Immunodetection of proteins in the innate immune system

Antibodies for nucleic acid sensing pathways.

The innate immune system is an ancient germ line–encoded eukaryotic defense mechanism that usually forms the first-line host response to infection. In vertebrates, it includes physical barriers and general defense mechanisms, as well as the execution of an immediate and nonspecific immune response to incoming pathogens. The innate immune pathway comprises several families of pattern recognition receptors (PRRs) and adapter and signaling molecules that together result in the induction of a potent inflammatory response. This response in turn activates the adaptive and cell-mediated immune systems, which promote programmed cell death through phagocytosis, apoptosis, and necroptosis pathways.

PRRs, which include RIG-I–like receptors (RLRs), toll-like receptors (TLRs), and NOD–like receptors (NLRs), recognize various pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). PAMPs are biomolecules that are typically signs of an infection, such as extranuclear DNA and RNA (including specific viral nucleic acid motifs), bacterial cell wall and membrane components, and bacterial flagellin. In contrast, DAMPs are host biomolecules expressed by damaged, dying, or cancerous cells in the absence of infection, and their ability to stimulate the innate immune system leads to sterile inflammation [1]. DAMPs can play a significant role in activating aberrant (auto-immune) responses or protective immune responses in reaction to cardiovascular or neurodegenerative diseases, cancer, or nucleic acid immunization. Recently, agonists of pathways activated by PRRs have been under intense focus as potential targets for cancer immunotherapy because they can directly induce programmed cell death specifically in the tumor microenvironment; there are several in preclinical trials [2,3]. Here we describe several Invitrogen™ antibodies that are proving

