NF-κB signaling in lymphocytes: a new cast of characters

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Summary

Cell-surface antigen receptors on B and T lymphocytes are complex, multisubunit assemblies that must recruit several accessory proteins and activate multiple signaling pathways in order to illicit a proper immune response. One pathway culminates in the activation of specific protein kinase C (PKC) isoforms, which is necessary for the ultimate activation of the NF-κB transcription factor. Since NF-κB plays a crucial role in the adaptive immune response (e.g. in lymphocyte proliferation and cytokine production), it is important to understand the molecular mechanisms by which NF-κB is regulated. Nevertheless, the connection between PKC activation and NF-κB has remained a mystery that has now been at least partly solved. Recent findings implicate a new scaffolding protein, Bimp3/CARMA1/CARD11, as a key factor in bridging PKC activation with the downstream activation of Bcl10 and MALT1, which ultimately stimulates NF-κB. Since some of these signaling components are lymphocyte specific, therapeutic agents that block this pathway could blunt the inappropriate proliferation of lymphocytes associated with certain inflammatory and neoplastic disorders. Alternatively, agents that specifically augment this pathway, thereby enhancing immune function in immunodeficiency, may be developed.

Key words: NF-κB, PKCθ, Bcl10, MAGUK

Introduction

In the past decade, much has been learned and much has been written regarding the mechanisms by which lymphocytes respond to foreign antigens, a critical process in the adaptive immune response. Numerous signaling pathways are activated in both B and T cells under these circumstances, and each may play an important role in the overall response. In recent years, activation of the nuclear factor (NF)-κB transcription factor has emerged as one of the preeminent steps in mounting an effective immune response. The NF-κB family of proteins consists of five members, which form various homo- and heterodimers and are normally sequestered in the cytoplasm through interaction with inhibitory (I)κB proteins (reviewed by Li and Verma, 2002). Upon stimulation of cell-surface receptors, including the Toll-like, tumor necrosis factor (TNF), interleukin (IL)-1 and antigen receptors, to name only a few, downstream signaling culminates in the activation of a multisubunit complex termed the IκB kinase (IKK) complex. The IKK complex is composed primarily of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (NEMO/IKKγ) (Li and Verma, 2002). Upon activation, the catalytic subunits phosphorylate IκB proteins, thereby marking them for ubiquitylation and subsequent degradation. This process frees NF-κB dimers, which then translocate into the nucleus and regulate expression of a wide variety of genes. Although numerous receptor systems ultimately activate NF-κB, the molecular pathways used to activate the IKK complex appear fairly specific to each receptor, a feature that could be capitalized on in the future design of pharmacological agents. By contrast, it appears that once the IKK complex is activated, the subsequent downstream events leading to NF-κB-activated gene transcription are shared, no matter what the initial stimulus.

In the adaptive immune system, foreign antigen is recognized by lymphocytes through either the B-cell receptor (BCR) or T-cell receptor (TCR). For example, antigens can be engulfed, processed into peptides and expressed on the surface of antigen-presenting cells (APCs) in conjunction with major histocompatibility complex (MHC) antigens. This combination is recognized by the TCR, in association with CD3 and costimulatory receptors, of which CD28 plays a particularly important role (Wulfing et al., 2002). Once engaged, the TCR/CD3 complex, in conjunction with CD28, initiates several distinct signaling cascades, but the importance of the NF-κB signaling pathway has been demonstrated repeatedly. Activation of NF-κB is required for antigen-induced proliferation, cytokine production and survival of T cells, and these processes form the cornerstone of the adaptive immune response (Kane et al., 2002).

Engagement of TCRs induces the formation of a highly ordered, membrane-associated complex termed the T-cell immunological synapse (Grakoui et al., 1999), in which a central signaling zone surrounds clustered TCRs. This is known as the supramolecular activation cluster (SMAC) (Monks et al., 1998) and is further subdivided into the central SMAC (cSMAC) and peripheral SMAC (pSMAC). The TCR/CD3 complex is localized in the cSMAC, but different sets of signaling proteins appear to be relegated to one or the other of these distinct regions, where they can generate unique signals (Monks et al., 1998). Most of the signaling intermediates relevant to NF-κB activation become clustered in the cSMAC.

Note that a similar synaptic structure is formed between B cells and APCs, and that many parallel signaling pathways are activated following this engagement (Batista et al., 2001). Because many of the downstream steps leading to the activation of the IKK complex have been investigated primarily in T cells, here we focus on recent developments in our understanding of NF-κB signaling in T lymphocytes.

PKCθ: a crucial mediator of TCR-induced NF-κB activation

The past two decades have witnessed the cloning and characterization of a series of serine/threonine protein kinases collectively known as protein kinase C (PKC) (Baier, 2003). This multigene family, which has nine known members, can be subdivided into three subfamilies: the conventional [Ca^{2+}- and diacylglycerol (DAG)-sensitive] isotypes, novel (Ca^{2+}-insensitive but DAG-sensitive) isotypes and atypical (Ca^{2+}- and DAG-insensitive) isotypes. PKCθ, a member of the novel PKC subfamily, was cloned in the early 1990s (Baier et al., 1993; Chang et al., 1993; Osada et al., 1992). Since then, a large body of data has implicated it as a crucial and specific mediator for the activation of NF-κB following stimulation of the TCR. Unlike most PKC isoforms, PKCθ has a highly restricted expression pattern, mostly limited to T cells and skeletal muscle (Baier et al., 1993; Meller et al., 1999). Stimulation of cultured T cells with antigen-pulsed APCs results in the translocation of PKCθ to the site of contact between the APC and T cell, specifically within the cSMAC (Monks et al., 1998; Monks et al., 1997). By contrast, five other PKC isoforms (β, δ, ε, η and ζ) that are present in the T cells fail to show stimulus-dependent subcellular translocation.

