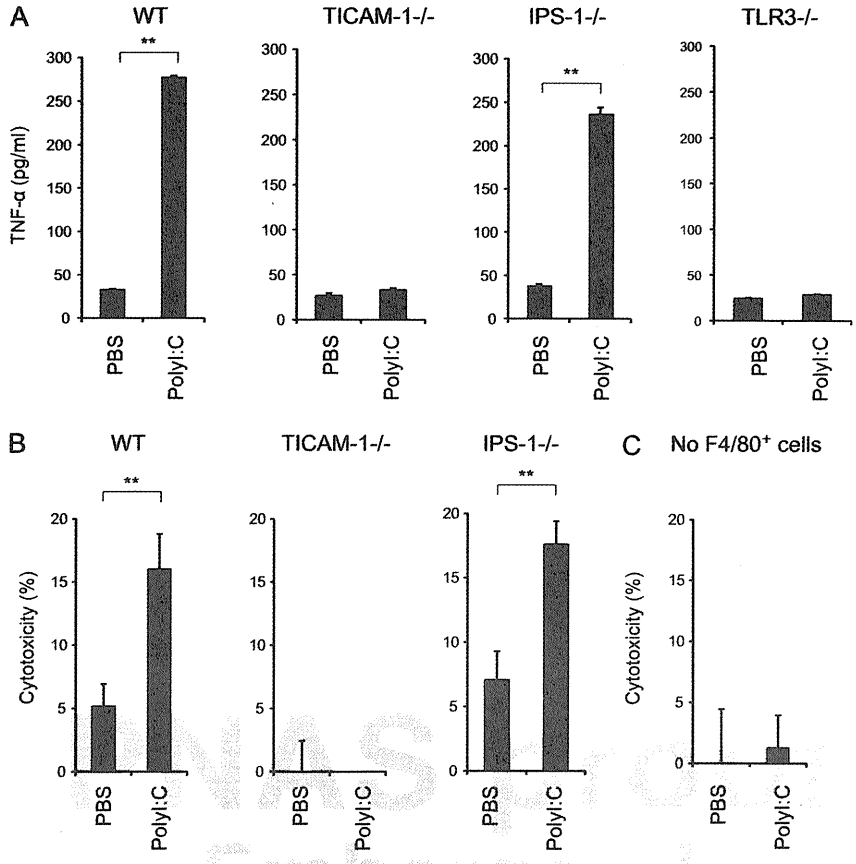

Fig. S8. In vitro poly I:C-stimulated F4/80$^+$ cells secrete TNF-α and have cytotoxic activity. (*A*) F4/80$^+$ cells isolated from 3LL tumor were stimulated with poly I:C (50 μg/mL) in vitro. After 24 h, the conditioned medium was collected and TNF-α concentration was determined by ELISA. (*B*) F4/80$^+$ cells isolated from tumor were mixed with ^{51}Cr-labeled 3LL tumor cells in the presence or absence of poly I:C (50 μg/mL). After 20 h, radioactivity of the conditioned medium was measured. E/T = 10. (*C*) ^{51}Cr-labeled 3LL tumor cells were incubated for 20 h in the presence or absence of poly I:C (50 μg/mL); $n = 3$. Data are shown as average ± SD. $^{**}P < 0.001$. A representative experiment of three with similar outcomes is shown.

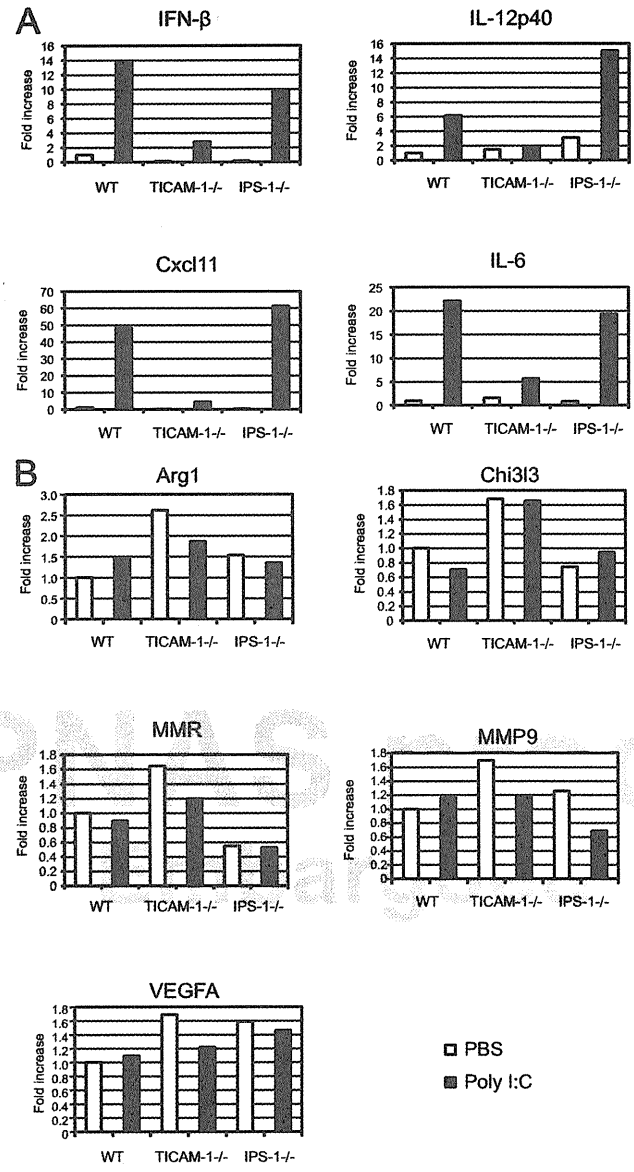

Fig. S9. Poly I:C induces the expression of M1 but not M2 macrophage-associated genes in tumor-infiltrated F4/80$^+$ cells through the TICAM-1 pathway. 3LL tumor-bearing mice were i.p injected with 200 μg poly I:C. After 3 h, tumors pooled from two mice treated with poly I:C or PBS were mixed. F4/80$^+$ cells were isolated from the mixed tumor, and the expression of (*A*) M1- and (*B*) M2-related genes was analyzed. A representative experiment of two with similar outcomes is shown.

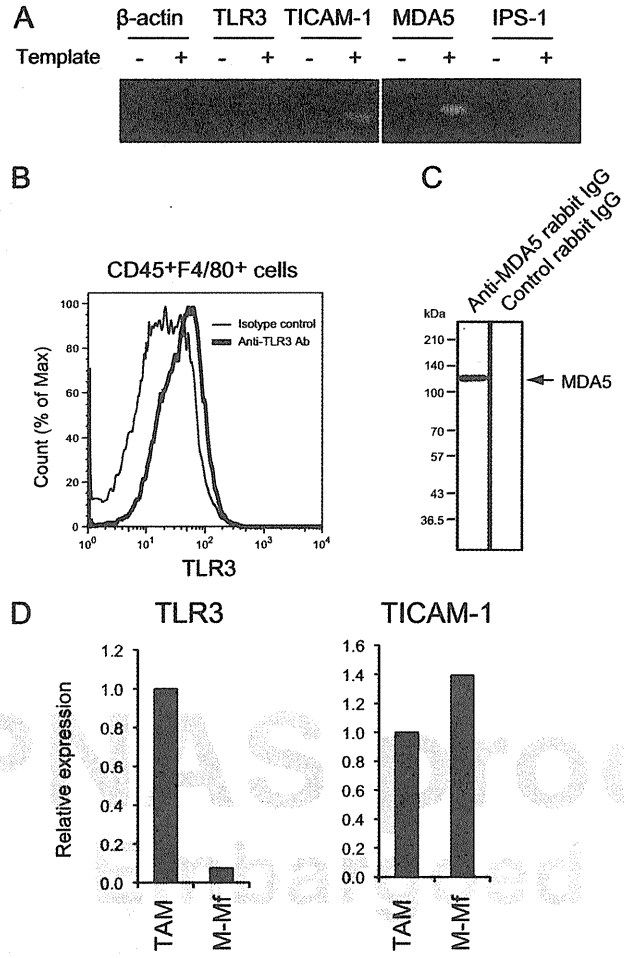

Fig. S10. Expression of TLR3 and MDA5 in 3LL tumor-associated F4/80$^+$ cells. (*A*) mRNA expression of TLR3, TICAM-1, MDA5, and IPS-1 in 3LL tumor-associated F4/80$^+$ cells. Total RNA (1 μg) of F4/80$^+$ cells isolated from 3LL tumor was used as a template for RT-PCR analysis. (*B*) Single-cell suspension of 3LL tumor was stained with FITC-labeled anti-CD45 and ~~APC~~-labeled anti-F4/80 antibody, followed by intracellular staining with Alexa 647-labeled anti-TLR3 antibody (11F8) or isotype control antibody (rat IgG2a). CD45$^+$F4/80$^+$ cells are shown. (*C*) Cytoplasmic extract of F4/80$^+$ cells was subjected to SDS/PAGE and immunoblotted with rabbit anti-MDA5 antibody or control IgG purified from rabbit serum. (*D*) mRNA expression of TLR3 and TICAM-1 in F4/80$^+$ tumor-associated macrophages (TAM) and macrophage colony-stimulating factor-induced bone marrow-derived macrophages (M-Mf).

