Dissection of Signaling in Inflammation: Three Novel Inflammatory Regulators

THORSTEN BERGER, MARY E. SAUNDERS, AND TAK W. MAK

The Campbell Family Institute for Breast Cancer Research and Ontario Cancer Institute, University Health Network, Toronto, Ontario M5G 2C1, Canada

Correspondence: tmak@uhnres.utoronto.ca

Uncontrolled inflammation is a feature of autoimmune diseases and autoinflammatory syndromes and may promote tumorigenesis. Thus, identifying molecules that regulate the signaling pathways triggering, mediating, and suppressing inflammation could be helpful in developing new therapeutic approaches for these debilitating diseases. In this review, we present new information on three molecules with important roles in controlling inflammation: MALT1, Ariadne-2, and acetylcholine. We summarize our current state of knowledge of how these molecules function, and how they are involved in pathways of NF- κ B activation or vagal nerve stimulation associated with inflammation.

Inflammation is a double-edged sword: essential for defense against incipient disease, but also harmful to the body if the response persists. Uncontrolled inflammation is a feature of autoimmune diseases and autoinflammatory syndromes, and evidence is accumulating that chronic inflammation sets up conditions that favor tumorigenesis. Thus, identifying molecules that regulate the signaling pathways initiating, mediating, and suppressing inflammation could be helpful in developing new therapeutic approaches for these debilitating diseases.

Signaling pathways triggered during a normal inflammatory response lead to the activation of numerous cell types mediating innate and adaptive immune responses. A key group of transcription factors involved in the activation and effector functions of many of these leukocytes is known as the NF- κ B family. Two pathways (Fig. 1) lead to the activation of NF- κ B family members: the classical or canonical pathway and the alternative or noncanonical pathway (Gasparini and Feldmann 2012). In both cases, the resulting transcriptionally active NF- κ B molecule is a dimer consisting of a family member such as p50 or p52, each of which possesses a Rel homology domain (RHD), and a family member such as RelA (p65), RelB, or c-Rel, each of which possesses both an RHD domain and a transcription activation domain. Transcriptionally active NF- κ B molecules initiate the transcription of specific sets of target genes that trigger not only the production of proinflammatory cytokines but also the recruitment, proliferation, and activation of innate and adaptive leukocytes. Tellingly, the NF- κ B pathway includes a self-control mechanism that eventually limits inflammation. Toward the end of the response, the expression of genes that promote leukocyte apoptosis or encode anti-inflammatory cytokines commences. It is when the control mechanisms governing NF- κ B signaling malfunction and fail to damp down inflammation, or are insufficient to limit rampant inflammation, that disease may be manifested.

Under steady-state conditions, inflammation does not occur because NF- κ B is prevented from reaching the nucleus and thus accessing the promoters of its target genes. This blockage is mediated by members of the “inhibitor of κ B” (I κ B) family of cytoplasmic inhibitors, which bind to NF- κ B proteins and sequester them in the cytoplasm. NF- κ B activation therefore generally requires the degradation of I κ B, which frees the NF- κ B protein and allows it to enter the nucleus and initiate transcription. The ubiquitination and subsequent proteasomal degradation of I κ B is triggered by phosphorylation of its I κ B α subunit, an event mediated by activation of the I κ B kinase (IKK) complex. IKK complex activation in turn depends on the CBM complex, which comprises three signaling proteins CARMA1 (caspase recruitment domain-containing protein 11; also known as CARD-containing MAGUK protein 1), BCL10 (B-cell lymphoma/leukemia 10), and MALT1 (mucosa-associated lymphoid tissue [MALT] lymphoma translocation gene 1) (Fig. 1). It is now becoming clear that MALT1 plays more than just a support role during NF- κ B activation and has profound effects on its outcome.