Formation of the T-cell immunological synapse is thought to occur in part through the rearrangement of the actin cytoskeleton and the movement of lipid rafts (detergent-insoluble membrane microdomains rich in glycosphingolipid) to the site of receptor engagement (Alonso and Millan, 2001; Simons and Ikonen, 1997). The rafts might therefore shuttle essential signaling components to the synapse, as a first step in assembling the required signaling machinery. Consistent with this notion, Bi et al. have shown that stimulation of Jurkat T cells by the combination of anti-CD3 and anti-CD28 antibodies results in the specific recruitment of PKCθ to the lipid raft fraction (Bi et al., 2001). Furthermore, they have shown that PKCθ colocalizes with lipid rafts at the immunological synapse in primary $CD4^+$ T cells incubated with antigen-pulsed APCs (Bi et al., 2001).

How is PKCθ selectively recruited to the TCR signaling complex? Recruitment of many PKC isoforms to the cell membrane appears to depend upon the phospholipase C (PLC)γ1-mediated production of DAG. This DAG is generated on the inner surface of the cell membrane, and might bind to various PKC isoforms through their C1 regulatory domains. However, because this is a general phenomenon, it is unlikely to account for the highly selective recruitment of PKCθ to the immunological synapse. Instead, a large body of work has demonstrated that a series of membrane-proximal signaling events set in motion by the TCR complex are responsible for this selective recruitment of PKCθ. Stimulation of the TCR results in a cascade of events including the activation of receptor-associated tyrosine kinases; among these are the Src-family kinases (Lck and Fyn), a Syk-family kinase (ZAP-70) and Tec-family kinases (Chu et al., 1998; Lucas et al., 2003; Takesono et al., 2002; Zamoyska et al., 2003). Subsequently, these enzymes phosphorylate and activate several downstream adaptor molecules, including SLP-76, Lat and Grb2, which play a major role in organizing a supramolecular signaling complex in the membrane microdomain associated with the TCR and CD28 (Fig. 1) (Acuto et al., 2003). Among the downstream signaling intermediates that are recruited to this cluster are the kinases phosphoinositide 3-kinase (PI3K) and Akt, as well as the Rho-family guanine nucleotide exchange factor Vav-1 (Acuto et al., 2003). Many of these, including Lck, ZAP-70, SLP-76, AKT, PI3K, Vav-1 and the small GTPase Rac have been shown to be involved in, and/or required for, the selective recruitment and activation of PKCθ following TCR/CD28 activation (Isakov and Altman, 2002). Indeed, some intermediates physically interact with PKCθ within the environment of the lipid raft. However, the precise mechanisms by which this myriad of signaling intermediates coordinates the recruitment and activation of PKCθ remains somewhat unclear, as does the relative importance of each factor.

Considerable biochemical evidence shows that, in T cells, PKCθ activation is crucial for stimulation of NF-κB. In these cells, a constitutively active mutant of PKCθ induces marked activation of both an NF-κB reporter gene and the CD28 response element of the gene encoding IL-2, a response element that is sensitive to both transcription factors AP-1 and NF-κB (Coudronniere et al., 2000; Lin et al., 2000). By contrast, constitutively active mutants of PKCα, δ, ζ or ε have no significant effect. In addition, whereas Gö6976 [a PKC inhibitor selective for Ca^{2+}-dependent PKC isoforms (α, β and γ)] has little effect on CD3/CD28 stimulation of NF-κB, rottlerin (an inhibitor of PCKδ and PKCθ) essentially blocks nuclear translocation of NF-κB (Coudronniere et al., 2000). Furthermore, expression of a kinase-defective mutant of PKCθ, or introduction of PKCθ antisense RNA, blocks CD3/CD28-mediated activation of NF-κB (Lin et al., 2000). Finally, CD3/CD28 stimulation has been shown to lead to PKCθ-dependent phosphorylation and activation of IKKβ in the lipid raft, possibly occurring through direct physical association of PKCθ with components of the IKK complex (Khoshnan et al., 2000; Lin et al., 2000). Nevertheless, as we discuss below, despite the apparently direct interaction between PKCθ and the IKK complex, several intermediate signaling events are probably required for the stimulation of IKKβ kinase activity.

Genetic studies also support the role of PKCθ as a crucial factor in TCR signaling. Sun et al. generated a PKCθ-deficient mouse strain and demonstrated that mature T cells from these mice show a striking defect in IκB-α degradation and NF-κB activation following exposure to crosslinking anti-CD3 and anti-CD28 antibodies (Sun et al., 2000). The lack of response correlates with markedly decreased cytokine production and T-cell proliferation, both of which depend upon efficient NF-κB signaling. The absence of PKCθ selectively disrupts the TCR-mediated NF-κB pathway, because stimulation of T cells by either TNF-α or IL-1 resulted in a robust NF-κB response. Highlighting the fact that multiple signals (the NF-κB pathway representing only one) probably diverge at the point of PKCθ activation, Sun et al. also showed that there is a marked diminution in the anti-CD3-mediated activation of AP-1 in the T cells of $PKC\theta^{-/-}$ mice (Sun et al., 2000).