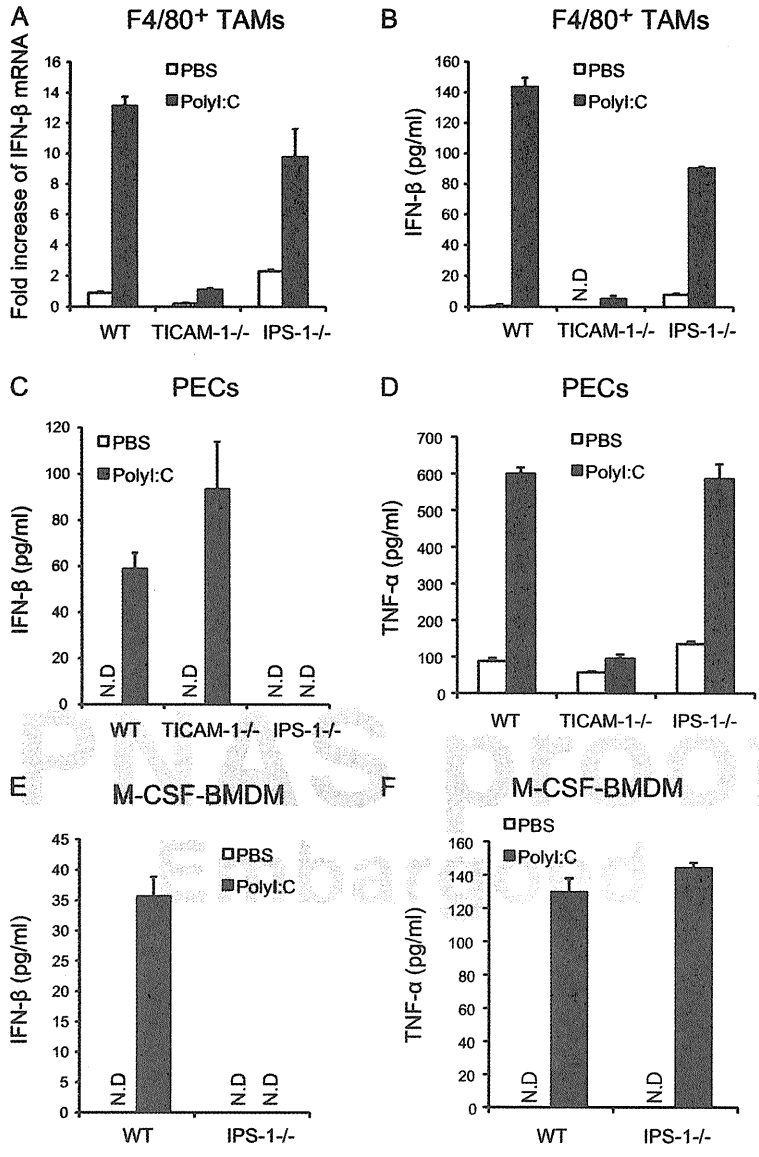

Fig. S11. In vitro stimulation with polyI:C increases the production of IFN-β and TNF-α by Mfs. (*A* and *B*) F4/80$^+$ cells were isolated from 3LL tumor implanted in WT, TICAM-1$^{-/-}$, and IPS-1$^{-/-}$ mice and stimulated with 50 μg/mL polyI:C. After 4 h, cells were harvested and IFN-β mRNA expression was analyzed by quantitative PCR analysis (*A*). After 20 h, IFN-β concentration in culture supernatant was determined by ELISA (*B*). (*C* and *D*) Peritoneal exudate cells (PECs) isolated from WT, TICAM-1$^{-/-}$, and IPS-1$^{-/-}$ mouse were stimulated with 50 μg/mL polyI:C for 20 h. The concentrations of IFN-β (*C*) and TNF-α (*D*) in culture supernatant were determined by ELISA. (*E* and *F*) Macrophage colony-stimulating factor (M-CSF)-induced bone marrow-derived macrophages (BMDM) were prepared from WT and IPS-1$^{-/-}$ mouse and cultured in the presence of 30% L929 supernatant containing M-CSF. After 6 d, adherent cells were harvested and stimulated with 50 μg/mL polyI:C for 20 h. The concentrations of IFN-β (*E*) and TNF-α (*F*) in culture supernatant were determined by ELISA. Data are shown as mean ± SD ($n = 3$). N.D., not detected. A representative experiment of two with similar outcomes is shown.

Table S1. Expression of various markers on 3LL and MC38 tumor cells

Surface marker	3LL	MC38
H2-K^b	–	++
H2-D^b	±	++
RAE1	++	Not determined
CD45	–	–

Expression of surface markers was analyzed by flow cytometry. Expression was evaluated by mean fluorescence shift: –, ~0.99 ; ±, 1~10 ; +, 11~100 ; ++, 101~.

Table S2. RT-PCR primers used in this study

	Forward primer (5′–3′)	Reverse primer (5′–3′)
IFN-β	CCAGCTCCAAGAAAGGACGA	CGCCCTGTAGGTGAGGTTGAT
IL-12p40	AATGTCTGCGTGCAAGCTCA	ATGCCCACTTGCTGCATGA
IL-6	GTGCATCATCGTTGTTCATACAATC	CTGGGAAATCGTGGAAATGAG
TNF-α	AGGGATGAGAAGTTCCCAAATG	GCTTGTCACTCGAATTTTGAGAAG
IL-1β	TGACGGACCCCAAAAGATGA	TGCTGCTGCGAGATTTGAAG
IL-10	GGCGCTGTCATCGATTTCTC	TGCTCCACTGCCTTGCTCTTA
Cxcl11	GGCTGCGACAAAGTTGAAGTGA	TCCTGGCACAGAGTTCTTATTGGAG
IRF4	AGCCCAGCAGGTTCATAACTACA	CCTCGTGGGCCAAACGT
IRF5	GGTCAACGGGGAAAAGAAACT	CATCCACCCCTTCAGTGTACT
Jmjd3	CGAGTGGTTCGCGGTACAT	GAAGCGGTAAACAGGAATATTGGA
Arg1	GGAATCTGCATGGGCAACCTGTGT	AGGGTCTACGTCTCGCAAGCCA
MMP9	CAAGTGGGACCATCATAACATCA	GATCATGTCTCGCGGCAAGT
VEGFA	GACATCTTCCAGGAGTACC	TGCTGTAGGAAGCTCATCT
Chi3l3	TCACTTACACACATGAGCAAGAC	CGGTTCTGAGGAGTAGAGACCA
Mrc1	CTCTGTTCAGCTATTGGACGC	CGGAATTTCTGGGATTCAGCTTC
Retnla	CCAATCCAGCTAACTATCCCTCC	ACCCAGTAGCAGTCATCCCA
GAPDH	GCCTGGAGAAACCTGCCA	CCCTCAGATGCCTGCTTCA

Table S3. Expression of surface markers on tumor-infiltrated F4/80$^+$ cells

Marker	Expression*
I-Ab	+
H2-D^b	+
H2-K^b	+
CD80	++
CD86	++
CD40	±
CD11c	±
CD3	−
CD4	−
CD8α	−
Gr1	+
B220	+
CD11b	+++
CD206 (MMR)	++

*Expression was evaluated by mean fluorescence shift: −, ~0.99 ; ±, 1 ~10 ; +, 11 ~100 ; ++, 101 ~1,000 ; +++, 1,001~.

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JB Review

Ubiquitin-mediated modulation of the cytoplasmic viral RNA sensor RIG-I

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RIG-I-like receptors, including RIG-I, MDA5 and LGP2, recognize cytoplasmic viral RNA. The RIG-I protein consists of N-terminal CARDs, central RNA helicase and C-terminal domains. RIG-I activation is regulated by ubiquitination. Three ubiquitin ligases target the RIG-I protein. TRIM25 and Riplet ubiquitin ligases are positive regulators of RIG-I and deliver the K63-linked polyubiquitin moiety to RIG-I CARDs and the C-terminal domain. RNF125, another ubiquitin ligase, is a negative regulator of RIG-I and mediates K48-linked polyubiquitination of RIG-I, leading to the degradation of the RIG-I protein by proteasomes. The K63-linked polyubiquitin chains of RIG-I are removed by a deubiquitin enzyme, CYLD. Thus, CYLD is a negative regulator of RIG-I. Furthermore, TRIM25 itself is regulated by ubiquitination. HOIP and HOIL proteins are ubiquitin ligases and are also known as linear ubiquitin assembly complexes (LUBACs). The TRIM25 protein is ubiquitinated by LUBAC and then degraded by proteasomes. The splice variant of RIG-I encodes a protein that lacks the first CARD of RIG-I, and the variant RIG-I protein is not ubiquitinated by TRIM25. Therefore, ubiquitin is the key regulator of the cytoplasmic viral RNA sensor RIG-I.