Another molecule recently found to be involved in regulating NF- κ B activation is Ariadne-2 (ARIH2). ARIH2 is a member of the RBR family of E3 ligases, a subset of degradative enzymes that are unusual in both their structure and method of extending ubiquitin chains (van der Reijden et al. 1999; Aguilera et al. 2000; Tan et al. 2000; Marin et al. 2004). The prototypical RBR E3 ligase TRIM-AD3 has been shown to be crucial for TLR degradation and thus the negative regulation of TLR-triggered NF- κ B activation leading to inflammation (Chuang and Ulevitch 2004; Fearn et al. 2006; Nakhaei et al. 2009). ARIH2 was first identified in flies and plants, and the mammalian form has only recently come under study.

It has been known since the 1980s that the body’s nervous system and immune system are linked (Levy et al.

Figure 1. Pathways of NF- κ B activation. The classical or canonical NF- κ B pathway is activated by numerous stimuli, including engagement of TCRs or BCRs. Such engagement leads to the activation of the classical I κ B kinase (IKK) complex through the CBM complex, which is composed of the three signaling proteins CARMA1, BCL10, and MALT1. IKK complex activation results in phosphorylation of its I κ B α subunit, triggering the ubiquitination and subsequent proteasomal degradation of I κ B; NF- κ B is then free to enter the nucleus. Activation of the noncanonical NF- κ B pathway leads to the phosphorylation of p100 and its partial proteasome-mediated degradation to p52, a process that is regulated through “NF- κ B-inducing kinase” (NIK) and IKK- α . In both pathways, transcriptionally active NF- κ B molecules initiate the transcription of specific sets of target genes. The proteins encoded by these genes are involved in cell survival, development, and differentiation, as well as in proinflammatory cytokine production.

1985; 1987), but elucidation of the precise connections has lagged. An intriguing circuit exists that involves the vagus nerve and inflammation. The vagus nerve arises in the brain and extends to the visceral organs, establishing a neural circuit that regulates the physiological responses of these tissues to injury, infection, or an altered environment. One of these responses is the suppression of proinflammatory cytokine production, leading to the naming of this circuit as the “inflammatory reflex” (Tracey 2002). It has been shown that electrical stimulation of the vagus nerve can reduce the tissue damage associated with the excessive inflammatory responses underlying sepsis or endotoxemia (Borovikova et al. 2000; Guarini et al. 2003; Huston et al. 2007; 2009). The suppressive signal is delivered when acetylcholine (ACh) released in response to vagus nerve stimulation binds to nicotinic acetylcholine receptors (nAChR) expressed on the surfaces of cytokine-producing splenic cells. Accordingly, cholinergic agonists that stimulate the vagus nerve and increase the production of ACh have decreased cytokine production and ameliorated inflammatory disease in animal models (van Westerloo et al. 2006; Giebelen et al. 2007; Pavlov et al. 2007; Yeboah et al. 2008; Kox et al. 2009;

Rosas-Ballina et al. 2009). However, exactly which cell types release ACh and how they participate in the inflammatory reflex has been challenging to dissect.

In this article, we examine the roles of three molecules newly recognized as important for the control of inflammation: MALT1, Ariadne-2, and ACh. We summarize our current state of knowledge of how these molecules function and how they are involved in NF- κ B or ACh–nAChR signaling pathways.

MALT1

Our laboratory has long been interested in NF- κ B signaling in lymphocytes. Our group was one of the first to show that MALT1 is a crucial component of antigen receptor-triggered signaling in T cells (Ruefli-Brasse et al. 2003; Ruland et al. 2003). Using gene-targeted MALT1-deficient (MALT1^{−/−}) mice, we showed that MALT1 is essential for T-cell activation, proliferation, and IL-2 production in response to TCR ligation and is strictly required for NF- κ B activation induced by TCR engagement (Ruland et al. 2003). We have continued our explorations of MALT1 functions in T cells and have

discovered that MALT1 is indispensable for the encephalitogenic potential of IL-17-producing T helper (Th17) cells. The normal function of Th17 cells is to battle certain bacteria and fungi, but uncontrolled activation of this subset results in excessive inflammation and symptoms of autoimmune disease (McGeachy and McSorley 2012) (Fig. 2). In our most recent work, we show that MALT1^{-/-} mice are resistant to the induction of experimental autoimmune encephalomyelitis (EAE), a well-established mouse model of multiple sclerosis (MS) (Brustle et al. 2012).

