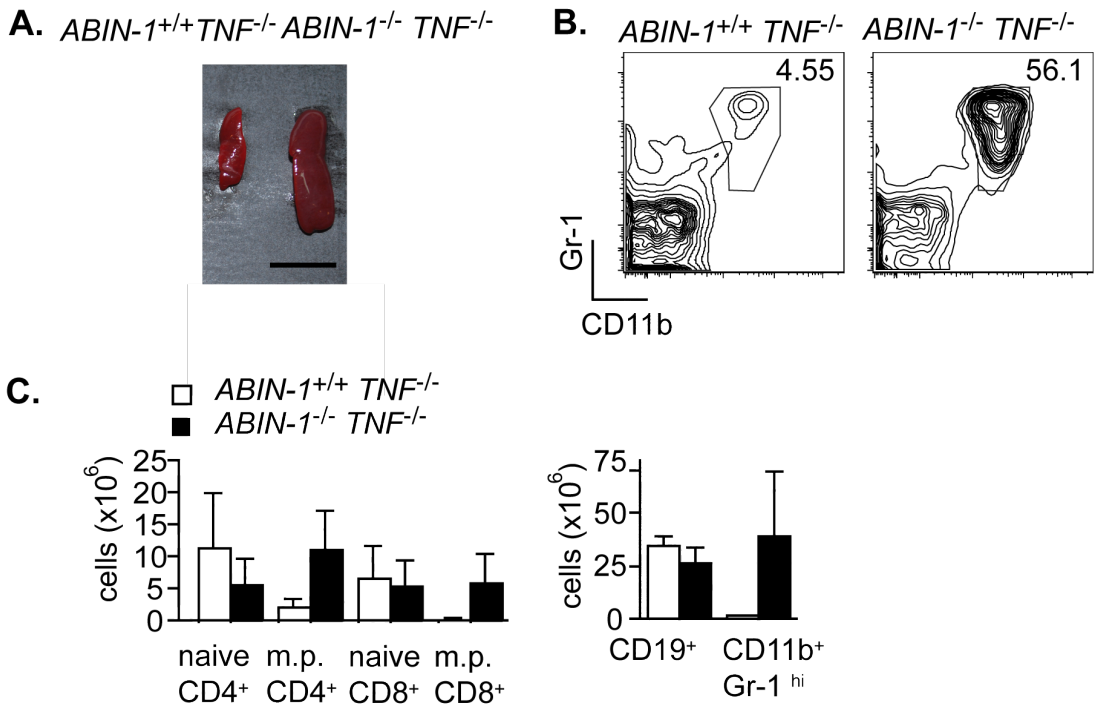


Figure 4. ABIN-1 restricts TNF-independent proinflammatory signals in the immune system.

(A) Photograph of ABIN-1$^{-/-}$ TNF$^{-/-}$ splenomegaly and ABIN-1$^{+/+}$ TNF$^{-/-}$ control spleen. Scale bar is 1 cm. (B) Total numbers of naïve T cells, memory phenotype (m.p. indicates CD44 ^{hi} CD62L ^{low}) T cells, CD19^{+} B cells, and CD11B^{+} Gr-1 ^{hi} (activated macrophages and granulocytes) myeloid cells in spleen of ABIN-1$^{-/-}$ TNF$^{-/-}$ mice compared with ABIN-1$^{+/+}$ TNF$^{-/-}$. (C) Flow cytometry displays increased frequency of CD11B^{+} Gr-1 ^{hi} cells in ABIN-1$^{-/-}$ TNF$^{-/-}$ spleen. Data for (A), (B), and (C) are representative of at least 5 mouse pairs age 2-3 months.

ABIN-1 regulates innate immune homeostasis.

To interrogate whether ABIN-1-deficient T cells and B cells were required for this loss of immune quiescence, we bred the ABIN-1$^{+/-}$ TNF$^{-/-}$ mice to mice deficient in RAG-1, therefore lacking mature T cells and B cells. ABIN-1$^{-/-}$ TNF$^{-/-}$ RAG-1$^{-/-}$ mice nevertheless developed severe splenomegaly within 4 months of birth (Figure 5A). This demonstrated that ABIN-1 restricts proinflammatory signals in an innate cell type.

ABIN-1 expression in hematopoietic cells regulates immune quiescence.

Recognizing that global deletion of TNF can disrupt signaling within multiple cell types and that ABIN-deletion in non-hematopoietic cell types could alter immune system function, we wanted to ask whether deletion of ABIN-1 alone strictly in hematopoietic cells was sufficient to reproduce splenomegaly and immune cell expansions. Additionally, we wanted to test whether TNF-deficiency was required to generate this phenotype. To answer these questions, we generated fetal liver chimera (FLC). By harvesting ABIN-1$^{-/-}$ and ABIN-1$^{+/+}$ fetal livers and injecting them into lethally irradiated, congenically marked adult recipients, we generated chimeric mice with adult TNF-competent immune systems. ABIN-1$^{-/-}$ FLC developed splenomegaly and consistently elevated numbers of memory phenotype T cells and granulocytes (Figure 5B-D). This approach simultaneously allowed us to exclude the possibility that ABIN-1 plays a vital role strictly during development of secondary lymphoid organ architecture or in non-hematopoietic cells critical for educating T cells and B cells. These experiments confirmed that

ABIN-1 expression in hematopoietic cells is required to maintain normal immune quiescence in the adult.

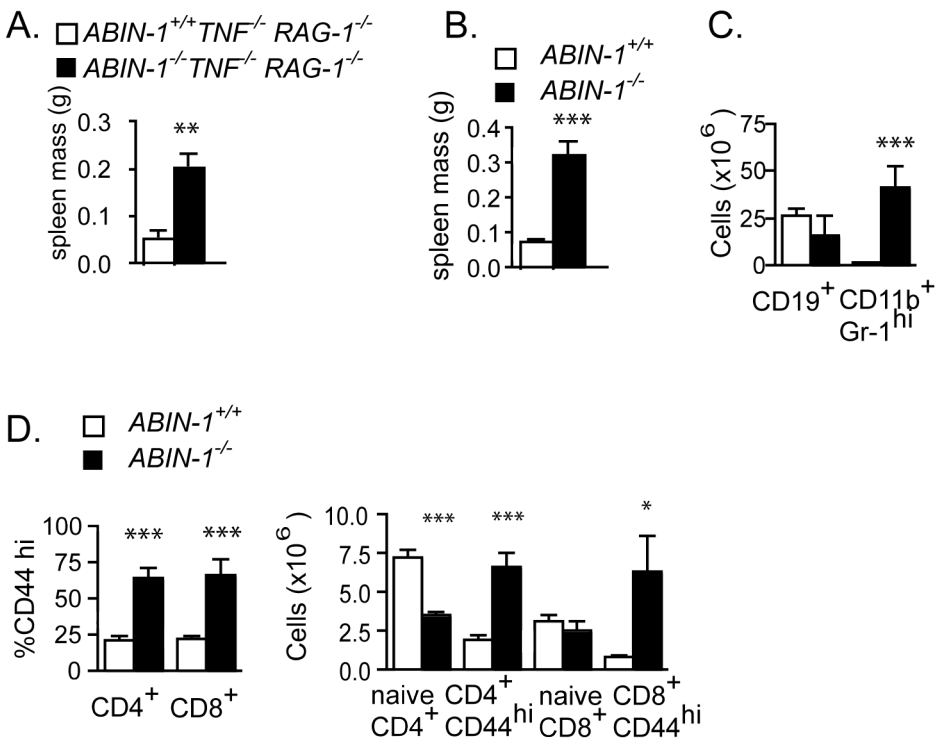


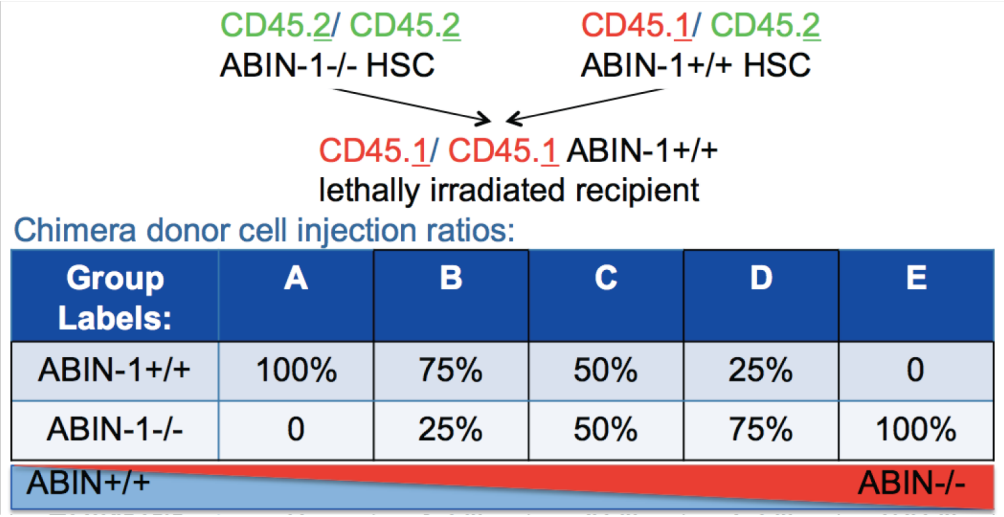
Figure 5. ABIN-1 expression in hematopoietic, myeloid cells preserves immune quiescence.

(A) Spleen masses in ABIN-1$^{-/-}$ TNF$^{-/-}$ RAG-1$^{-/-}$ and ABIN-1$^{+/+}$ TNF$^{-/-}$ RAG-1$^{-/-}$ mice. Data are representative of 3 mice age 2-4 months. (B-D) Spleen masses, cell type percentages and/ or numbers from chimeric mice generated by transfer of ABIN-1$^{-/-}$ or ABIN-1$^{+/+}$ fetal liver HSCs into lethally irradiated C57BL/6J mice. Two to 4 months after reconstitution, spleens were analyzed by flow cytometry to calculate cell numbers. Data are representative of 3 independent experiments with at least 2 pairs of mice per experiment.

Cell extrinsic and cell intrinsic roles of ABIN-1 In hematopoietic cells.

Myeloid cells regulate T and myeloid cell activation through cell extrinsic and intrinsic mechanisms; it is possible that ABIN-1 restricts activated T cell and myeloid accumulation cell-intrinsically. To test whether ABIN-1-deficient T and myeloid cells are abnormally susceptible to activating or survival signals *in vivo*, we generated FLC that had received both ABIN-$1^{+/+}$ and ABIN-$1^{-/-}$ fetal liver HSC coinjected at titrated ratios (Figure 6A). In ABIN-deficient FLC, the percentage of granulocytes in spleen increases, and an abnormally high percentage of CD4 T cells are memory phenotype $CD44^{hi}$. In the mice that received titrated progressively higher fractions of ABIN-$1^{-/-}$ donor HSC, the percentage of $CD11b^{+}$ $Gr1^{hi}$ cells increased (as did percentage of $CD11b^{+}$ myeloid cells). Granulocytes from chimera with mixed ABIN-$1^{+/+}$ and ABIN-$1^{-/-}$ donor HSC were comprised of both donors' cells. Since these granulocytes show no preferential origination from ABIN-$1^{-/-}$ cells, this expansion is not intrinsic to ABIN-$1^{-/-}$ granulocytes (Figure 6B-C).

A.

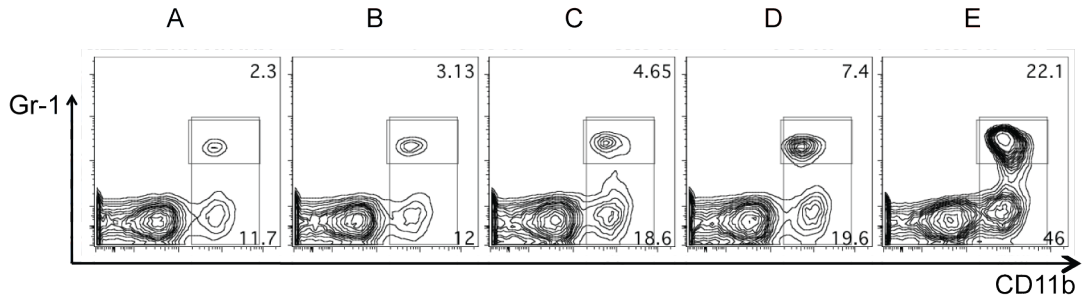


Chimera donor cell injection ratios:

Group Labels:	A	B	C	D	E
ABIN-1+/+	100%	75%	50%	25%	0
ABIN-1-/-	0	25%	50%	75%	100%

ABIN+/+ ABIN-/-

B. Chimera ratio group:



C. Chimera ratio group:

Percentage of $CD11b^{+}$ $Gr\text{-}1^{+}$ myeloid cells derived from each donor:

	A	B	C	D	E
$ABIN\text{-}1^{+/+}$	100%	89%	65%	41%	-
$ABIN\text{-}1^{-/-}$	-	6%	32%	50%	100%

Figure 6. Accumulation of expanded granulocyte populations is not intrinsic to $ABIN\text{-}1^{-/-}$ cells.

(A) Experiment layout and congenic marker designation of cells from each donor.

(B) Flow cytometry plots of $CD11b^{+}$ $Gr\text{-}1^{hi}$ and $CD11b^{+}$ myeloid cells in each ratio

group. Numbers at upper right of flow plots corresponds to percentage of total cells that are $CD11b^{+}$ $Gr1^{hi}$. Numbers at lower right corresponds to percentage of total cells that are $CD11b^{+}$. (C) Percentage of $CD11b^{+}$ $Gr\text{-}1^{hi}$ splenocytes from each donor. Results are representative of 2-3 independent experiments.

Similarly, we determined whether the $CD4^{+}$ $CD44^{hi}$ T cell accumulation was cell-intrinsic. We determined what fraction of either ABIN-$1^{+/+}$ or ABIN-$1^{-/-}$ CD4 T cells within the same FLC becomes $CD44^{hi}$. ABIN-$1^{-/-}$ CD4 T cells showed higher percentages of $CD44^{hi}$ cells accumulating (contrasted against ABIN-$1^{+/+}$ CD4 T cells in the same mouse). As the fraction of coinjected ABIN-$1^{-/-}$ HSC increased, the percentage of $CD44^{hi}$ cells within the ABIN-$1^{+/+}$ CD4 T cells increased (Figure 7A-C). These results suggest that ABIN-$1^{-/-}$ are more prone than ABIN-$1^{+/+}$ CD4 T cells to accumulate memory phenotype cells (when coinjected into the same mouse). In addition, increasing the ratio of ABIN-$1^{-/-}$ to ABIN-$1^{+/+}$ fetal liver cells injected affected the ABIN-$1^{+/+}$ CD4 T cells in a manner reflecting compromised immune quiescence within the ABIN-$1^{+/+}$ T cell compartment. So, ABIN-1 restricts memory phenotype T cell accumulation through both cell-intrinsic and extrinsic roles.

