

Breaking the tolerance for tumor Targeting negative regulators of TLR signaling

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Keywords: tumor tolerance, immunoediting, immunosurveillance, toll-like receptors, IRAK-M, TGF β

Abbreviations: TLR, toll-like receptors; sTLR, soluble TLR; sMyD88, short form of MyD88; IRAK, interleukin-1 receptor-associated kinase; TGF, transforming growth factor; NF κ B, nuclear factor kappa B; PAMP, pathogen associated molecular pattern; DAMP, danger associated molecular pattern; TNF, tumor necrosis factor; LPS, lipopolysaccharide; NOD, nucleotide-binding oligomerization domain protein; SOCS, suppressor of cytokine signaling; TOLLIP, toll-interacting protein; FADD, Fas-associated death domain; TRAF, tumor necrosis factor associated factor; PI3K, phosphatidylinositol 3-kinase; SIGIRR, single immunoglobulin interleukin-1-related receptor; TRAILR, tumor necrosis factor-related apoptosis inducing ligand receptor; TRIF, toll/IL-1 receptor-domain containing adaptor protein inducing IFN β ; TRAM, TRIF-related adaptor molecule; LLC, Lewis lung carcinoma

Submitted: 10/03/11

Accepted: 10/16/11

<http://dx.doi.org/10.4161/onci.18434>

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Tumors arise and progress in immunocompetent hosts presumably by activating tolerance mechanisms critical for normal homeostasis. Host immune cells can mount anti-tumor responses by activation of Toll-like receptors (TLRs). However, emerging data suggests that molecules that negatively regulate TLRs are exploited by tumors to induce tolerance and mitigate the host immunosurveillance. Targeting these negative regulators can be a potential new immunotherapeutic strategy.

As an important extrinsic tumor suppressive mechanism, immune system can identify and destroy nascent tumor cells through a process known as cancer immunosurveillance. However, tumors still arise in immune-competent hosts, after progressing through three distinct phases of a process known as immunoediting.¹ In the first phase termed elimination, which also encompasses immunosurveillance, the innate and adaptive immune systems work together to detect the presence of a developing tumor and destroy it. Malignant cell variants that survive the elimination phase enter the equilibrium phase, in which the adaptive immune system prevents outgrowth of tumor and sculpts the immunogenicity of the malignant cells. During the escape phase, malignant cells acquire the ability to circumvent immune recognition and manifest into visible tumors. Tumors do so by exploiting several different tolerance mechanisms, which are also employed by the host to maintain the normal immune homeostasis.¹

Host immune cells can launch anti-tumor responses through activation of cell

surface receptors, including toll-like receptors (TLRs), which are capable of sensing exogenous and endogenous danger signals. To strike an immunological balance between activation and inhibition and to avoid triggering inappropriate inflammatory responses, the immune system tightly regulates TLR signaling through multiple negative regulatory mechanisms.² Molecules that negatively regulate TLR signaling may be exploited by tumors to induce immune tolerance and mitigate host immunosurveillance. Recently, we demonstrated that tumor cells induce the expression of IRAK-M, a negative regulator of TLR signaling, in tumor associated macrophages, promoting an immunosuppressive M2 phenotype. Tumor cell induced IRAK-M is mediated by the cytokine TGF β , which serves as a key mechanism by which lung tumors may circumvent anti-tumor responses of macrophages promoting tumor immunotolerance.³ In this article, we discuss the implications, potential role in immunoediting and prospects for IRAK-M and other negative regulators of TLR signaling for potential therapeutic targeting in oncoimmunology.