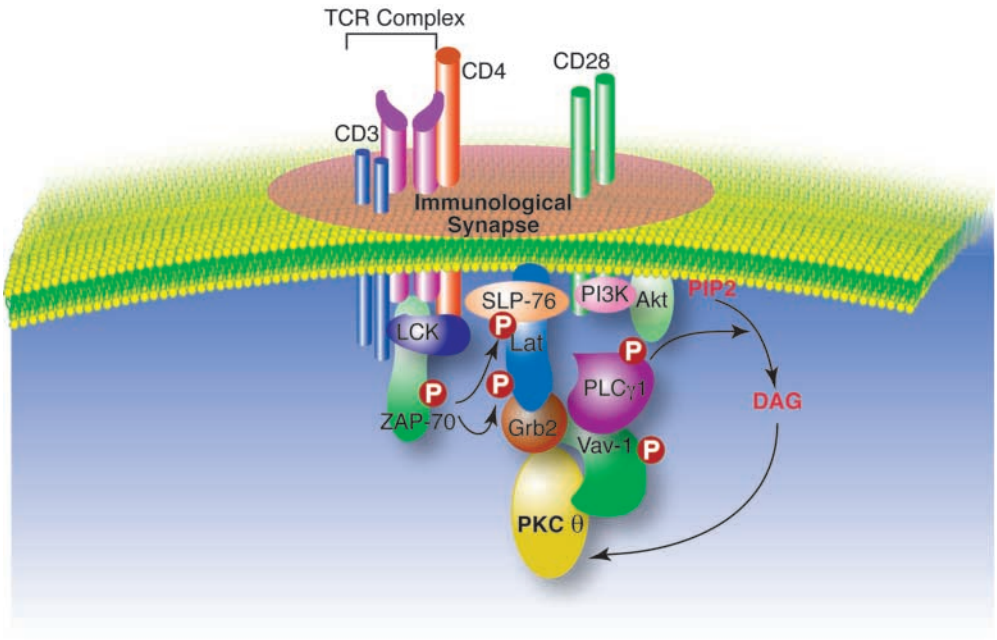

Fig. 1. Proximal signaling factors recruited to the SMAC. Several receptor-associated tyrosine kinases and adaptor proteins are recruited to the SMAC upon engagement of the antigen receptor complex. Key players are represented here. DAG is also generated through the action of PLCγ1 and subsequently serves to activate PKCθ, which is the isoform of PKC that is specifically recruited to the SMAC in T cells.

Despite the accumulating evidence implicating a crucial and selective role for PKCθ in mediating NF-κB activation in response to TCR stimulation, there remain some doubts. Specifically, Baier and co-workers have challenged the results of the first published report of a PKCθ$^{-/-}$ model (Baier, 2003; Pfeifhofer et al., 2003). These investigators have produced a second PKCθ$^{-/-}$ model through the germline introduction of a somewhat different PKCθ-targeting vector. This second mouse model shows only a modest diminution of NF-κB and AP-1 activation following CD3/CD28 stimulation (Pfeifhofer et al., 2003). Instead, these mice showed a complete block in CD3/CD28-responsive activation of NF-AT, which was entirely unaffected in the PKCθ$^{-/-}$ mice generated by Sun et al. (Pfeifhofer et al., 2003; Sun et al., 2000). These results suggest that other PKC isoforms participate or substitute for PKCθ in the NF-κB pathway and that the crucial role for PKCθ might lie elsewhere. The reasons for these discrepancies are not understood, but might stem from differences in the gene-targeting strategies employed by the two groups. Baier has suggested that the targeting strategy of Sun et al. could produce a truncated mutant of PKCθ, which might act in a dominant-negative manner under some circumstances (Baier, 2003). Clearly, further work will be required to resolve these issues but, at the moment, the majority of data point to PKCθ as playing a central role in coordinating the TCR-mediated activation of NF-κB.

Filling the black box: the Bcl10 connection

Despite the wealth of information implicating PKCθ as a critical mediator of NF-κB activation in T cells, a veritable 'black box' has existed downstream of this kinase, until only recently. Analysis of mice lacking the signaling protein Bcl10 led Ruland et al. to suggest that Bcl10 is a crucial link between PKCθ and NF-κB in this pathway (Ruland et al., 2001). Bcl10 (also known as CIPER, CARMEN, CLAP, mE10 and c-E10) was identified almost simultaneously by several laboratories in 1999 (Costanzo et al., 1999; Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999; Willis et al., 1999; Yan et al., 1999; Zhang et al., 1999). Sequence analysis revealed that it contains an N-terminal caspase-recruitment domain (CARD), which is a protein-protein interaction domain present within several signaling intermediates involved in apoptosis machinery. Typically, CARD-mediated homotypic interactions allow proteins to interact physically in specific signaling pathways (Hofmann et al., 1997). Although some initial data suggested a role for Bcl10 in apoptosis, the preponderance of evidence indicated that overexpression of Bcl10 results in robust NF-κB activation, and this depends on CARD-mediated self-oligomerization. In common with other activators of NF-κB, Bcl10 works by stimulating the IKK complex (Lucas et al., 2001).

