NK-activating capacity of BMDC is usually linked to their maturation, neither cytokines in NK activation, including IFN-α and IL-12p40, nor co-stimulators such as CD40 and CD86, were specifically induced in mDC by INAM expression (Fig. S6). INAM has a C-terminal cytoplasmic stretch (Fig. 4A), and we tested the function of this region by a deletion mutant (C-del INAM). This region in BMDC barely participates in driving NK activation, since no decrease of IFN-γ induction by NK cells was observed with IRF-3-/- BMDC supplemented with C-del INAM compared to control INAM. Thus far, no significant signal alteration has been detected in BMDC supplemented with INAM by lentivirus.

On the other hand, INAM-transduced IRF-3-/- NK cells produced IFN-γ in concert with BMDC like WT NK cells (Fig. 5F). However, introduction of C-del INAM into IRF-3-/- NK cells did not result in high induction of IFN-γ in response to BMDC (Fig. 4C). Together with the data on INAM expression in BMDC, these infer that the INAM cytoplasmic region signals for NK activation in NK cells. The one way role of the cytoplasmic tail in NK activation will be an issue to be further analyzed.

In this study, IL-15 was found to be up-regulated by polyI:C in BMDC. The remaining NK activity in the resting population of NK cells co-cultured with TICAM-1-/- BMDC and polyI:C (Fig. 1B), suggests that IL-15 has some effect in our system and other report (9, 36-38). However, we did not observe decreased IL-15 expression in the TICAM-1-/- BMDC thatcould not activate NK cells (Fig. 1E). *In vitro* co-culture systems (Figs. S1) suggest that the IPS-1 pathway in BMDC has a pivotal role in not only type I IFN induction but also mDC-driven NK activation. A number of molecules such as B7-H6/NKp30 (39), CD48/2B4 (40) and NKG2D ligands/NKG2D (32) have been identified as ligand/receptor molecules in mDC-NK reciprocal activation by *in vitro* co-culture. INAM identified here serves a unique function in the *in vivo* induction of NK activation, and may offer a tool to investigate the reported mDC-mediated NK activation.

Rae-1 was reported as a molecule with MHC-like structure (41), and later identified as a mouse NKG2D ligand (32). Although Rae-1 is a GPI-anchored protein with no cytoplasmic sequences (42), it can act as an NK-activating ligand (32, 43, 44). Murine BaF3 cells become NK-sensitive after forced expression of Rae-1α (44). Actually, mouse macrophages induce Rae-1 expression in response to TLR stimuli (45). In contrast, INAM-expressing stable BaF3 cell lines (INAM/BaF3) did not reveal a function as an NK cell-activating ligand. NK cell cytotoxicity is directed against Rae-1α/BaF3 cells, but not against INAM/BaF3 cells (Fig. S8). Therefore, INAM does not represent a typical NK activating ligand. For NK activation, INAM on BMDC appears to require other molecules that are expressed in BMDC, but not in BaF3.





INAM has four transmembrane regions, similar to the cell adhesion tetraspanins, which may support cell-cell contact (46). Tetraspanins provide a scaffold that facilitates complex formation with associated proteins. INAM on BMDC and NK cells may use cell-cell interaction to assemble in a synaptic formation to activate NK cells. Since the protein constituents of the tetraspanin complexes are cell-specific, we are interested in finding partners for INAM that might participate in efficient BMDC/NK interaction. TLR-inducible cell-cell contact may occur through INAM in an immune cell-specific manner. Knockout of this INAM will facilitate clarifying this issue. The identification of INAM defines a novel pathway in mDC-NK reciprocal interaction. This study will lead to further research on the molecules that form complexes with INAM on BMDC and NK cells and lead to efficient BMDC-NK interaction.

## EXPERIMENTAL PROCEDURE

### Mice

All mice were backcrossed with C57BL/6 mice >7 times before use. TICAM-1-/- (8) and IPS-1-/- mice were generated in our laboratory. Exact information about the IPS-1 mice we established will be presented elsewhere. IRF-3-/- (47) and IRF-7-/- mice (48) were kindly provided by T. Taniguchi (Univ. Tokyo). All mice were maintained under specific pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine. Animal experiments were performed according to the guidelines set by the animal safety center, Japan.

### Cells

B16D8 cell line was established in our laboratory as a subline of B16 melanoma (49). This subline was characterized by its low or virtually no metastatic properties when injected subcutaneously (s.c.) into syngeneic C57BL/6 mice. B16D8 was cultured in RPMI 1640/10% fetal calf serum (FCS). The mouse B cell line, BaF3 was obtained from ATCC and cultured in RPMI 1640/10% FCS/2 μM 2ME/5 ng/ml IL-3. Mouse NK cells ($DX5^+$ cell) were positively isolated with MACS Beads (Miltenyi Biotec, Auburn, CA). Mouse BMDC were prepared as previously reported (8).

For purification of cells from spleen or LN, these tissues were treated with 400IU MandleU/ml collagenase D (Roche) at 37°C for 25min in HBSS (SIGMA). Then EDTA was added, and the cell suspension was incubated for additional 5min at 37°C. After removal of RBC with ACK lysis buffer, Splenocytes and Lymph node cells were stained

with CD45-FITC, CD3ε-PE, CD19-PE, DX5-PE, CD11b-FITC (eBioscience) and CD11c-FITC (Biolegend), and sorted by FACS Aria II (BD Biosciences). The purity of sorted cells were >96%.

**Construction and expression**

Mouse INAM cDNA (A630077B13Rik) was obtained from RIKEN (Tokyo, Japan) and placed into expression vector pEFBOS and pLenti-IRES-hrGFP, both of which provide the specialized components needed for expression of a recombinant C-terminal FLAG fusion (8). For construction of shRNA-expressing lentivirus vector, The ClaI/XhoI flagment of pLenti6-blockit-dest (Invitrogen, Carlsbad, CA) was inserted into pLenti-IRES-hrGFP at the site of ClaI and XhoI. This vector was named as pLenti-dest-IRES-hrGFP (pLDIG). INAM sequence 5'-CTTCTCTCCGGTTAGTTATCT-3' was targeted for INAM knockdown (shINAM/pLDIG) and 5'-AGTCTGACATACTTATACTTA-3' was used for negative control (shCont/pLDIG). We used a gene-expression kit, Lenti-viral system (Invitrogen) as previously described (8). Four plasmids (one of these pLenti vectors, pLP1, pLP2, pLP/VSVG) were transfected into 293 FT packaging cells, and the viral particles for transfection were prepared according to the manufacturer's protocol. The 100x concentrated virus particles were produced after centrifugation of 8000g at 4 °C for 16 h. Lentivirus produced by pLenti-IRES-hrGFP and pLDIBG could be tittered by GFP expression using flow cytometry. Since this lentivirus vector, pLenti-IRES-hrGFP, has the IRES-GFP region, we prepared negative control virus by pLenti-IRES-hrGFP without construct. Infection efficiency for BMDC was high with the control vector compared to the INAM-expressing lentivector as in Fig. S4.