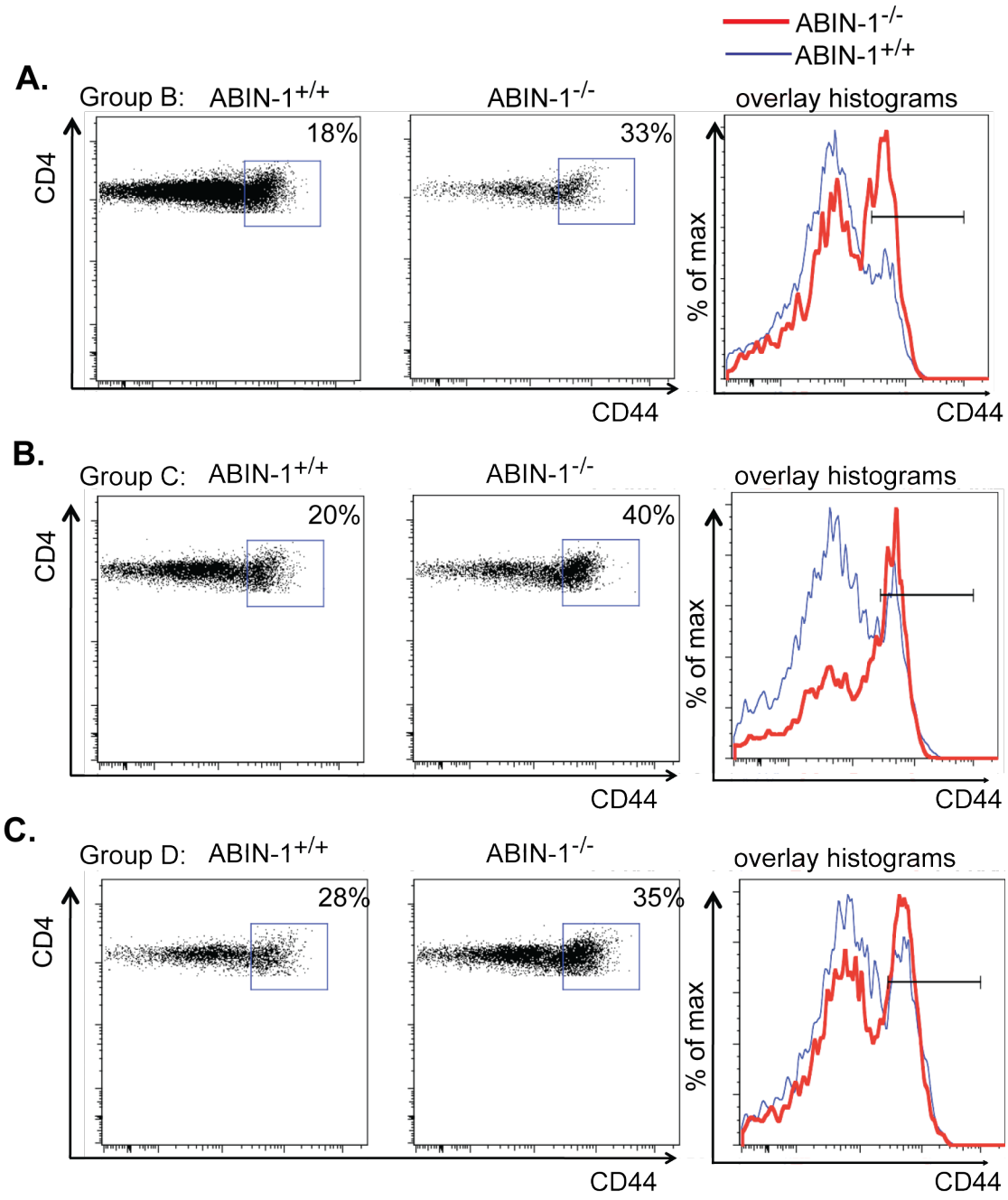


Figure 7. ABIN-1 restricts accumulation of aberrant CD44 ^{hi} T cells through partially cell-intrinsic and extrinsic mechanisms.

(A) CD44 expression on CD4 T cells from mixed chimera in ratio group B, equally mixed ratio group C (in part B), and ratio group D (in part C). Percentages shown

on dot plots indicate the percentage of each donor's $CD4^+$ T cells found within the $CD44^{hi}$ gate drawn. Brackets in histograms indicate $CD44^{hi}$ cells. Representative plots are shown. Data are representative of 3 independent experiments.

Discussion

Our studies presented the first ABIN-1-deficient mouse and established that ABIN-1 inhibits TNF-induced apoptosis *in vivo* and *in vitro*. My colleague, Dr. Shigeru Oshima, demonstrated a novel role for ABIN-1 in using it's newly discovered ubiquitin-binding function to disrupt FADD-Caspase 8-containing complexes. ABIN-1 could still protect $A20^{-/-}$ cells against apoptosis, suggesting that ABIN-1 can inhibit cell death in the absence of A20 (Oshima et al., 2009).

Demonstration of an in vivo requirement for ABIN-1 during embryogenesis emphasizes the physiological importance of ABIN-1. We characterized how and on what timescale ABIN-$1^{-/-}$ cells die. Our *ex vivo* assays included sectioning through entire embryos during late stage embryonic development to screen for affected organs. The embryonic liver was the only tissue that showed substantive damage. We had hypothesized that the liver may be an area of demonstrable death based on report that $RelA^{-/-}$ fetal livers show exaggerated apoptosis. Considering that TNF is produced in many tissues during embryonic development, it is uncertain why the liver shows the clearest increased death. Others have reported that perhaps Kuppfer cells in the developing liver produce an exceptionally high local concentration of TNF (Doi et al., 1999). Our finding that ABIN-$1^{-/-}$ fetal livers undergo degeneration underscores that ABIN-1 is critical for developing organisms.

TNF is one of many signals *in utero*, and it remains unknown whether ABIN-1 protects against additional TNF-independent potentially cytotoxic factors *in utero* and after birth. We demonstrated that ABIN-1$^{-/-}$ TNF$^{-/-}$ mice are born at closer to Mendelian ratios, however ABIN-1$^{-/-}$ TNF$^{-/-}$ are still slightly lower than Mendelian ratios predict. We demonstrated that adult ABIN-1$^{-/-}$ TNF$^{-/-}$ mice are severely inflamed by early adulthood. In data not shown, we looked for the earliest primary defect in these mice. As young as 4 weeks old, both aberrant memory phenotype T cells and granulocytes accumulated. It is possible that even at birth, these phenotypic elements are already developing. Even juvenile ABIN-1$^{-/-}$ TNF$^{-/-}$ mice with an immature immune system show signs of inflammation. This suggests that fully mature B cell populations are not required for these T cell and myeloid cell expansions. It was still formally possible that ABIN-deficient non-hematopoietic cells contributed to producing an environment that lead to aberrant immune cell activation/ accumulation (e.g., through cytokine production or aberrant organization/ development of secondary lymphoid organs).

Ongoing studies have and will investigate which TNF-independent signals ABIN-1 restricts to maintain immune quiescence. ABIN-1 could restrict TLR/ IL-1R-induced signals. Commensal microflora are constantly in contact with skin/ epithelial barriers. Ubiquitin-editing enzymes (e.g., A20) restrict proinflammatory signals to maintain immune quiescence (Turer et al., 2008). A20 strongly regulates TLR-induced signals, so we hypothesized that ABIN-1 may also. In data not shown, tests of LPS-induced acute signaling responses and cytokine

production in ABIN-1$^{-/-}$ TNF$^{-/-}$ and ABIN-1$^{+/+}$ TNF$^{-/-}$ bone marrow derived macrophages (BMDM) and bone marrow derived dendritic cells (BMDC) repeatedly showed mild differences. Analyses used immunoblot to analyze signals in NF-κB, ERK, JNK, and P38 pathways. Cytokines detected in ELISA or multiplex immunoassay included IL-6, IL-12, and TNF. It remains possible that TNF-deficiency alters the development of ABIN-1$^{-/-}$ TNF$^{-/-}$ myeloid cells or dampens their *in vitro* signaling responses. Future testing may include generation of TNF-competent ABIN-1$^{-/-}$ myeloid cells or may utilize additional readouts (e.g. QPCR).

It is also possible that ABIN-1 restricts the production of or intensity of response to a TNF-independent growth factor/ cytokine that expands myeloid cells and granulocytes *in vivo*. We tested ABIN-1$^{-/-}$ TNF$^{-/-}$ and ABIN-1$^{+/+}$ TNF$^{-/-}$ serum for granulocyte and macrophage colony stimulating factor (GM-CSF) concentration, but found no ABIN-dependent difference. Additionally, we measured serum levels of IFN-γ and IL-6, hypothesizing those may activate macrophages *in vivo* and lead to inflammation or myeloid expansion. We detected no significant exaggeration of these cytokines in ABIN-1$^{-/-}$ TNF$^{-/-}$ serum. Alternately, it remained possible that ABIN-deficient cells hyperrespond to IFN-γ or GMCSF. Stimulation of BMDM and/ or BMDC and use of immunoblot to detect phosphorylation of STATs or MAPK proteins did not support that hypothesis. These tests did not support a role for ABIN-1 in restricting production of or response to these growth factors and cytokines (data not shown). Future experiments may utilize a

different cell type or different analysis method to achieve greater sensitivity (e.g. QPCR).

Aberrant memory phenotype T cell accumulation remains a consistent, striking phenotype of ABIN-1-deficient mice, and the cause of this accumulation is unknown. We used mixed donor HSC chimera to test whether strictly ABIN-$1^{-/-}$ T cells assume this aberrant $CD44^{hi}$ memory phenotype in a mixed genotype environment. ABIN-$1^{-/-}$ T cells are overrepresented among $CD44^{hi}$ T cells, but both genotypes' $CD44^{hi}$ percentages increase in mice receiving substantial fractions of ABIN-$1^{-/-}$ HSC. This suggests partially cell-intrinsic and partially cell-extrinsic roles for ABIN-1 in maintaining immune quiescence in T cell populations. Determining the identity of cell-extrinsic factors that may promote the T cell phenotype in ABIN-deficient mice will be an area of future study. Serum screening might benefit from analyzing serum levels of cytokines IL-2 and IL-7 across several ages. IL-2 promotes T cell growth, proliferation, and differentiation. DC-secreted IL-7 could also support T cell survival. Overrepresentation of ABIN-$1^{-/-}$ T cells among the $CD44^{hi}$ fraction suggests that ABIN-$1^{-/-}$ T cells may also be hyper-responsive to an *in vivo* signal (e.g., costimulatory molecule expression on myeloid cells, engagement of TCR by APC-presented MHC-peptide complexes). Immunoblot interrogation of acute signaling intensity following ABIN-$1^{-/-}$ T cells' or thymocytes' encounter of activating/ pro-survival cytokines may help identify a role for ABIN-1 in maintaining immune quiescence through modulating T cells. Finally, generation

of T cell lineage-specific ABIN-1-deficient mice could be a valuable step toward determining whether any role of ABIN-1 in T cells protects immune quiescence.

One of our key goals was to identify how ABIN-1 regulates immune homeostasis. Analyzing ABIN-1$^{-/-}$ TNF$^{-/-}$ RAG$^{-/-}$ mice demonstrated that splenomegaly persists in absence of mature T and B cells. This strongly suggests that ABIN-1 preserves immune quiescence by restricting proinflammatory signals in innate immune cells. This may be relevant to human disease, as SNPs link TNIP1 to multiple human diseases (Ma and Malynn, 2012). It will be important to interrogate myeloid cell type-specific roles of ABIN-1 in maintaining normal immune system function and in protecting against disease.

Methods and Materials.

Mice.

Generation of ABIN-1 (*tnip1*) deficient mice.

Recombineering was used to generate a gene targeting construct from a bacterial artificial chromosome (BAC) containing the *tnip1* gene (C57Bl/6J inbred strain). C57Bl/6 inbred PRX-B6T ES cells were transfected with this construct, and successfully targeted ES cells were identified by Southern blot analysis. Blastocyst injections of targeted ES cells were performed by the UCSF Transgenic Core. Mice bearing this targeted allele in the germline were interbred with E2a-Cre transgenic mice to delete intervening sequences including exons 12-15 and generate the null allele. These mice were then bred to B6 mice to eliminate the E2a-Cre transgene and generate ABIN-$1^{+/-}$ mice. Chimera were generated by reconstitution of lethally irradiated, congenic CD45.1^{+} C57BL/6 mice with wild type and/ or mutant donor (C57BL/6, CD45.2^{+}) bone marrow or fetal liver cells. Recipients were at least 8 weeks old, and were lethally irradiated prior to injection of new HSC through the retroorbital sinus. Unless otherwise noted, mice were analyzed between 6 and 30 weeks of age. All mouse experiments were approved by the institutional care and use committee.

Cell preparation and analyses.

Single-cell suspensions were prepared from lymph nodes or spleens. Spleens and peripheral lymph nodes were mashed on glass slides or mashed in nylon mesh. Cell counts were determined using hemacytometer or NucleoCounter.

Cells were incubated on ice with the designated fluorophore-conjugated antibodies. All antibodies were purchased from BD Biosciences. Cells were analyzed by flow cytometry using an LSRII (BD Biosciences) and FlowJo software (Tree Star).

Programmed cell death assays.

ABIN-1 competent and deficient cells were treated with TNF (10-40 ng/ml) plus cycloheximide (10mg/ml) and assayed for programmed cell death by flow cytometric quantitation of Annexin V reactive cells using an LSRII flow cytometer and FlowJo software. Cell death signaling in these cells was assessed by preparing whole cell lysates in lysis buffer (50 mM Hepes, 120 mM NaCl, 1mM EDTA, 0.1%NP-40) and immunoblotting for expression and cleavage of cell death proteins using commercial antibodies (caspase 3 [Cell Signaling], BID [Santa Cruz]).

Cell signaling assays.

Activation of cell signaling cascades was assessed by stimulating cells with the indicated ligands (e.g., TNF), preparing whole cell lysates in lysis buffer (above), and immunoblotting for the expression of various proteins using commercial antibodies (phospho-IkBa, IkBa, ABIN-1 anti-serum custom made by Antagene, actin [Calbiochem]). A20 protein was detected using a polyclonal anti-murine A20 antibody (Boone et al., 2004).

Real Time-PCR assays of mRNA expression.

RT-PCR analyses were performed using TaqMan Gene Expression kit (Applied Biosystems), according to manufacturer's instructions.

Lentiviral shRNA knock-down of ABIN-1 in mouse cell lines.

Lentiviral constructs bearing shRNA sequences specific to ABIN-1 or A20 were purchased from Open Biosystems. Constructs were co-transfected with expression constructs for helper virus R89.1, VSV-G, and tat into 293T cells, and replication deficient virus from the resulting supernatants was used to infect MEF cells. The degree of ABIN-1 or A20 protein reduction was assessed by immunoblotting of whole cell lysates or RNA transcript reduction was assessed by Taqman RT-PCR. Control cells included cells infected with lentiviruses devoid of coding sequences, but which had puromycin resistance conferred by the pLKO plasmid DNA. Cells that were not transfected were killed by puromycin.

Chapter 3

ABIN-1 Protects Against Psoriasis by Restricting MyD88 Signals In Dendritic Cells

ABIN-1 Protects Against Psoriasis by Restricting MyD88 Signals In Dendritic Cells[1]

Running title: ABIN-1 Expression in DC Protects Against Psoriasis

Joseph A. Callahan[2], Gianna E. Hammer[2], Alexander Agelidis[2], Bao H. Duong[2], Shigeru Oshima[2], Jeffrey North[3], Rommel Advincula[2], Nataliya Shifrin[2], Hong-An Truong[3], Jonathan Paw[3], Julio Barrera[2], Anthony DeFranco[4], Michael D. Rosenblum[3], Barbara A. Malynn[2], and Averil Ma[2*]

[1]This work was supported by grants from the NIH (A.M.) and by the Histopathology and Immunology cores of the UCSF Liver Center. G.E.H. was supported by T32 DK007007. J.A.C. was partially supported by an NSF predoctoral fellowship.

[2] Department of Medicine, University of California, San Francisco, San Francisco, CA 94143 [3] Department of Dermatology, University of California, San Francisco, San Francisco, CA 94143 [4] Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA 94143

* To whom correspondence should be addressed:
University of California, San Francisco, 513 Parnassus Ave, S-1057, San Francisco, CA 94143-0451. EM: Averil.ma@ucsf.edu

Abbreviations: ABIN-1, A20 Binding and Inhibitor of NF-|B-1; DC, dendritic cell; cDC, conventional DC; pDC, plasmacytoid DC.