Bcl10$^{-/-}$ mice are grossly normal at birth but quickly become susceptible to infection (Ruland et al., 2001). Despite possessing normal numbers of total peripheral T and B cells, they exhibit significantly fewer activated T cells and a dramatic impairment of the humoral and cellular immune response when challenged with viral infection. In vitro analysis showed that, when stimulated with anti-CD3 and anti-CD28 antibodies, isolated T cells from Bcl10$^{-/-}$ mice are defective in both their proliferative response and their production of IL-2. Since these are effects known to be dependent on NF-κB activation, Ruland et al. focused on this signaling pathway and discovered that isolated Bcl10$^{-/-}$ T cells cannot respond to TCR stimulation with an increase in IKK complex activation, IκB phosphorylation and degradation, and nuclear NF-κB translocation. Nevertheless, the cells demonstrate a robust NF-κB response when challenged with either TNF-α or IL-1, both of which are cytokines that do not operate through the TCR. Also, since Bcl10$^{-/-}$ T cells can activate AP-1 in response to TCR stimulation, the defect in TCR signaling appears to be specific to the NF-κB pathway.

Ruland et al. also tested the integrity of more-proximal TCR signaling events and showed that stimulation of isolated Bcl10$^{-/-}$ T cells with anti-CD3 results in normal intracellular tyrosine phosphorylation patterns and Ca^{2+} currents, steps

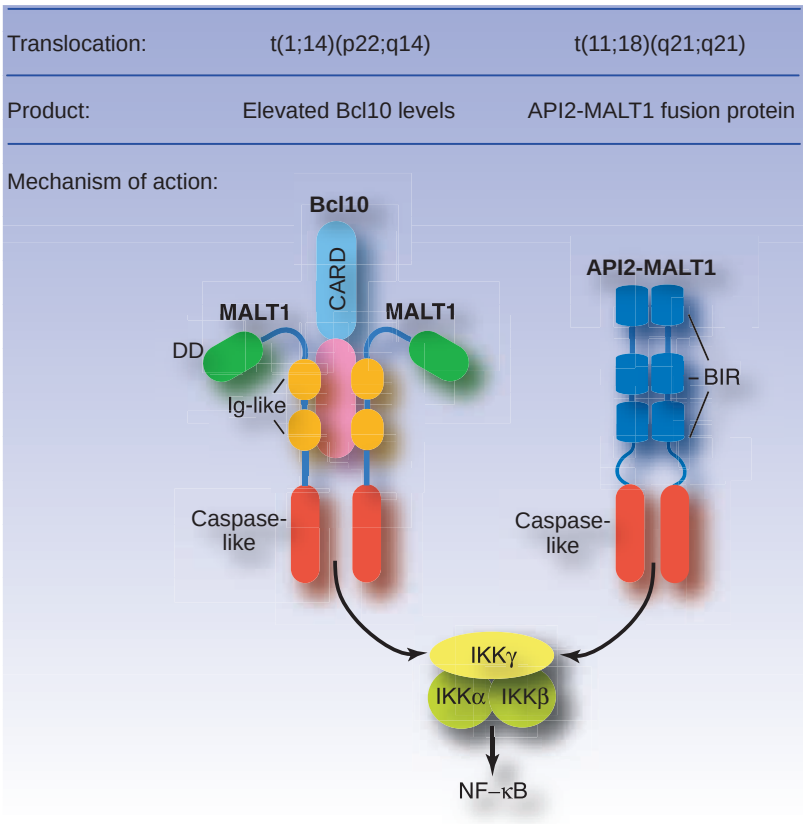

Fig. 2. Chromosomal translocations in MALT lymphoma. Shown are the products of two recurrent chromosomal translocations seen in MALT B-cell lymphomas. Overexpression of either wild-type Bcl10 or the API2-MALT1 fusion protein is thought to result in excessive NF-κB activation, although Bcl10 might require an intact downstream factor, MALT1. In both cases, oligomerization-dependent activation of the MALT1 protease (caspase-like) domain appears to play an important role for the subsequent stimulation of the IKK complex.

which should contribute to the activation of PKC (Ruland et al., 2001). Nevertheless, phorbol myristate acetate (PMA) and a Ca^{2+} ionophore (ionomycin), which together bypass early TCR signaling steps and directly stimulate PKC family members, are ineffective at inducing NF-κB and promoting proliferation of these cells. Together, these findings suggested a block in the NF-κB signaling pathway downstream of PKCθ in these mice, thus identifying Bcl10 as the first component of the 'black box' linking PKCθ activation to the canonical IKK-mediated NF-κB activation pathway.

Of considerable importance is the demonstration by Ruland et al. of similar deficiencies in NF-κB signaling downstream of the BCR in isolated $Bcl10^{-/-}$ B cells. A parallel pathway seems to operate in these cells, but apparently depends on the activation of a different PKC isoform, PKCβ (Saijo et al., 2002; Saijo et al., 2003; Su et al., 2002).