A key conundrum in the EAE model has been the identity of the pathogenic CD4⁺ T-cell population responsible for the disease symptoms. Historically, Th1 cells were thought to be the culprit, in line with the elevated Th1 cytokines observed in relapsing MS patients (Imam et al. 2007). However, mice lacking IFN- γ are more, not less, susceptible to EAE induction (Krakowski and Owens 1996). Subsequent work has established that Th17 cells are in fact the main player in EAE/MS, and that the Th17-inducing cytokine IL-23, rather than the Th1-inducing cytokine IL-12, is crucial for EAE induction (Cua et al. 2003). Th17 cells have thus been studied intensively in vitro as well as in vivo in the context of EAE/MS (Langrish et al. 2005; Park et al. 2005; Littman and Rudensky 2010). Examinations in mouse models of the expression of Th17-specific cytokines (IL-23, IL-17, and GM-CSF) and transcription factors (ROR γ t and ROR α) and their impact on EAE have solidified the premise that it is Th17 cells that are the main drivers of EAE/MS (Dardalhon et al. 2008).

The dependence of EAE on both Th17 cells and MALT1 prompted us to examine the properties of MALT1-deficient Th17 cells. We showed that Th17 cells derived from MALT1^{-/-} mice were still capable of infiltrating the central nervous system but did not cause disease (Brustle et al. 2012). Accordingly, these non-pathogenic T cells showed a marked decrease in vitro and in vivo in their production of the encephalitogenic cytokines IL-17 and GM-CSF. However, no alterations to the expression of lineage-specific transcription factors were observed. These findings implied that MALT1^{-/-} mice do not have a defect in Th17 cell differentiation per se, but rather that these cells cannot perform normal Th17 effector functions and so are not encephalitogenic. We then investigated the scaffolding function of MALT1 by examining the differentiation of naïve wild-type (WT) T cells into Th1 or Th17 cells in the presence or absence of the pharmacological inhibitor “Nemo-binding domain” (NBD), which prevents IKK assembly and therefore blocks canonical NF- κ B activation (May et al. 2000). We found that Th17-polarized WT Th cells differentiating in the presence of NBD showed a drastic reduction in IL-17 secretion, whereas secretion of IFN- γ by Th1-polarized cells differentiating in the presence of NBD was not affected (Brustle et al. 2012). These data indicate that canonical NF- κ B activation involving MALT1 is more important for Th17 cells than for Th1 cells (Fig. 3).

In addition to its scaffolding function, MALT1 possesses paracaspase activity that cleaves specific protein substrates to facilitate T-cell activation (Coornaert et al. 2008; Rebeaud et al. 2008; Hailfinger et al. 2011). In contrast to

Figure 2. Role of MALT1 in pathways of T helper cell subset differentiation. Naïve T helper (Th) cells that undergo activation because of TCR/CD28 engagement differentiate into distinct effector cell subsets depending on the cytokine milieu. The presence of TGF- β plus IL-6 drives the differentiation of Th17 cells, a process that depends on the transcription factor ROR γ t and results in the production of IL-17 and GM-CSF. Although these molecules are helpful in combatting certain extracellular pathogens, they also contribute to the inflammation associated with diseases such as MS and arthritis. MALT1 is critical for this proinflammatory cytokine production. Th17 cells derived from MALT1^{-/-} mice show a marked decrease in IL-17 and GM-CSF production but no alterations to lineage-specific transcription factors.

Figure 3. Both canonical NF-κB activation and MALT1 protease activity are crucial for inflammatory cytokine production by activated Th17 cells. MALT1 has both a structural scaffolding function and a catalytic protease function. Treatment of WT T cells with the pharmacological inhibitor NBD, which prevents IKK assembly and therefore blocks canonical NF-κB activation, leads to a drastic reduction in IL-17 secretion. In contrast, NBD has no effect on IFN-γ secretion by Th1 cells. Similarly, treatment of WT T cells with the peptide caspase inhibitor Z-VPR-FMK, which inhibits MALT1's paracaspase activity, reduces IL-17 production by Th17 cells but has no impact on IFN-γ secretion by Th1 cells.