Summary

Psoriasis is a chronic, inflammatory skin disease caused by a combination of environmental and genetic factors. The *Tnip1* gene encodes ABIN-1 (A20 Binding and Inhibitor of NF-|B-1) protein and is strongly associated with susceptibility to psoriasis in humans. ABIN-1, a widely expressed ubiquitin binding protein, restricts TNF and TLR induced signals. We report here that mice lacking ABIN-1 specifically in dendritic cells (DCs), ABIN-1^{Flox} CD11c-Cre mice, exhibit perturbed immune homeostasis. ABIN-1 deficient DCs display exaggerated NF-|B and MAP kinase signaling and produce more IL-23 than normal cells in response to TLR ligands. Challenge of ABIN-1^{Flox} CD11c-Cre mice with topical TLR7 ligand leads to greater numbers of TH17 and TCR$\gamma\delta$ T cells and exacerbated development of psoriaform lesions. These phenotypes are reversed by DC-specific deletion of the TLR adaptor MyD88. These studies link ABIN-1 with IL-23 and IL-17, and provide cellular and molecular mechanisms by which ABIN-1 regulates susceptibility to psoriasis.

Introduction

Psoriasis is a common immune mediated skin disorder whose complex pathophysiology may involve environmental factors, including microbes, as well as host susceptibility factors, including genetically determined immunologic propensities (Nestle et al., 2009b). Abnormalities in keratinocyte function, adaptive and innate immune cells, and cytokine production have been implicated in psoriasis (Capon et al., 2012). Therapeutic responses of psoriasis patients to anti-TNF, anti-IL-12/23 receptor, anti-IL-17, and anti-IL-22 imply that these cytokines are involved in psoriasis pathogenesis (Johnson-Huang et al., 2012).

Recent genome wide association studies (GWAS) have further highlighted specific proteins and pathways likely to be involved in disease pathogenesis. Single nucleotide polymorphisms (SNPs) of immunoregulatory genes (HLA-C, IL-12B, *TNIP1*/ABIN-1, *TNFAIP3*/A20, IL-23A and IL-23R) are strongly linked to susceptibility to psoriasis in humans (Johnson-Huang et al., 2012). Some of these polymorphisms are also linked to therapeutic responses of psoriasis patients to specific therapies (Tejasvi et al., 2012). These findings emphasize the immunological nature of psoriasis. Moreover, they implicate specific aspects of immune functions, including HLA-C mediated antigen presentation and IL-12/IL-23 dependent innate immune signals. As innate immune cells such as DCs are outstanding antigen presenting cells, and as IL-12 and IL-23 are secreted by innate immune cells to amplify T cell activation and differentiation events, these genetic clues suggest that aberrant DC and T cell functions are integral to

psoriasis.

TNIP1, which encodes ABIN-1 protein, was found to be strongly linked to psoriasis in GWAS in both European (combined genome-wide P value of 1×10^{-20}) and Chinese (combined genome-wide P value of 3.8×10^{-21}) populations (Nair et al., 2009; Strange et al., 2010; Sun et al., 2010). ABIN-1 restricts several NF-|B signaling cascades and regulates cell survival (Heyninck et al., 1999; Wullaert et al., 2005; Wagner et al., 2008; Oshima et al., 2009; Nanda et al., 2011). In vitro studies suggest that ABIN-1 can bind NEMO/IKKγ and inhibit TNF induced NF-|B signaling (Mauro et al., 2006). ABIN-1 can also bind ubiquitin chains, and ubiquitin binding by ABIN-1 is important for ABIN-1's ability to restrict TNF and TLR signals (Oshima et al., 2009; Nanda et al., 2011). Global loss or mutation of ABIN-1 leads to either embryonic lethality or spontaneous inflammation and autoimmunity (Oshima et al., 2009; Nanda et al., 2011; Zhou et al., 2011). These studies indicated that ABIN-1 plays critical roles in regulating TNF and TLR signals and preventing autoimmune disease. As ABIN-1 is expressed in multiple cell types, the cellular mechanisms by which ABIN-1 preserves physiological immune homeostasis and psoriasis susceptibility are unknown.

DCs have long been recognized as important cells for triggering immune responses during overt immunizations or infections (Steinman, 2012). Recent studies suggest that DCs also preserve immune homeostasis under basal conditions (Chen et al., 2006; Stranges et al., 2007; Birnberg et al., 2008;

Ohnmacht et al., 2009; Hammer et al., 2011; Kool et al., 2011). DCs may also regulate susceptibility to psoriasis (Nestle et al., 2009a). The exquisite sensitivity of DCs to TLR ligands such as nucleic acids or bacterial cell wall components may underlie the efficacy of the TLR7 ligand imiquimod in enhancing immune responses for a variety of dermatological conditions (Cantisani et al., 2012). DCs may contribute to psoriasis by secreting type I interferons, TNF, or other pro-inflammatory cytokines, and stimulating skin T cells. Given the potential importance of DCs to immune homeostasis in the skin and the potential importance of ABIN-1 polymorphisms to psoriasis, we have investigated whether ABIN-1 expression in DCs may regulate psoriasis susceptibility.

Results

To investigate whether ABIN-1 expression in DCs regulates immune functions in vivo, we generated mice bearing LoxP sites flanking exons 12-15 of ABIN-1 (ABIN-1^{FL}) mice and bred them with CD11c-Cre transgenic mice to create mice lacking ABIN-1 in DCs (Figure 1A) (Caton et al., 2007).

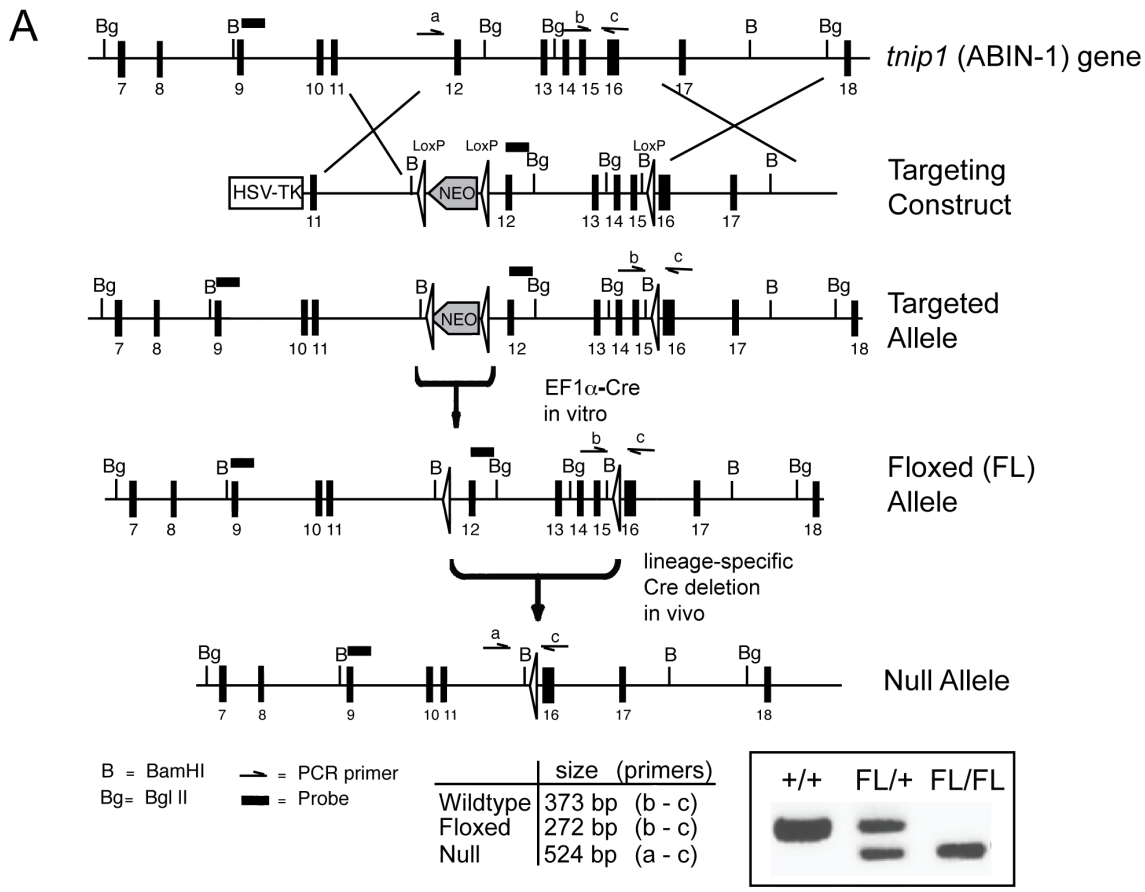


Figure 1. Generation of loxP flanked ABIN-1 (*Tnip1*) allele.

(A) Schematic diagram of gene targeting strategy for generating ABIN-1^{FL} mice. PCR confirmation of germline transmission (using tail DNA) of in vitro Cre-mediated deletion of loxP sites, generating ABIN-1^{FL} allele.

ABIN-1$^{FL/FL}$ CD11c-Cre mice appeared grossly normal for up to six months of age. Conventional DCs (cDCs, CD11c^{high} MHC-II^{+}) and plasmacytoid DCs (pDCs, CD11c^{Lo} B220^{Hi}) were present in slightly elevated numbers in spleens from ABIN-1$^{FL/FL}$ CD11c-Cre mice. These cells expressed relatively normal levels of activation markers, with the exception of minimally elevated levels of CD40 on cDCs and slightly lower CD80 levels on pDC (Figure 2A-C). Thus, ABIN-1 expression in DCs is not required for DC development but does modestly restrict DC activation under basal conditions.

ABIN-1 expression in DCs is necessary to preserve immune homeostasis.

As DCs play important roles in regulating basal lymphoid homeostasis, we asked whether ABIN-1 expression in DCs is important for this function. ABIN-1$^{FL/FL}$ CD11c-Cre mice developed splenomegaly and lymphadenopathy by 3-4 months of age with significant accumulation of myeloid (CD11b^{+} Gr-1^{+}) cells (Figure 2D-E). While thymic development was normal in ABIN-1$^{FL/FL}$ CD11c-Cre mice, these mice accumulated higher numbers of memory phenotype CD4^{+} and CD8^{+} T cells in spleens and peripheral lymph nodes relative to control CD11c-Cre mice (Figure 2F-G, and data not shown). Thus, ABIN-1 expression in DCs is necessary to preserve myeloid and lymphoid immune homeostasis.

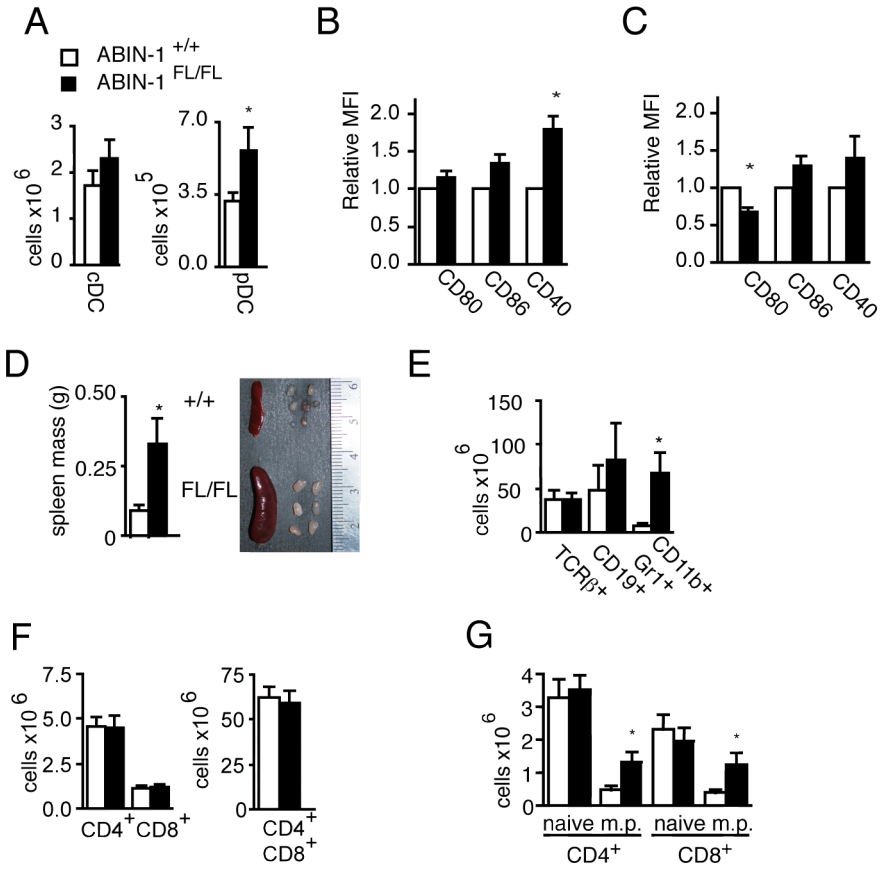


Figure 2. ABIN-1 expression in DCs is necessary to preserve immune homeostasis.

(A) Flow cytometric quantitation of MHC II^{+} CD11c^{hi} conventional DCs (cDCs) and MHC II^{+} CD11c^{dim} B220^{+} plasmacytoid DCs (pDCs) in spleens from indicated mice. (B, C) Flow cytometric analyses of expression of indicated maturation markers on conventional (B) and plasmacytoid (C) DCs. Data in (B, C) represent 4-10 CD11c-Cre^{+} mice age 1.5-6 months. (D) Spleens from indicated mice. (E) Quantitation of myeloid, T cell, and B cell splenic subpopulations. (F) Thymocyte subpopulations from indicated mice. (G) Quantitation of naïve (CD44^{lo}) and

memory phenotype ($CD44^{hi}$ or m.p.) T cells. Error bars represent SEM. All mice in (A-G) are CD11c-Cre^{+}. Open bars indicate ABIN-$1^{+/+}$ CD11c-Cre^{+} mice, while shaded bars indicate control ABIN-$1^{FL/FL}$ CD11c-Cre^{+} mice. Data in (D-G) are representative of 5-6 pairs of 8-16 week old mice in 3-7 independent experiments. * indicates $p < 0.05$ by Student's t-test.

ABIN-1 restriction of MyD88-dependent signals in DCs is required to maintain immune homeostasis.

DCs are activated by TLR ligands during overt immunizations and infections and may also respond to MyD88-dependent signals under basal conditions (Hammer et al., 2011). We asked whether ABIN-1 preserves immune homeostasis by restricting MyD88-dependent signals in DCs by generating ABIN-1$^{FL/FL}$ MyD88$^{FL/FL}$ CD11c-Cre mice that lack both ABIN-1 and MyD88 specifically in DCs (Hou et al., 2008). Remarkably, in contrast to ABIN-1$^{FL/FL}$ CD11c-Cre mice, the spontaneous accumulation of myeloid cells and activated T lymphocytes observed in ABIN-1$^{FL/FL}$ CD11c-Cre mice was abrogated in ABIN-1$^{FL/FL}$ MyD88$^{FL/FL}$ CD11c-Cre mice (Figure 3A-D). These results indicate that ABIN-1 restricts basal Myd88-dependent intracellular signals in DCs, thereby preserving immune homeostasis in unperturbed mice.