Downstream of Bcl10

When Bcl10 was first cloned and characterized, it quickly gained prominence because the *Bcl10* gene was identified as the target of the recurrent t(1;14) translocation seen in a subset of mucosa-associated lymphoid tissue (MALT) B-cell lymphomas (Willis et al., 1999; Zhang et al., 1999). This translocation places the entire *Bcl10* coding region downstream of the strong immunoglobulin enhancers on chromosome 14. As a result, Bcl10 is overexpressed, presumably leading to heightened NF-κB activation and a survival advantage for affected B cells (Fig. 2). Interestingly, a seemingly unrelated translocation, t(11;18), is seen in an even greater percentage of MALT lymphomas. This leads to the production of a fusion protein composed of the N-terminal portion of API2, one of the inhibitors of apoptosis, linked to the C-terminus of MALT1, a newly described protein identified as a distant relative of the caspases (Fig. 2) (Akagi et al., 1999; Dierlamm et al., 1999; Morgan et al., 1999; Uren et al., 2000).

Surprisingly, the API2-MALT1 fusion protein is also a potent activator of NF-κB, which suggests that both translocations target a common signaling pathway (Lucas et al., 2001; Uren et al., 2000). With this realization, we hypothesized that Bcl10 and MALT1, a protein that had no ascribed physiological role, might act in a common mechanistic capacity. Indeed, Bcl10 and MALT1 bind tightly to one another, Bcl10 serving to oligomerize MALT1 (Lucas et al., 2001; Uren et al., 2000). Furthermore, MALT1 synergistically enhances Bcl10-mediated NF-κB activation (Lucas et al., 2001). These findings suggested that oligomerization of MALT1 is required for Bcl10 to carry out its function, and we subsequently found that artificial oligomerization of the C-terminus of MALT1 is sufficient for the activation of NF-κB (Lucas et al., 2001). MALT1 thus appears to function as a downstream effector of Bcl10 in the activation of NF-κB (Fig. 2). Unlike activation by wild-type MALT1, NF-κB activation mediated by API2-MALT1 is not dependent on Bcl10-induced oligomerization. The fusion protein therefore seems to represent a gain-of-function mutant that can self-activate the MALT1 effector domain, even in the absence of Bcl10 (Fig. 2). Thus, seemingly unrelated chromosomal translocations involving the genes encoding Bcl10 and MALT1 perturb a common step in this signaling pathway, and both translocations are now thought to contribute to MALT lymphoma pathogenesis by causing inappropriate activation of NF-κB.

Since the C-terminus of MALT1 contains a proteolytic active site similar to that in caspases, upon oligomerization MALT1 might cleave some unknown substrate that then serves as a signaling factor that directly or indirectly activates the IKK complex (Lucas et al., 2001; Uren et al., 2000). Indeed, mutation of the conserved cysteine residue in the MALT1 caspase-like active site results in a reduction in NF-κB activation, suggesting that proteolytic activity is essential for this function. Still, the identity of such a factor, or even the substrate sequence specificity of MALT1, remains unknown. The generation of MALT1-deficient mice might provide additional information regarding the precise role MALT1 plays in this pathway. It is also unclear whether Bcl10 promotes NF-

κB activation by other mechanisms, independent of MALT1. Thus, a smaller 'black box' remains between the Bcl10/MALT1 complex and the IKK complex. Filling in the details will be a major challenge for the future.

MAGUK family members operate upstream of Bcl10

Another major step in the understanding of TCR-mediated NF-κB activation came in 2001, when several groups identified a new subfamily of membrane-associated guanylate kinase (MAGUK) proteins that bind to, and operate upstream of, Bcl10 (Bertin et al., 2001; Gaide et al., 2001; McAllister-Lucas et al., 2001; Wang et al., 2001). Members of the MAGUK superfamily function as molecular scaffolds by using multiple discreet protein interaction domains to cluster receptors and cytosolic signaling molecules at the cell membrane (Dimitratos et al., 1999; Fanning and Anderson, 1999). As such, all MAGUKs contain three defining interaction domains: the PSD-95/Dlg/Z0-1 homologous (PDZ) domain, the Src-homology (SH3) domain and the guanylate kinase (GUK)-like domain (Fig. 3). The new subfamily is further distinguished by the presence of a coiled coil domain and an N-terminal CARD, which appears to interact specifically with the CARD of Bcl10. Therefore, we have termed these new members Bimps, for Bcl10-interacting MAGUK proteins, but they have also been given other names, including CARMAs for CARD-containing MAGUKs (Fig. 3). Because these proteins are now most commonly referred to as CARMAs in the literature, we will use this designation hereafter.

The three CARMAs show distinct tissue distributions, but share 50-60% sequence identity in the CARD and coiled-coil domains, and 20-30% identity in the PDZ, SH3 and GUK domains. (Bertin et al., 2001; Gaide et al., 2001; McAllister-Lucas et al., 2001; Wang et al., 2001). Similar to the combination of Bcl10 and MALT1, overexpression of all CARMA family members results in NF-κB activation through stimulation of the canonical IKK pathway. Furthermore, in cellular extracts, CARMAs not only bind Bcl10 but also recruit MALT1 through formation of a ternary complex (McAllister-Lucas et al., 2001). This interaction appears to be essential for NF-κB activation, because CARMAs are unable to stimulate NF-κB in mouse embryonic fibroblasts derived from Bcl10-deficient mice (McAllister-Lucas et al., 2001).