**Real-time PCR**

BMDC were harvested after 4 h stimulation by LPS (100 ng/ml), PolyI:C (50 μg/ml), $Pam_3CSK_4$ (Pam3: 1 μg/ml), mycoplasmal macrophage-activating lipopeptide-2 (Malp-2: 100 nM), CpG (10 μg/ml), and IFN-α (2000 IU/ml) (50). Mouse tissues (heart, stomach, small intestine, large intestine, lung, brain, muscle, liver, kidney, thymus and spleen) were collected from C57BL/6. Splenocytes were stained by CD3-PE, CD19-PE, DX5-PE, CD11b-PE, CD11c-FITC and PDCA1-PE (eBioscience, San Diego, SA) and sorted by BD FACS Aria. Purity was over 98% in each population. For RNA extraction, we used the RNeasy kit (Invitrogen). After removal of genomic DNA by treatment with DNase, randomly primed cDNA strands were generated with Moloney murine leukemia virus-reverse transcriptase (Promega, Madison, WI). RNA expression was quantified by quantitative RT-PCR with gene-specific primers (IL-15 forward: TTAACTGAGGCTGGCATTCATG, IL-15 reverse: ACCTACACTGACACAGCCCAAA, INAM forward: CAACTGCAATGCCACGCTA, INAM reverse: TCCAACCGAACACCTGAGACT, β-actin forward: TTTGCAGCTCCTTCGTTGC β-actin reverse: TCGTCATCCATGGCGAACT, HPRT forward: GTTGGATACAGGCCAGACTTTGTTG, HPRT reverse: GAAGGGTAGGCTGGCCTATAGGCT) and values were normalized to the expression of β-actin mRNA or HPRT mRNA.

Other primers for PCR were designed using Primer Express software (Perkin Elmer Applied Biosystems, Foster City, CA, USA) for another experiments. The following primers were used for PCR: ß-actin forward (Fwd), 5'-CCTGGCACCCAGCACAAT-3' and reverse (Rev), 5'-GCCGATCCACACGGAGTACT-3'; Granzyme B (Gzmb) Fwd, 5'-TCCTGCTACTGCTGACCTTGTC-3' and Gzmb Rev, 5'-ATGATCTCCCCTGCCTTTGTC-3'; IFN-α4 Fwd, 5'-CTGCTGGCTGTGAGGACATACT-3' and Rev, 5'-AGGCACAGAGGCTGTGTTTCTT-3'; TRAIL (Tnfsf10), Fwd: 5'-CTTCACCAACGAGATGAAGCAG-3' and Rev, 5'-TCCGTCTTTGAGAAGCAAGCTA-3'; IL-12p40 (Il12b), Fwd: 5'-AATGTCTGCGTGCAAGCTCA-3' and Rev, 5'-ATGCCCACTTGCTGCATGA-3'.

**Anti-INAM pAb**

C-terminal-INAM (cINAM: 191-314 aa) was subcloned between Nde I and Sal 1 sites of pCold I vector (TAKARA, Tokyo, Japan). 6x His-tagged cINAM protein was expressed in BL21 by manufacturer's methods. The cells were sonicated in 20 mM Tris-HCl, 150 mM NaCL 1mM PMSF and 7M Urea (pH 7.4) on ice. Expression products of cINAM were purified using the HisTrap™ HP Kit (GE Healthcare). The extracted proteins were refolded by stepwise dialysis against decreasing amounts of urea. Rabbit anti-cINAM polyclonal Ab was produced with the cINAM proteins by standard protocol. IgG was purified by precipitation with 33% ammonium sulfate, dialyzed against PBS

**Surface labeling with biotin**

Biotinylation of cell-surface proteins was performed according to the reported method (51). Briefly, cells (~$10^8$) were suspended in 1 ml of HEPES-buffered saline (HBS), pH 8.5, and incubated with 10 ml of 10 mg/ml NHS-sulfobiotin (Vector Co. Ltd) for 1 h at room temperature. Cells were washed in HBS three times, then solubilized with lysis buffer containing 1% NP-40, pH 7.4. The cell lysate was immnoprecipitated with

avidin-labeled Abs as described previously (51).

**Immunoblot analysis**

Lysates were harvested 24 h after transfection of Flag-tagged-INAM/pEFBOS into 293FT cells and treated with N-Glycosidase F (PNGaseF; NEB, Ipswich, MA) by manufacturer's method in some experiments. Protein samples were separated on SDS-PAGE and immunoblotted by anti-Flag M2 Ab (Sigma, St. Louis, MO).

**Confocal microscopy**

BMDC and NK cells were infected with control or INAM-expressing lentivirus as described previously (Fig. S5). 24 hrs later, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with PBS containing 0.5% saponin for 30min at room temperature. Fixed cells were stained with anti–FLAG mAb and Alexa568-conjugated secondary Ab. Stable Ba/F3 transfectants expressing INAM were treated with Cytofix/Cytoperm (BD) according to manufacturer. Then cells were stained with PE-phalloidin and rabbit anti-INAM pAb followed by Alexa 488-conjugated secondary Ab. Cells were analyzed on LSM510META confocal microscope (Zeiss, Jena, Germany) for the detection of INAM.



**BMDC/NK interaction**

BMDC were co-cultured with freshly isolated NK cells (BMDC:NK = 1:2~1:5) with or without polyI:C (10 μg/mL) for 24 h (8). In some experiments, function of BMDC and NK cells was modified by lentivirus vector before BMDC/NK coculture. IRF-3-/- BMDC were transfected by control lentivirus and INAM-expressing lentivirus (INAM/pLenti-IRES-hrGFP) and incubated with polybrene (6 μg/mL) for 24 h before co-culture. WT BMDC were transfected with shRNA expressing lentivirus (shCont/pLDIG or shINAM/pLDIG) and incubated with polybrene (6 μg/mL) for 48 h before co-culture. Freshly isolated NK cells were transfected with control lentivirus and INAM-expressing lentivirus (INAM/pLenti-IRES-hrGFP) and cultured with polybrene (6 μg/mL) in the presence of IL-2 (500 IU/mL) for 72 h before co-culture. Activation of NK cells was assessed by concentration of IFN-γ (ELISA: GE-Healthcare, Buckinghamshire, UK) in the medium and by NK cytotoxicity against B16D8. Cytotoxicity was determined by standard $^{51}$Cr release assay as described previously (8).