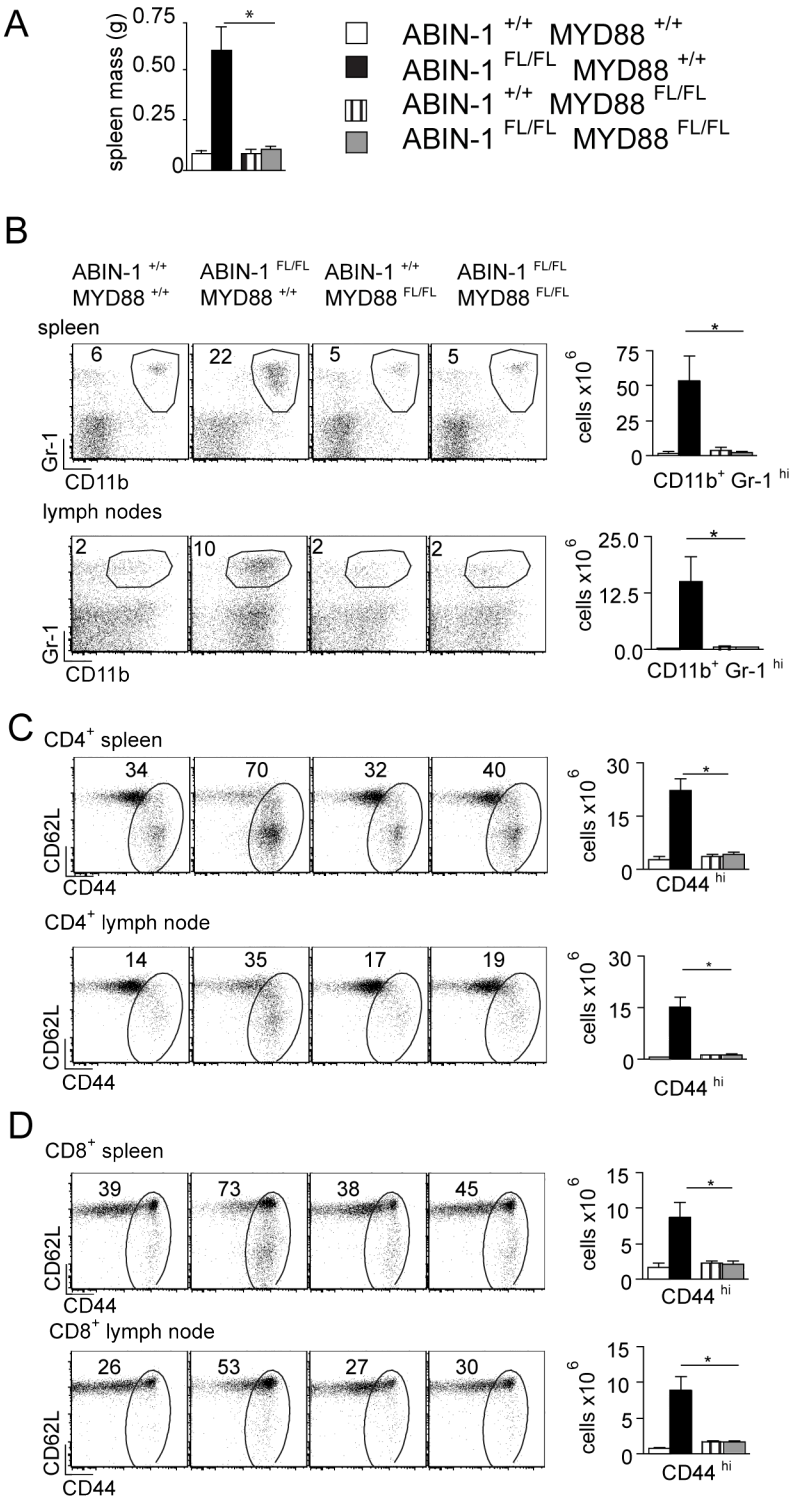


Figure 3. ABIN-1 restriction of MyD88-dependent signals in DCs is required to maintain immune homeostasis.

(A) Spleen mass of indicated genotypes of mice. (B) Flow cytometric analyses of myeloid cell populations in spleens and lymph nodes from indicated mice. Numbers in plots indicate percentages of cells within gate. Bar graphs show total number of indicated cell type in corresponding organs. (C-D) Flow cytometric analyses and total numbers of T cell subpopulations in spleens and pooled axillary, brachial, and inguinal lymph nodes from indicated mice. Representative flow cytometry of $CD4^+$ (C) and $CD8^+$ (D) T cells, including analysis of memory phenotype ($CD44^{hi}$) T cell subsets, of indicated genotypes of mice. All mice in (A-D) are 3-4 months old and CD11c-Cre^+. Data are representative of 3 independent analyses, including 6 pairs of ABIN-1 $^{FL/FL}$ Myd88 $^{+/+}$ and ABIN-1 $^{FL/FL}$ Myd88 $^{FL/FL}$ mice. Significant differences were determined using 1-way ANOVA. Error bars represent SEM. * indicates $p< 0.05$ by Student's t-test.

ABIN-1 restricts LPS responses in DCs.

To interrogate how ABIN-1 restricts MyD88 dependent signals in DCs, we tested the responses of ABIN-$1^{-/-}$ and ABIN-$1^{+/+}$ bone marrow derived DCs (BMDCs) to the TLR4 ligand LPS. LPS stimulated ABIN-$1^{-/-}$ BMDCs secreted more TNF, IL-6, IL-12, and IL-23 than control BMDCs (Figure 4A). These experiments demonstrate that ABIN-1 restricts TLR induced cytokine responses of DCs. To determine how ABIN-1 regulates TLR responses in DCs, we tested TLR signaling pathways in these cells. After LPS stimulation, ABIN-$1^{-/-}$ BMDCs exhibited exaggerated NF-κB, JNK and p38 (but not ERK) signaling, compared to control BMDCs (Figure 4B-C). These studies indicate that ABIN-1 regulates TLR responses in DCs by directly restricting TLR induced NF-κB and some MAP kinase signals.

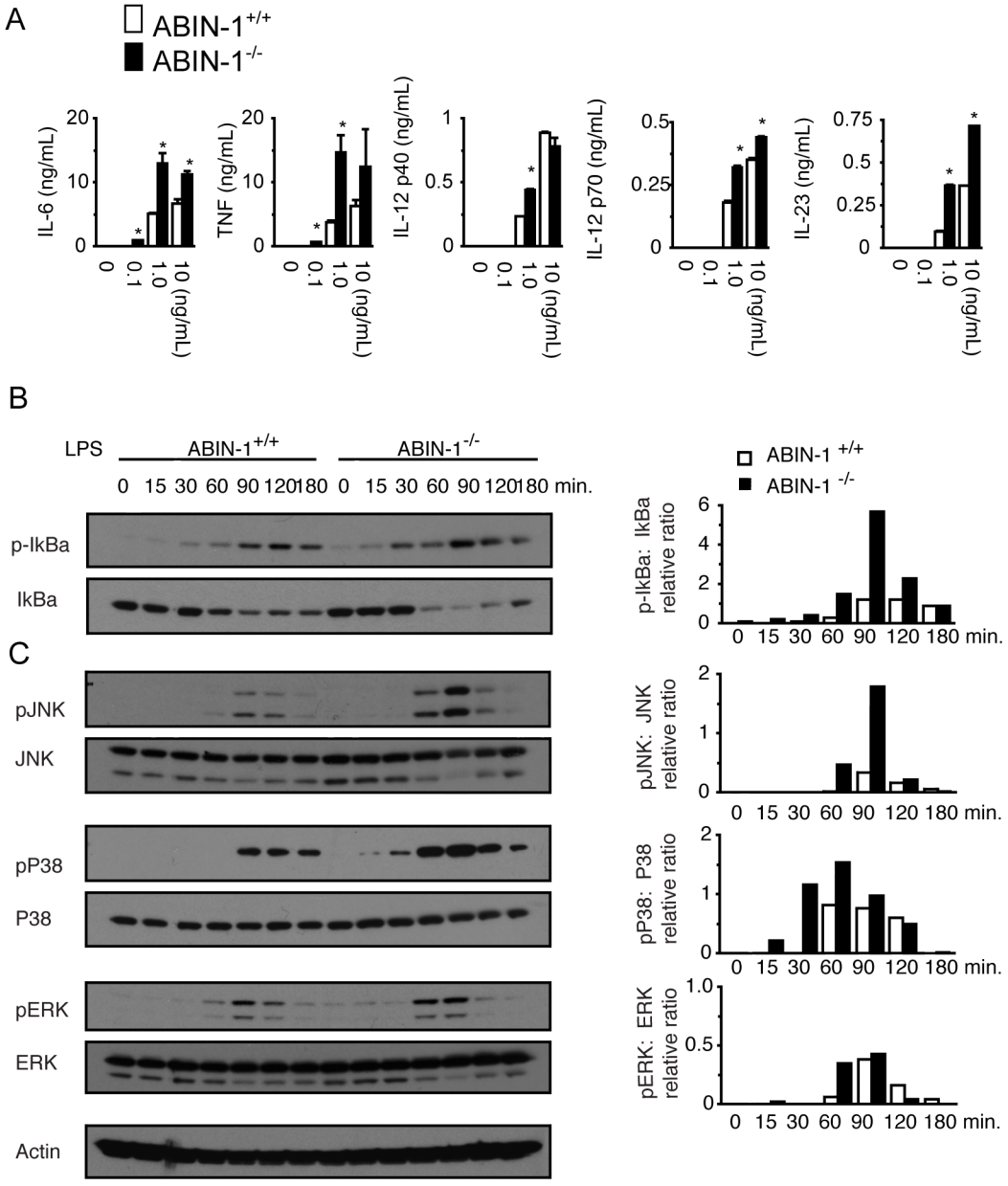


Figure 4. ABIN-1 restricts LPS responses in DCs.

(A) ELISA or multiplex Luminex analyses of cytokine production from ABIN-1$^{-/-}$ and ABIN-1$^{+/+}$ BMDCs after treatment with the indicated doses of LPS. (B) Immunoblot analyses of NF-|B and (C) MAP kinase signaling activity in ABIN-1$^{-/-}$

and control ABIN-$1^{+/+}$ BMDCs treated with 10 ng/ml LPS. pIκBα: IκBα ratios determined by densitometry and shown beside IκBα immunoblots. Actin protein levels shown below as loading controls. All data are representative of 3 independent experiments. Error bars represent SD. * indicates $p < 0.05$ by Students t-test.

ABIN-1 restricts imiquimod responses in DCs and prevents imiquimod induced psoriasis.

Given the genetic linkage of ABIN-1 to psoriasis and the exaggerated production of IL-12 and IL-23 by ABIN-$1^{-/-}$ DCs, we asked whether ABIN-1 expression in DCs might regulate susceptibility to experimental psoriasis. Topical treatment of human patients with the TLR7 ligand imiquimod can cause a psoriasis-like condition in human patients, and imiquimod induces similar lesions in mice (Fanti et al., 2006; van der Fits et al., 2009). To selectively interrogate the functions of radiation sensitive DCs, we generated radiation chimera bearing HSCs from ABIN-$1^{FL/FL}$ CD11c-Cre or ABIN-$1^{+/+}$ CD11c-Cre mice. Treatment of mice with imiquimod caused markedly increased erythema, scaling, and skin thickening in ABIN-$1^{FL/FL}$ CD11c-Cre chimera, which combine to yield increased composite psoriasis scores in ABIN-$1^{FL/FL}$ CD11c-Cre mice compared to control chimera (Figure 5A). Histologic examination of skin sections from these mice revealed epidermal hyperplasia, hypogranulosis, hyperkeratosis, and parakeratosis with neutrophils--all stereotypical histologic findings of human psoriasis—in ABIN-$1^{FL/FL}$ CD11c-Cre but not control mice (Figure 5B-G). Treatment of mice with a topical emollient as control did not lead to significant clinical responses (Figure 5H, I). Hence, ABIN-1 expression in DCs prevents susceptibility to imiquimod induced experimental psoriasis.

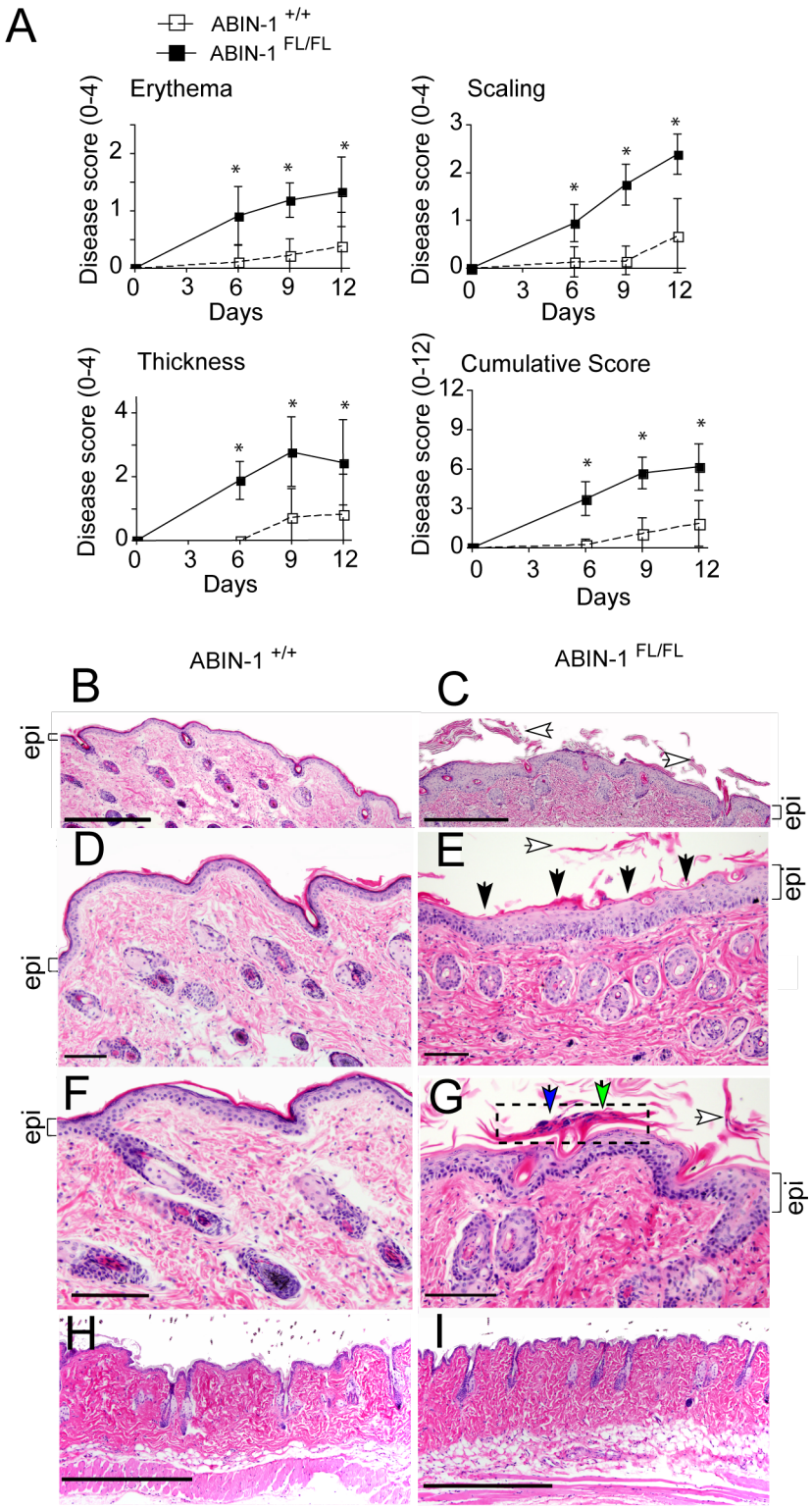

A
ABIN-1 +/+
ABIN-1 FL/FL
Erythema
Scaling
Thickness
Cumulative Score
Disease score (0-4)
Disease score (0-12)
Days
ABIN-1 +/+
ABIN-1 FL/FL
B
C
D
E
F
G
H
I
epi

Figure 5. ABIN-1 restricts imiquimod responses in DCs and prevents imiquimod induced psoriasis.

(A) Individual and combined clinical scores of imiquimod induced skin inflammation in ABIN-$1^{FL/FL}$ CD11c-Cre^{+} and ABIN-$1^{+/+}$ CD11c-Cre^{+} (control) mice at indicated days of imiquimod treatment. (B-I) Hematoxylin and eosin stained sections of back skin of mice of indicated genotypes from areas treated with imiquimod (B-G) or lotion (H, I). The epithelial layer (labeled "epi") is indicated by brackets (B-G), and epidermal hyperplasia (thickening of epidermal layer) is evident in ABIN-$1^{FL/FL}$ (C,E,G), compared to ABIN-$1^{+/+}$ (B,D,F). In (C), (E), and (G), open arrows show hyperkeratotic scaling. In (E), broad areas of hypogranulosis (abnormal loss of purple keratohyaline granules in the skin's granular layer) are indicated by closed arrows. In (G), neutrophils (blue arrow) and parakeratosis (abnormal retention of nuclei in the outermost layer of skin, green arrow) are shown in Munro's microabscess (dotted boxed area). For (D-G), scale bar = 0.1 mm. For (B-C) and (H-I), scale bar = 0.5 mm. All data are representative of 3-7 independent experiments. Error bars represent SD. * indicates $p < 0.05$ by Student's t-test.