Keywords: RIG-I/type I interferon/ubiquitin/virus.

Abbreviations: CARD, caspase activation and recruitment domain; CTD, C-terminal domain; dsRNA, double-stranded RNA; RLR, RIG-I-like receptor; pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; MEF, mouse embryonic fibroblast cell; BM, bone-marrow; Mf, macrophage; IFN, interferon; ISG, interferon-stimulated gene; TRIM, tripartite motif; RNF, RING finger.

Recognition of viral RNA

Type I interferons (IFNs) are inflammatory cytokines that possess strong anti-viral activity. During viral infection, type I IFNs are produced from dendritic cells (DC), macrophages (Mf) and fibroblast cells (Fig. 1A). Viral RNA is mainly recognized by Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). TLRs are type I transmembrane proteins. TLR3, 7 and 8, which are members of the TLR family, are localized to endosomes, and are responsible for the recognition of viral RNA (*1*). RLRs are DExD/H box RNA helicases and recognize viral RNA in the cytoplasmic region (Fig. 1B). There are three members of the RLR family: RIG-I, MDA5 and LGP2. RIG-I has the ability to recognize various types of viruses, and MDA5 mainly recognizes picornaviruses (*2*). LGP2 promotes RIG-I and MDA5-mediated signalling (*3*).

A cytoplasmic sensor for the detection of viral RNA

RIG-I, a cytoplasmic sensor for viral RNA, is induced by viral infection, polyIC and type I IFN stimulation (*4*). This protein is composed of two N-terminal caspase recruitment domains (CARDs), a central DExD/H box helicase/ATPase domain and a C-terminal regulatory domain (CTD) (Fig. 2). N-terminal CARDs are responsible for the binding to the adaptor molecule IPS-1/MAVS/VISA/Cardif, which is located on the outer membrane of the mitochondria (*5–8*). In the absence of viral RNA, RIG-I CTD represses the interaction between RIG-I CARDs and IPS-1 CARD (*9*). RIG-I CTD recognizes the 5′ triphosphate of short double-stranded RNA, leading to multimerization of RIG-I and IPS-1 (*10–13*). IPS-1 triggers signaling to induce type I IFN and other inflammatory cytokines through STING (also called MITA) protein, which is localized to the endoplasmic reticulum or the mitochondria (*14–17*). STING then activates transcription factors, such as IRF-3, IRF-7 and NF-κB (*15*, *18*).

Knockout of RIG-I abrogates the production of type I IFNs and inflammatory cytokines from mouse embryonic fibroblasts (MEFs), conventional DC and Mfs in response to viral infections, including infections caused by vesicular stomatitis virus (VSV), Sendai virus (SeV), influenza A virus, Newcastle disease virus, hepatitis C virus and Japanese encephalitis virus (*2*, *19*). However, RIG-I is not necessary for the production of type I IFNs by plasmacytoid dendritic cells (pDCs), which are strong inducers of type I IFNs *in vivo* (*19*). In pDCs, TLR7 is responsible for the detection of viral RNA (*20*). In addition, knockout of IPS-1 and STING inhibits the production of type I IFNs from MEFs, Mfs and cDCs, but not from pDCs (*15–18*). Once type I IFNs are produced from these cells, IFN production is secondly amplified via the IFNAR (*21*). The deficiency of the RIG-I-dependent pathway causes a reduction in early type I IFN production *in vivo* but shows only a marginal effect on late type I IFN production (*15–18*). Knockout of RIG-I increases the

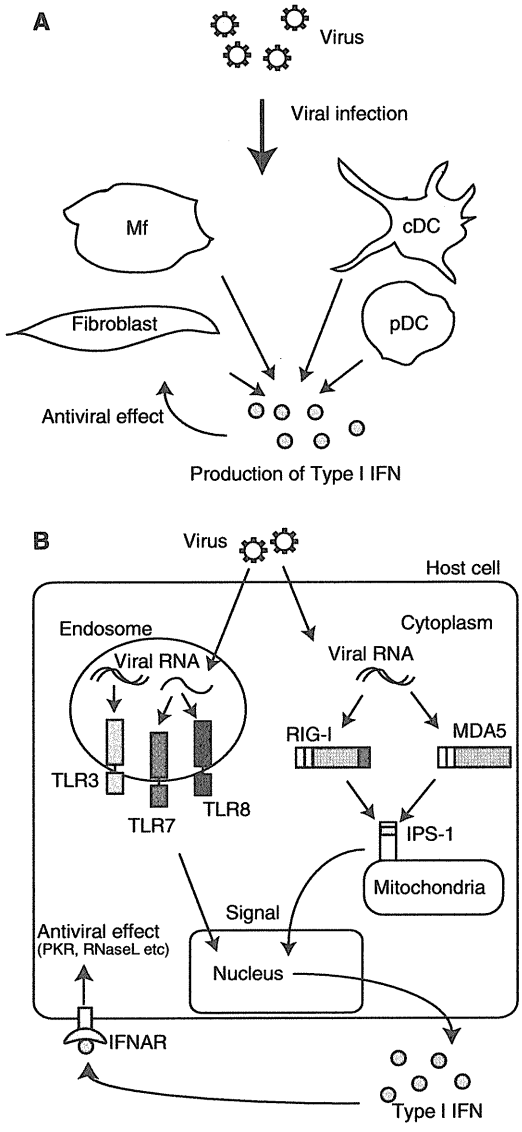

Fig. 1 Production of type I IFN in response to viral infection. (A) Type I IFN is a cytokine that possesses strong anti-viral activity. Type I IFN is produced from fibroblast cells, cDC, pDC and Mf in response to viral infection. (B) TLR3, 7 and 8 are localized to endosomes and are responsible for the recognition of viral RNA. Viral RNA in the cytoplasmic region is recognized by RIG-I and MDA5, leading to the activation of the adaptor molecule IPS-1. IPS-1 triggers the signal to induce type I IFNs. Type I IFNs binds to an IFN receptor, IFNAR, leading to the activation of anti-viral factors, such as PKR and RNaseL.

mortality due to viral infections (*2*, *19*). Thus, RIG-I-dependent pathways are necessary for efficient early type I IFN production and are required for protection against viral infections (*18*).

TRIM25 ubiquitin ligase is a positive factor for the RIG-I activation

During viral infection, the RIG-I protein has a modified form of ubiquitin. TRIM25 (also called Efp)

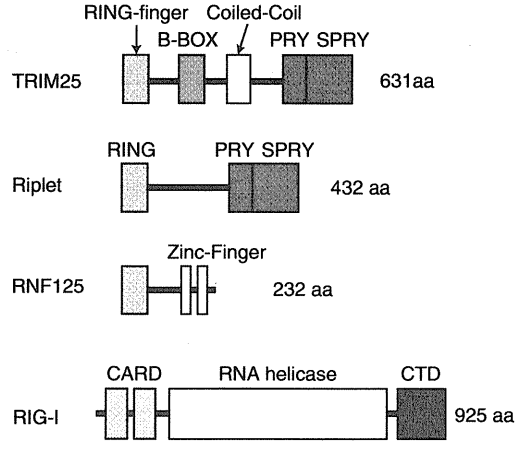

Fig. 2 Domain structures of TRIM25, Riplet, RNF125 and RIG-I. TRIM25 consists of RING finger, B-box, coiled-coil, PRY and SPRY domains. Riplet is similar to TRIM25 and consists of RING-finger, PRY and SPRY domains. RNF125 consists of RING-finger and two zinc-finger domains. Three proteins mediate the polyubiquitination of RIG-I. RIG-I consists of two N-terminal CARDs, central RNA helicase and CTDs.

is a ubiquitin ligase (*22*, *23*), and its domain structure is described in Fig. 2. This protein interacts with the first CARD of RIG-I (*22*, *24*). T55I mutation of the first CARD of RIG-I is found in RIG-I-deficient HuH7.5 cells. T55 of RIG-I is critical for the interaction between TRIM25 and RIG-I (*9*, *24*, *25*). Gack et al. detected the polyubiquitination of the K99, K169, K172, K181, K190 and K193 residues of RIG-I CARDs by mass spectrometry analysis (*22*), and the K172R mutation alone causes a near-complete loss of the polyubiquitination of RIG-I CARDs (*22*). TRIM25 delivers the K63-linked polyubiquitin moiety to the K172 residue of the second CARD of RIG-I, leading to efficient interaction with IPS-1/MAVS/VISA/Cardif (*22*, *24*). On the other hand, Zeng *et al.* reported another mechanism of the activation of RIG-I by ubiquitin. They reconstituted RIG-I pathway *in vitro* and showed that RIG-I CARDs sense unanchored polyubiquitin chains mediated by TRIM25, and the binding of RIG-I CARDs to the unanchored polyubiquitin chains leads to the activation of RIG-I (*26*). Knockout of TRIM25 abrogates IFN-β production from MEF in response to viral infection (*22*). Thus, ubiquitination or polyubiquitin binding is essential for the activation of RIG-I (Fig. 2).