***Ex vivo* NK activation**

Mice were intraperitoneally (i.p.) injected with 250 μg polyI:C. After 24 h, spleen cells were harvested and then NK cells ($DX5^+$ cells) were positively isolated with MACS



system (Miltenyi Biotec, Auburn, CA). The $DX5^+$ NK cells were suspended in RPMI1640 with 10% FCS, and mixed with $^{51}$Cr-labeled B16D8 cells at indicated E/T ratios. After 4h, supernatants were harvested and $^{51}$Cr release was measured. Specific lysis was calculated by (specific release - spontaneous release) / (max release - spontaneous release). In some experiments, blood was drawn from the eyes of mice 8 h after polyI:C administration for cytokine measurement.

**Test for *in vivo* NK activation in LN**

WT BMDC ($5x10^5$) incubated with or without polyI:C (10 μg/ml) for 24 h, or IRF-3-/- BMDC ($5x10^5$) infected with control virus or INAM-expressing lentivirus and allowed to stand for 24 h, were injected into the footpads of WT C57BL/6 mice. 48 h later, cells in their inguinal LN were harvested, and stained with PE-DX5 and sorted by FACS AriaII. RNA was extracted from the DX5-positive cells with TRIzol.

**Dendritic cell therapy**

DC therapy against mice with B16D8 tumor burden was described previously (8). C57BL/6 mice (N = 3) were shaved at the flank and injected subcutaneously with 6 x $10^5$ syngeneic B16D8 melanoma cells (indicated as Day 0). For DC therapy, BMDC were prepared by transfecting control lentivirus or INAM-expressing lentivirus (INAM/pLenti-IRES-hrGFP) and cultured for 24 h. At the time point indicated in the figures, 1 x $10^6$ cells of BMDC were injected subcutaneously near the tumor. To deplete NK cells *in vivo*, mice were i.p. injected with hybridoma ascites of anti-NK1.1 mAb (PK136) (8). Tumor volumes were measured using a caliper every 1 or 2 days. Tumor volume was calculated using the formula: Tumor volume ($cm^3$) = (long diameter) x (short diameter) x (short diameter) x 0.4.

**Statistical analysis**

Statistical analyses were made with the Student's *t*-test. The P value of significant differences is reported. Unless otherwise stated, plotted data represent mean ± standard deviation (s.d.).

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**Abbreviations list**

BMDC, bone marrow-derived DC; GFP, geen fluorescence protein; INAM, IRF-3-inducible NK activation molecule (also named FAM26F); IRF, interfern regulatory factor; IPS-1, Interferon promoter stimulator-1; mDC, myeloid DC; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; Rae-1, retinoic acid-inducible gene 1; TICAM-1, Toll/IL-1 receptor homology domain-containing adaptor molecule 1.





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**Figure legends**

Figure 1. IRF-3 in BMDC controls the capacity to activate NK cells in response to polyI:C. (A, B, C) WT or TICAM-1 -/- NK cells were co-cultured with WT, TICAM-1 -/-, IRF-3 -/- or IRF-7 -/- BMDC in the presence of polyI:C (10 μg/mL) for 24 h. NK cytotoxicity against B16D8 was determined by standard $^{51}Cr$ release assay. (D) ELISA of IFN-α in WT, TICAM-1-/-, IRF-3-/-, and IRF-7-/- BMDC treated with polyI:C (10 μg/mL) for 24 h. (E) Quantitative RT-PCR for IL-15 expression in BMDC stimulated with polyI:C (10 μg/mL). All data are means ± s.d. of duplicate or triplicate samples from one experiment that is representative of three.

Figure 2. Sequence alignment of INAM and expression of INAM. (A) Sequence alignment of human and mouse INAM. Asterisks, identical residues; double dots, conserved substitutions; single dots, semi-conserved substitutions; box, N-glycosylation site; underline, trans-membrane motif. (B) Immunoblot analysis of lysates of 293FT cells transfected with plasmid encoding Flag-tagged INAM. PNGase, N-Glycosidase. 2ME, 2-Mercaptoethanol. (C, D) Quantitative RT-PCR for INAM expression in mouse tissue. $CD3^+$, $CD19^+$, $DX5^+$, $CD11b^+$, $CD11c^+$, mDC (myeloid DC: $CD11c^+PDCA1^+$) and pDC (plasmacytoid DC: $CD11c^+PDCA1^+$) cells were isolated from splenocytes by cell sorting. Data shown are means ± s.d. of triplicate samples from one experiment that is representative of three. (E) Augmented INAM expression in lymph node cells after polyI:C stimulation. WT mice were i.p. injected with polyI:C (100 μg) or control buffer. After 24 h, inguinal, axillar and mesenteric LN were harvested and RNA was extracted from the LN cells. The levels of the INAM mRNA were measured by real-time PCR.

Figure 3. INAM on BMDC participates in DC-mediated NK activation. (A) Quantitative RT-PCR for INAM expression in WT, TICAM-1-/-, IRF3-/-, and IRF-7-/- BMDC stimulated with polyI:C (10 μg/mL). (B) Quantitative RT-PCR for INAM expression in WT BMDC stimulated by LPS (100 ng/ml), polyI:C (10 μg/ml), Pam3 (1 μg/ml), Malp-2 (100 nM), CpG (10 μg/ml), and IFN-α (2000 IU/ml) for 4 h. (C) BMDC were transduced with Flag-tagged INAM-expressing lentivirus, or control lentivirus. GFP expression in the BMDC was determined by flowcytometry, and subcellular localization of INAM was examined by immunofluorescence assay using anti-Flag mAb. Shaded peak, non-infected control; Blank peak, infected BMDC. (D, F) ELISA of IFN-γ induced by NK cells co-cultured with WT BMDC or IRF-3-/- BMDC tranfected with control lentivirus, or INAM-expressing lentivirus with/without polyI:C (10 μg/mL). In some experiments, a transwell was inserted between the INAM-transduced BMDC and NK cells to separate the cells. (E) Cytotoxicity against



B16D8 by NK cells co-cultured with BMDC transfected with control or INAM-expressing lentivirus with/without polyI:C (10 μg/mL) for 24 h. (G) Quantitative RT-PCR for expression of INAM in BMDC transduced with INAM-shRNA (INAM) or scrambled shRNA (Control) and cultured for 48 h. (H) IFN-γ production by WT NK cells determined using ELISA after co-culturing with control or the shRNA transfected-BMDC (INAM) and polyI:C (10 μg/mL) for 24 h. All data shown are means ± s.d. of triplicate samples from one experiment that is representative of three.