Imiquimod induced psoriasis involves IL-23 dependent production of IL-17 (van der Fits et al., 2009). We thus measured the levels of inflammatory cytokines produced by ABIN-$1^{-/-}$ BMDCs compared to control BMDCs in response to imiquimod. Imiquimod stimulated higher levels of IL-23, IL-6, IL-12p70 and TNF secretion from ABIN-$1^{-/-}$ BMDCs compared to wild type BMDCs, while IL-12p40 levels were similar (Figure 6A). We next tested the induction of IL-17 expression in imiquimod treated mice. While the total numbers of T cells in skin draining lymph nodes were similar in ABIN-$1^{FL/FL}$ CD11c-Cre and ABIN-$1^{+/+}$ CD11c-Cre chimera, increased numbers of $CD4^+$ TH17 cells were observed in ABIN-$1^{FL/FL}$ CD11c-Cre mice. Many IL-17 producing T cells in imiquimod-treated mice are epidermal TCRγ/δ^+ T cells (Cai et al., 2011). Consistent with this notion, increased percentages and numbers of TCRγ/δ^+ T cells were noted in draining lymph nodes from ABIN-$1^{FL/FL}$ CD11c-Cre mice. In contrast, analyses of the skin draining lymph nodes from IMQ-treated ABIN-$1^{FL/FL}$ CD11c-Cre mice showed approximately normal TH1 cell numbers and no distinct TH2 population (Figure 6B). Thus, ABIN-1 expression in DCs restricts IL-23 secretion, TH17 cell differentiation, neutrophilic inflammation, and psoriatic lesions after imiquimod treatment.

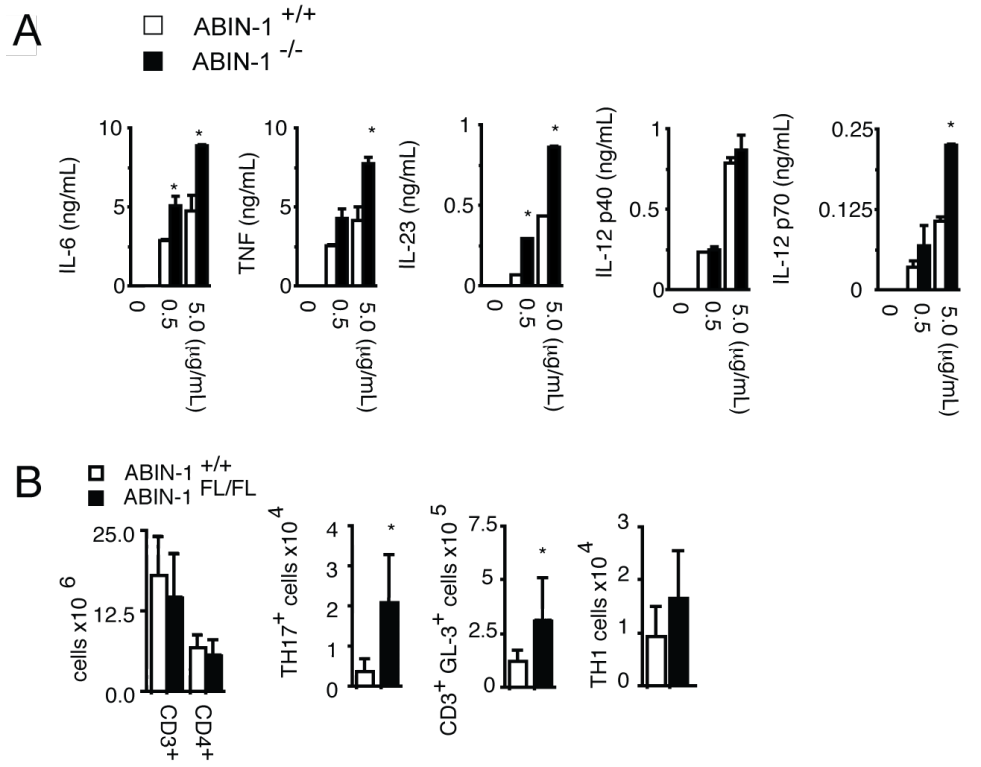


Figure 6. ABIN-1 restricts imiquimod induced proinflammatory cytokines and cell expansions

(A) ELISA and multiplex Luminex analyses of cytokine production from ABIN-$1^{-/-}$ and ABIN-$1^{+/+}$ BMDCs after treatment with the indicated doses of imiquimod. (B) Numbers of $CD3^+$ and $CD4^+$ T cells from skin draining lymph nodes from imiquimod treated mice of indicated genotypes. Numbers of TH17 ($CD4^+$ IL-17^+), TCRγ/$δ^+$ ($CD3^+$ GL-3^+), and TH1 ($CD4^+$ IFN$γ^+$) T cells from skin draining lymph nodes from imiquimod treated mice. Distinct TH2 ($CD4^+$ IL-4^+) populations were not detected. All mice in (B) are CD11c-Cre+ radiation chimera. All data are representative of 3-7 independent experiments. Error bars represent SD. * indicates $p < 0.05$ by Student's t-test.

ABIN-1 restricts MyD88 signals in DCs to prevent imiquimod induced psoriasis.

To determine whether ABIN-1 dependent regulation of DC TLR responses to imiquimod was integral to disease pathogenesis, we tested the responses of chimera generated from ABIN-1$^{FL/FL}$ MyD88$^{FL/FL}$ CD11c-Cre compound mutant and control hematopoietic stem cells. Double mutant ABIN-1$^{FL/FL}$ MyD88$^{FL/FL}$ CD11c-Cre mice exhibited much less psoriasis than ABIN-1$^{FL/FL}$ CD11c-Cre mice. Indeed, ABIN-1$^{FL/FL}$ MyD88$^{FL/FL}$ CD11c-Cre mice exhibited similar clinical responses to MyD88$^{FL/FL}$ CD11c-Cre and wild type chimera (Figure 7A). Histological studies confirmed the reduced inflammation observed in ABIN-1$^{FL/FL}$ MyD88$^{FL/FL}$ CD11c-Cre mice compared with ABIN-1$^{FL/FL}$ CD11c-Cre mice (Figure 7B). Thus, ABIN-1 dependent regulation of MyD88 dependent signals in DCs regulates susceptibility to experimental psoriasis.

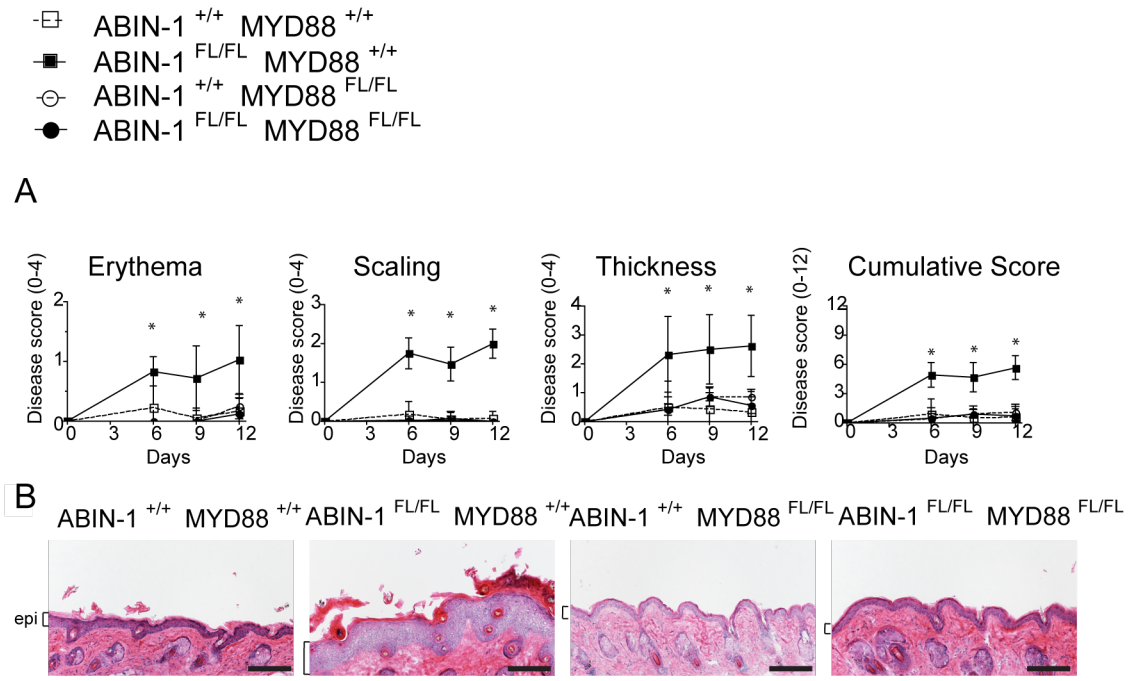


Figure 7. ABIN-1 restricts MyD88 signals in DCs to prevent imiquimod induced psoriasis.

(A) Clinical skin inflammation scores in the indicated genotypes of mice at the indicated days of imiquimod treatment. (B) Hematoxylin and eosin stained sections of back skin of mice of indicated genotypes from areas treated with imiquimod. Epithelial layer denoted by brackets and "epi." All mice are CD11c-Cre+. Error bars represent SD; * indicates $p < 0.05$ by Student's t-test. Scale bar = 0.1 mm. Data are representative of 3 independent experiments.

Discussion

Our studies reveal potent immunoregulatory functions for ABIN-1 in DCs. We have discovered that ABIN-1 mediated inhibition of MyD88-dependent signals in DCs is critical for preserving immune homeostasis and preventing imiquimod induced psoriasis. ABIN-$1^{-/-}$ DCs elaborate exaggerated levels of IL-23 after TLR engagement, and mice lacking ABIN-1 selectively in DCs accumulate more TH17 cells and clinical psoriaform lesions. Our findings linking ABIN-1 deficiency in DCs with psoriasis provide pathophysiological mechanisms by which human ABIN-1/*Tnip1* SNPs may contribute to psoriasis susceptibility.

Our findings indicate that ABIN-1 restricts TLR induced NF-κB and MAPK signals, consistent with a prior study (Nanda et al., 2011). By contrast, another study described ABIN-1's role in restricting TLR induced C/EBPβ signals (Zhou et al., 2011). These differences may be due to the different regions of the ABIN-1 gene targeted in these studies, background strain, and/or and environmental differences—including differences in microbiome constitution.

ABIN-1 SNPs are strongly linked to susceptibility to psoriasis in multiple ethnic groups, and globally ABIN-1 deficient mice develop spontaneous autoimmunity and inflammation (Oshima et al., 2009; Nanda et al., 2011; Zhou et al., 2011). However, the pleiotropic cellular expression of ABIN-1 and the diverse signaling pathways regulated by this protein leave a broad array of potential mechanisms

by which ABIN-1 SNPs might confer susceptibility to psoriasis. Recent studies suggest that TLR ligands engage DCs under basal conditions and that the ubiquitin modifying enzyme A20 restricts these signals, preventing autoimmune and inflammatory diseases (Hammer et al., 2011; Kool et al., 2011). Our data suggest that ABIN-1, originally identified as an A20 binding partner, also restricts MyD88-dependent signals in DCs in unperturbed mice. While the TLR ligands that trigger DCs under homeostatic situations are incompletely defined, the observation that imiquimod induces similar psoriasiform lesions in mice and humans suggests that this TLR ligand, or similar ligands derived from commensal microbes or host nucleic acids may normally be restrained by ABIN-1. The failure to properly regulate intracellular DC signals may cause DCs to aberrantly trigger inflammatory cascades and activate T cells.

Our studies suggest that ABIN-1 expression in DCs restricts TLR induced NF-|B and JNK signals, thereby limiting DC expression of IL-23 and other cytokines. IL-23 supports the accumulation of IL-17 and IL-22 producing T cells. IL-17 induces epidermal neutrophil infiltration, and IL-22 alters keratinocyte proliferation and differentiation. Thus, exaggerated IL-23 expression likely leads to characteristic dermal lesions of psoriasis. Our studies mechanistically link ABIN-1, a major psoriasis susceptibility gene, with IL-23A and IL-23R, two other major psoriasis susceptibility genes. This linkage suggests that ABIN-1 and IL-23-dependent inflammation may be part of a common dominant pathophysiological pathway leading to psoriasis. These cellular and molecular insights into how ABIN-1

prevents psoriasis provide mechanistic credence to genetic suggestions about psoriasis pathophysiology. Moreover, mice bearing ABIN-1 mutations should be extremely valuable models for studying psoriasis pathophysiology and treatment.

Methods and Materials

Mice. The ABIN-1 gene targeting construct and initial targeting of the *Tnip1/ABIN-1* gene have previously been described (Oshima et al., 2009). The initial targeting of the *Tnip1* gene in C57BL/6N PRX-B6T ES cells has previously been described (Oshima et al., 2009). Appropriately targeted ES cells were transfected with an EF1α-Cre expression construct (Hammer et al., 2011), and colonies were screened by Southern blot for deletion of the neomycin gene and retention of exons 12-15 flanked by LoxP sites (floxed allele). ES cells with the floxed *Tnip1* allele were injected into blastocysts cells by the UCSF Transgenic Core. Mice bearing this targeted allele in the germline were interbred with CD11c-Cre transgenic mice to delete intervening sequences including exons 12–15 specifically in DCs (Caton et al., 2007). Genotypes were initially confirmed by Southern blot analyses and subsequently identified by PCR with the following primers:
TTGATTCCCCTTCGCCCATTCCAGC; CCTCAAACAGCAGAAGAGGAAAGC; ATGGGTGGGTAGGCATAGGGATAG. $MyD88^{FL}$ mice were described previously (Hou et al., 2008). Unless otherwise noted, mice were analyzed between 12 and 24 weeks of age.
All mouse experiments were approved by the UCSF Institutional Care and Use Committee.

Cell preparation and analyses. Cell preparations and flow cytometric analyses were performed as previously described (Hammer et al., 2011). Spleens were treated with 0.2 ug/mL Liberase Blendzyme II digestion for isolation of DCs. $TER119^+$ erythrocyte-lineage cells were depleted using antibody-coated magnetic beads (Dynabead). All antibodies were purchased from BD Biosciences. Cells were analyzed by flow cytometry using an LSRII (BD Biosciences) and Flowjo software (Tree Star). Electronic removal of $CD3^+$, $CD19^+$, and $NK1.1^+$ cells in a dump channel was used for analyses of MHC-II, CD11c, and B220 expression on pDCs and cDCs. Serum cytokines were determined using Beadlyte Mouse Multi-Cytokine Detection System assay (Millipore) and Bio-Plex 200 System (Biorad). Cytokine levels in BMDC supernatants were measured after 7 hrs (imiquimod) or 24 hrs (LPS) by ELISA (BD Biosciences). Immunoblots were performed as described (Oshima et al., 2009).

Preparation and analyses of BMDCs. Bone marrow derived dendritic cells (BMDCs) were derived from bone marrow cells cultured in RPMI medium containing GMCSF for 10 days. BMDCs were replated overnight and then rested for 2 hours in media containing 0.1%FCS prior to treatment with TLR ligands. Cytokine responses were measured after 7 hrs (imiquimod) or 24 hrs (LPS) by ELISA (BD Biosciences). For signaling studies, cells were lysed in 2X NuPAGE LDS sample buffer (Invitrogen) containing protease inhibitors (Roche) and phosphatase inhibitors (0.5 mM NaF, 0.8 mM beta-glycerophosphate, 1 mM

PNPP, 0.08 mM NaVO3). Whole cell lysates were sonicated and heated 5 min. at 70^{o}C prior to SDS PAGE separation (NOVEX System, Invitrogen). Immunoblots were probed for actin (Calbiochem), phospho-IkBa, IkBa, phospho-Erk, Erk, phospho-JNK, and JNK (Cell Signaling Technologies). LPS (Sigma) and imiquimod (Invivogen) were purchased.

