EBV LMP1: New and shared pathways to NF-κB activation

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The canonical NF-κB pathway plays a pivotal role in regulating a variety of essential processes, such as immunity, cell survival, and proliferation, and therefore must be tightly regulated to ensure a transient response to infection or other stimuli. When dysregulated, persistent NF-κB activation may fuel chronic inflammation and tumorigenesis in certain settings. NF-κB–activating stimuli converge at the level of the IκB kinase (IKK) complex consisting of catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ (also known as NEMO). IKK phosphorylates the NF-κB inhibitor IκBα to trigger its ubiquitination and proteasomal degradation, thus allowing NF-κB subunits to translocate to the nucleus and activate target genes (1, 2). A large number of both positive and negative regulators of NF-κB have been identified, although there are likely many more to be found. An effective and powerful approach to finding new mediators of NF-κB activation is used by Gewurz et al. (3) in a study published in PNAS, whereby the authors conduct genome-wide screening using an siRNA library to identify novel regulators of EBV-encoded latent membrane protein 1 (LMP1)-induced activation of NF-κB.

NF-κB Is a Key Target for EBV-Induced Tumorigenesis

EBV (also known as human herpes virus 4) transforms resting B lymphocytes into proliferating lymphoblastoid cells by inducing a constitutive activation of NF-κB through the LMP1 protein (4, 5). LMP1 is a transmembrane protein that activates NF-κB using specific domains—known as transformation effector sites (TES) 1 and 2—that recruit and usurp cytoplasmic signaling effector proteins and adaptors, such as TNFRSF1A-associated via death domain (TRADD), TNF-receptor-associated factors (TRAFs), and receptor-interacting protein 1 (RIP1). TES1 and -2 are both required for B-lymphocyte transformation, although TES2 preferentially activates the canonical NF-κB pathway, whereas TES1 activates the noncanonical NF-κB pathway that controls processing of the NF-κB2/p100 precursor (6–11). Blockade of NF-κB triggers the death of EBV-transformed lymphoblastoid cells, underscoring the importance of NF-κB in the survival of these malignant cells and indicating NF-κB as a viable target for therapeutic inhibitory molecules.

LMP1 Shares Signaling Components with the IL-1 and TNF Pathways

The study by Gewurz et al. (3) uses an NF-κB–dependent GFP reporter gene to screen an siRNA library upon inducible expression of LMP1 in human embryonic kidney 293 cells. The study identified 155 unbiased proteins regulating LMP1-mediated NF-κB activation, 118 of which were also essential for IL-1β– and 79 for TNF-α–induced NF-κB signaling. Importantly, known LMP1-induced mediators of NF-κB, such as the E3 ligase TRAF6, the NF-κB subunit RelA, and IKK, were identified in the siRNA screen. Interestingly, LMP1 signaling to NF-κB bears a stronger resemblance to IL-1β signaling rather than the TNF-α pathway, and indeed several core components of IL-1β signaling, including TRAF6, the E2 ubiquitin-conjugating enzyme Ubc13, and IKKγ, were essential for LMP1. Several components common to both LMP1 and IL-1β include the phosphatase 4 subunit SMEK1 and the vesicle protein VMP1. In contrast, a subset of proteins were common for LMP1 and TNF-α, including the heat shock protein Hsp27, the phosphatase PPAP2B, the nuclear receptor NR1H4, the PDZ actin stress fiber binding and LIM domain protein (PDLIM1), and the RUN and SH3 domain protein RUSC2. PDLIM1 and RUSC2 may be important for the transport and/or signaling of LMP1 and TNF receptor-1 (TNFR1). Finally, a subset of proteins was unique to the LMP1 pathway, such as the kinase TPL2 and the protooncogene PIM3, both of which are known to be upregulated in EBV-associated malignancies.

Gewurz et al. establish a comprehensive map of NF-κB signaling components downstream of LMP1, IL-1βR, and TNFR1.

The authors also investigated whether the essential LMP1 components were upstream or downstream of IKK using an IκBα-*Photinus* luciferase fusion protein that permits quantitation of IκBα degradation. As expected, essential regulators of NF-κB were identified both upstream and downstream of IKK. Most of the essential components downstream of LMP1 were enzymes or components of enzyme complexes, and the expression of those proteins was also found in lymphoid cells, where LMP1 is commonly expressed and biologically active. As expected, siRNA-mediated depletion of IKKγ, TRAF6, Ubc13, and other core components of the NF-κB pathway impaired LMP1-induced degradation of IκBα. As described below, several classes of proteins were found to be important for the activation of NF-κB.