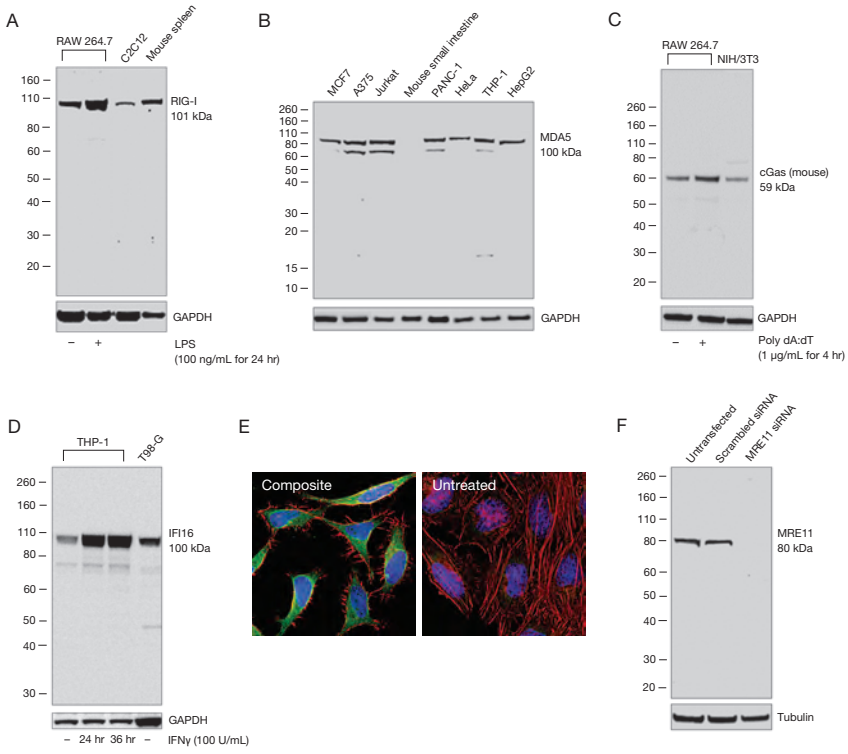

Figure 1. Assessment of the specificity of Invitrogen DNA and RNA sensor antibodies. (A) Western blot analysis of RIG-I antibody (clone 35H2L48, Cat. No. 700366) using whole-cell extracts from various cell lines and tissues. A 101 kDa band corresponding to RIG-I was observed in the cell lines and tissue tested, and this signal was enhanced in RAW 264.7 cells upon LPS treatment, which is known to increase RIG-I expression. **(B)** Western blot analysis of MDA5 antibody (clone 33H12L34, Cat. No. 700360) using whole-cell extracts from various cells and tissues. **(C)** Western blot analysis of cGAS (mouse) antibody (clone 10H1L5, Cat. No. 703149), showing increased expression of cGAS (mouse) in RAW 264.7 cells upon transfection with poly dA:dT (1 µg/mL for 4 hr). **(D)** Western blot analysis of IFI16 antibody (clone 8H37L1, Cat. No. 703147), showing increased expression of IFI16 in THP-1 cells treated with 100 U/mL IFNγ for 24 hr (lane 2) and for 36 hr (lane 3). **(E)** Immunofluorescence analysis of IFI16 using IFI16 polyclonal antibody (green, Cat. No. PA5-76462), showing increased expression of IFI16 in HeLa cells upon IFNγ treatment. **(F)** Western blot analysis of MRE11 polyclonal antibody (Cat. No. PA3-16527) using whole-cell extracts from A431 cells transfected with MRE11 siRNA. For the western blot analyses, primary antibodies were probed with Invitrogen™ Goat Anti–Rabbit IgG Superclonal™ Recombinant Secondary Antibody, HRP (Cat. No. A27036), detected using the Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (Cat. No. 32106), and imaged using the Invitrogen™ iBright™ FL1000 Imaging System.

useful for the study of the nucleic acid sensing pathways, highlighting the strategies we employ to ensure target specificity for each antibody.

Antibody detection of nucleic acid sensors

Nucleic acid sensors, an important class of PRRs, are characterized according to whether they recognize RNA or DNA. The RIG-I–like receptors (RLRs), for example, are a family of cytosolic, structurally related RNA helicases that include RIG-I, MDA5, and LGP2. These receptors recognize "nonself" RNA structures in the cytoplasm, such as double-stranded RNA (dsRNA) and 5′-triphosphate RNA (5′-3pRNA), as well as aberrantly localized RNA that can result, for example, from ionizing radiation or chemotherapy. Such RNA-based PAMPs are commonly associated with viral infection, and mice lacking either RIG-I or MDA5 show increased susceptibility to viruses [4]. Furthermore, injection of 5′-3p–containing siRNAs into mice tumor models has been shown to induce RIG-I–dependent pathways and the recruitment of NK or $CD8^+$ T cells to the tumor site, leading to the production of IFNγ, tumor regression, and a prolonged life span [5,6]. Similarly, injection of the synthetic ligand poly I:C into epithelial ovarian cancer cells has been shown to activate MDA5, trigger tumor apoptosis, induce inflammatory cytokines, and enhance expression of HLA class 1 molecules [7]. Figures 1A and 1B show validation* data for Invitrogen™ rabbit recombinant antibodies that recognize RIG-I and MDA5; target specificity for the RIG-I antibody has been verified by cell treatment with lipopolysaccharide (LPS), which is known to increase RIG-I expression.

Like RNA sensors, DNA sensors also produce an inflammatory response that can lead to programmed cell death and may be harnessed to improve patient outcomes after ionizing radiation or chemotherapy [8]. DNA sensors specifically detect the presence of cytoplasmic DNA, which is indicative of infection or cell damage; nuclear and mitochondrial DNA do not activate these pathways. The DNA sensor cGAS recognizes dsDNA and synthesizes the second messenger cyclic GMP-AMP (2′3′-cGAMP) upon ligand binding. Injection of cyclic dinucleotides into mice tumor models results in cGAS activation, improved tumor clearance, and enhanced survival [3]. The DNA sensor IFI16 recognizes single-stranded DNA (ssDNA) from human cytomegalovirus and HSV-1 in the cytoplasm of human macrophages and is essential for induction of type I interferon response in these infections. MRE11 is a DNA damage sensor that recognizes dsDNA in the cytoplasm and activates downstream signaling [8]. Figures 1C–1F show validation data for antibodies that recognize the cGAS, IFI16, and MRE11 DNA sensors; target specificity for each antibody was verified using cell treatments known to increase expression of target proteins (cGAS and IFI16 antibodies) or siRNA to silence expression (MRE11 antibody).