TLR Signaling and Tolerance for Tumors

Toll like receptors are critical components of innate immunity and are broadly distributed on cells of the immune system. TLRs are evolutionarily conserved to recognize molecular patterns associated with pathogens (PAMPs) such as bacterial lipopolysaccharides (LPS), hypomethylated DNA, flagellin, dsRNA. In addition, molecular patterns associated with tissue damage (danger associated molecular

patterns or DAMPs) including heat shock proteins, high mobility group box proteins and dsDNA can directly activate TLRs.⁴ TLR signaling is initiated by dimerization of TLRs, forming homodimers or heterodimers. All TLRs, with the exception of TLR3, recruit and utilize the adaptor protein MyD88 for signaling upon receptor activation. This allows the recruitment and activation of a family of kinases, namely IRAKs (IL-1 receptor-associated kinases) 1, 2 and 4. IRAK-4 is initially recruited to the complex, becomes activated, and then phosphorylates IRAK-1. These kinases interact with MyD88 through the death domains common to both proteins, resulting in a cascade of interactions culminating in the activation of further downstream kinases, including inhibitor of $\text{NF}\kappa\text{B}$ ($\text{I}\kappa\text{B}$) kinases (IKKs). Activation of $\text{I}\kappa\text{B}$ releases $\text{NF}\kappa\text{B}$, allowing $\text{NF}\kappa\text{B}$ translocation to the nucleus to mediate an increase in inflammatory cytokine gene expression.^{4,5} The specificity and diversity of TLR function is conferred in part by the selective interaction with the adaptor molecules. For example, the adaptor MAL is vital for both TLR2 and TLR4 activation of $\text{NF}\kappa\text{B}$, whereas, TLR3 uses the adaptor TRIF to induce interferon- β ($\text{IFN}\beta$) synthesis and TLR4 uses both TRIF and TRAM to activate the IRF-3 signaling pathway.⁵ There are other signaling pathways that contribute to TLR function, such as Jun N-terminal kinase (JNK) and the mitogen-activated protein kinases (MAPKs). The relationship and interaction between these various signaling pathways is a major subject of interest in TLR biology.

In the tumor microenvironment, the exact trigger(s) of TLR signaling in host immune cells is not known. However, the tumor microenvironment is rich in molecules that can potentially activate TLR signaling to trigger anti-tumor responses. This includes heat shock proteins, high mobility group proteins, double stranded DNA from necrotic tumor cells, and hyaluronic acid.^{6,7} Tumors may activate the same negative regulatory mechanisms that are critical for normal homeostasis of the immune system, and induce immune tolerance to cancer cells. Dynamic interactions between cancer cells and tumor associated host immune cells initiate and

maintain tumor immune tolerance which eventually predominates and overcomes effective host immune response.^{7,8} The nature of the interactions between cancer cells and immune cells, and the molecular mechanisms underlying tumor-induced immune tolerance is poorly understood. Even though a number of molecules that negatively regulate TLR signaling has been identified and been shown to play an important role in both limiting excessive inflammation as well as immune tolerance, their role in tumor immunology has just started to emerge.

Negative Regulators of TLR Signaling

Negative regulators of TLRs can selectively inhibit one or more TLRs or target common components of the TLR-signaling pathway that function to block the effects of the entire TLR family (for a comprehensive review see ref. 2). The selective use of negative regulators by different TLRs, sometimes in a tissue specific manner, might therefore contribute to the functional specificity and diversity of TLR responses. In order to attenuate signaling events downstream of TLRs, at least three levels of negative regulation have so far been reported. These include extracellular decoy receptors to intracellular inhibitors, and membrane-bound suppressors (Fig. 1).² In addition, several miRNAs have been identified as negative regulators of TLR signaling.⁹ However, functional studies establishing their roles are lacking and for that reason these molecules will not be addressed in this article. Moreover, while a number of TLR inhibitors have been shown to promote a tolerance phenotype in monocyte/macrophage populations under certain experimental conditions, only IRAK-M has been directly linked to macrophage tolerance within the tumor microenvironment.