Imiquimod (IMQ) treatment and scoring of skin inflammation. Radiation chimera were reconstituted with ABIN-1$^{FL/FL}$ CD11c-Cre or ABIN-1$^{+/+}$ CD11c-Cre bone marrow HSCs 2-3 months prior to the start of IMQ treatments. IMQ treatments were performed largely as previously described (van der Fits et al., 2009). Each mouse received a daily topical dose of 12.5 ug of IMQ cream (5%) (Perrigo) or lotion control on the shaved back and right ear for 12 consecutive days. PBS was administered intraperitoneally on days 6 and 7 to provide hydration. Skin inflammation was scored using a previously described scoring system (van der Fits et al., 2009). Histological sections were prepared by the UCSF VAMC Pathology Core.

Acknowledgements

We thank Sandra Huling and Ivy Hsieh of the UCSF VAMC Pathology Core for excellent assistance with histological studies. This work was supported by grants from the NIH (A.M.) and by the Histopathology and Immunology cores of the UCSF Liver Center. J.A.C. was partially supported by an NSF predoctoral fellowship.

Chapter 4

Summary and Future Directions

Introduction

The mammalian immune system is critical for protecting host immunity. Multiple diseases are initiated or exacerbated by damaging inflammation or autoimmune responses. GWAS have implicated SNPs in multiple genes that typically regulate these potent proinflammatory immune cell signaling pathways. A20 susceptibility SNPs have linked A20 to multiple autoimmune and/ or inflammation-mediated diseases, including: psoriasis, SSc (Systemic Sclerosis), SLE (Systemic lupus erythematosus), rheumatoid arthritis, type 1 diabetes, coeliac disease, Crohn's disease, coronary artery disease in type 2 diabetes (Boonyasrisawat et al., 2007). SNPs in ABIN-1 confer susceptibility to a partially overlapping set of diseases: psoriasis, SSc, SLE, psoriatic arthritis, myasthenia gravis (Adrianto et al., 2012; Allanore et al., 2011; Bossini-Castillo et al., 2013; Bowes et al., 2011; Gateva et al., 2009; Gregersen et al., 2012; Han et al., 2009; Kawasaki et al., 2010; Nair et al., 2009; Sun et al., 2010). The connection of both of these genes to inflammation-mediated human disease suggested that interrogation of ABIN-1 may provide valuable insights into mechanisms that protect against damaging inflammation and autoimmune pathophysiology.

Our studies demonstrate ABIN-1's role as an ubiquitin sensor that restricts TNF-induced apoptosis in vitro and in vivo. In addition, we demonstrated that ABIN-1 restricts TNF-independent signals in vivo to maintain immune homeostasis.

ABIN-1-/-TNF-/-RAG1-/- mice still develop splenomegaly, supporting that ABIN-1 restricts immune activating signals in myeloid cells.

Using DC-specific deletion of ABIN, we demonstrated that ABIN-1 expression in DC protects against a MyD88-dependent immune activation phenotype and experimental psoriasis. Together, these studies provide pathophysiological insights into how ABIN-1 restricts potentially fatal apoptotic cell death signaling and both local and systemic inflammation.

ABIN-1 uses ubiquitin sensing mechanisms to restrict TNFR signaling.

Our studies have implicated ABIN-1 in restricting TNF-induced apoptotic signaling, and future research will be needed to determine the identity of the direct target of ABIN-1 binding. We and others discovered that ABIN-1 uses its ubiquitin sensing function to restrict TNF-induced signals (Oshima et al., 2009; Wagner et al., 2008). We demonstrated that a point mutant, ABIN-1 QQ477EE, lacking this ubiquitin sensing function fails to protect against TNF-induced apoptosis. Heterologous expression and immunoprecipitation experiments showed that ABIN-1 QQ477EE loses its ability to disrupt complexes containing FADD and Caspase 8 (Oshima et al., 2009). These studies support that ABIN-1 restricts apoptosis by sensing a ubiquitinated target.

Multiple ubiquitinated species have been found in the TNFR-proximate signaling complexes, including FADD, caspase 8, and RIP1 (Jin et al., 2009; Lee Ew Fau - Kim et al.; Lee et al., 2012; O'Donnell et al., 2007). Interrogating whether ABIN-1 can interact with complexes containing ubiquitinated FADD or ubiquitinated Caspase 8 may help identify ABIN-1's targets in restriction of proapoptotic signals. ABIN-1 may use its ubiquitin-binding function to bind ubiquitinated species that mediate disruption of FADD-Caspase 8 containing complexes. It remains possible that ubiquitinated FADD and/or ubiquitinated Caspase 8 are ABIN-1's targets. Caspase 8 ubiquitination was recently shown to mediate proapoptotic signaling. A20 can reverse this ubiquitination, which would be consistent with A20's previously published antiapoptotic role. Ubiquinated Caspase 8 may also represent a ubiquitinated species to which ABIN-1 could bind and perhaps mask a surface that interacts with other pro-apoptotic signaling factors, potentially including FADD. Development of additional cell-free binding assays or in vitro TNF-stimulated immunoprecipitation experiments will be necessary to test these possibilities. Cell-free binding assays could utilize ubiquitinated FADD or ubiquitinated Caspase 8. Mass spectrometry could identify components of ABIN-1-containing protein complexes. Determination of ABIN-1's direct target(s) in apoptotic signaling will help determine how ABIN-1 protects at a molecular level and whether ABIN-1 may protect from additional extrinsic signals.

ABIN-1 may restrict necroptosis.

Future studies could investigate whether ABIN-1 may work with Caspase 8 or FADD to regulate alternative non-apoptotic cell death signaling pathways, such as necroptosis. Necroptosis is a RIP1 and RIP3-kinase dependent cell death pathway. Proper regulation of necroptosis is required for maintenance of immune homeostasis and embryonic survival (Bonnet et al., 2011; Han et al., 2011; Kaiser et al., 2011; Lee et al., 2012; Vanlangenakker et al., 2012; Zhang et al., 2011). FADD and Caspase 8 are linked to necroptosis. Doubly deficient mice lacking key mediators of apoptosis were generated to test whether the interplay of mediators of necroptosis lead to the death of Caspase $8^{-/-}$ or $FADD^{-/-}$ embryos. Caspase 8 has a protective role against necroptosis (Wrighton, 2011). Caspase $8^{-/-}$ mice die during embryogenesis. Caspase $8^{-/-}$ $RIP3^{-/-}$ embryos survive (Kaiser et al., 2011; Oberst et al., 2011). This suggests that death of Caspase 8-deficient embryos is caused by a RIP3-dependent mechanism. This study links the activities of Caspase 8 and RIP3 in regulating embryonic survival. Analogously, $FADD^{-/-}$ mice die during embryogenesis, and $FADD^{-/-}$ $RIP1^{-/-}$ mice are viable. This again links a mediator of necroptosis (RIP1) with a regulator of apoptosis (FADD) in the context of embryonic survival (Zhang et al., 2011). During embryogenesis, FADD and caspase 8 promote survival by suppressing the function of RIP1 and RIP3, and thus suppress necroptosis. We demonstrated that ABIN-1 protects embryonic survival and restricts apoptosis at or upstream of FADD-Caspase8 interaction (Oshima et al., 2009). Like FADD and Caspase 8, ABIN-1 might also regulate RIP3-dependent necroptosis.

Interrogation of whether ABIN-1 regulates necroptosis could simultaneously use in vitro and in vivo experiments. In vitro, ABIN-1$^{-/-}$ MEFs are hypersensitive to apoptotic stimuli. This sensitivity could be contrasted with necroptotic stimulation. These experiments would utilize specialized death inhibitors. For example, Z-VAD-FMK protects against apoptosis, but not necroptosis. If ABIN-1 does regulate necroptosis, one could interrogate whether ABIN-1 regulates formation of FADD-containing complexes or Caspase 8-containing complexes. If necroptosis is partially restricted by ABIN, one could test whether ABIN-1's intact ubiquitin-binding domain is necessary. Utilizing cell-free binding assays or the above-mentioned stimulation and candidate-based IP approach could help identify additional targets of ABIN-binding. If ABIN-1 is implicated in necroptosis signaling, one could also generate the ABIN-1$^{-/-}$RIP3$^{-/-}$ or ABIN-1$^{-/-}$ RIP1$^{-/-}$ embryos to test whether either of these RIP kinase deficiencies rescues ABIN-1$^{-/-}$ embryonic lethality. This investigation could implicate ABIN-1 in regulating additional high-profile cell death pathways and help demonstrate the mechanisms by which ABIN-1 protects embryogenesis.

ABIN-1 may use ubiquitin sensing to restrict TLR signaling.

Future studies will investigate whether ABIN-1 modulates TLR-proximate signaling complexes to restrict TLR-induced NF-κB. We and others have shown

that ABIN-1 acutely restricts MyD88-mediated NF-κB signaling (Callahan et al., 2013; Nanda et al., 2011). It is possible that ABIN-1 recruits A20 to TLR-proximate signaling complexes, and there may be additional mechanisms. Mice with a ubiquitin-binding disrupting mutation in ABIN-1's UBAN caused severe autoimmunity (Nanda et al., 2011). Human ABIN-1 D472N point mutant lost ability to bind linear and K63-polyubiquitin (pUB) chains. Upon testing complementary mouse ABIN-1 D485N point mutant's binding efficiency in LPS-stimulated macrophages, ABIN-1 D485N lost its ability to bind pUb-IRAK1, suggesting that modified IRAK1 may be a target of ABIN-1 following TLR stimulation.

Further investigation could help determine whether this interaction between ABIN-1 and Ub-IRAK1 alters recruitment of negative regulators of polyubiquitinated IRAK1 stability. Following TLR/IL-1 stimulation IRAK1 is K63 polyubiquitinated, but it is also eventually degraded (Ordureau et al., 2008; Windheim et al., 2008). Recently, K48 ubiquitination of IRAK1 was described as a potential subsequent step leading to signal propagation and IRAK1 degradation. SCF (Skp1–Cullin1–F-box)–β-TrCP complex was shown to function as a K48-linked ubiquitination E3 ligase for IRAK1 (Cui et al., 2012). Future experiments could test whether ABIN-1 alters the kinetics of recruitment of Cullin1 or β-TrCP to K63-pUb-IRAK1 and whether ABIN-deficiency alters the stability, length, or type of ubiquitin (K63- or K48-linked) chain on IRAK1. Competition between ABIN-1 and SCF–β-TrCP complex for K63-pUb-IRAK1

binding might hinder subsequent K48-ubiquitination of IRAK1 and restrict efficient activation of TAK1 and IKK complexes. Resolving whether these pUB-IRAK1 complexes form with altered kinetics or chain configurations will help determine whether IRAK1-proximate signaling steps are acutely regulated by ABIN.

Future studies may also interrogate whether ABIN-1 displaces or competes with NF-κB positive regulators for binding to TLR-proximate signaling complexes. NEMO uses its ubiquitin sensing function to bind TLR/IL-1R-induced polyubiquitinated IRAK1 and promote IKK complex activation. Mutating NEMO's ubiquitin-binding domain to prevent ubiquitin binding inhibits IKK activation and cytokine production downstream of TNF and TLR stimulation (Ea et al., 2006; Windheim et al., 2008; Wu et al., 2006). Loss of NEMO's ubiquitin-binding function correlates with immunodeficiency in humans (Doffinger et al., 2001). Disrupting efficient ubiquitin-binding of NF-κB signaling positive regulators can dramatically compromise host immunity. It remains possible that ABIN-1 uses its ubiquitin-binding domain to bind polyubiquitinated IRAK1 (following TLR stimulation) and displace or hinder NEMO's interaction with proteins in this complex.

Combining mass spectrometry and immunoblot validation may detect rare or transient interactions with positive regulators. IL-1 stimulated MEFs or LPS-stimulated BMDC could be immunoprecipitated with recently developed anti-ABIN-1 antibodies and interrogated by mass spectrometry. Zhou et al. used

mass spectrometry to identify that ABIN-1 interacts with a MyD88-containing complex (Zhou et al., 2011). Future IP (of MyD88 or pUB-IRAK1) and immunoblot experiments could determine whether inducible TLR-proximate complexes include exaggerated levels of positive regulators (e.g. TAK1, TAB2, TAB3, TRAF6) in absence of ABIN. Increased stability of complexes containing TAK1 and ubiquitinated IRAK1 might correspond with the increased pro-inflammatory signaling in ABIN$^{-/-}$ cells. Experiments could explore the kinetics of complex formation and degradation/ disassembly. Ubiquitination status of these targets could also be interrogated using this candidate-based approach. IRAK1 ubiquitination, TRAF6 ubiquitination, and NEMO ubiquitination could be compared between ABIN$^{-/-}$ and ABIN$^{+/+}$ cells. Finally, cell-free binding experiments could be used to confirm suspected direct interaction of ABIN-1 with targets. Identifying the direct targets of ABIN-1 in TLR-proximate signaling and determining the consequences of those interactions will help craft molecular-level understanding of how ABIN-1 protects against inflammation and disease.

ABIN-1 may protect from psoriasis by restricting production of IL-1 family members.

ABIN-1's restriction of TLR signaling reduces proinflammatory cytokine production (e.g. IL-23, IL-6, TNF, and IL-12 p70). Future experiments may explore whether aberrant TLR and/ or TNFR-induced signaling in ABIN$^{-/-}$ DC may produce additional cytokines (e.g., IL-36, IL-1α) that directly activate

keratinocytes or fibroblasts. Investigating this hypothesis may link ABIN-1 to additional psoriasis-mediating cytokines. IL-1 family member IL-36 contributes to psoriasis pathogenesis (Tortola et al., 2012). Mutations in IL-36 antagonist (*IL36RN)* were recently described in patients with pustular psoriasis, and removing this IL-36 antagonist gene from mice predisposes transgenic keratin promoter driven (K14)-IL-36α mice to skin abnormalities (Blumberg et al., 2007; Marrakchi et al., 2011; Onoufriadis et al., 2011). A recent study demonstrated that IL-36R-/- mice are more effectively protected from experimental psoriasis than mice deficient for IL-23, IL-17α, or IL-22 (Tortola et al., 2012). Targeted therapeutic inhibition of the IL-23/ IL-17 axis has proven effective in clinical psoriasis (Griffiths et al., 2010; Leonardi et al., 2008; Papp et al., 2008). This striking comparison suggests IL-36 is critical for experimental psoriasis pathogenesis even when contrasted with the potent IL-23 axis.

Compromised regulation of IL-36 production could increase severity of psoriasis consistent with experimental psoriasis observed in ABIN-1 DCKO mice. ABIN-deficient DC produced exaggerated levels of multiple TNF-induced or NF-κB-induced proinflammatory cytokines. TNF can induce DC production of IL-36. IL-36 can upregulate IL-1α expression (Blumberg et al., 2007; Vigne et al., 2011). IL-1α can induce fibroblast production of GM-CSF and fibroblast growth factor 7 to induce keratinocyte proliferation (Szabowski et al., 2000). This crosstalk between DC-derived IL-36 and keratinocyte proliferation could underlie part of the complex skin inflammation. The combination of IL-23 and IL-1 are essential

for expansion of IL-17-producing γδ T cell (Cai et al., 2011; Sutton et al., 2009). In this way, misregulated IL-36 signals may stimulate multiple pathways of T cell-dependent and T cell-independent skin inflammation.

Future experiments could assay DC-derived IL-36 production. ABIN-1 restricts proinflammatory signaling downstream of TNF, and ABIN-deficient DC are severely proinflammatory in experimental psoriasis. Future experiments could include TNF stimulation of ABIN$^{-/-}$ DC to test IL-36 (and IL-1α) production in vitro by ELISA or QPCR. These tests could also include parallel conditions of LPS with or without TNF-neutralizing antibody to test whether the TLR-induced signals are sufficient to induce IL-36 in absence of ABIN. Simultaneously, skin biopsies from ABIN-1 DC KO mice treated for experimental psoriasis might yield detectable IL-36 for QPCR detection, although DCs are not the only source of cytokines detected in skin. ABIN-dependent restriction of IL-36 in BMDC experiments could suggest ABIN-1 has a previously unappreciated protective role and provide a rationale for using experimental psoriasis in studies investigating roles of ubiquitin sensors in regulating IL-1 family members.