Antibody detection of adapter and signaling molecules

The nucleic acid sensor proteins associate with various adapter proteins, which in turn signal downstream components. For example, RLRs activate a protein called MAVS (mitochondrial antiviral signaling protein), which is localized in mitochondria and peroxisomes, leading to its oligomerization and filament formation [9]. MAVS knockout →

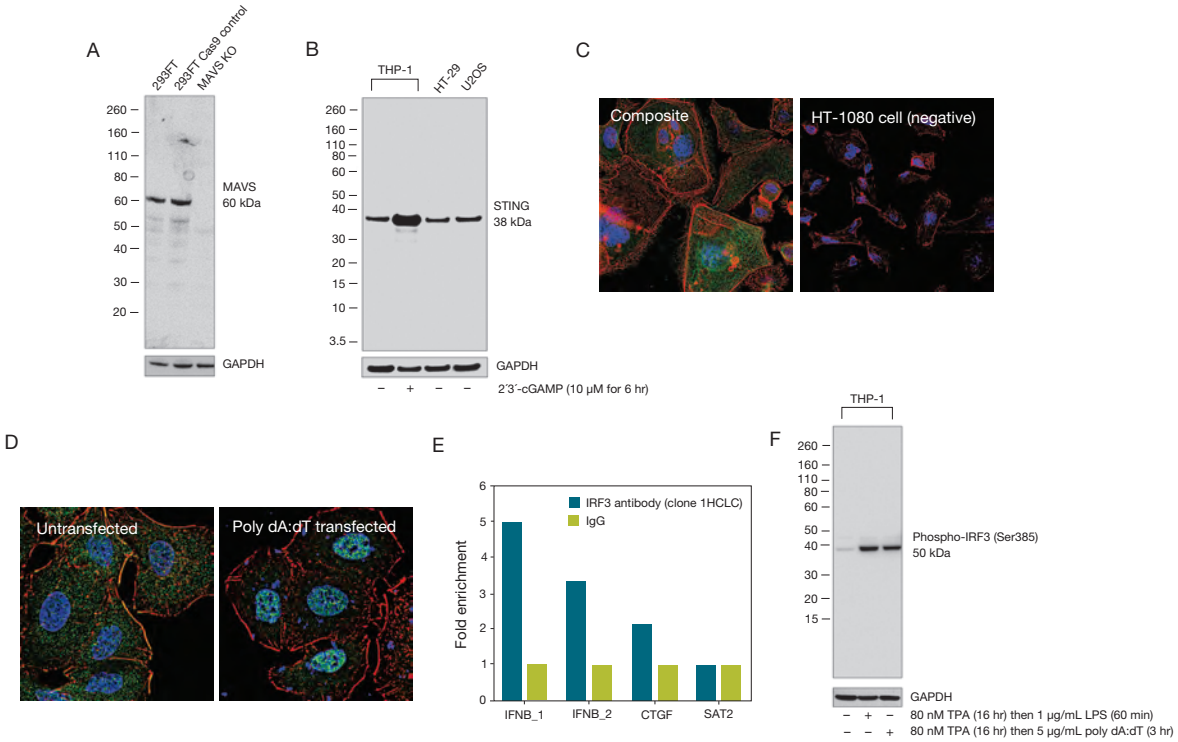

Figure 2. Assessment of the specificity of Invitrogen adapter and signaling protein antibodies. (A) Western blot analysis of MAVS antibody (clone 20H41L5, Cat. No. 703153) using cell membrane–enriched extracts from a CRISPR-Cas9 mediated knockout (KO) of MAVS in 293FT cells. **(B)** Western blot analysis of STING antibody (clone 2H1L5, Cat. No. 702993), showing increased STING expression in THP-1 cells upon treatment with 2′3′-cGAMP. **(C)** Immunofluorescence analysis of MyD88 polyclonal antibody (Cat. No. PA5-19919), showing increased MyD88 expression in SK-BR-3 cells as compared with HT-1080 cells. **(D)** Immunofluorescence analysis of IRF3 antibody (green; clone 3H32L10, Cat. No. 703682), showing IRF3 expression in the nucleus of A549 cells transfected with poly dA:dT and in the cytoplasm in untransfected control cells. **(E)** Chromatin immunoprecipitation (ChIP) analysis of IRF3 (clone 1HCLC, Cat. No. 712217) using PCR primer pairs for two different promoter regions in the IFNB gene, for the CTGF promoter (positive control), and for SAT2 satellite repeats (negative control). ChIP analysis demonstrated IRF3 antibody specificity through the detection of IRF3 enrichment at specific gene loci. **(F)** Western blot analysis of phospho-IRF3 (pSer385) polyclonal antibody (Cat. No. PA5-36775), showing an increase in phospho-IRF3 (pSer385) upon treatment of THP-1 cells with TPA and LPS, and with TPA and poly dA:dT. For the western blot analyses, primary antibodies were probed with Invitrogen™ Goat Anti–Rabbit IgG Superclonal™ Recombinant Secondary Antibody, HRP (Cat. No. A27036), detected using the Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (Cat. No. 32106), and imaged using the Invitrogen™ iBright™ FL1000 Imaging System.