Extracellular decoy receptors. There are at least two soluble forms of TLRs (sTLRs) identified so far. These naturally produced decoy sTLRs may serve as first line of negative regulation to prevent overactivation of host response against microbial threat. Even though TLR4 and TLR2 are encoded by single distinct genes, multiple different mRNA products

are detected for each in various mammalian hosts, including humans, indicating the presence of different isoforms. Recombinant form of sTLR4 inhibited LPS-induced $\text{NF}\kappa\text{B}$ activation and $\text{TNF}\alpha$ production by macrophages *in vitro*.¹⁰ Similarly, sTLR2 isoforms, with molecular weights from 20–85 kDa, were detected in human milk and plasma, resulting from the post-translational modification of the transmembrane receptor.¹¹ Similarly, sTLR2 inhibited IL-8 and $\text{TNF}\alpha$ production by monocytes stimulated with the TLR2 agonist, bacterial lipopeptide. sTLR4 and sTLR2 may inhibit signaling by blocking the interaction between TLR4 or TLR-2 and other co-receptors, particularly MD2 and CD14 (Fig. 1).

Intracellular inhibitors. Intracellular molecules constitute the largest group of negative regulators that inhibit TLR signaling in both humans and mice. These molecules include short form of MyD88 (MyD88s), TRIAD3A, A20, NOD2, PI3K, SOCS1, TOLLIP, FADD, IRAK-2c, IRAK-2d and IRAK-M (Fig. 1).

Similar to the decoy sTLRs, an alternatively spliced MyD88s was identified that acts as a dominant negative inhibitor of MyD88. In contrast to the ubiquitous MyD88 expression, MyD88s was predominantly expressed in spleen and brain. MyD88s overexpression results in MyD88s-MyD88 heterodimers, rather than MyD88-MyD88 homodimers required for IRAK-4 recruitment. This prevents IRAK-1 phosphorylation by IRAK-4, resulting in the inhibition of MyD88-dependent signaling. Consequently, MyD88s inhibits LPS and IL-1 induced $\text{NF}\kappa\text{B}$ activation *in vitro*.^{12,13}

TRIAD3A is a member of E3-ubiquitin ligases that promotes ubiquitylation and degradation of TLR4 and 9 but not TLR2. Overexpression of TRIAD3A reduced LPS- and CpG-induced $\text{NF}\kappa\text{B}$ activity, whereas knockdown of TRIAD3A enhances TLR expression and increases signaling *in vitro*.¹⁴ Conversely, A20 is a deubiquitylating enzyme that blocks both MyD88-dependent and independent TLR signaling by deubiquitylating TRAF6, a common signaling component shared by all TLRs.¹⁵