Figure 4. Role of the cytoplasmic tail of INAM. (A) Construction of INAM and a mutant which lacks C-terminal region of INAM (C-del INAM). (B) C-terminal region was not required for BMDC-mediated NK activation. ELISA of IFN-γ by WT NK cells co-cultured with IRF-3-/- BMDCs transfected with control lentivirus (CV) or INAM (intact INAM or C-del INAM)-expressing lentivirus with/without polyI:C (10 μg/mL). Data shown are means ± s.d. of triplicate samples from one experiment representative of three. (C) The cytoplasmic tail of INAM is indispensable for NK IFN-γ induction. INAM or C-del INAM (see panel A) was expressed on IRF-3-/- NK cells. CV was used as control. The INAM (or C-del INAM) -expressing IRF-3-/- NK cells were incubated with WT BMDC for 24 h. IFN-γ levels in the supernatants were determined by ELISA. One representative result out of several similar experiments is shown.

Figure 5. INAM on NK cell works for efficient NK activation mediated by mDC. (A, B) Quantitative RT-PCR for INAM expression in WT, TICAM1 -/- or IRF-3 -/- NK cells stimulated with polyI:C (50 μg/mL). Data shown are means of duplicate or triplicate samples from one experiment that is representative of three. (C) IRF-3-/- BMDC were transfected with control lentivirus, or INAM-expressing lentivirus before treatment with polyI:C (10 μg/mL) for 4 h. BMDC in some wells were washed to remove polyI:C before wild-type (WT) NK cells were added. IFN-γ production by NK cells was determined by ELISA after 24 h culture. (D) ELISA of IFN-γ in co-culture of WT or IRF-3-/- NK cells and WT BMDC with/without polyI:C (10 μg/mL). (E, F) NK cells were transfected with control lentivirus, or INAM-expressing lentivirus and cultured with IL-2 (500 IU/mL) for 3 days. After determining transfection efficiency by GFP intensity using flow cytometry, cells were cultured with/without BMDC for 24 h and IFN-γ production in the supernatant determined by ELISA. Shaded peak, non-infected control; Blank peak, infected BMDC. All data are means ± s.d. of triplicate samples from one experiment that is representative of three.

Figure 6. INAM on BMDC retarded B16D8 tumor growth in a NK-dependent manner.

(A) Tumor volume after dendritic cell (DC) therapy using BMDC expressing INAM. B16D8 cells were subcutaneously injected into C57BL/6 mice and 11-13 d later, medium only (○) or BMDC (1 x $10^6$/mouse) transfected with control lentivirus (◆) or those with INAM-expressing lentivirus (■) were administered subcutaneously near the tumor at the time indicated by the open arrow. (B) Abrogation of INAM-dependent tumor regression by administration of NK1.1 Ab. For depletion of NK cells, antiNK1.1 mAb was injected intraperitoneally 1 d before treatment of BMDC (arrow head). Tumor volume in every mouse group was sequentially monitored. Data represent mean ± s.d. (n = 3), and are representative of two experiments. Statistical analyses were made with the Student's *t*-test.

Figure 7. INAM-mediated induction of TRAIL and granzyme B in BMDC. (A) *In vitro* induction of TRAIL (Tnfsf10) and granzyme B (Gzmb) mRNA by INAM-expressing BMDC. BMDC(IRF-3-/-) were infected with INAM-expressing virus or CV as in Fig. S4. After 24 h, the BMDC(IRF-3-/-) were incubated with WT NK cells at DC:NK=1:2. 8 h later, DX5+ cells were collected by FACS sorting and their RNA were extracted to determine the mRNA levels of the indicated genes. A representative result of three similar experiments are shown. (B) *In vivo* induction of TRAIL and granzyme B mRNA by INAM-expressing BMDC. WT BMDC were stimulated with polyI:C (10 μg/ml) or medium only. IRF-3-/- BMDC were infected with CV or INAM-expressing vector. These BMDC were allowed to stand for 24 h and harvested for injection ($5x10^5$ cells) into footpads of WT mice. After 48 h, $DX5^+$ cells were collected from the inguinal LN by FACS sorting. RNA of the cells were extracted and the levels of the indicated mRNA were determined by real time PCR. Data show one of two experiments with similar results.