These findings led to the hypothesis that a CARMA serves as a scaffolding protein to integrate upstream molecules involved in TCR signaling with the downstream factors Bcl10 and MALT1. To test this hypothesis, we constructed a dominant-negative CARMA lacking the CARD, which severs the connection with downstream Bcl10. In a T-cell hybridoma cell line, this dominant-negative mutant completely blocks NF-κB activation induced by the combination of PMA and ionomycin, or by anti-CD3 antibodies, but does not affect activation of NF-κB by TNF-α or Toll-like receptor 2 (TLR2) (McAllister-Lucas et al., 2001). These findings were the first to suggest that a CARMA protein may indeed be a crucial link between PKCθ and Bcl10.

Subsequently, several studies definitively established that CARMA1 (also known as Bimp3 and CARD11), the family member expressed in the spleen, thymus and peripheral blood leukocytes, is an important component of the antigen-induced NF-κB signaling pathway in T cells. First, dominant-negative CARMA1 and appropriate short interfering (si)RNA both block CD3-mediated NF-κB activation in Jurkat T cells (Gaide et al., 2002; Pomerantz et al., 2002). Second, CARMA1-deficient T cells, generated through somatic mutagenesis, exhibit impaired activation of NF-κB following TCR stimulation (Wang et al., 2002). In the latter study, Wang et al. used the acylation agent, ICR191, to chemically mutagenize Jurkat T cells, and isolated a monoclonal mutant cell line termed JPM50.6 that failed to activate NF-κB in response to CD3/CD28 stimulation (Wang et al., 2002). Further analysis demonstrated that JPM50.6 cells could not activate IKK or induce IκB-α degradation following CD3/CD28 stimulation, but that both occurred following TNF-α treatment. Nevertheless, other signaling pathways activated by CD3/CD28, such as the JNK, Erk and Akt pathways, were unaffected. To determine where the defect in signaling was occurring, the authors tested whether PMA treatment or overexpression of a constitutively active form of PKCθ could activate NF-κB in JPM50.6 cells (Wang et al., 2002). Interestingly, neither of these stimuli was effective. These results indicate that the mutant cell line is defective in NF-κB signaling at a point distal to the CD3/CD28-mediated activation of PKCθ. Reconstitution of JPM50.6 cells with

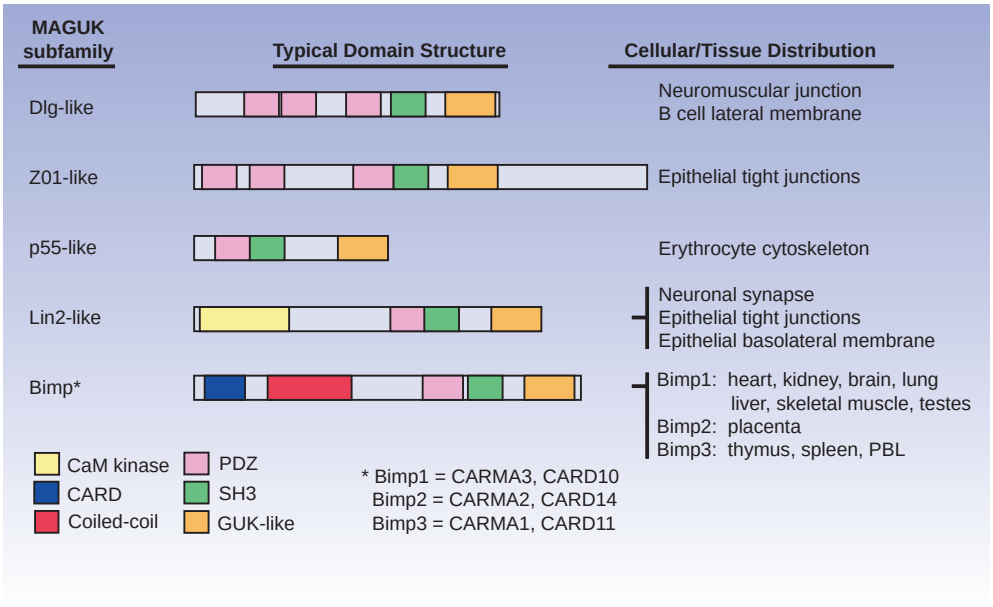

Fig. 3. MAGUK subfamily classification. All MAGUKs are defined by the presence of at least one PDZ, SH3 and GUK-like domain. The various subfamilies are distinguished by the presence of additional domains (e.g. the CARD and coiled-coil domains of the Bimp subfamily), or by unique linear arrangements of the key domains.

CARMA1 fully rescues the defect, and western blotting has confirmed that the cells indeed lack endogenous CARMA1.

Co-immunoprecipitation and confocal microscopy reveal that CARMA1 physically associates with the TCR-CD3 complex following CD3 stimulation (Gaide et al., 2002). These results essentially parallel previous studies regarding the recruitment of PKCθ to the immunological synapse. Significantly, following TCR stimulation, at least some Bcl10 is recruited to lipid rafts, and Gaide et al. have presented evidence supporting the movement of Bcl10 to the membrane-associated signaling complex (Gaide et al., 2002).