NOD2 is an intracellular pathogen pattern recognition receptor in the Nod-like

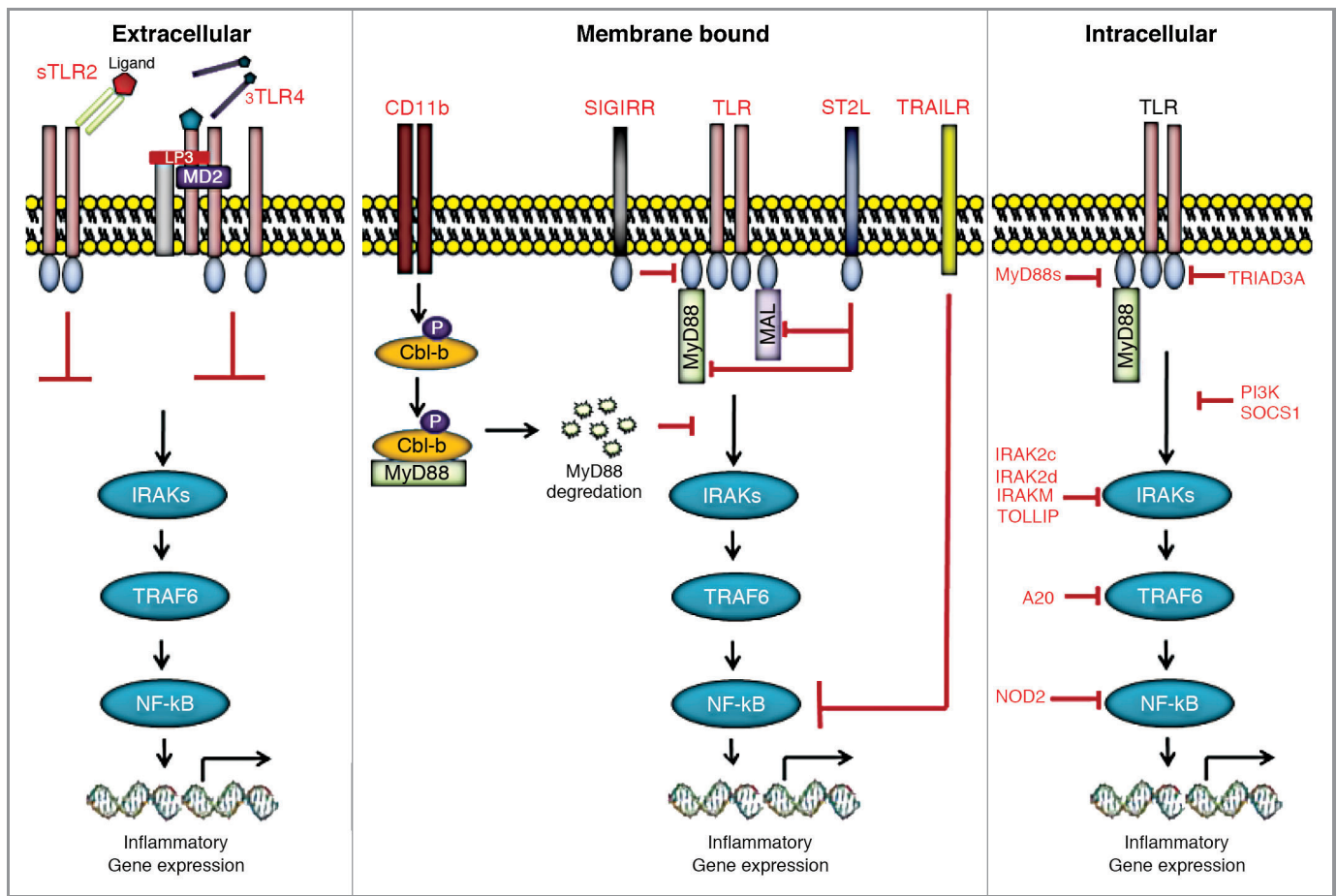


Figure 1. Negative regulation of Toll-like receptor signaling. Toll-like receptor (TLR) signaling pathways are tightly regulated in multiple different ways by several endogenous regulators to prevent hyperactivation. To attenuate the signaling from TLRs, at least three levels of negative regulation have so far been reported. These range from intracellular inhibitors to extracellular decoy receptors, and membrane-bound suppressors.

receptor family that recognizes bacterial muramyl dipeptide (MDP). Interestingly, ligation of NOD2 has been shown to specifically inhibit TLR2-mediated signaling. NOD2-deficient mice demonstrated enhanced TH1 responses when stimulated with TLR2 ligands, but not other TLR ligands.¹⁶ PI3K, a ubiquitously expressed signaling molecule that functions as an early event in many cellular responses, was also shown to inhibit TLR signaling. Unlike NOD2, PI3K-deficient mice show enhanced TH1 responses when stimulated with several TLR agonists, including TLR2, TLR4 and TLR9 ligands.¹⁷ The mechanism(s) by which NOD2 and PI3K inhibit TLR signaling is not well defined, but both were shown to suppress NFκB activation downstream of TLR activation.^{16,17}

An additional group of intracellular negative regulators are adaptor molecules

that target IRAK-1. One such molecule, suppressor of cytokine signaling 1 (SOCS1), inhibits TLR4 and TLR9 signaling by suppressing IRAK-1 expression.¹⁸ The Toll-interacting protein (TOLLIP) inhibits autophosphorylation of IRAK-1 to negatively regulate both TLR2 and TLR4 signaling.¹⁹ The death receptor binding adaptor protein, FADD, impairs IRAK-1 interaction with MyD88 to block TLR2 and TLR4 mediated signaling in endothelial cells.²⁰ IRAK-2c and IRAK-2d are the alternatively spliced variants of IRAK-2 that lack death domains and have been shown to inhibit LPS-induced NFκB activation.²¹ Lastly, IRAK-M (also referred to as IRAK-3) is the inactive kinase member of the IRAK family, one of the first identified and most studied negative regulators of TLR signaling.²² A more detailed discussion on IRAK-M in the context of tumor

immunosuppression will be discussed further.