MALT1's scaffolding function, this protease activity is not crucial for IκBα degradation and acts downstream from IKK (Duwel et al. 2009). We showed that inhibition of MALT1's paracaspase activity by the peptide caspase inhibitor Z-VPR-FMK reduced IL-17 production by Th17-polarized WT Th cells but had no impact on Th1-cell differentiation (Fig. 3) (Brustle et al. 2012). Thus, both canonical NF-κB activation and MALT1 protease activity are crucial for the activation of Th17 cells but not Th1 cells. These data further suggest that Th17-cell activation is regulated by two independent pathways that both involve MALT1.

Th1 and Th17 cells can overlap in their cytokine production profiles, a plasticity (ability to convert from one subset to another) that is critical in various mouse and human inflammatory diseases (Lock et al. 2002; Kurschus et al. 2010; Feng et al. 2011; Hirota et al. 2011). We investigated whether Th17/Th1 plasticity was affected by MALT1 deficiency. We injected WT mice with MOG-specific, *in vitro*-generated Th17 cells derived from WT or MALT1^{-/-} mice and determined the frequencies of Th1 and Th17 cells in these animals 18 days later. In mice receiving WT Th17 cells, a small population of Th cells producing both IFN-γ and IL-17 was detected, indicative of Th17/Th1 plasticity (Brustle et al. 2012). In contrast, in mice receiving MALT1^{-/-} Th17 cells, no Th cells with the capacity to produce both IFN-γ and IL-17

were observed, and only a few Th cells able to produce IFN-γ alone were detected. This lack of double IFN-γ/IL-17 producers in the absence of MALT1 suggests a defect in Th1/Th17 plasticity that could explain the inability of MOG-specific MALT1^{-/-} Th17 cells to cause EAE in WT mice. Hirota et al. have shown using a fate-mapping system for IL-17-producing cells that almost all IFN-γ-producing cells in the spinal cords of mice with severe EAE were formerly Th17 cells (Hirota et al. 2011). However, we have found comparable levels of IFN-γ producers in the brains and spinal cords of WT and MALT1^{-/-} mice subjected to EAE induction, even though Th17 cells are missing in MALT1^{-/-} mice and these animals do not develop EAE. These data suggest that MALT1^{-/-} T cells are capable of differentiating normally into Th1 cells (but not Th17 cells) during EAE induction.

Many different mechanisms by which Th17 cells could be converted into Th1 cells have been proposed in the literature (Coomes et al. 2013). These mechanisms include not only T-cell-intrinsic factors such as micro-RNAs, transcription factor regulation, and epigenetic modifications but also extrinsic factors such as accessory innate leukocytes, the cytokine microenvironment, receptor regulation, and metabolic pathways. An example of an intrinsic conversion factor is the up-regulation of T-bet, the Th1-specific transcription factor that drives IFN-γ production and blocks IL-17 expression (Mathur et al. 2006). Another example that involves both intrinsic and extrinsic factors is the production by Th17 cells of IL-17A. In a colitis model, IL-17A treatment induced IL-12 production by innate leukocytes and therefore facilitated the conversion of Th17 cells into Th1 cells (Feng et al. 2011). The surrounding microenvironment alone can sometimes promote the generation or conversion of Th1 cells. For example, nitric oxide production by macrophages induces the expression of IL-12Rβ on T cells and therefore favors Th1 differentiation (Niedbala et al. 1999). Nevertheless, our MALT1^{-/-} Th17 cells did not convert into Th1 cells even in a WT microenvironment. The reason for this lack of plasticity remains unknown, as the transcriptional profile of MALT1^{-/-} T cells is not abnormal (Brustle et al. 2012). Our laboratory continues to investigate the properties of MALT1 and its perhaps surprisingly broad regulatory influence on inflammation.