The expression of a splice variant of RIG-I mRNA is robustly up-regulated upon viral infection (*24*). This splice variant encodes a protein that lacks the first 36–80 amino acid region within the first CARD of RIG-I; therefore, the RIG-I splice variant (RIG-I SV) protein loses TRIM25 binding, CARD ubiquitination and downstream signalling ability (Fig. 3) (*24*). RIG-I SV inhibits the multimerization of the wild-type RIG-I protein and IPS-1 interaction and shows a dominant negative effect on the RIG-I-mediated anti-viral IFN response (*24*). Thus, RIG-I SV acts as the off switch regulator of its own signalling pathway (*24*).

In addition to the IPS-1 adaptor molecule, RIG-I also binds to the inflammasome adaptor apoptosis-associated speck-like protein containing a CARD domain (ASC), also known as Pycard, in response to viral infection (*27*). ASC activates caspase-1, leading to the proteolytic processing of pro-IL-1β into mature, bioactive IL-1β (*28*). TRIM25 activity is dispensable for caspase-1 activation through ASC (*27*). Thus, RIG-I polyubiquitination by TRIM25 is dispensable for ASC inflammasome adaptor activation (*27*).

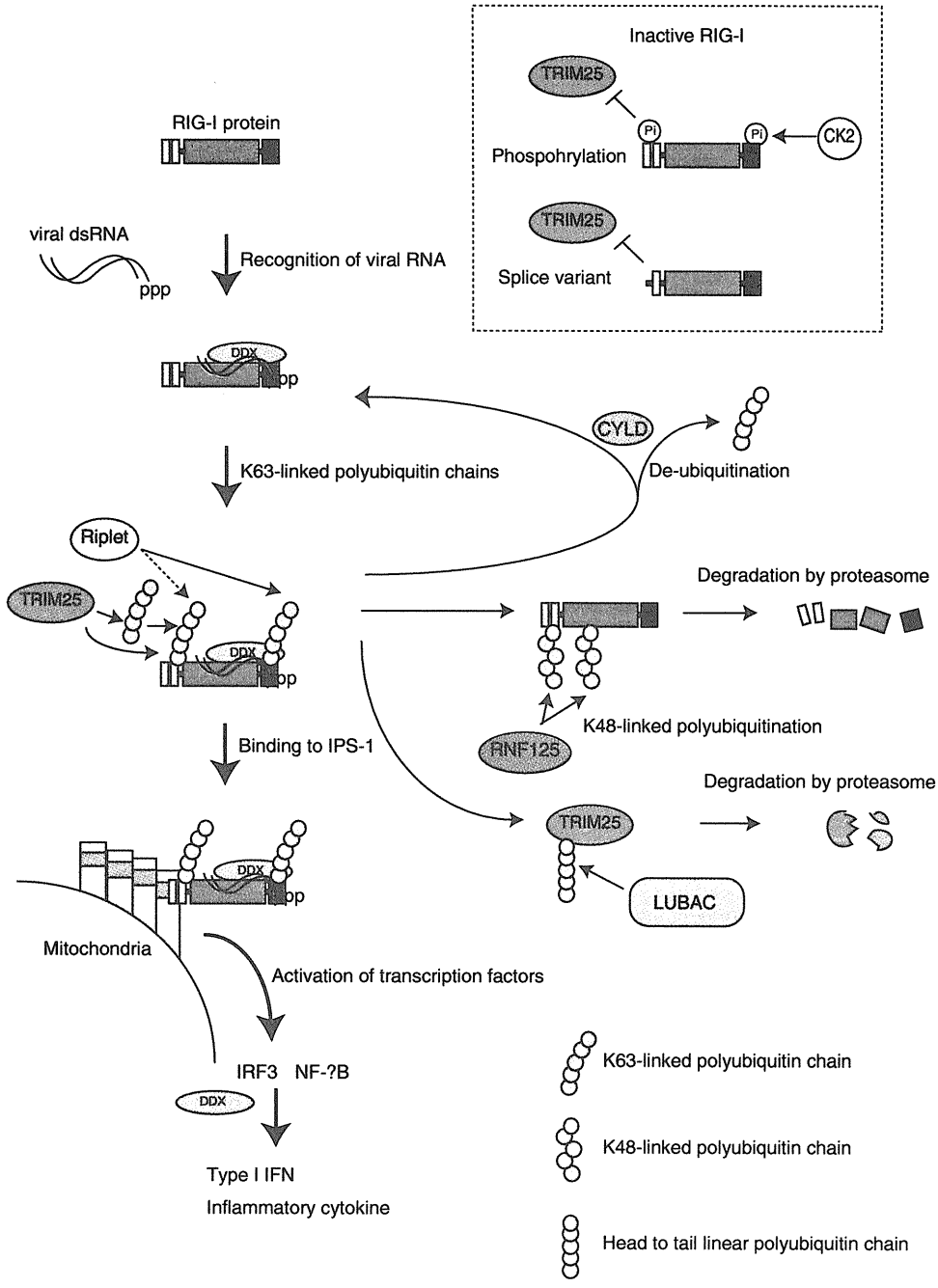

Fig. 3 Regulation of RIG-I by the ubiquitin chain. RIG-I binds to viral RNA together with other cofactors, such as DDX3. After the recognition of viral RNA, RIG-I changes its conformation and harbours K63-linked polyubiquitination by TRIM25 and Riplet. Polyubiquitination causes the activation of IPS-1, leading to the production of type I IFN. CYLD, a deubiquitin enzyme, removes the polyubiquitin chain of RIG-I. CK2 and other unknown kinase phosphorylate RIG-I, and the phosphorylated RIG-I protein is not polyubiquitinated by TRIM25. In addition, splice variant RIG-I (SV RIG-I) is not polyubiquitinated by TRIM25, and the SV RIG-I protein acts as a dominant negative form. RNF125 mediates the K48-linked polyubiquitinatioin of RIG-I, which causes the degradation of RIG-I by proteasomes. The LUBAC protein complex suppresses TRIM25 function by mediating the head-to-tail polyubiquitination of TRIM25.

However, RIG-I polyubiquitination is essential for NF-κB activation by RIG-I, which is required for IL-1β mRNA expression; thus, knockout of TRIM25 reduces the production of mature IL-1β (*4*, *19*, *27*).

Riplet ubiquitin ligase is essential for the activation of RIG-I

Riplet (also called Reul or RNF135) was isolated by yeast two-hybrid screening to isolate RIG-I CTD binding proteins (*29*). The Riplet protein is composed of N-terminal RING finger, C-terminal SPRY and PRY domains, and is similar to TRIM25 (Fig. 2). However, this protein lacks B-box, which is a typical feature of TRIM family proteins. Thus, the protein does not belong to the TRIM family. Riplet expression is observed in various tissues and cells such as DC, Mfs and MEF (*29*, *30*). Hu *et al.* (*31*) detected endogenous Riplet protein in human DC lysates. Riplet expression is induced in mouse bone marrow-derived DCs (BM-DCs) by polyIC stimulation, which is a double-stranded RNA analog; however, its expression is not changed in human fibroblast and HeLa cells (*29*).

The Riplet protein physically interacts with RIG-I CTD, and in some experimental conditions, it binds to RIG-I CARDs (*29*, *32*). The Riplet C-terminal region is responsible for this binding. Riplet mediates K63-linked polyubiquitination of RIG-I CTD, leading to the activation of RIG-I (Fig. 3) (*29*). The five CTD lysine residues at 849, 851, 888, 907 and 909 are important for the polyubiquitination and activation of RIG-I (*29*, *30*). In contrast, Gao *et al.* (*32*) reported that Riplet mediates K63-linked polyubiquitination of K154, K164 and K172 of RIG-I CARDs in their experimental conditions (Fig. 3).