Membrane-bound suppressors. Transmembrane proteins, such as ST2, SIGIRR, TRAILR and CD11b constitute another group of important negative regulators of TLR signaling. ST2, and SIGIRR are the two orphan receptors that block NFκB activation in response to IL-1, TLR4 and TLR9 ligands but not to TLR3 agonists.^{23,24} ST2 exists as two alternately spliced transmembrane forms (ST2L) expressed in hematopoietic cells, and a soluble form (sST2) that is present in both hematopoietic and non-hematopoietic cells. ST2-deficient mice were no different than wild-type mice in their response to endotoxin-induced shock, but failed to develop LPS tolerance.²³ This suggests that ST2 may play an important role in regulating sustained TLR responses. ST2L suppresses IL-1 and TLR signaling

by sequestering MyD88 and MAL through TIR domain. In addition, sST2 was shown to inhibit mRNA expression of TLR4 and TLR1 in response to LPS. SIGIRR is a transmembrane protein with an extracellular domain that interferes with TLR4, TLR5, and TLR9 ligand binding, and a long non-signaling intracellular domain that interferes with recruitment of IRAK-1 and TRAF6. Interestingly, SIGIRR is most highly expressed by epithelial cells, including in kidney, gut, and lung. SIGIRR deficient mice show exaggerated response to TLR4 and TLR9 activation and enhanced susceptibility to endotoxin shock.²⁴ Interestingly, mice deficient in the membrane-bound negative regulator, TRAILR, also showed enhanced viral clearance and increased cytokine production in response to TLR2, TLR3, and TLR4 but not to TLR9.²⁵ Another novel mechanism identified in macrophages is TLR-induced activation of CD11b, which inhibits TLR signaling by targeting MyD88 and TRIF for Cbl-b-mediated proteolytic degradation (Fig. 1). Consistent with other negative regulators, CD11b-deficient mice also show enhanced inflammatory cytokines in circulation and are susceptible to septic shock.²⁶

IRAK-M and Tumor-Induced Tolerance

IRAK-M is an intracellular negative regulator of TLR signaling and probably the only molecule to date investigated in the context of tumor immunology. It is an inactive kinase that antagonizes TLR signaling through protein-protein interactions preventing activation of IRAK-1. IRAK-M is regarded as a key negative regulator of TLR signaling in macrophages preventing excessive inflammatory responses.²² Unfortunately, in pathologies such as sepsis and cancer, immune suppressive function of IRAK-M is exploited to evade host immune surveillance. Enhanced expression of IRAK-M was observed in alveolar macrophages during sepsis-induced immune suppression²⁷ and in PBMCs isolated from patients with chronic myeloid leukemia.²⁸

We have shown that tumor associated macrophages (TAMs) express significantly higher levels of IRAK-M compared with