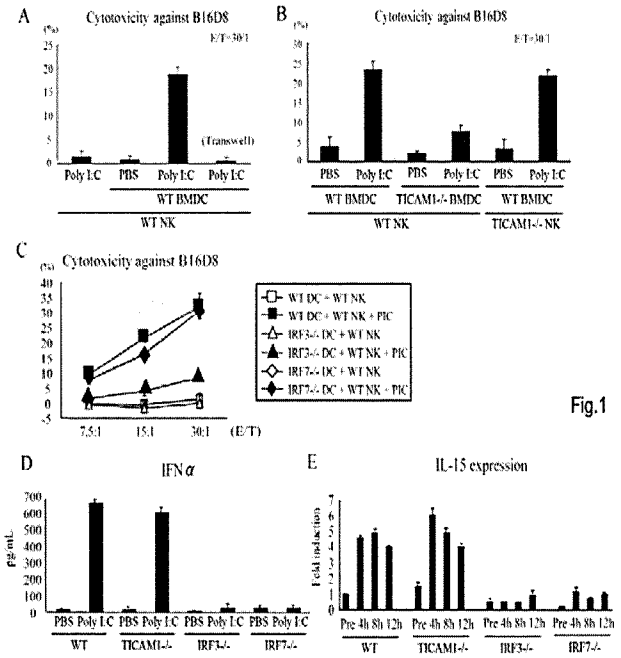



Fig.1

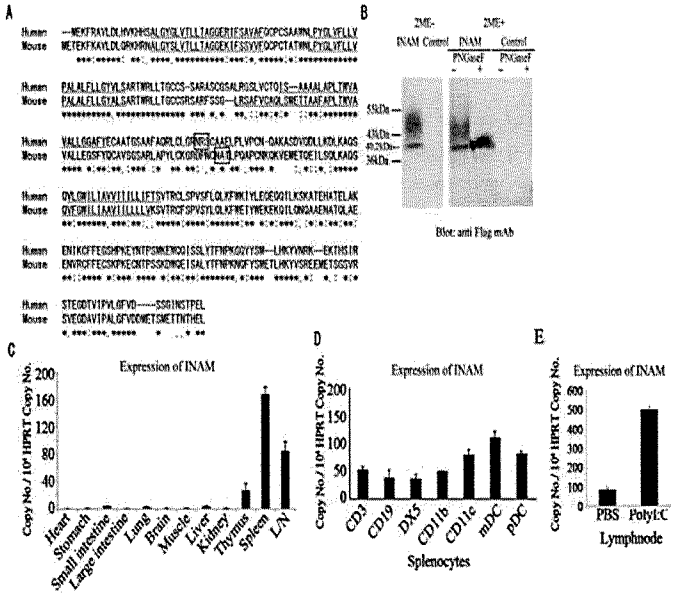

A
Human
Mouse
B
2ME-
2ME+
INAM Control
INAM
Control
PNGaseF
55kDa
43kDa
36kDa
Blot: anti Flag mAb
C
Expression of INAM
Copy No./ 10⁶ HPRT Copy No.
Heart
Stomach
Small intestine
Large intestine
Lung
Brain
Muscle
Liver
Kidney
Thymus
Spleen
LN
D
Expression of INAM
CD3
CD19
DX5
CD11b
CD11c
mDC
pDC
Splenocytes
E
Expression of INAM
PBS
PolyI:C
Lymphnode

Fig.2

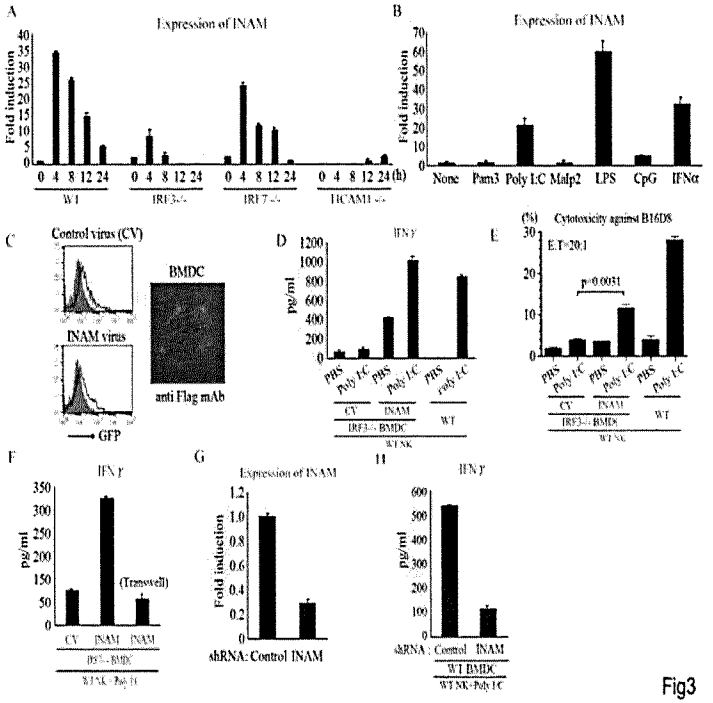

A
Expression of INAM
Fold induction
0 4 8 12 24 (h)
WT
IRF3-/-
IRF7-/-
ICAM1-/-
B
Expression of INAM
None
Pam3
Poly I:C
Malp2
LPS
CpG
IFNα
C
Control virus (CV)
INAM virus
BMDC
anti Flag mAb
GFP
D
IFN β
pg/ml
PBS
Poly I:C
CV
INAM
IRF3-/- BMDC
WT NK
WT
E
(%) Cytotoxicity against B16D8
E:T=20:1
p=0.0031
F
IFN β
(Transwell)
G
Expression of INAM
shRNA: Control INAM
H
IFN β
shRNA : Control
INAM
WT BMDC
WT NK+Poly I:C

Fig3

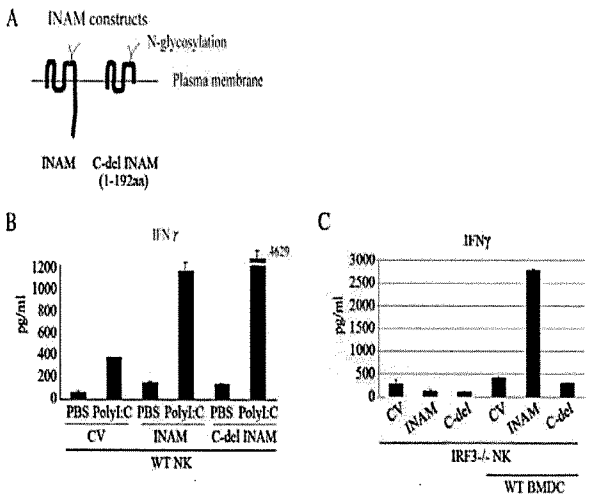

A
INAM constructs
N-glycosylation
Plasma membrane
INAM
C-del INAM
(1-192aa)
B
IFNγ
pg/ml
1200
1000
800
600
400
200
0
4629
PBS PolyI:C PBS PolyI:C PBS PolyI:C
CV
INAM
C-del INAM
WT NK
C
IFNγ
pg/ml
3000
2500
2000
1500
1000
500
0
CV
INAM
C-del
CV
INAM
C-del
IRF3-/- NK
WT BMDC