ABIN-1 SNPs and expression levels correlate with human disease susceptibility.

ABIN-1 may protect against inflammation-driven autoimmune disease in humans.

Multiple genome wide association studies (GWAS) link ABIN-1 (*TNIP1*) to diseases: psoriasis, SSc, SLE, psoriatic arthritis, and myasthenia gravis (Adrianto et al., 2012; Allanore et al., 2011; Bossini-Castillo et al., 2013; Bowes et al., 2011; Gateva et al., 2009; Gregersen et al., 2012; Han et al., 2009; Kawasaki et al., 2010; Nair et al., 2009; Sun et al., 2010). SNPs in *TNFAIP3, TNIP1,* IL23α, IL12β, and IL23R have been linked to psoriasis in European populations. This study's results suggest that compromised regulation of the IL-23 axis and NF-κB signaling may predispose to psoriasis (Nair et al., 2009). A second independent study in the Chinese population also found that SNPs in ABIN-1 correlate with increased psoriasis susceptibility (Sun et al., 2010). In sample collections from the UK and Ireland, SNPs in *TNIP1* correlated with increased incidence of psoriatic arthritis (Bowes et al., 2011). Multiple studies suggest that ABIN-1 SNPs correlate with compromised regulation of NF-κB signaling and skin inflammation.

Recent reports have suggested that ABIN-1 susceptibility SNPs may also lead to reduced ABIN-1 expression. In European populations, *TNIP1* SNPs were linked to SSc. Samples with ABIN-1 susceptibility SNPs yielded reduced expression of ABIN-1 mRNA and protein (Allanore et al., 2011). SNPs in the region of *TNIP1* have been associated with SLE and correspond with reduced levels of ABIN-1 expression in B cells (Adrianto et al., 2012). Disease-associated susceptibility SNPs frequently reside outside of the coding region of the associated gene. These studies are consistent with a role for ABIN-proximal susceptibility SNPs

reducing the level of expression of ABIN. In the future, samples from these ABIN-1 SLE susceptibility SNP studies could be used to test whether these SNPs that correlate with reduced *TNIP1* expression in B cells also show reduced *TNIP1* expression in DC. Reduced ABIN-1 in susceptibility SNP-bearing DC would be consistent with a role for ABIN-1 acting in DC to restrict psoriasis. Additional tests might interrogate whether DC bearing ABIN-1 psoriasis susceptibility produce exaggerated levels of proinflammatory cytokines. Reducing expression of negative regulators of pro-inflammatory signaling – perhaps in a tissue specific way - could give rise to exaggerated proinflammatory signals. These human SNP studies support that decreased levels of ABIN-1 may predispose individuals to disease. Enhancing the activity of ABIN-1 may be a promising therapeutic strategy to restrict exaggerated inflammatory signaling that exacerbates many human diseases.

References

Adrianto, I., Wang, S., Wiley, G.B., Lessard, C.J., Kelly, J.A., Adler, A.J., Glenn, S.B., Williams, A.H., Ziegler, J.T., Comeau, M.E.*, et al.* (2012). Association of two independent functional risk haplotypes in TNIP1 with systemic lupus erythematosus. Arthritis Rheum *64*, 3695-3705.

Allanore, Y., Saad, M., Dieude, P., Avouac, J., Distler, J.H., Amouyel, P., Matucci-Cerinic, M., Riemekasten, G., Airo, P., Melchers, I.*, et al.* (2011). Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. PLoS Genet *7*, e1002091.

Beutler, B., and Cerami, A. (1988). Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Annu Rev Biochem *57*, 505-518.

Beutler, B., and Cerami, A. (1989). The biology of cachectin/TNF--a primary mediator of the host response. Annu Rev Immunol *7*, 625-655.

Beutler, B.A. (2009). TLRs and innate immunity. Blood *113*, 1399-1407.

Birnberg, T., Bar-On, L., Sapoznikov, A., Caton, M.L., Cervantes-Barragan, L., Makia, D., Krauthgamer, R., Brenner, O., Ludewig, B., Brockschnieder, D.*, et al.* (2008). Lack of conventional dendritic cells is compatible with normal

development and T cell homeostasis, but causes myeloid proliferative syndrome. Immunity *29*, 986-997.

Blasius, A.L., and Beutler, B. (2010). Intracellular toll-like receptors. Immunity *32*, 305-315.

Blumberg, H., Dinh, H., Trueblood, E.S., Pretorius, J., Kugler, D., Weng, N., Kanaly, S.T., Towne, J.E., Willis, C.R., Kuechle, M.K.*, et al.* (2007). Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. J Exp Med *204*, 2603-2614.

Bonnet, M.C., Preukschat, D., Welz, P.S., van Loo, G., Ermolaeva, M.A., Bloch, W., Haase, I., and Pasparakis, M. (2011). The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity *35*, 572-582.

Boone, D.L., Turer, E.E., Lee, E.G., Ahmad, R.C., Wheeler, M.T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsu, O.*, et al.* (2004). The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat Immunol *5*, 1052-1060.

Boonyasrisawat, W., Eberle, D., Bacci, S., Zhang, Y.Y., Nolan, D., Gervino, E.V., Johnstone, M.T., Trischitta, V., Shoelson, S.E., and Doria, A. (2007). Tag

polymorphisms at the A20 (TNFAIP3) locus are associated with lower gene expression and increased risk of coronary artery disease in type 2 diabetes. Diabetes *56*, 499-505.

Bossini-Castillo, L., Martin, J.E., Broen, J., Simeon, C.P., Beretta, L., Gorlova, O.Y., Vonk, M.C., Ortego-Centeno, N., Espinosa, G., Carreira, P.*, et al.* (2013). Confirmation of TNIP1 but not RHOB and PSORS1C1 as systemic sclerosis risk factors in a large independent replication study. Ann Rheum Dis *72*, 602-607.

Bowes, J., Orozco, G., Flynn, E., Ho, P., Brier, R., Marzo-Ortega, H., Coates, L., McManus, R., Ryan, A.W., Kane, D.*, et al.* (2011). Confirmation of TNIP1 and IL23A as susceptibility loci for psoriatic arthritis. Ann Rheum Dis *70*, 1641-1644.

Brint, E.K., Xu, D., Liu, H., Dunne, A., McKenzie, A.N., O'Neill, L.A., and Liew, F.Y. (2004). ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. Nat Immunol *5*, 373-379.

Cai, L.Q., Wang, Z.X., Lu, W.S., Han, J.W., Sun, L.D., Du, W.H., Zhang, S.M., Zuo, X.B., Zhang, X.J., and Yang, S. (2010). A single-nucleotide polymorphism of the TNFAIP3 gene is associated with systemic lupus erythematosus in Chinese Han population. Mol Biol Rep *37*, 389-394.

Cai, Y., Shen, X., Ding, C., Qi, C., Li, K., Li, X., Jala, V.R., Zhang, H.G., Wang, T., Zheng, J*., et al.* (2011). Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. Immunity *35*, 596-610.

Callahan, J.A., Hammer, G.E., Agelides, A., Duong, B.H., Oshima, S., North, J., Advincula, R., Shifrin, N., Truong, H.A., Paw, J*., et al.* (2013). Cutting Edge: ABIN-1 Protects against Psoriasis by Restricting MyD88 Signals in Dendritic Cells. J Immunol *191*, 535-539.

Cantisani, C., Lazic, T., Richetta, A.G., Clerico, R., Mattozzi, C., and Calvieri, S. (2012). Imiquimod 5% cream use in dermatology, side effects and recent patents. Recent Pat Inflamm Allergy Drug Discov *6*, 65-69.

Capon, F., Burden, A.D., Trembath, R.C., and Barker, J.N. (2012). Psoriasis and other complex trait dermatoses: from Loci to functional pathways. J Invest Dermatol *132*, 915-922.

Caton, M.L., Smith-Raska, M.R., and Reizis, B. (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. J Exp Med *204*, 1653-1664.

Chen, G., and Goeddel, D.V. (2002). TNF-R1 signaling: a beautiful pathway. Science *296*, 1634-1635.

Chen, J., and Chen, Z.J. (2013). Regulation of NF-kappaB by ubiquitination. Curr Opin Immunol *25*, 4-12.

Chen, M., Wang, Y.H., Wang, Y., Huang, L., Sandoval, H., Liu, Y.J., and Wang, J. (2006). Dendritic cell apoptosis in the maintenance of immune tolerance. Science *311*, 1160-1164.

Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. Mol Cell *33*, 275-286.

Cui, W., Xiao, N., Xiao, H., Zhou, H., Yu, M., Gu, J., and Li, X. (2012). beta-TrCP-mediated IRAK1 degradation releases TAK1-TRAF6 from the membrane to the cytosol for TAK1-dependent NF-kappaB activation. Mol Cell Biol *32*, 3990-4000.

Consortium, W.T.C.C. (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature *447*, 661-678.

Doffinger, R., Smahi, A., Bessia, C., Geissmann, F., Feinberg, J., Durandy, A., Bodemer, C., Kenwrick, S., Dupuis-Girod, S., Blanche, S*., et al.* (2001). X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. Nat Genet *27*, 277-285.

Doi, T.S., Marino, M.W., Takahashi, T., Yoshida, T., Sakakura, T., Old, L.J., and Obata, Y. (1999). Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality. Proc Natl Acad Sci U S A *96*, 2994-2999.

Ea, C.K., Deng, L., Xia, Z.P., Pineda, G., and Chen, Z.J. (2006). Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell *22*, 245-257.

Elsby, L.M., Orozco, G., Denton, J., Worthington, J., Ray, D.W., and Donn, R.P. (2010). Functional evaluation of TNFAIP3 (A20) in rheumatoid arthritis. Clin Exp Rheumatol *28*, 708-714.

Eyre, S., Hinks, A., Bowes, J., Flynn, E., Martin, P., Wilson, A.G., Morgan, A.W., Emery, P., Steer, S., Hocking, L.J.*, et al.* (2010). Overlapping genetic susceptibility variants between three autoimmune disorders: rheumatoid arthritis, type 1 diabetes and coeliac disease. Arthritis Res Ther *12*, R175.

Fanti, P.A., Dika, E., Vaccari, S., Miscial, C., and Varotti, C. (2006). Generalized psoriasis induced by topical treatment of actinic keratosis with imiquimod. Int J Dermatol *45*, 1464-1465.

Fung, E.Y., Smyth, D.J., Howson, J.M., Cooper, J.D., Walker, N.M., Stevens, H., Wicker, L.S., and Todd, J.A. (2009). Analysis of 17 autoimmune disease-associated variants in type 1 diabetes identifies 6q23/TNFAIP3 as a susceptibility locus. Genes Immun *10*, 188-191.

Gallagher, J., Howlin, J., McCarthy, C., Murphy, E.P., Bresnihan, B., FitzGerald, O., Godson, C., Brady, H.R., and Martin, F. (2003). Identification of Naf1/ABIN-1 among TNF-alpha-induced expressed genes in human synoviocytes using oligonucleotide microarrays. FEBS Lett *551*, 8-12.

Gateva, V., Sandling, J.K., Hom, G., Taylor, K.E., Chung, S.A., Sun, X., Ortmann, W., Kosoy, R., Ferreira, R.C., Nordmark, G.*, et al.* (2009). A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. Nat Genet *41*, 1228-1233.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. Cell *109 Suppl*, S81-96.

Graham, R.R., Cotsapas, C., Davies, L., Hackett, R., Lessard, C.J., Leon, J.M., Burtt, N.P., Guiducci, C., Parkin, M., Gates, C.*, et al.* (2008). Genetic variants near TNFAIP3 on 6q23 are associated with systemic lupus erythematosus. Nat Genet *40*, 1059-1061.

Gregersen, P.K., Kosoy, R., Lee, A.T., Lamb, J., Sussman, J., McKee, D., Simpfendorfer, K.R., Pirskanen-Matell, R., Piehl, F., Pan-Hammarstrom, Q.*, et al.* (2012). Risk for myasthenia gravis maps to a (151) Pro-->Ala change in TNIP1 and to human leukocyte antigen-B*08. Ann Neurol *72*, 927-935.

Griffiths, C.E., Strober, B.E., van de Kerkhof, P., Ho, V., Fidelus-Gort, R., Yeilding, N., Guzzo, C., Xia, Y., Zhou, B., Li, S.*, et al.* (2010). Comparison of ustekinumab and etanercept for moderate-to-severe psoriasis. N Engl J Med *362*, 118-128.

Hammer, G.E., Turer, E.E., Taylor, K.E., Fang, C.J., Advincula, R., Oshima, S., Barrera, J., Huang, E.J., Hou, B., Malynn, B.A.*, et al.* (2011). Expression of A20 by dendritic cells preserves immune homeostasis and prevents colitis and spondyloarthritis. Nat Immunol *12*, 1184-1193.

Han, J., Zhong, C.Q., and Zhang, D.W. (2011). Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nat Immunol *12*, 1143-1149.

Han, J.W., Zheng, H.F., Cui, Y., Sun, L.D., Ye, D.Q., Hu, Z., Xu, J.H., Cai, Z.M., Huang, W., Zhao, G.P.*, et al.* (2009). Genome-wide association study in a

Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. Nat Genet *41*, 1234-1237.

Heyninck, K., De Valck, D., Vanden Berghe, W., Van Criekinge, W., Contreras, R., Fiers, W., Haegeman, G., and Beyaert, R. (1999). The zinc finger protein A20 inhibits TNF-induced NF-kappaB-dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF-kappaB-inhibiting protein ABIN. J Cell Biol *145*, 1471-1482.

Hou, B., Reizis, B., and DeFranco, A.L. (2008). Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity *29*, 272-282.

Hsu, H., Huang, J., Shu, H.B., Baichwal, V., and Goeddel, D.V. (1996). TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity *4*, 387-396.

Huang, G., Wang, Y., and Chi, H. (2012). Regulation of TH17 cell differentiation by innate immune signals. Cell Mol Immunol *9*, 287-295.

Hughes, L.B., Reynolds, R.J., Brown, E.E., Kelley, J.M., Thomson, B., Conn, D.L., Jonas, B.L., Westfall, A.O., Padilla, M.A., Callahan, L.F.*, et al.* (2010). Most common single-nucleotide polymorphisms associated with rheumatoid arthritis in

persons of European ancestry confer risk of rheumatoid arthritis in African Americans. Arthritis Rheum *62*, 3547-3553.

Iwasaki, A., and Medzhitov, R. (2010). Regulation of adaptive immunity by the innate immune system. Science *327*, 291-295.

Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V.C., Lill, J.R., and Ashkenazi, A. (2009). Cullin3-Based Polyubiquitination and p62-Dependent Aggregation of Caspase-8 Mediate Extrinsic Apoptosis Signaling. Cell *137*, 721-735.

Joffre, O., Nolte, M.A., Sporri, R., and Reis e Sousa, C. (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. Immunol Rev *227*, 234-247.

Johnson-Huang, L.M., Lowes, M.A., and Krueger, J.G. (2012). Putting together the psoriasis puzzle: an update on developing targeted therapies. Dis Model Mech *5*, 423-433.

Kaiser, W.J., Upton, J.W., Long, A.B., Livingston-Rosanoff, D., Daley-Bauer, L.P., Hakem, R., Caspary, T., and Mocarski, E.S. (2011). RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature *471*, 368-372.

Kang, S.J., Liang, H.E., Reizis, B., and Locksley, R.M. (2008). Regulation of hierarchical clustering and activation of innate immune cells by dendritic cells. Immunity *29*, 819-833.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol *18*, 621-663.

Kawai, T., and Akira, S. (2006). TLR signaling. Cell Death Differ *13*, 816-825.

Kawasaki, A., Ito, S., Furukawa, H., Hayashi, T., Goto, D., Matsumoto, I., Kusaoi, M., Ohashi, J., Graham, R.R., Matsuta, K.*, et al.* (2010). Association of TNFAIP3 interacting protein 1, TNIP1 with systemic lupus erythematosus in a Japanese population: a case-control association study. Arthritis Res Ther *12*, R174.