Most recently, Jun et al. have applied a whole animal chemical mutagenesis approach, analogous to that used to create the JPM50.6 mutant Jurkat cell line, to prove the importance of CARMA1 in antigen receptor signaling in vivo (Jun et al., 2003). These authors created a library of mice that have random mutations generated by treatment with the chemical mutagen ethylnitrosourea. One pedigree has a phenotype somewhat similar to that of the Bcl10-knockout strain (Ruland et al., 2001). This strain, termed *unmodulated*, harbors a single T→A nucleotide substitution in the *CARMA1* gene. This mutation results in a Leu→Gln substitution within the coiled-coil domain of CARMA1, and is predicted to disrupt the ability of this domain to function as a protein-interaction domain (Jun et al., 2003). T and B cells from these mice show evidence of defective antigen receptor signaling through the NF-κB pathway. Whereas early events in antigen receptor signaling remain intact, such as tyrosine phosphorylation of receptor-proximal targets, elevation of intracellular Ca^{2+} levels, and activation of both NF-AT and the ERK mitogen-activated protein (MAP) kinase cascades, there is a significant block in IκB-α degradation. This defect is observed whether cells are stimulated by antigen receptor engagement or direct activation of PKC by PMA and ionomycin. Physiologically, the block in NF-κB activation correlates with an abnormally blunted proliferative response in both B and T cells and a decrease in the antigen-induced expression of CD25, a subunit of the IL-2 receptor whose expression depends on NF-κB. Somewhat surprisingly, the lymphocytes from these mice also show defects in JNK signaling, which suggests a more pervasive role for CARMA1 in antigen receptor signaling. This finding echoes similar observations by Gaide et al., who found that a dominant-negative CARMA1 can also impair JNK signaling in Jurkat cells (Gaide et al., 2002).

Finally, Hara et al. have used a classic knockout approach to study the requirement for CARMA1 in lymphocyte signaling and function (Hara et al., 2003). In a very thorough analysis, these authors provide definitive evidence for the role of CARMA1 in both B- and T-cell signaling; again, activation of antigen receptors in both types of cells from mice lacking the protein results in normal receptor-proximal signaling events. However, there is a complete block in IκB-α degradation and NF-κB-DNA complex formation, steps that are completely intact following stimulation by TNF-α. The defect in antigen receptor signaling was mapped to a position downstream of PKC, as would be expected from previous work. As in the *unmodulated* strain, these mice show a defective lymphoproliferative response, and their T cells show decreased IL-2 production, both of which depend upon efficient NF-κB activation. Also consistent with other results (Gaide et al., 2002; Jun et al., 2003) is the finding that the mice have a defect in JNK activation following stimulation of T cells by anti-CD3/CD28 or stimulation of B cells by anti-IgM (Hara et al., 2003).

Adding to the complexity of this topic, the CARMA knockout mice have two additional surprises (Hara et al., 2003). First, inactivation of CARMA1 apparently also leads to an impairment in innate immunity; splenic B cells from the knockout mice display a cell-cycle block and fail to proliferate in response to lipopolysaccharide (LPS), which activates TLR4 (reviewed by Beutler, 2000). This finding contrasts with that of Jun et al., who found that B cells from *unmodulated* mice have normal proliferative responses to LPS (Jun et al., 2003). However, the differences in phenotype might simply reflect a residual functional activity retained by the mutated CARMA1 of the *unmodulated* strain, which is lost upon complete deletion of the gene. If the involvement of CARMA1 in the LPS response holds true, this will prove to be the first indication that this MAGUK protein has a role beyond its now well-established function in integrating antigen-mediated PKC activation with downstream signaling pathways. The second surprise provided by the analysis of CARMA knockout mice is the finding that CARMA1 is essential for efficient development of other immune-system-related cell types; not only is there impaired development of $CD5^+$ peritoneal B cells, but natural killer (NK)-cell development is also abnormal (Hara et al., 2003). The precise mechanisms underlying this requirement remain to be determined.

Conclusions and perspectives

In only a short time, a flurry of research activity has filled many of the gaps in our understanding of antigen receptor signaling, particularly as it pertains to the activation of NF-κB. It is clear that analogous signaling pathways exist in T and B cells, and that these are required for the proliferative and adaptive responses of both cell types when challenged by foreign antigen. It is also clear that perturbations in these pathways may have profound pathological consequences. As an example, many cases of MALT B-cell lymphoma appear to arise as a consequence of chromosomal translocations that cause unregulated NF-κB activation. In the case of the t(11;18) translocation, expression of the API2-MALT1 fusion protein results in constitutive NF-κB activation through direct or indirect stimulation of the IKK complex. Accordingly, evidence suggests that lymphomas characterized by this translocation show a proliferative phenotype that is independent of antigen receptor signaling, probably because receptor-proximal steps such as the activation of PKC and the recruitment of Bcl10 are simply bypassed by the ability of the API2-MALT1 protein to self-dimerize and thereby activate its caspase-like domain (Fig. 4). By contrast, MALT lymphomas characterized by the t(1;14) translocation show overexpression of Bcl10. In this case, affected B cells may be ‘supersensitized’ to antigen-receptor-induced proliferation, and this could explain the initial dependence of such lymphomas on the continued presence of an inflammatory signal (Fig. 4). The most prominent example of this phenomenon is the dependence of many MALT lymphomas of the stomach on an ongoing *Helicobacter pylori* infection, and the ability to treat such lymphomas with antibiotic therapy (Cavalli et al., 2001).