the peritoneal macrophages (PEMs) from the same tumor bearing mice. Subcutaneous implantation of LLC cells in IRAK-M null mice resulted in a five-fold reduction in tumor growth, as compared with tumors in wild type (WT) animals. TAMs isolated from IRAK-M-deficient mice displayed a classically-activated M1 phenotype rather than M2 phenotype observed in TAMs from WT mice. Human lung cancer cells induced IRAK-M expression in human PBMCs when co-cultured together. Tumor cell-induced expression of IRAK-M was TGF β dependent.³ However, the mechanism by which TGF β induced IRAK-M expression is not known. Bioinformatics analysis of human and mouse IRAK-M promoter identified Smad3 binding elements (unpublished observations). Ongoing studies in our lab will verify the direct binding of Smad3 to IRAK-M promoter and its functional significance in regulating TGF β -induced IRAK-M expression. Importantly, enhanced IRAK-M expression in primary lung tumors correlated with poor survival in patients with lung cancer.³ Collectively, our data demonstrated that TGF β -dependent induction of IRAK-M expression is an important, clinically relevant mechanism by which tumors may circumvent anti-tumor responses of macrophages. Recent studies implicating a role for IRAK-M in regulating TLR responses in other cell types raising the question as to whether changes in macrophage phenotype in the IRAK-M deficient mice is solely responsible for inhibitory effects on tumor growth. Studies are underway in our laboratory to address this issue by using bone marrow chimera mouse models. The major deficiency in many of the earlier studies, including ours, was use of existing cell lines that are already immunoeedited. To gain precise insights into the role of IRAK-M in the process of immunoeediting, it is necessary to investigate by using a chemical or genetic model of carcinogenesis in an immunocompetent host. To this end we recently developed a mouse model by crossing the inducible K-ras mouse model of lung cancer into IRAK-M-deficient background (unpublished studies).

A Potential New Class of Therapeutic Targets for Oncoimmunology

To develop more effective immunotherapies, immunologists must identify the cellular and molecular mechanisms that either eliminate or promote cancer development. For this reason, inhibitors of TLR signaling may be therapeutically useful in switching the nature of the tumor microenvironment from one of tumor-promoting inflammation to that of tumor-eliminating immunity. Surprisingly, with the exception of IRAK-M, the expression or the role of other TLR negative regulators has not been investigated in the context of tumor immunology. In a preliminary analysis, we assessed the mRNA expression of some of the negative regulators discussed above, in a spectrum of human cancers using OncoPrint database (www.oncoPrint.com).²⁹ Interestingly, five out of six molecules assessed showed upregulation in the majority of unique analyses comparing cancer cells vs. normal tissue (Fig. 2). For example, A20 was upregulated (within top 10% with FC > 2 and p-value < 0.0001) in 17 unique analyses (sample sets) compared with its downregulation in 8 (Fig. 2). This shows a trend toward increased expression in tumors, where head and neck cancers stand out with upregulation in six unique analyses. This trend is consistent with our recent finding that high IRAK-M expression in tumors correlated with poor survival in patients with lung cancer.³ By comparison, for NOD2 expression there was a reverse correlation with two up and seven down. It should be cautioned that the mRNA data in the oncoPrint data sets is derived from entire tumors, without any consideration or separation for their immune component. Therefore, it's worth noting that these correlations may not truly represent a proof-of-principle but provides a rationale and starting point for further investigation. However, the lack-of or negative correlation in this analysis does not preclude a molecule from further investigation.

In light of emerging IRAK-M data, it's reasonable to expect an important role for other negative regulators in conferring tumor tolerance. It is necessary to