Kim, I., Rao, H., Li, W., and Ye, Y. (2006). What's Ub chain linkage got to do with it? Polyubiquitin chains: functions, structures, and mechanisms. Sci STKE *2006*, pe18.

Kobayashi, K., Hernandez, L.D., Galan, J.E., Janeway, C.A., Jr., Medzhitov, R., and Flavell, R.A. (2002). IRAK-M is a negative regulator of Toll-like receptor signaling. Cell *110*, 191-202.

Kool, M., van Loo, G., Waelput, W., De Prijck, S., Muskens, F., Sze, M., van Praet, J., Branco-Madeira, F., Janssens, S., Reizis, B.*, et al.* (2011). The ubiquitin-editing protein A20 prevents dendritic cell activation, recognition of apoptotic cells, and systemic autoimmunity. Immunity *35*, 82-96.

Koumakis, E., Giraud, M., Dieude, P., Cohignac, V., Cuomo, G., Airo, P., Hachulla, E., Matucci-Cerinic, M., Diot, E., Caramaschi, P.*, et al.* (2012). Brief report: candidate gene study in systemic sclerosis identifies a rare and functional variant of the TNFAIP3 locus as a risk factor for polyautoimmunity. Arthritis Rheum *64*, 2746-2752.

Krikos, A., Laherty, C.D., and Dixit, V.M. (1992). Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. J Biol Chem *267*, 17971-17976.

Larsen, G.L., and Henson, P.M. (1983). Mediators of inflammation. Annu Rev Immunol *1*, 335-359.

Lee, E.G., Boone, D.L., Chai, S., Libby, S.L., Chien, M., Lodolce, J.P., and Ma, A. (2000). Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science *289*, 2350-2354.

Lee, E.W., Kim, J.H., Ahn, Y.H., Seo, J., Ko, A., Jeong, M., Kim, S.J., Ro, J.Y., Park, K.M., Lee, H.W.*, et al.* (2012). Ubiquitination and degradation of the FADD

adaptor protein regulate death receptor-mediated apoptosis and necroptosis. Nat Commun *3:978.*, 10.1038/ncomms1981.

Leonardi, C.L., Kimball, A.B., Papp, K.A., Yeilding, N., Guzzo, C., Wang, Y., Li, S., Dooley, L.T., and Gordon, K.B. (2008). Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). Lancet *371*, 1665-1674.

Li, W., and Ye, Y. (2008). Polyubiquitin chains: functions, structures, and mechanisms. Cell Mol Life Sci *65*, 2397-2406.

Ma, A., and Malynn, B.A. (2012). A20: linking a complex regulator of ubiquitylation to immunity and human disease. Nat Rev Immunol *12*, 774-785.

Marrakchi, S., Guigue, P., Renshaw, B.R., Puel, A., Pei, X.Y., Fraitag, S., Zribi, J., Bal, E., Cluzeau, C., Chrabieh, M.*, et al.* (2011). Interleukin-36-receptor antagonist deficiency and generalized pustular psoriasis. N Engl J Med *365*, 620-628.

Matzinger, P. (2002). The danger model: a renewed sense of self. Science *296*, 301-305.

Mauro, C., Pacifico, F., Lavorgna, A., Mellone, S., Iannetti, A., Acquaviva, R., Formisano, S., Vito, P., and Leonardi, A. (2006). ABIN-1 binds to NEMO/IKKgamma and co-operates with A20 in inhibiting NF-kappaB. J Biol Chem *281*, 18482-18488.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.

Musone, S.L., Taylor, K.E., Lu, T.T., Nititham, J., Ferreira, R.C., Ortmann, W., Shifrin, N., Petri, M.A., Kamboh, M.I., Manzi, S.*, et al.* (2008). Multiple polymorphisms in the TNFAIP3 region are independently associated with systemic lupus erythematosus. Nat Genet *40*, 1062-1064.

Musone, S.L., Taylor, K.E., Nititham, J., Chu, C., Poon, A., Liao, W., Lam, E.T., Ma, A., Kwok, P.Y., and Criswell, L.A. (2011). Sequencing of TNFAIP3 and association of variants with multiple autoimmune diseases. Genes Immun *12*, 176-182.

Nair, R.P., Duffin, K.C., Helms, C., Ding, J., Stuart, P.E., Goldgar, D., Gudjonsson, J.E., Li, Y., Tejasvi, T., Feng, B.J.*, et al.* (2009). Genome-wide scan reveals association of psoriasis with IL-23 and NF-kappaB pathways. Nat Genet *41*, 199-204.

Nanda, S.K., Venigalla, R.K., Ordureau, A., Patterson-Kane, J.C., Powell, D.W., Toth, R., Arthur, J.S., and Cohen, P. (2011). Polyubiquitin binding to ABIN1 is required to prevent autoimmunity. J Exp Med *208*, 1215-1228.

Nestle, F.O., Di Meglio, P., Qin, J.Z., and Nickoloff, B.J. (2009a). Skin immune sentinels in health and disease. Nat Rev Immunol *9*, 679-691.

Nestle, F.O., Kaplan, D.H., and Barker, J. (2009b). Psoriasis. N Engl J Med *361*, 496-509.

O'Donnell, M.A., Legarda-Addison, D., Skountzos, P., Yeh, W.C., and Ting, A.T. (2007). Ubiquitination of RIP1 Regulates an NF-kB-Independent Cell-Death Switch in TNF Signaling. Current Biology *17*, 418-424.

Oberst, A., Dillon, C.P., Weinlich, R., McCormick, L.L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G.S., and Green, D.R. (2011). Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature *471*, 363-367.

Ohnmacht, C., Pullner, A., King, S.B., Drexler, I., Meier, S., Brocker, T., and Voehringer, D. (2009). Constitutive ablation of dendritic cells breaks self-

tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. J Exp Med *206*, 549-559.

Onoufriadis, A., Simpson, M.A., Pink, A.E., Di Meglio, P., Smith, C.H., Pullabhatla, V., Knight, J., Spain, S.L., Nestle, F.O., Burden, A.D.*, et al.* (2011). Mutations in IL36RN/IL1F5 are associated with the severe episodic inflammatory skin disease known as generalized pustular psoriasis. Am J Hum Genet *89*, 432-437.

Opipari, A.W., Jr., Boguski, M.S., and Dixit, V.M. (1990). The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. J Biol Chem *265*, 14705-14708.

Ordureau, A., Smith, H., Windheim, M., Peggie, M., Carrick, E., Morrice, N., and Cohen, P. (2008). The IRAK-catalysed activation of the E3 ligase function of Pellino isoforms induces the Lys63-linked polyubiquitination of IRAK1. Biochem J *409*, 43-52.

Oshima, S., Turer, E.E., Callahan, J.A., Chai, S., Advincula, R., Barrera, J., Shifrin, N., Lee, B., Benedict Yen, T.S., Woo, T.*, et al.* (2009). ABIN-1 is a ubiquitin sensor that restricts cell death and sustains embryonic development. Nature *457*, 906-909.

Oudshoorn, D., Versteeg, G.A., and Kikkert, M. (2012). Regulation of the innate immune system by ubiquitin and ubiquitin-like modifiers. Cytokine Growth Factor Rev *23*, 273-282.

Papp, K.A., Langley, R.G., Lebwohl, M., Krueger, G.G., Szapary, P., Yeilding, N., Guzzo, C., Hsu, M.C., Wang, Y., Li, S.*, et al.* (2008). Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 52-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 2). Lancet *371*, 1675-1684.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu Rev Biochem *70*, 503-533.

Pickart, C.M., and Fushman, D. (2004). Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol *8*, 610-616.

Plenge, R.M., Cotsapas, C., Davies, L., Price, A.L., de Bakker, P.I., Maller, J., Pe'er, I., Burtt, N.P., Blumenstiel, B., DeFelice, M.*, et al.* (2007). Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. Nat Genet *39*, 1477-1482.

Reis e Sousa, C. (2006). Dendritic cells in a mature age. Nat Rev Immunol *6*, 476-483.

Rubartelli, A., and Lotze, M.T. (2007). Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. Trends Immunol *28*, 429-436.

Shimane, K., Kochi, Y., Horita, T., Ikari, K., Amano, H., Hirakata, M., Okamoto, A., Yamada, R., Myouzen, K., Suzuki, A.*, et al.* (2010). The association of a nonsynonymous single-nucleotide polymorphism in TNFAIP3 with systemic lupus erythematosus and rheumatoid arthritis in the Japanese population. Arthritis Rheum *62*, 574-579.

Steinman, R.M. (1991). The dendritic cell system and its role in immunogenicity. Annu Rev Immunol *9*, 271-296.

Steinman, R.M. (2012). Decisions about dendritic cells: past, present, and future. Annu Rev Immunol *30*, 1-22.

Strange, A., Capon, F., Spencer, C.C., Knight, J., Weale, M.E., Allen, M.H., Barton, A., Band, G., Bellenguez, C., Bergboer, J.G.*, et al.* (2010). A genome-wide association study identifies new psoriasis susceptibility loci and an interaction between HLA-C and ERAP1. Nat Genet *42*, 985-990.

Stranges, P.B., Watson, J., Cooper, C.J., Choisy-Rossi, C.M., Stonebraker, A.C., Beighton, R.A., Hartig, H., Sundberg, J.P., Servick, S., Kaufmann, G.*, et al.* (2007). Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. Immunity *26*, 629-641.

Sun, L.D., Cheng, H., Wang, Z.X., Zhang, A.P., Wang, P.G., Xu, J.H., Zhu, Q.X., Zhou, H.S., Ellinghaus, E., Zhang, F.R.*, et al.* (2010). Association analyses identify six new psoriasis susceptibility loci in the Chinese population. Nat Genet *42*, 1005-1009.

Sun, S.C. (2008). Deubiquitylation and regulation of the immune response. Nat Rev Immunol *8*, 501-511.

Sutton, C.E., Lalor, S.J., Sweeney, C.M., Brereton, C.F., Lavelle, E.C., and Mills, K.H. (2009). Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity *31*, 331-341.

Szabowski, A., Maas-Szabowski, N., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., Fusenig, N.E., and Angel, P. (2000). c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. Cell *103*, 745-755.

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. Annu Rev Immunol *21*, 335-376.

Tavares, R.M., Turer, E.E., Liu, C.L., Advincula, R., Scapini, P., Rhee, L., Barrera, J., Lowell, C.A., Utz, P.J., Malynn, B.A.*, et al.* (2010). The ubiquitin modifying enzyme A20 restricts B cell survival and prevents autoimmunity. Immunity *33*, 181-191.

Tejasvi, T., Stuart, P.E., Chandran, V., Voorhees, J.J., Gladman, D.D., Rahman, P., Elder, J.T., and Nair, R.P. (2012). TNFAIP3 gene polymorphisms are associated with response to TNF blockade in psoriasis. J Invest Dermatol *132*, 593-600.

Thomson, W., Barton, A., Ke, X., Eyre, S., Hinks, A., Bowes, J., Donn, R., Symmons, D., Hider, S., Bruce, I.N.*, et al.* (2007). Rheumatoid arthritis association at 6q23. Nat Genet *39*, 1431-1433.

Tian, B., Nowak, D.E., Jamaluddin, M., Wang, S., and Brasier, A.R. (2005). Identification of direct genomic targets downstream of the nuclear factor-kappaB transcription factor mediating tumor necrosis factor signaling. J Biol Chem *280*, 17435-17448.

Tortola, L., Rosenwald, E., Abel, B., Blumberg, H., Schafer, M., Coyle, A.J., Renauld, J.C., Werner, S., Kisielow, J., and Kopf, M. (2012). Psoriasiform dermatitis is driven by IL-36-mediated DC-keratinocyte crosstalk. J Clin Invest *122*, 3965-3976.

Trynka, G., Zhernakova, A., Romanos, J., Franke, L., Hunt, K.A., Turner, G., Bruinenberg, M., Heap, G.A., Platteel, M., Ryan, A.W.*, et al.* (2009). Coeliac disease-associated risk variants in TNFAIP3 and REL implicate altered NF-kappaB signalling. Gut *58*, 1078-1083.

Turer, E.E., Tavares, R.M., Mortier, E., Hitotsumatsu, O., Advincula, R., Lee, B., Shifrin, N., Malynn, B.A., and Ma, A. (2008). Homeostatic MyD88-dependent signals cause lethal inflamMation in the absence of A20. J Exp Med *205*, 451-464.

van der Fits, L., Mourits, S., Voerman, J.S., Kant, M., Boon, L., Laman, J.D., Cornelissen, F., Mus, A.M., Florencia, E., Prens, E.P.*, et al.* (2009). Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. J Immunol *182*, 5836-5845.

Vanlangenakker, N., Vanden Berghe, T., and Vandenabeele, P. (2012). Many stimuli pull the necrotic trigger, an overview. Cell Death Differ *19*, 75-86.

Vigne, S., Palmer, G., Lamacchia, C., Martin, P., Talabot-Ayer, D., Rodriguez, E., Ronchi, F., Sallusto, F., Dinh, H., Sims, J.E.*, et al.* (2011). IL-36R ligands are potent regulators of dendritic and T cells. Blood *118*, 5813-5823.

Wagner, S., Carpentier, I., Rogov, V., Kreike, M., Ikeda, F., Lohr, F., Wu, C.J., Ashwell, J.D., Dotsch, V., Dikic, I.*, et al.* (2008). Ubiquitin binding mediates the NF-kappaB inhibitory potential of ABIN proteins. Oncogene *27*, 3739-3745.

Wang, Y., Tang, Y., Teng, L., Wu, Y., Zhao, X., and Pei, G. (2006). Association of beta-arrestin and TRAF6 negatively regulates Toll-like receptor-interleukin 1 receptor signaling. Nat Immunol *7*, 139-147.

Wertz, I.E., O'Rourke, K.M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D.L.*, et al.* (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature *430*, 694-699.

Wilkinson, K.D. (1997). Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. Faseb J *11*, 1245-1256.

Windheim, M., Stafford, M., Peggie, M., and Cohen, P. (2008). Interleukin-1 (IL-1) induces the Lys63-linked polyubiquitination of IL-1 receptor-associated kinase

1 to facilitate NEMO binding and the activation of IkappaBalpha kinase. Mol Cell Biol *28*, 1783-1791.

Wrighton, K.H. (2011). Cell death: A killer puts a stop on necroptosis. Nat Rev Mol Cell Biol *12*, 279.

Wu, C.J., Conze, D.B., Li, T., Srinivasula, S.M., and Ashwell, J.D. (2006). Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat Cell Biol *8*, 398-406.

Wullaert, A., Wielockx, B., Van Huffel, S., Bogaert, V., De Geest, B., Papeleu, P., Schotte, P., El Bakkouri, K., Heyninck, K., Libert, C.*, et al.* (2005). Adenoviral gene transfer of ABIN-1 protects mice from TNF/galactosamine-induced acute liver failure and lethality. Hepatology *42*, 381-389.

Zhang, H., Zhou, X., McQuade, T., Li, J., Chan, F.K., and Zhang, J. (2011). Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature *471*, 373-376. doi: 310.1038/nature09878. Epub 02011 Mar 09872.

Zhou, J., Wu, R., High, A.A., Slaughter, C.A., Finkelstein, D., Rehg, J.E., Redecke, V., and Hacker, H. (2011). A20-binding inhibitor of NF-kappaB (ABIN1) controls Toll-like receptor-mediated CCAAT/enhancer-binding protein beta

activation and protects from inflammatory disease. Proc Natl Acad Sci U S A *108*, E998-1006.

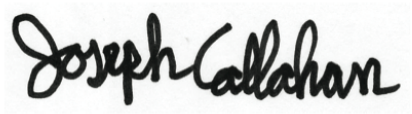
Zhu, J., Yamane, H., and Paul, W.E. (2010). Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol *28*, 445-489.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.



Author Signature

Sept. 5, 2013

Date