In some strain backgrounds, RIG-I-deficient mice are embryonic lethal, but Riplet knockout mice are born at expected Mendelian ratios from Riplet$^{+/-}$ mice (*19*, *30*, *33*). Moreover, the development of DCs and Mfs is also normal in Riplet-deficient mice (*30*). Douglas *et al.* (*30*, *34*) reported that Riplet/RNF135 haploinsufficiency causes an overgrowth syndrome and learning disabilities in human: however, knockout of the Riplet gene in mice does not cause any apparent defects with regard to development. Knockout of Riplet severely reduces the production of type I IFN and abrogates the activation of RIG-I and RIG-I CTD polyubiquitination (*30*). Riplet knockout mice are more susceptible to VSV infection than wild-type mice. As IPS-1 and STING, Riplet is necessary for efficient, early type I IFN production *in vivo*, but it is dispensable for late type I IFN productions (*30*). This indicates the essential role that Riplet plays in the RIG-I-dependent innate immune response against RNA virus infection. Genetic evidence shows that knockout of either Riplet or TRIM25 destroyed the RIG-I-dependent innate immune response; therefore, both ubiquitin ligases are required for the activation of RIG-I in response to RNA virus infection (*22*, *30*). RLR pathways contribute to type I IFN expression in response to cytoplasmic DNA (*35–37*). However, Riplet-independent type I IFN expression pathway in response to cytoplasmic DNA exists in MEF (*30*).

Ubiquitin ligases target several proteins. For example, TRIM25 targets the proteolysis of 14-3-3 σ, a negative cell cycle regulator that causes G2 arrest, and thus, promotes breast tumour growth (*23*). Proteome analysis reveals that Riplet binds to the TRK-fused gene (TFG), which is a target of chromosome translocation in lymphoma (*38–40*). Pasmant *et al.* (*41*) reported that the Riplet/RNF135 gene is down-regulated in tumour Schwann cells from malignant peripheral nerve sheath tumours, and their study suggested the involvement of Riplet/RNF135 in an increased risk of malignancy observed in NF1 microdeletion patients. Thus, it is possible that Riplet targets not only RIG-I but also other proteins.

Negative regulators of RIG-I

The RNF125 (also called TRAC1) protein possesses a RING finger domain and functions as a ubiquitin ligase (*42*). Arimoto *et al.* (*43*) isolated RNF125 by yeast two-hybrid screening to obtain the protein that binds to UbcH8, which is an E2 ubiquitin-conjugating enzyme, and found that RNF125 also binds to RIG-I. Unlike Riplet and TRIM25, RNF125 ubiquitin ligase mediates K48-, but not K63-linked polyubiquitination of RIG-I, leading to the degradation of RIG-I by proteasomes (Fig. 3) (*43*). UbcH5c is possibly an E2 enzyme, which cooperates with RNF125, and UbcH8 acts as a negative factor in the RNF125-mediated polyubiquitination of RIG-I (*43*, *44*). Furthermore, RNF125 ubiquitinates MDA5, a member of RLRs, and the expression of RNF125 impairs MDA5-mediated signalling (*43*). RNF125 expression is induced by type I IFN and polyIC treatment. The increase in RNF125 mRNA expression correlates temporally with the decrease in RIG-I expression (*43*). Knockdown of RNF125 increases the type I IFN expression in response to viral infection (*43*). Since RNF125 is enhanced by type I IFN, the function of RNF125 constitutes a negative regulatory loop circuit for type I IFN production.

CYLD is a deubiquitinase that cleaves the K63-linked polyubiquitin chain. This protein acts as a negative regulator of NF-κB and Jun N-terminal kinase signalling pathways by cleaving the K63-linked polyubiquitin chains of NEMO, TRAF2 and BCL3 (*45–48*). Friedman *et al.* (*49*) performed a microarray analysis and found that the expression profile of RIG-I is correlated with that of CYLD. Moreover, they found that the CYLD protein physically interacts with RIG-I, TBK1 and IKKε, and deubiquitinates these proteins. CYLD inhibits SeV-induced type I IFN production. Thus, it is expected that CYLD attenuates the establishment of an anti-viral state (Fig. 3).

There are host and viral negative regulators for TRIM25. HOIL-1L and HOIP are members of the RING-IBR-RING (RBR) E3 ubiquitin ligase family and form complexes (*50*). HOIL-1L and HOIP form ubiquitin polymers through the linkage between the C- and N-termini of the ubiquitin molecules in order to assemble a head-to-tail linear polyubiquitin chain; thus,

the protein complex is designated as LUBAC (linear ubiquitin assembly complex) (*50*). LUBAC has the ability to induce polyubiquitination of TRIM25; it specifically suppresses TRIM25-mediated RIG-I ubiquitination by inducing TRIM25 degradation and inhibiting TRIM25 interaction with RIG-I (Fig. 3) (*51*). Excessive production of IFNs or inflammatory cytokines is destructive rather than protective; thus, an absolute regulation of the immune signalling pathway is essential for a successful immune response against viral infections. HOIL-1L- and HOIP-mediated suppression of TRIM25 would be important for the absolute regulation of an immune response (*51*).

Viruses have evolved sophisticated mechanisms to evade the host IFN system. There are several virus-encoded IFN antagonists that inhibit host innate anti-viral responses. NS1 of the influenza A virus is one of the IFN antagonists (*52*, *53*). It sequestrates viral dsRNA from cellular sensors including RIG-I (*52*). In addition, it interacts with the coiled-coil region of TRIM25 and blocks TRIM25 multimerization and RIG-I CARD polyubiquitination (*54*).

Perspectives

Several ubiquitin-like proteins (UBLs) exist. ISG15 is a UBL and is induced in response to viral infection (*55*). Several anti-viral proteins are modified by ISG15, including RIG-I (*44*, *55*). UbcH8 is an E2 enzyme that promotes ISG15 conjugation to RIG-I (*44*). However, ISG15 knockout mice do not either reduce immunological functions or decrease anti-viral activity (*56*). Thus, the physiological role of ISG15 conjugation to RIG-I remains unknown.

In addition, the RIG-I protein is modified by phosphorylation. The T170 residue of RIG-I is phosphorylated under normal conditions, and phosphorylation is reduced after SeV infection (*24*). Phosphorylation of RIG-I CARDs inhibits the TRIM25-mediated polyubiquitination (Fig. 3). Thus, Gack *et al.* suggested that dephosphorylation of RIG-I permits the TRIM25 binding and TRIM25-mediated polyubiquitination of RIG-I, allowing RIG-I to form a stable complex with IPS-1 in order to trigger an IFN-mediated anti-viral innate immune response. However, the kinase and phosphatase that target RIG-I N-terminal CARDs are still unknown. In addition to RIG-I CARDs, RIG-I CTD is regulated by phosphorylation. In resting cells, casein kinase II (CK2) phosphorylates T770, and S854 and S855 (*57*). The phosphorylation of RIG-I CTD suppresses the RIG-I-mediated signalling (Fig. 3) (*57*). Following viral infection, phosphatases cause dephosphorylation of the RIG-I CTD, leading to the activation of RIG-I-mediated signalling (*57*).

RIG-I requires several cofactors. High mobility group box proteins are required for the RIG-I to recognize viral RNA (*58*). DDX3 and DDX60 are non-RLR helicases that are involved in RLR signallings, and play pivotal roles in RIG-I-mediated signalling (Fig. 3) (*59–62*). It remains to be determined whether the post-translational modification of RIG-I affects the interaction with those co-factors.

Riplet ubiquitinates RIG-I CTD. The molecular mechanism of how the Riplet-dependent polyubiquitination of RIG-I CTD triggers the downstream signalling remains to be determined yet. RIG-I CTD has two functions. In the absence of viral RNA, RIG-I CTD suppresses the activation of RIG-I CARDs. Following viral infection, RIG-I CTD binds to viral RNA, leading to the conformational changes and ultimately removal of the suppression. It is possible that CTD polyubiquitination affects both functions of RIG-I CTD.

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Conflict of Interest
None declared.

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Review Article

Mycobacterium bovis Bacille Calmette-Guérin as a Vaccine Vector for Global Infectious Disease Control

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Mycobacterium bovis bacille Calmette-Guérin (BCG) is the only available vaccine for tuberculosis (TB). Although this vaccine is effective in controlling infantile TB, BCG-induced protective effects against pulmonary diseases in adults have not been clearly demonstrated. Recombinant BCG (rBCG) technology has been extensively applied to obtain more potent immunogenicity of this vaccine, and several candidate TB vaccines have currently reached human clinical trials. On the other hand, recent progress in the improvement of the BCG vector, such as the codon optimization strategy and combination with viral vector boost, allows us to utilize this bacterium in HIV vaccine development. In this paper, we review recent progress in rBCG-based vaccine studies that may have implications in the development of novel vaccines for controlling global infectious diseases in the near future.

1. Introduction

Mycobacterium bovis bacille Calmette-Guérin (BCG) is the only licensed vaccine that has substantially helped controlling tuberculosis (TB) for more than 80 years. This vaccine affords ~80% protection against TB meningitis and miliary TB in infants and young children [1]. However, the BCG-induced protective effects against pulmonary diseases over all ages are variable; the escalation of the worldwide TB epidemic is evidence that the vaccine does not work well to prevent pulmonary TB [2]. Recently, studies on the advanced molecular biology and genomics of mycobacteria have revealed that the BCG genome has various mutations and deletions compared with the original virulent strain of *Mycobacterium tuberculosis* and *M. bovis* [3]. Interestingly, there are substantial differences in the genomic DNA even among BCG substrains [4, 5] that can cause biological differences in the population of BCG vaccines.