mice can no longer induce interferon and other inflammatory cytokines in response to viral infections. The DNA sensors cGAS and IFI16 both engage the endoplasmic reticulum–localized protein STING (stimulator of interferon genes). MyD88 (myeloid differentiation marker 88) is an adapter that plays an important role downstream of endosomal DNA sensor TLR9 [8]. Figures 2A–2C show the validation data for MAVS, STING, and MyD88 antibodies; antibody specificities have been verified using a CRISPR knockout model (MAVS antibody), cell treatment with 2′3′-cGAMP (STING antibody), and relative expression (MyD88 antibody).

Activated MAVS, STING, and MyD88 lead to the phosphorylation and activation of the protein kinase TBK1. Phosphorylated TBK1 further phosphorylates and activates the transcription factor IRF3. IRF3 usually resides in the cytoplasm; however upon TBK1 phosphorylation, it dimerizes and translocates from the cytoplasm to the nucleus. In the nucleus, it binds to the promoters of genes containing the ISRE (interferon-stimulated response element) sequence and activates the transcription of IFNβ and a subset of interferon-stimulated genes. Phosphorylated TBK1 also activates the transcription factor NFkB, leading to its nuclear translocation and transcriptional activation of

proinflammatory cytokines [8,9]. Figures 2D–2F show the validation data from three IRF3 antibodies, two that recognize total IRF3 and one that recognizes phospho-IRF3 (pSer385). Target specificity for the two Invitrogen™ IRF3 rabbit recombinant polyclonal antibodies is shown by observing the poly dA:dT–mediated translocation of IRF3 from the cytoplasm to the nucleus using immunofluorescence (Figure 2D) and by detecting pull down of IFN promoter sequences but not those from the SAT2 locus using chromatin immunoprecipitation (ChIP) (Figure 2E). The Invitrogen™ polyclonal antibody for phospho-IRF3 (pSer385) was used to detect phosphorylated IRF3 on western blots in extracts from cells treated with TPA followed by either LPS or poly dA:dT (Figure 2F).

Antibody tools for investigating the immune system

Nucleic acid sensing pathways are increasingly being investigated for their therapeutic potential in treating infection, cancer, and degenerative diseases. Table 1 lists Invitrogen™ antibodies that recognize several components of these pathways. Search for these and other target-verified, application-tested antibodies at **thermofisher.com/antibodies**. To learn more about our validation methodology, visit **thermofisher.com/antibodyvalidation**. ■

*The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

References

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Table 1. Invitrogen™ antibodies for nucleic acid sensing pathways.

Target protein	Rabbit monoclonal, recombinant monoclonal, or recombinant polyclonal Ab (Cat. No.)	Rabbit polyclonal Ab (Cat. No.)	Mouse monoclonal Ab (Cat. No.)
DNA and RNA sensors			
RIG-I	700366		
MDA5	700360		
cGAS (mouse)	703149	PA5-56820	
IFI16	703147	PA5-39180, PA5-51690, PA5-76462	
MRE11		PA3-16527, PA5-31262	
DHX9		PA5-19542, PA5-55754	
Adapter proteins			
STING	702993		
MAVS	703153		14-9835-82
MyD88		PA5-19919	
Signaling proteins			
IRF3	703682, 712217	PA5-20087, PA5-78026	14-9947-82
Phospho-IRF3 (pS385)		PA5-36775	
NFkB p50	710450	51-3500	MA5-15860, MA5-15870
NFkB p65	710048	PA1-186, PA5-16545, 51-0500	33-9900
Phospho-NFkB p65 (pS536)	MA5-15160		
NFkB p52		PA5-27340	

*Go to **thermofisher.com/antibodies** for detailed information about each of these antibodies, including advanced verification data. To learn more about our two-part antibody validation methods—target specificity verification and functional application validation—visit **thermofisher.com/antibodyvalidation**.