With this greater understanding of antigen receptor

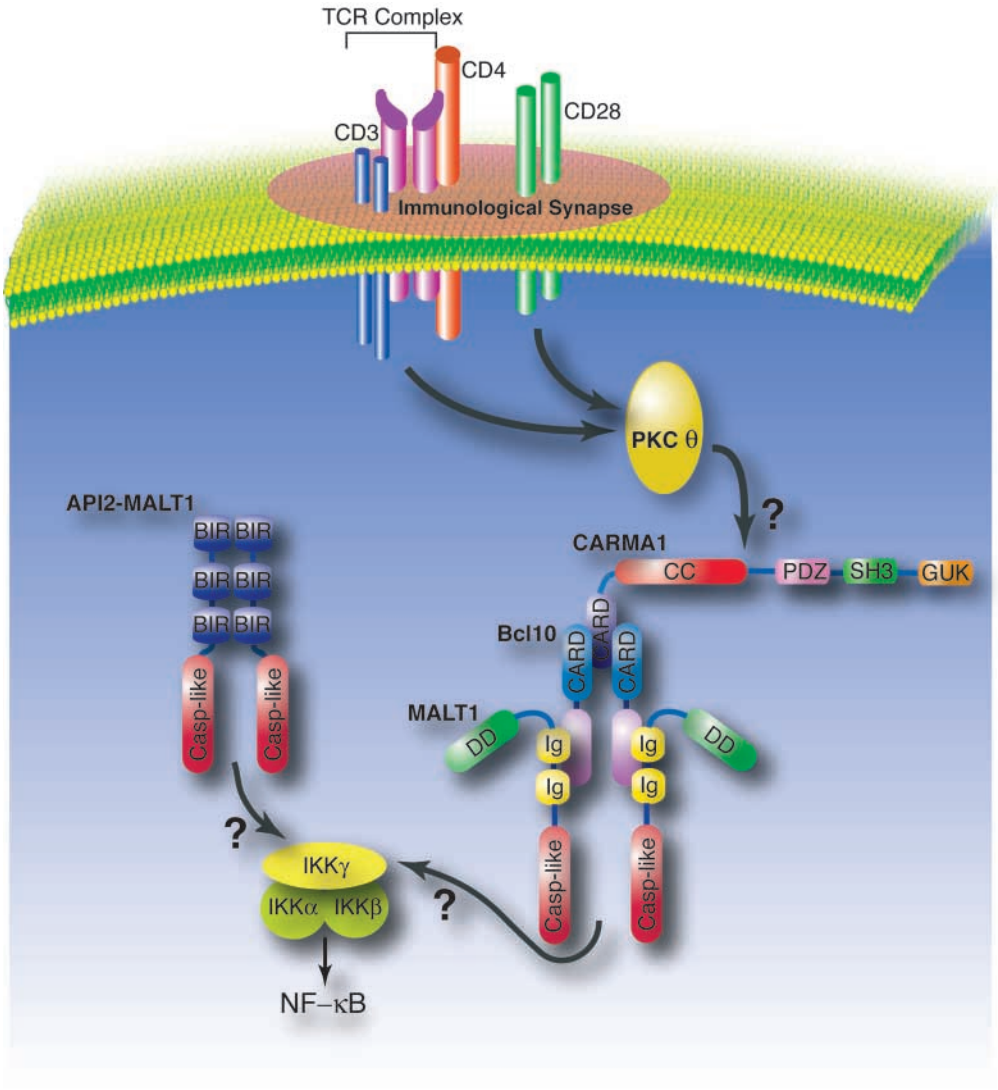

Fig. 4. Downstream events in the TCR-mediated activation of NF-κB. Recent studies have implicated several new players in the activation of NF-κB, including CARMA1, Bcl10 and MALT1. However, as indicated in the text, numerous unanswered questions remain.

signaling, there is promise for the development of more-specific therapeutic agents aimed at selectively inhibiting or augmenting downstream signaling events. Whereas general NF-κB inhibitors have been developed to treat a variety of neoplastic, autoimmune, or inflammatory conditions, such agents will probably produce a myriad of side-effects related to the fact that NF-κB activation is essential to numerous physiological processes and not just those perturbed by a given pathological condition. The characterization of PKCθ and CARMA1 as essential, lymphocyte-specific mediators of antigen-receptor-dependent NF-κB activation might provide the first really promising targets for the development of specific therapeutic agents aimed at blocking this pathway.

Despite the explosion of knowledge regarding this private pathway for NF-κB activation in lymphocytes, many questions remain (Fig. 4). How does MALT1 communicate with the IKK complex and are there other, MALT1-independent, mechanisms by which Bcl10 can activate NF-κB? How does CARMA1 integrate upstream signaling events, such as the activation of PKC, with the downstream process of recruiting and activating Bcl10 and MALT1? In addition, we have only scratched the surface with regard to the role of CARMA1 in JNK signaling, and essentially nothing is known as to how CARMA1 might mediate signals emanating from TLR4. Certainly this is an exciting time to be studying lymphocyte biology, and it is made only more exciting by the knowledge that we have an opportunity to uncover basic biochemical processes that, when perturbed, can contribute to the development of inflammatory, immunodeficient, autoimmune and neoplastic disorders.

Note added in proof

While this Commentary was under review, two additional reports were published that provide further support for the essential role of CARMA1 in lymphocyte signaling (Egawa et al., 2003; Newton and Dixit, 2003). The reports independently describe the phenotype of CARMA1$^{-/-}$ mice, or mice harboring a CARD-deficient mutant of CARMA1. The results are fully consistent with those of the knockout studies reviewed above.

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