Since a host-vector system in mycobacteria was developed in 1987 [6], recombinant BCG (rBCG) technology has been extensively applied in the development of vaccines against a variety of infectious diseases, including bacterial, viral, and parasitic infections in addition to TB [7, 8]. BCG is attractive as a vaccine vector because of its extensive safety record in humans, heat stability, low production cost, induction of long-lasting type 1 helper T cell (Th1) immunity, $CD8^+$ T-cell triggering, adjuvant activity, usability in newborns and its mucosal immune induction by oral administration. Taking the current situation of serious epidemics of emerging and reemerging diseases mainly in developing African and Asian countries into account, a new global vaccine should be affordable in such areas. Therefore, the low price and heat stability of BCG-based vaccines would be desirable. In this paper, we review various efforts to develop novel BCG vector-based vaccines mainly for controlling TB and HIV/AIDS.

2. Immunological Properties of BCG Vector

The immune responses induced by BCG are outlined in Figure 1. The most characteristic response to BCG is the induction of innate (nonspecific) immunity by cell wall components through toll-like receptors (TLRs) 2 and 4 on dendritic cells and macrophages [9]. After phagocytosis,

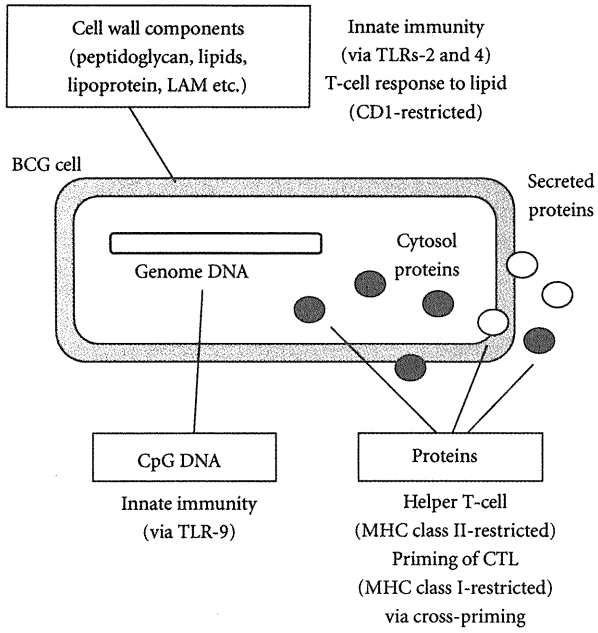

FIGURE 1: Outline of immune responses by BCG. Both innate immunity via TLRs and antigen-specific immunity via MHC- or CD1-restricted antigen presentation to T cells are induced by various BCG cell components.

BCG is degraded by lysosomal enzymes, and the processed antigen can be presented to the host immune system via various pathways. DNA fragments containing the CpG motif may activate innate immunity via the TLR9 route [10]. Lipids such as mycolic acid presented by CD1 stimulate CD1-restricted $CD8^+$ T cells [11]. Protein antigens, such as antigen 85 complex produced by BCG, induce Th1 response through presentation by major histocompatibility complex (MHC) class II. This pathway is the major route of BCG-induced responses and is indispensable for protective immunity against *M. tuberculosis* infection via protective cytokine interferon (IFN)-γ production. On the other hand, the processing and presentation of protein antigens via the MHC class I pathway are also elicited in the BCG-infected antigen presenting cell (APC). As reported by Goonetilleke et al. [12], immunizing BCG-sensitized animals with recombinant vaccinia virus MVA expressing antigen 85A greatly enhances the MHC class I-restricted CTL response against antigen 85A, indicating that BCG priming could be a novel type of prime-boost vaccine. This immunological feature of BCG vector allows its application in vaccines against chronic viral infectious diseases such as HIV/AIDS. In addition, the strong Th1 induction by BCG would be favorable to aid the maturation and maintenance of CTL [13]. Thus, the BCG vector is expected to induce effective cell-mediated immunity against a targeted antigen.

3. TB Vaccine

3.1. Background of the Global TB Epidemic. TB kills 1.7 million people worldwide each year; someone dies from TB every 19 seconds [14]. Although the TB treatment protocol was established a long time ago, the recent increase of multidrug-resistant *M. tuberculosis* infection has generated a serious situation. New vaccines are urgently needed to eliminate TB as a public health threat and should be a major global public health priority. TB is a disease that is spread from person to person through the air. Furthermore, the terrible synergy between TB and HIV makes this disease even more dangerous, especially in sub-Saharan African countries. For instance, according to the World Health Organization's (WHO) Global TB report 2010 [14], South Africa had nearly 400,000 new TB cases in 2009 with an incidence rate of an estimated 806 cases per 100,000; TB is one of the leading causes of death in both adults and children of this country. The case fatality rate has increased from 3% in 1993 to 24.3% in 2007. A major reason for the increased fatality rate is South Africa's concurrent HIV epidemic. The prevalence of HIV infection in South Africa in 2009 was approximately 7%, which has been decreasing as a result of various efforts toward prevention. TB is a common opportunistic infection among people living with HIV, and 60% of new TB cases occurred in persons who were also infected with HIV in 2009 [14]. We can observe similar critical situations in the countries surrounding South Africa. Regarding the vaccination, such situation has raised concerns about the safety of using BCG vaccine in HIV-infected infants because between 10 and 30% of pregnant women are HIV infected in many sub-Saharan African countries.

3.2. Current Efforts toward New TB Vaccine Development. The global plan to stop TB 2011–2015 report [15] offers 7 objectives as follows: (i) to maintain a robust TB vaccine pipeline by supporting research and discovery, (ii) to conduct research to identify correlates of protection and preclinical studies to assess new TB vaccine candidates, (iii) to ensure the availability of vaccine production capacity by expanding manufacturing facilities for TB vaccines, (iv) to build capacity for large-scale clinical trials (phases II and III) of TB vaccine candidates at field sites in TB-endemic countries, (v) to conduct phase I, II, and III clinical trials of TB vaccine candidates, (vi) to develop delivery, regulatory, and access strategies for new TB vaccines, (vii) to build support for TB vaccine development and uptake through advocacy, communications, and resource mobilization. All these objectives are important to realize new TB vaccine development.

The main goal of vaccine development in the Global Plan to Stop TB 2006–2015 is for 2 vaccines to be in proof-of-concept trials by 2010 and that 1 new and safe vaccine is available by 2015. As of 2009, 12 TB vaccine candidates had entered clinical trials. Of these, 9 are still being tested (Table 1) : 5 are in phase I clinical trials, 2 are in phase II trials, and 2 are in phase IIb proof-of-concept trials [15]. One vaccine has produced estimates of safety and effectiveness in a targeted HIV-infected population. At least 6 TB vaccine candidates are in preclinical development, and at least 21 additional next-generation candidates are in the vaccine discovery phase [15]. As mentioned earlier, the current BCG vaccine has limited and variable effectiveness against TB.

Table 1: Summary of candidate TB vaccines in clinical trials 2009. Nine candidate preventive TB vaccines are currently in clinical phases.

Status	Products	Product description	Sponsor
Phase IIb	MVA85A/AERAS-485	Vaccinia virus MVA	OETC/AERAS
Phase IIb	AERAS-402/Crucell Ad35	rBCG/adenovirus 35	Crucell/AERAS
Phase II	Hybrid-I + IC31	Ag85B/ESAT6 + adjuvant	SSI/TBVI
Phase II	M72	Fusion protein + adjuvant	GSK/AERAS
Phase I	AdAg85A	adenovirus 5/Ag85A	McMaster Univ.
Phase I	VPM 1002	rBCG/listeriolysin::ΔureC	Max Planck/TBVI
Phase I	Hyvac 4/AERAS-404	Fusion protein + adjuvant	SSI/Sanofi/AERAS
Phase I	RUTI	Fragmented Mtb cell	Archivel Farma
Phase I	Hybrid-I + CAF01	Ag85B/ESAT6 + adjuvant	SSI

Abbreviations in the sponsors: AERAS, AERAS Global TB Vaccine Foundation; GSK, GlaxoSmithKline; OETC, The Oxford-Emergent Tuberculosis Consortium Ltd.; SSI, Staten Serum Institute; TBVI, Tuberculosis Vaccine Initiative.