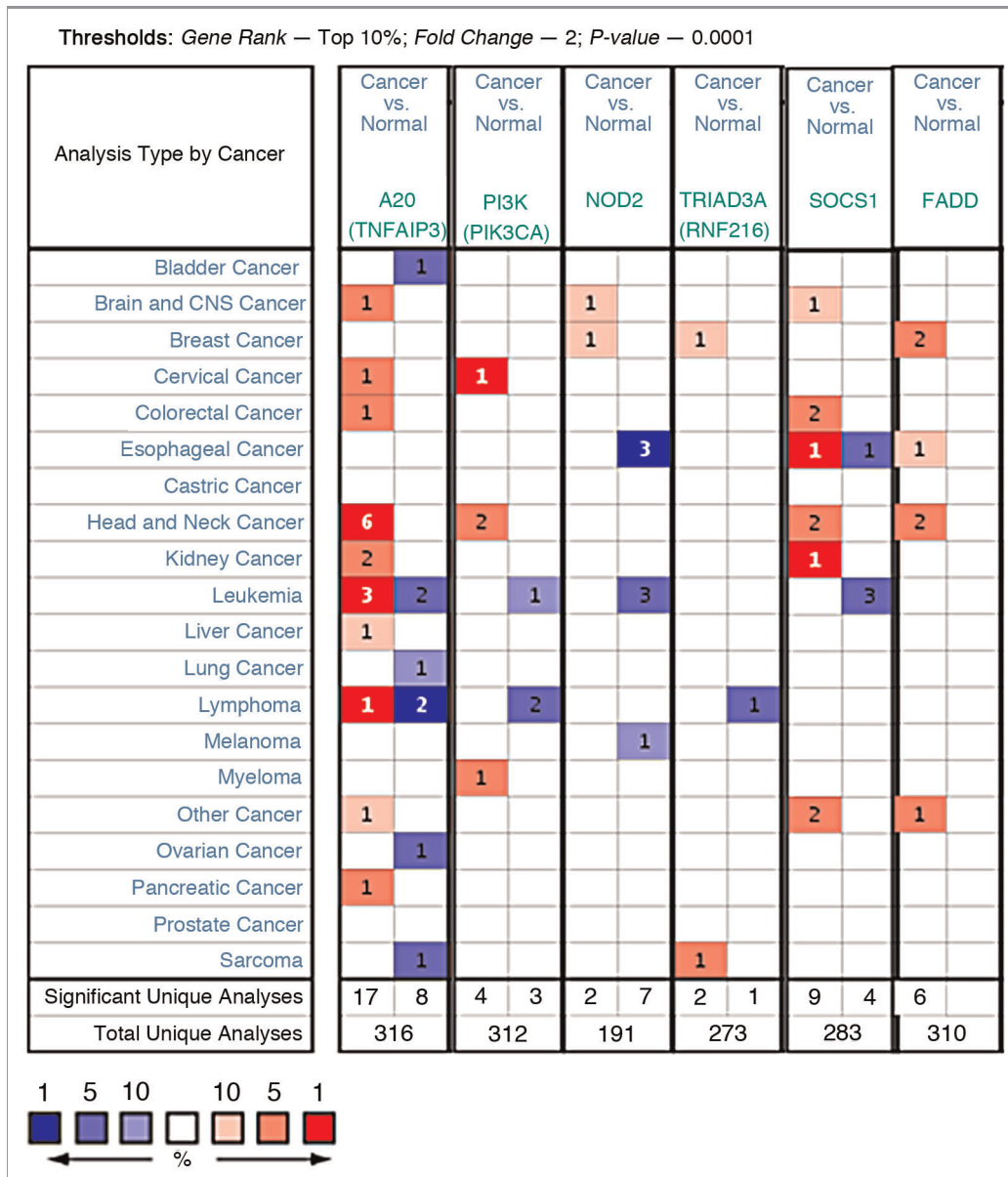


Figure 2. Analysis and visualization of A20, PI3K, NOD2, TRIAD3A, SOCS1 and FADD gene expressions in a spectrum of human cancers using OncoPrint database. Each individual gene was analyzed separately for differential expression between cancers vs. normal by applying indicated thresholds and compiled together for visualization. Cell color is determined by the best gene rank percentile for the analyses within the cells. Red, upregulation; Blue, downregulation.

investigate the effect of each of these molecules on immunoeediting, starting with molecules for which knockout mouse models are currently available. Given the TGFβ regulation of IRAK-M, it is important to explore the regulation of other negative regulators by TGFβ or other immunosuppressive cytokines in the tumor microenvironment. The tissue or cell type-specific expression of some regulators suggests that it would not be

surprising to see tumor type specific mechanisms in operation. In summary, understanding the functional control of immune-suppressive networks, including the role of TLR signaling and their negative regulators, may offer new opportunities to shift the balance between tolerance and immunity. The identification and targeting such nodes and molecules will facilitate the elimination of tolerance phenomena in the tumor microenvironment and aid

the development of effective cancer immunotherapeutic strategies.

Acknowledgments

This research is funded by NIH/NCI (R01 CA132571-01), and American Cancer Society (RSG-CSM-116801) grants to V.G.K. We thank Dr. Ajay Kumar Reka for the art work in **Figure 1** and Viritha Kaza for her help with the OncoPrint analysis.

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