Fig.4

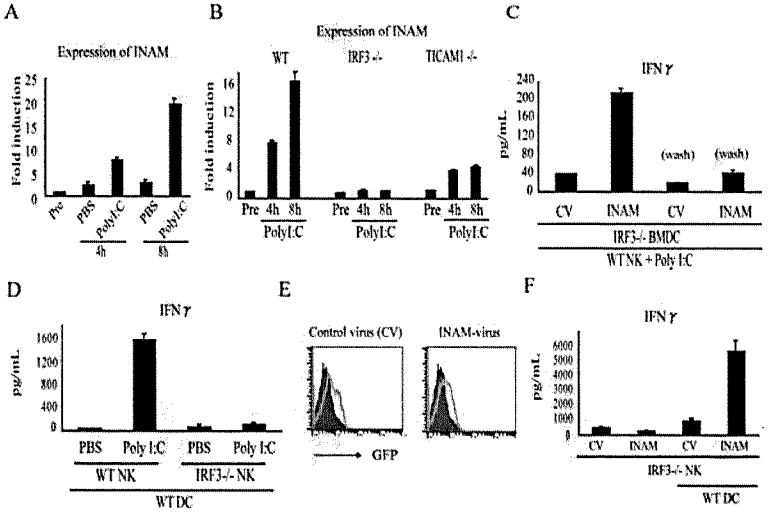

A
Expression of INAM
Fold induction
25
20
15
10
5
0
Pre
PBS
PolyI:C
PBS
PolyI:C
4h
8h
B
Expression of INAM
WT
IRF3 -/-
TICAM1 -/-
Fold induction
16
12
8
4
0
Pre 4h 8h
PolyI:C
Pre 4h 8h
PolyI:C
Pre 4h 8h
PolyI:C
C
IFNγ
pg/mL
240
200
160
120
80
40
0
(wash)
(wash)
CV
INAM
CV
INAM
IRF3-/- BMDC
WT NK + Poly I:C
D
IFNγ
pg/mL
1600
1200
800
400
0
PBS
Poly I:C
PBS
Poly I:C
WT NK
IRF3-/- NK
WT DC
E
Control virus (CV)
INAM-virus
GFP
F
IFNγ
pg/mL
6000
5000
4000
3000
2000
1000
0
CV
INAM
CV
INAM
IRF3-/- NK
WT DC

Fig.5

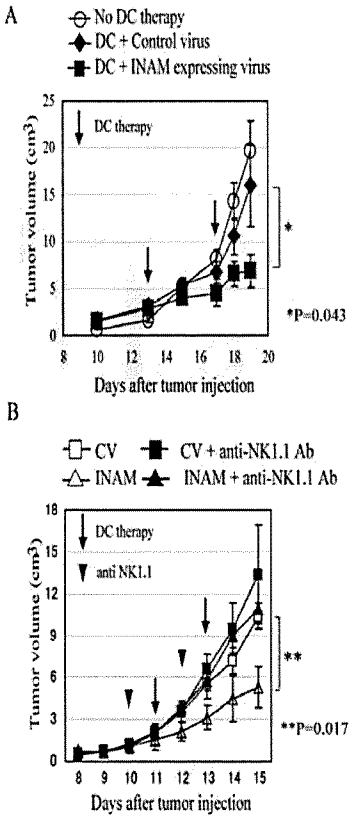

A
No DC therapy
DC + Control virus
DC + INAM expressing virus
DC therapy
Tumor volume (cm3)
Days after tumor injection
*P=0.043
B
CV
CV + anti-NK1.1 Ab
INAM
INAM + anti-NK1.1 Ab
DC therapy
anti NK1.1
**P=0.017

Fig.6

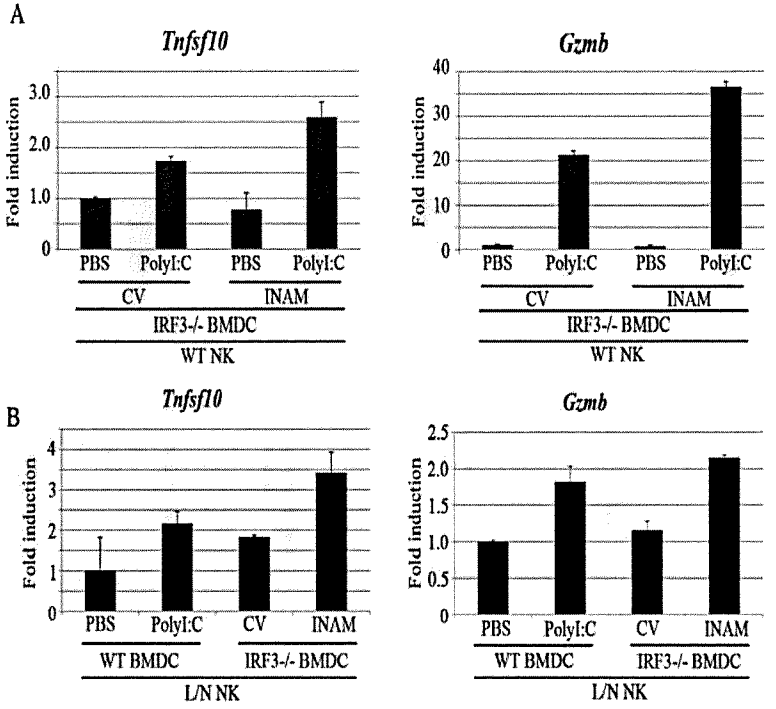

A
Tnfsf10
Gzmb
Fold induction
PBS
PolyI:C
CV
INAM
IRF3-/- BMDC
WT NK
B
WT BMDC
L/N NK

Fig.7

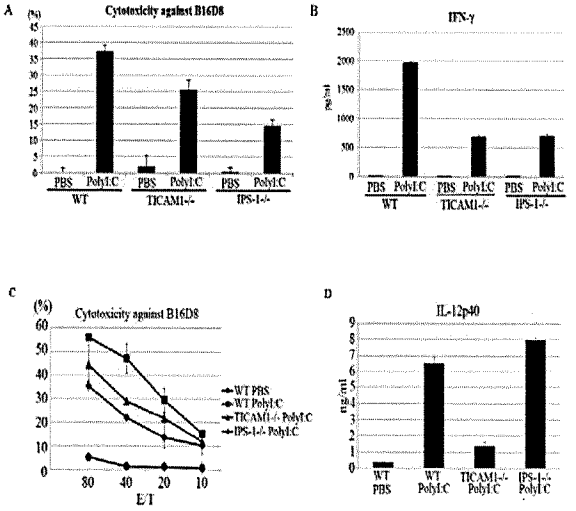

A
Cytotoxicity against B16D8
(%)
PBS
Poly I:C
WT
TICAM1-/-
IPS-1-/-
B
IFN-γ
pg/ml
C
Cytotoxicity against B16D8
WT PBS
WT Poly I:C
TICAM1-/- Poly I:C
IPS-1-/- Poly I:C
E/T
D
IL-12p40
ng/ml