Therefore, the first choice of strategy may be improving BCG by using recombinant DNA technology even though it may imply safety issue of vaccination in HIV-infected individuals. Overproduction against a protective antigen of TB in BCG (rBCG30) exhibited enhanced immunogenicity in humans [16]. Moreover, the expression of the listeriolysin gene in BCG (rBCG/*hly*$^+$::Δ*ureC*) is proven to be more potent in the induction of TB-specific cellular immune responses [17]. Another strategy for improving BCG vaccines is boosting BCG immunity with protein [18, 19] or viral vector vaccine such as modified vaccinia virus Ankara (MVA) strain [20] and adenovirus type 35 [21]. BCG-prime and recombinant MVA-antigen 85A boost regimen [22] exhibited efficient immune responses in humans and have entered the first phase IIb trial in newborns. Furthermore, a combination of such strategies in which 3 major antigens are overproduced and the perfringolysin gene is incorporated into BCG and boosted with a recombinant adenovirus vaccine has been developed [23]. However, it is unknown whether such strategies are relevant for developing vaccines that are effective against adult pulmonary TB. It is necessary to test whether these candidate vaccines effectively induce mucosal immunity and protect against lung disease.

4. HIV/AIDS Vaccine

4.1. Background of the Global HIV Epidemic. In 2009, there were an estimated 2.6 million people who became newly infected with HIV. This is more than 21% less than the estimated 3.2 million who became infected in 1997, the year in which annual new infections peaked. In 33 countries, the incidence of HIV has decreased by more than 25% between 2001 and 2009; 22 of these countries are in sub-Saharan Africa. This trend reflects a combination of factors including the impact of HIV prevention efforts and the natural course of HIV epidemics [24].

Although highly activated antiretroviral therapy apparently contributes to control HIV replication in infected individuals [25], several problems remain to be resolved. These problems include: (i) the following viral load recovers soon after the interruption of treatment; (ii) chronic toxicities cause abnormalities in lipid metabolism and mitochondria; (iii) drug-resistant viruses increase during long period of treatment; (iv) long-term treatment carries a risk of carcinogenesis [26]; (v) expensive drugs are still difficult to access in developing countries. Even in developed countries, the high cost of antiretroviral drugs produces a sense of impending crisis in public health policy [27]. In such circumstances, although the rate of new infections with HIV-1 is gradually decreasing, an effective preventive vaccine is still urgently needed to stem further spread of the virus [28]. Even though considerable recent progress has been made in the development of an HIV vaccine [29, 30], the immune correlate of viral protection is not fully elucidated due to the complicated interaction of viral, immunological, and genetic factors [31, 32]. Since it is known that some populations of HIV-1-infected people do not present disease progression when HIV-1 replication is regulated by host immunity [33, 34], targeted vaccine immunogens are designed to closely mimic the long-lasting protective immunity induced in the long-term human survivors of natural infection [35, 36]. Due to safety issues, a live-attenuated HIV vaccine is not practical. This inevitably led the trend of HIV vaccine development to component- and vector-based vaccines.

4.2. Current Trends in HIV/AIDS Vaccine Research. The first large-scale efficacy trial of an HIV/AIDS vaccine was conducted by a US company, Vaxgen Co., in which a genetically engineered surface envelope (Env) glycoprotein, gp120, vaccine was tested in humans. Although the vaccine was targeted toward inducing effective virus-neutralizing antibodies, the phase III efficacy trial revealed its ineffectiveness [37, 38]. The failure of the gp120 vaccine changed the trend of HIV/AIDS vaccine research from an antibody-targeted strategy to a cell-mediated immunity-targeted strategy. Because HIV-1 causes chronic infection due to its cell-associated features, cellular immunity especially virus-specific cytotoxic T lymphocyte (CTL) should be a more important arm of the host immune system. Indeed, immune deficiency virus-specific cell-mediated immunity has been suggested to effectively control viral replication during the natural course of viral infections [39–41]. Based on these findings, various vaccine modalities, including live viral vectors and DNA vaccines, have been used to elicit strong CTL and Th1 type

responses in nonhuman primate models. Although single-vaccine delivery systems sometimes exhibit insufficient immune responses, boosting with viral vector vaccines such as vaccinia virus [40, 41], adenovirus [42, 43], and Sendai virus [44] in DNA-primed individuals strongly amplified CTL responses and resulted in the effective control of simian immunodeficiency virus (SIV) replication. Among such viral vectors, adenovirus type 5 (Ad5) had the strongest CTL enhancement effect, and the DNA-prime and recombinant Ad5 boost vaccine strategy is recognized as the most promising. However, in 2007, Merck Co. reported that a recombinant Ad5 vaccine expressing HIV-1 Gag, Pol, and Nef antigens did not demonstrate any protective efficacy in a phase IIB clinical trial [45]. Surprisingly, the vaccinated group exhibited a significantly higher HIV-1 infection rate than the placebo group [45], suggesting that the recombinant Ad5 immunization may have some unknown effect in enhancing HIV-1 infection. Thus, we were aware that T-cell vaccine approaches may involve certain risks and limitations; this paradigm appears to have reached an impasse.

In September 2009, there was ground-breaking news that the RV144 large-scale efficacy trial in Thailand demonstrated a partial effect of reducing HIV-1 infection rate in the recipients of ALVAC (canarypox)/gp120 prime-boost vaccine [46]. Although the results demonstrated limited effects, they demonstrated the possibility of preventing HIV infection with the active immunization for the first time. Furthermore, although there was no apparent correlation between protection and virus-specific cellular immune response or neutralizing antibody levels in the vaccinees, more detailed analyses of the total host responses are expected in the future. Taking the vaccine formulation with the gp120 protein boost into account, some antibody-mediated reactions may be involved in this partial protection. On the other hand, a new T-cell-targeted vaccine also demonstrated protective efficacy in a macaque study in the same year. A rhesus cytomegalovirus-vectored vaccine expressing SIV Gag, Rev-Tat-Nef, and Env persistently infected rhesus macaques, primed, and maintained robust SIV-specific $CD4^+$ and $CD8^+$ effector memory T-cell responses in the absence of neutralizing antibodies [47]. The report suggests that T cell vaccines may have greater potential than previously estimated. Although the importance of broadly neutralizing antibody production would not change despite tremendous difficulties, cellular immunity-targeted candidate vaccines should be also clinically tested for proofs of concept.

4.3. BCG-Vectored HIV Vaccine. The most practical advantage of the BCG vector is its high safety. In addition to being effective at inducing protective immunity, an HIV-1 vaccine regimen must be shown to be safe, affordable, and compatible with other vaccines before it can be considered promising [39]. In this respect, vectors that have already been used in humans without serious complications and with low cost should be utilized for HIV vaccines. BCG is a unique live vaccine vector because of its easy antigen delivery to the professional APC to be presented to T cells. Therefore, this bacterium is expected to be an important vector for HIV vaccine development.

At the early stage of rBCG research in the 1990s, Aldovini and Young [48] demonstrated immunogenicity of rBCG against genetically engineered HIV-1 antigens in mice. We independently worked on an rBCG-vectored anti-HIV vaccine simultaneously. First, we demonstrated effective cellular immune induction against SIV Gag antigen by the rBCG vector in rhesus macaques [49, 50]. Furthermore, we cloned an extracellular α antigen (antigen 85B) gene from both BCG [51] and *Mycobacterium kansasii* [52], and established a foreign antigen secretion system in mycobacteria [53]. Based on this system, we extensively evaluated several rBCG constructs for candidate HIV vaccines and reported that an rBCG-HIV vaccine could induce protective humoral immune responses in guinea pigs [54]. These studies suggest that rBCG-based vaccines are feasible as AIDS vaccines. However, the CTL activity did not reach protective levels with a single injection of rBCG-HIV vaccine in the macaque model. To overcome the low immunogenicity of the rBCG vaccine in CTL induction, we utilized various strategies for enhancing the immune potential of the BCG vector.

4.4. Prime-Boost Regimen for Enhancing Immune Responses. The first strategy by which we tried to improve the potential of the rBCG-HIV vaccine was the use of a safe recombinant viral vector for a booster vaccine. With respect to safety, traditional live vaccines, which have been administered safely to both the healthy and the HIV-infected individuals, may be the vectors of choice for HIV-1 vaccines. To fully take advantage of the benefits of such traditional vaccines in the development of anti-HIV vaccines, we studied BCG Tokyo 172 strain and the replication-deficient vaccinia vaccine strain DIs [55, 56] both of which have been shown to be nonpathogenic when inoculated into immune-deficient animals as live recombinant vaccine vehicles [57]. The vaccinia virus DIs have been tested clinically as a smallpox vaccine in Japanese infants and proved to be quite safe. We chose this highly attenuated virus as a booster vaccine vector and constructed recombinant DIs (rDIs) expressing the HIV *gag* [58] or SIV *gag-pol* gene [59]. Both rDIs constructs were found to be effective in eliciting HIV- or SIV-Gag-specific immunity in mice. When they were administered as a booster antigen after priming with an SIV-DNA vaccine, the cellular immunity to SIV Gag was greatly enhanced [59]. In brief, we tested a new combination regimen: priming with rBCG-SIV Gag followed by boosting with rDIs-SIV Gag.