LMP1 Usurps Multiple Classes of Proteins to Activate NF-κB Signaling

Given the importance of ubiquitin in regulating NF-κB pathways, it is reassuring that several E3 ubiquitin ligases, including RNF11, RNF34, FBX041, and DDA1, were required for LMP1 to activate NF-κB. Although RNF11 has previously been identified as a negative regulator of NF-κB by associating with the A20 ubiquitin-editing complex (12), it is possible that LMP1 may have hijacked RNF11 to instead activate NF-κB. Depletion of RNF11 and RNF34 led to a significant increase of IκBα-*Photinus* levels, indicating that their function is upstream of the IKK complex. In addition, RNF31 and RBCK1, subunits of the LUBAC E3 ligase complex that catalyzes linear polyubiquitin chains, were also essential for LMP1-induced NF-κB activation when simultaneously knocked down with siRNA. This finding further emphasizes the importance of linear ubiquitination in multiple NF-κB signaling pathways.

Although deubiquitinating enzymes (DUBs) have predominantly been found to negatively regulate the NF-κB pathway (13), the study by Gewurz et al. (3) and

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another recent study (14) indicate that DUBs may also function as positive regulators for NF-κB activation. For example, the authors demonstrated that diminished expression of USP11, USP21, USPL1, or USP43 interfered with LMP1-induced degradation of IκBα. It is possible that these DUBs remove degradative ubiquitin chains from key NF-κB signaling components.

Kinases were another important class of LMP1-induced NF-κB regulatory components. As expected, IKKα and IKKβ knockdown induced a strong stabilization of IκBα-*Photinus*, but only when they were combined because of partial redundancy, as previously demonstrated (15, 16). LMP1-induced NF-κB regulatory kinases identified include PIM3, PKN3, and RIPK4, all of which function upstream of IKK. PKN3 and RIPK4 were previously demonstrated to interact with TRAFs, suggesting that their effects may be due to the regulation of TRAF molecules. TPL2 and STK40 were also found to be important for NF-κB activation, although these kinases seem to function downstream of IκBα.

In addition to DUBs, phosphatases play a central role in the negative regulation of NF-κB. Surprisingly, Gewurz et al. (3) find numerous catalytic and regulatory phosphatase subunits, such as PTPRS, PPP1CB, and PPP2R5E, which play essential roles in NF-κB activation upstream of IKK. However, the exact function of these phosphatase subunits in NF-κB signaling remains unknown.

ZC3H zinc finger family RNA binding proteins regulate gene expression through posttranscriptional mechanisms to negatively regulate mRNAs regulating NF-κB (17). Surprisingly, in this study two ZC3H proteins, ZC3H13 and ZC3H18, were found to act as positive regulators for LMP1-induced NF-κB activation. Whereas ZC3H18 functioned upstream of IKK, ZC3H13 was downstream of IκBα degradation.

LMP1 Signaling: How Do the Pieces of the Puzzle Fit?

Although the study by Gewurz et al. (3) greatly enhances our knowledge of the pathways and signaling components downstream of LMP1, IL-1βR, and TNFR1, many outstanding questions remain. The precise function of many of the newly identified NF-κB signaling components remains unknown. Future mechanistic studies are needed to determine how each of these proteins regulates NF-κB activation and where they function in the pathways. Gene-targeted mouse models will also be necessary to delineate the in vivo functions and potential tissue-specific roles of these proteins. It may also be worthwhile to determine whether any of the LMP1-required components play a role in the noncanonical NF-κB pathway. Along the same lines, a whole-genome siRNA screen to identify LMP1 TES1-induced activators of the noncanonical NF-κB pathway will be useful to better understand how LMP1 activates the noncanonical pathway. Finally, it will be interesting to determine whether other viral oncogenes, such as human T-cell leukemia virus type 1 Tax or Kaposi's sarcoma herpesvirus vFLIP, share many components with LMP1 for NF-κB activation.

In summary, the results from Gewurz et al. (3) establish a comprehensive map of NF-κB signaling components downstream of LMP1, IL-1βR, and TNFR1. Therefore, the results from this study have expanded the list of potential NF-κB regulators, some of which may be mutated in lymphoid malignancies such as B-cell lymphomas and multiple myeloma (18). Given that many of the newly identified NF-κB regulators have enzymatic activity, it may be possible to identify small-molecule inhibitors that effectively target malignant cells that rely on NF-κB for their survival.

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