ARIADNE-2

Our group recently collaborated with that of Dr. Marc Pellegrini of the Walter and Eliza Hall Institute to determine the physiological function of ARIH2 in mammals. We generated ARIH2-deficient (ARIH2^{-/-}) mice and found that only 3% of progeny on a mixed genetic background were able to avoid embryonic lethality (Lin et al. 2013). Although these animals survived beyond weaning, they died soon after reaching maturity because of massive multiorgan inflammation. To determine which pathway underlay this inflammation, we crossed ARIH2^{-/-} mice to animals lacking the TLR signaling adaptor MyD88. Combined deficiency of ARIH2 and MyD88

(but not IFN- γ or TNF) delayed but did not prevent death (Lin et al. 2013). These data implicated TLR-MyD88 signaling in the fatal inflammation killing ARIH2^{-/-} mice.

To circumvent the early lethality of ARIH2^{-/-} mice, we constructed chimeric animals bearing ARIH2^{-/-} hematopoietic cells. These mice once again developed severe inflammation characterized by high levels of serum proinflammatory cytokines and immunoglobulins. Lymphoid organs showed prominent infiltration by granulocytes, macrophages, and DCs, and other organs showed infiltration by T cells and myeloid cells. Subsequent examination of isolated leukocyte subsets revealed that ARIH2^{-/-} T cells and B cells did not have any intrinsic signaling defects. Although regulatory T-cell numbers were increased in ARIH2-deficient chimeras, these cells showed a normal capacity to inhibit responses of conventional effector T cells (Lin et al. 2013). Thus, the runaway inflammation in ARIH2^{-/-} mice and chimeras was not due to a T-cell defect.

Examination of DCs isolated from ARIH2^{-/-} mice established that ARIH2 plays no role in DC generation or differentiation, at least at the early stages. However, ARIH2^{-/-} DCs showed greater surface concentrations of MHC class II and T-cell costimulatory molecules and spontaneously produced high levels of proinflammatory cytokines, including IL-6, IL-12, and TNF. In collaboration with Dr. Pamela Ohashi's group of the Campbell Family Institute for Breast Cancer Research, we investigated whether ARIH2^{-/-} DCs could induce diabetes in the RIP-GP system (Ohashi et al. 1991; Oldstone et al. 1991). In RIP-GP mice, disease induction depends on DC maturation typically triggered by prior addition of a TLR ligand. Surprisingly, ARIH2^{-/-} DCs successfully induced diabetes in all mice tested in the absence of any TLR ligand, indicating that these cells had spontaneously become activated and had matured enough to activate autoreactive T cells. Furthermore, the transfer of ARIH2^{-/-} DCs alone into WT mice resulted in the rapid death of these animals due to excessive inflammation (Lin et al. 2013). These results indicated that ARIH2 is a crucial negative regulator of the inflammatory response.

Our next set of experiments explored signaling pathways in ARIH2^{-/-} DCs. NF- κ B signaling was found to be hyperactivated and sustained in these cells. Previous reports have established that, in contrast to the suppressive effects of cytoplasmic I κ B, a nuclear hypophosphorylated form of the I κ B β subunit of I κ B can bind to and activate nuclear p65, promoting NF- κ B signaling (Suyang et al. 1996; Rao et al. 2010; Scheibel et al. 2010). This signaling in turn leads to proinflammatory cytokine production. Our investigations revealed that ARIH2 physically binds to newly synthesized nuclear hypophosphorylated I κ B β and facilitates its ubiquitination and proteasomal degradation, thereby decreasing I κ B-mediated protection of p65 and promoting NF- κ B activation. In the absence of ARIH2, the unrestrained NF- κ B signaling in DCs leads to their spontaneous maturation and consequently uncontrolled inflammation (Lin et al. 2013). Thus, ARIH2 is a novel and essential regu-

lator of DC maturation and activation that is required to regulate the inflammatory response.