In the macaque study, we found that BCG/DIs vaccination induced a long-lasting and effective cellular immunity that was able to control a highly pathogenic virus SHIV C2/1 [60], after mucosal challenge [61]. A possible mechanism of effective Gag-specific cell-mediated immunity is shown in Figure 2. The strong Th1 response induced by the BCG vector may contribute to eliciting the Gag-specific CTL response. How these immune inductions are correlated with protective efficacy requires further investigation. In this study, the BCG/DIs vaccination developed high levels of cellular immunity in the macaques that were protected against the loss of $CD4^+$ T lymphocytes with reduced viral RNA levels after virus challenge. Furthermore, the BCG/DIs group showed no evidence of clinical diseases or mortality

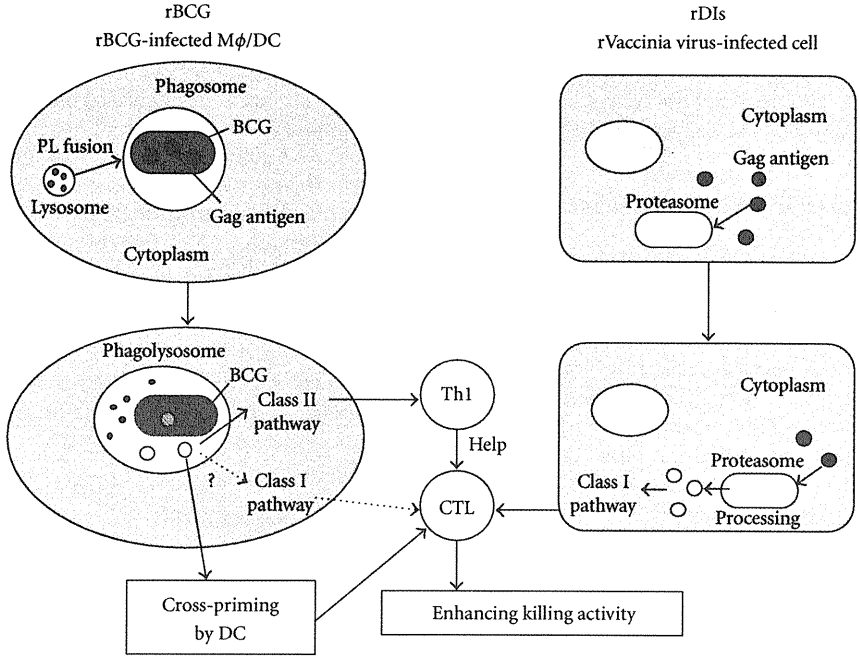

FIGURE 2: A possible mechanism of effective Gag-specific cell-mediated immunity induction with the rBCG/rDIs prime-boost vaccine. Abbreviations: DC, dendritic cell; Mϕ, macrophage; PL, phagosome-lysozome; Th1, type 1 helper T cell; CTL, cytotoxic T lymphocyte.

after viral challenge during the 1-year observation period [61]. These results suggest that the BCG/DIs prime-boost regimen might be a potential candidate for an effective and safe anti-HIV vaccine. Recent studies in macaques subjected to BCG/Ad5 [62] and BCG/MVA [63] regimens strongly support the effectiveness of the BCG vector. In the latter study, a hemolysin-expressing BCG strain, which was devised for more efficient antigen presentation to the CTL precursor, elicited a robust and broad range of HIV-1 specific T-cell responses along with recruitment of multiple T-cell clonotypes into the memory pool.

4.5. Codon Optimization Strategy. The major issue with BCG vehicle vaccines is the low expression level of the foreign antigen gene in BCG cells. In general, sufficient levels of foreign antigen-specific immune responses are obtained with high doses of rBCG between 10- and 100-fold greater than that needed for a practical dose against TB in humans [54]. This is considered the main limitation for the clinical use of rBCG-based vaccines. To address this substantial issue, we applied a codon optimization strategy for foreign genes in the rBCG system to increase its expression level. The aims of the study were to increase the immunogenicity of the foreign antigen, decrease inoculation dosages as small as the conventional BCG vaccine against TB, avoid adverse reactions, prevent possible association with Th2-type immune responses, and ward off the exacerbation of retroviral infections.

First, we determined the in vitro effects of codon optimization of the HIV gene in rBCG. Although the effect of codon optimization in mammalian cells is well documented [64–66], its effect in rBCG vehicle had never been fully elucidated. We targeted the HIV-1 *gag p24* gene as a model antigen to clarify the effect of codon optimization in the rBCG system. A specially designed synthetic p24 gene consisting of mycobacterial-preferred codons resulted in an increase in their GC content from 43.4% to 67.4%. Furthermore, codon-optimized rBCG was generated without any detectable changes in its characters including the growth rate. This rBCG exhibited a dramatic increase in Gag p24 antigen production approximately 40-fold greater than the non-optimized rBCG. Moreover, we successfully obtained data regarding the enhancement of immune responses in codon-optimized rBCG-immunized mice [67]. Inoculation of mice with a single low dose of the codon-optimized bacteria elicited effective cellular immunity. In the ELISPOT assay, the number of Gag-specific IFN-γ spot-forming cells elicited by codon-optimized rBCG was significantly greater than that elicited by non-optimized recombinants [67]. These cellular immune responses would decrease if the $CD8^+$ T cells were depleted. The results also suggest that effective MHC-class I-restricted CTL responses are inducible by vaccination with codon-optimized rBCG. Furthermore, Gag-specific lymphocyte proliferative responses were also detected in the codon-optimized rBCG-immunized mice [67].

We also applied this strategy to an SIV Gag construct and successfully generated an rBCG harboring the codon-optimized SIV *gag* gene with an expression 10-fold greater than that of the native *gag* gene. In the macaque study, compared with a native *gag* gene construct, a low-dose (10^6 bacilli) injection of this construct induced optimal priming of Gag-specific $CD4^+$ and $CD8^+$ T cells and prolonged the maintenance of memory T-cell response after vaccinia DIs

boost [68]. These results imply that the quality of the priming vaccine is a critical factor for inducing a desirable immune response against immunodeficiency viruses. Thus, the codon optimization strategy should generally be applied to other foreign genes in rBCG-based vaccine development.

5. Vaccine for Other Infectious Diseases

There were various candidate rBCG vaccines targeting infectious diseases other than TB or HIV. Stover et al. [69] reported that the rBCG system would be useful in Lyme disease vaccine development; the vaccine incorporated with the surface protein of *Borrelia burgdorferi* first reached clinical phase I trials. However, the vaccine was rejected due to its low antibody production response [70]. Two groups [71, 72] applied rBCG in malaria vaccine development and demonstrated efficacy in a mouse model. Malaria is recognized as one of the three major infectious diseases as well as TB and AIDS. Although there is a long history of malaria vaccine development, we have not seen any licensed vaccine. The strategy to induce cellular immunity against conserved antigens using BCG vector could be effective to overcome substantial difficulties in producing vaccine due to antigenic diversity and unique life cycle of this parasite. In addition, BCG vector was tested for vaccine discovery against some viral diseases. A rBCG expressing the measles virus nucleoprotein demonstrated protection against measles virus pneumonia in macaques [73]. Furthermore, we demonstrated that a rBCG with a single hepatitis C virus (HCV) NS5 CTL epitope into antigen 85B induced HCV-specific CTL response in mice [74]. HCV is recognized as one of the major infectious pathogens of which the global infection rate is ~3%. Although the priority for preventive HCV vaccine development has become lower because of the remarkable progress in the treatment, BCG vector of targeting CTL induction may have implication for therapeutic vaccine against this disease. All these candidates at the early stage of rBCG study could not proceed to further development stages at those times. The rBCG-based vaccine development for these diseases should be reconsidered because the advanced technology that enhances the potential of BCG vectors has become currently available.

6. Conclusion and Future Perspective

As described in Section 3, several rBCG-based candidate vaccines are currently being evaluated for the development of TB vaccines. Such human trials would provide a greater insight into the paradigm of immune correlation in *M. tuberculosis* infection. In addition, the application of the codon optimization strategy enables us to utilize this bacterial vector as a primer of a heterologous prime-boost regimen for a preventive HIV vaccine. These results could suggest that the BCG vector is possible divalent vaccine controlling both TB and HIV/AIDS with a single construct; such study may help resolve the serious public health problem in the sub-Saharan African countries in which both diseases are highly prevalent [14].

Another potential outcome is the utility of the BCG vector for infant vaccines. One of the largest advantages of rBCG vaccines is their applicability to newborns. Because BCG as a TB vaccine is integrated into the expanded program on immunization in many countries, we have the earliest chance to immunize newborns with BCG within 3 months of birth before they are exposed to a variety of infectious pathogens. Substituting the current BCG with a novel rBCG vaccine possessing protective antigens against pathogens that cause serious diseases in infants, such as severe diarrhea and respiratory diseases, could be effective in developing countries. Such vaccine concepts should be also tested in appropriate animal models before they are tested in humans. Thus, after much trial and error in the last 2 decades, rBCG-based vaccines may contribute to the control of global infectious diseases in the near future.

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