Fig.S1

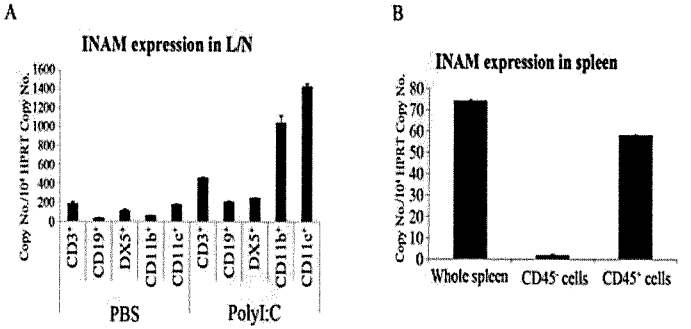
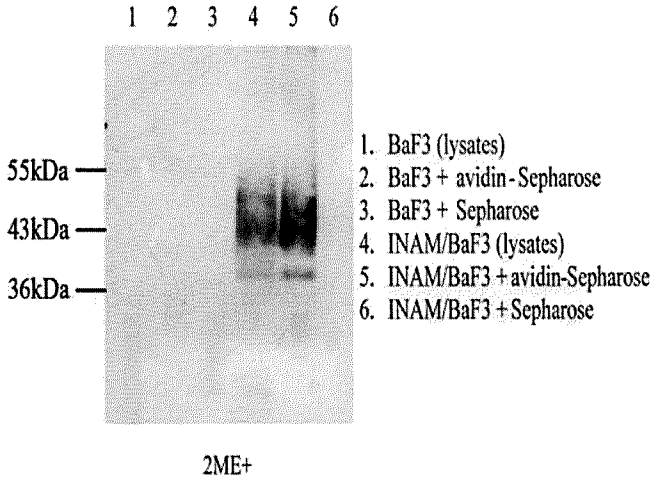

A
INAM expression in L/N
Copy No./10⁴ HPRT Copy No.
CD3⁺
CD19⁺
DX5⁺
CD11b⁺
CD11c⁺
PBS
PolyI:C
B
INAM expression in spleen
Whole spleen
CD45⁻ cells
CD45⁺ cells

Fig.S2


1 2 3 4 5 6
55kDa
43kDa
36kDa
1. BaF3 (lysates)
2. BaF3 + avidin-Sepharose
3. BaF3 + Sepharose
4. INAM/BaF3 (lysates)
5. INAM/BaF3 + avidin-Sepharose
6. INAM/BaF3 + Sepharose
2ME+

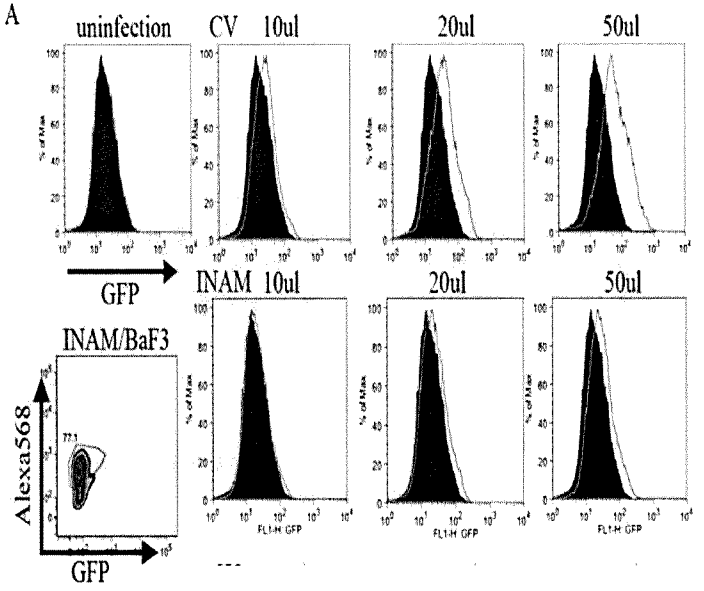

A
uninfection
CV 10ul
20ul
50ul
GFP
INAM 10ul
20ul
50ul
INAM/BaF3
Alexa568
GFP

Fig.S3

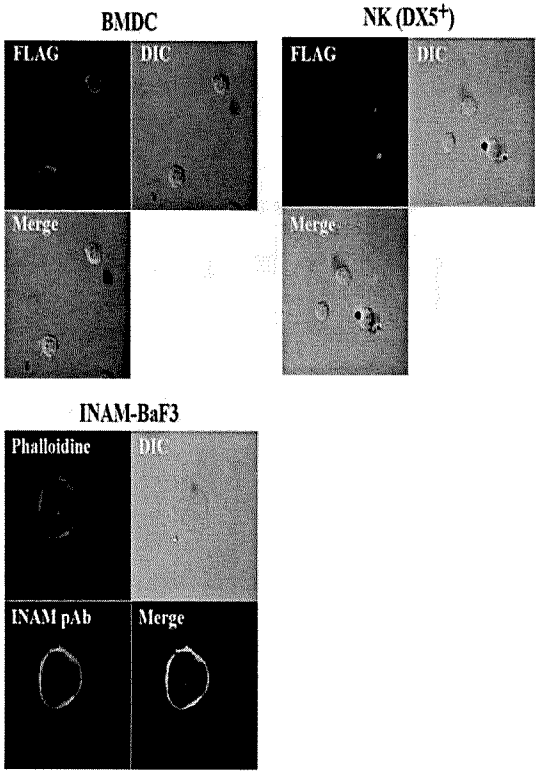
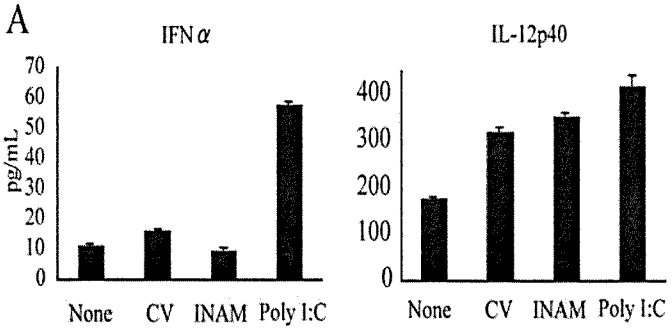

BMDC
NK (DX5+)
FLAG
DIC
Merge
FLAG
DIC
Merge
INAM-BaF3
Phalloidine
DIC
INAM pAb
Merge

Fig.S5


A
IFN α
IL-12p40
pg/mL
70
60
50
40
30
20
10
0
400
300
200
100
0
None
CV
INAM
Poly I:C
None
CV
INAM
Poly I:C