ARIH2 may be an attractive therapeutic target for drugs intended to reduce inflammation or autoimmune symptoms because the structure of this E3 ligase is comparatively unusual. An agonist that could increase ARIH2 activity and thus damp down DC activation and inflammation might have little effect on other E3 ligases essential for normal homeostasis. Such drugs could represent a boon for those suffering from immune hypersensitivities or autoimmune disease symptoms. Conversely, antagonists of ARIH2 could stimulate DC activity in situations where it might be beneficial, such as in conjunction with administration of an anticancer vaccine or where a patient is battling a persistent infection. We continue to investigate the properties of this novel inflammatory regulator with these goals in mind.

ACETYLCHOLINE

As noted above, it was known as early as the mid-1980s that a neural circuit existed in which vagal nerve fibers were connected to the splenic nerve enervating the spleen (Levy et al. 1985; 1987). Electrical stimulation of this circuit resulted in the release of ACh that shut down TNF production, reducing inflammation. However, it was unclear exactly which cells were the source of this ACh. Subsequent work revealed that lymphocytes could produce ACh (Kawashima et al. 1989; Rinner and Schauenstein 1993), a result we have capitalized on to determine whether the T and B cells in the white pulp of mouse spleen could produce sufficient ACh to suppress inflammation. In collaboration with Dr. Kevin Tracy's group, we used *nude* mice, which lack functional T cells, and showed that vagal nerve stimulation in these mutant animals did not reduce TNF production in response to endotoxin injection, in contrast to endotoxin-injected WT mice (Rosas-Ballina et al. 2009). Examinations of reporter mice expressing EGFP under the control of the promoter of the gene encoding choline acetyltransferase (ChAT), the enzyme that synthesizes ACh, showed that ACh was produced by only a small subset of CD4⁺ T cells (~4% of the total) bearing a memory surface marker phenotype. Furthermore, ChAT expression and ACh release were increased if these particular T cells were activated by anti-CD3 antibody (Rosas-Ballina et al. 2009).

Immunofluorescence analyses confirmed that ACh-producing T cells were located in areas of the splenic white pulp bearing nerve endings, and that these nerve endings were incapable of ChAT expression themselves. Importantly, the neural synapse glycoprotein synaptophysin was found to be localized next to ChAT-EGFP⁺ T cells in the white pulp. In addition, these T cells expressed mRNAs encoding the β_1 - and β_2 -adrenergic receptors, which have been shown to transduce splenic nerve signaling (Feldman et al. 1987; Kohm and Sanders 2001; Riether et al. 2011).

To test *in vivo* the validity of our hypothesis that the inflammatory reflex depends on ACh-producing splenic

T cells, we transferred the appropriate ChAT-EGFP⁺ CD4⁺ T-cell population (or control ChAT-EGFP⁻ CD4⁺ T cells) into *nude* mice, treated the animals with endotoxin, and stimulated the vagus nerve. Only mice bearing T cells capable of ChAT expression were able to activate the inflammatory reflex and suppress serum TNF production. Moreover, the adoptively transferred T cells migrated to the splenic white pulp and took up residence near splenic neurons known to respond to vagus nerve stimulation (Rosas-Ballina et al. 2009).

We confirmed that the effects we observed were indeed dependent on ChAT by using specific siRNA to “knock down” expression of ChAT in splenic CD4⁺ T cells. These T cells (or control T cells treated with scrambled siRNA) were adoptively transferred into *nude* mice, which were then subjected to endotoxin injection and vagus nerve stimulation. We found that mice with reduced ChAT expression were unable to invoke the inflammatory reflex and could not suppress TNF production (Rosas-Ballina et al. 2009). Thus, the ACh produced by splenic memory T cells is a novel regulator of inflammation, and manipulation of this neural circuit may yield therapeutic benefits. We continue to examine other secondary lymphoid tissues for the presence of ACh-producing T cells as well as nerve endings that may govern their responses to various stimuli.

CONCLUSION

Uncontrolled inflammation is a feature of many serious human disorders, and the pinpointing of molecules that can reduce inflammation is an important pursuit of modern biomedicine. We are hopeful that our identification of MALT1, ARIH2, and T-cell-produced ACh as regulators of inflammatory signaling will contribute to the design of new anti-inflammatory therapies.

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Thorsten Berger, Mary E. Saunders and Tak W. Mak

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