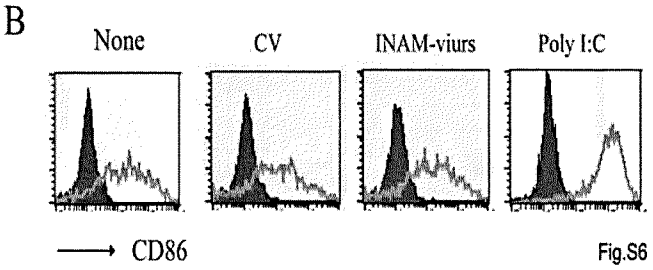

B
None
CV
INAM-viurs
Poly I:C
CD86

Fig.S6

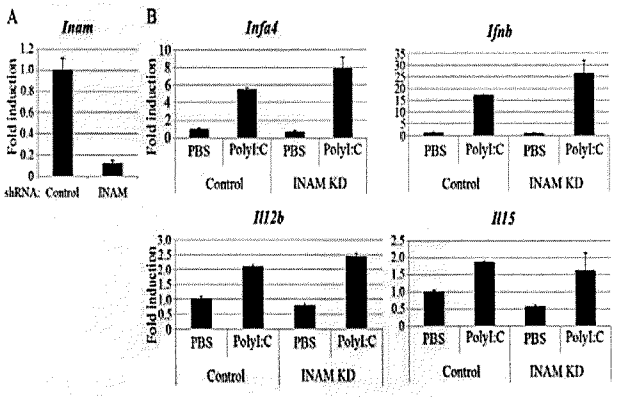

A
Inam
Fold induction
shRNA: Control INAM
B
Infa4
Ifnb
Il12b
Il15
Fold induction
PBS
PolyI:C
Control
INAM KD

Fig.S7

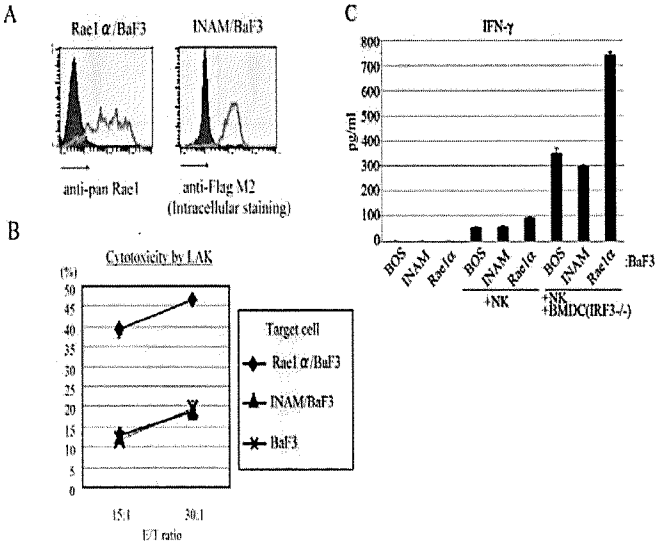

A
Rae1 α/BaF3
INAM/BaF3
anti-pan Rae1
anti-Flag M2
(Intracellular staining)
B
Cytotoxicity by LAK
(%)
Target cell
Rae1 α/BaF3
INAM/BaF3
BaF3
15:1
30:1
E/T ratio
C
IFN-γ
pg/ml
BOS
INAM
Rae1α
+NK
+NK
+BMDC(IRF3-/-)
:BaF3

Fig.S8

# Innate immunity and vaccine

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Running title: Prologue

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Keywords: TLR, MyD88, TICAM-1 (TRIF), dendritic cells, NK activation.

Abstract

Immune adjuvant is an artificial pathogen-associated molecular patterns (PAMP) for potentiating various immune responses. Vaccine represents one event that is capable of inducing immune response caused by antigen and PAMP stimuli, which act on antigen-presenting dendritic cells (mDCs). Here, we introduce the pathways by which CTL and NK cells are driven through mDC maturation in response to adjuvants.

Microbial pattern molecules (PAMP, pathogen-associated molecular patterns) are agonists of pattern-recognition receptors, a representative of which is Toll-like receptor. Adjuvants belong to non-infectious artificial PAMP, typically administered with the target antigen (Ag) in order to enhance the host immune response (1). However, the mechanism by which these reagents enhance immunity had not clearly been understood, until the recent progress on elucidation of the ligand properties of Toll-like receptors (TLRs) and TLR-mediated DC maturation (2). The accumulated evidence on TLR-dependent DC maturation has solidified the current understanding that DC TLRs participate in determining what kind of effector cells are driven by the DCs that present antigens. Now, we hold that Ags determine the object toward which immune cells are proliferated whereas adjuvant determines what effectors will be selected for immunological output (1). The fundamental concepts of the immune system should be re-evaluated through the understanding of TLR-mediated DC immune responses, which will also revolutionize the concepts related to vaccination.

In myeloid DCs (mDCs), a representative Ag-presenting cells, the two major arms of the innate immune signaling pathway, the MyD88 and TICAM-1 (Toll-IL-1 receptor homology domain-containing adaptor molecule, also named TRIF) pathways, have been identified through the investigation of TLR signaling (2). TLR3 represents the sensor of dsRNA of viral origin and recruits TICAM-1 (3). TICAM-1 links the type I IFN-inducing pathways in mDCs of both human and mouse (2,3). TLR4 recruits both MyD88 and TICAM-1 (2). TLRs other than TLR3 can take the MyD88 pathway. Hence, the representative inflammatory responses in TLR pattern-recognition are rooted in the properties of the adaptors MyD88 and TICAM-1. In myeloid DCs, these pathways play a significant role in differential maturation.

Using BCG-CWS as the TLR2/4 adjuvant, we found that MyD88 is an adaptor essential for induction of cross-priming in mDCs (4). MyD88 -/- mice have been reported to far less induction of CTL against exogenous Ags and TLR2/4 adjuvants (4). Cytokines and NF-κB-inducing factors may be required for mDC cross-priming, although the molecular mechanism whereby MyD88 can induce responses related to cross-presentation in mDCs.

We have used polyI:C for evaluating the TICAM-1 (TRIF) potential in mDC maturation (5). The TICAM-1 pathway allows mDCs to activate IRF-1 and IRF-3, which in turn activate the IFN-β promoter as well as unidentified NK-driving factors. The data imply

that the cross-priming and the NK-driving signals are also dependent upon TICAM-1, but the transcription factors utilized by TICAM-1 are wholly distinct from those of MyD88. We found that mDC TICAM-1-mediated NK activation largely relies on the IRF-3-derived NK-activating molecule (INAM) which promotes mDC-NK cell contact (6), in addition to the reported soluble mediators IL-15, IFN-α, and IL-12p70. Thus, the mode by which mDCs matured differs in the MyD88 and the TICAM-1 pathways. If an appropriate adjuvant is conjugated with vaccine, NK cells can be activated for eradication of microbes.

We have analyzed how mDCs acquire effector-driving functions by focusing on the innate immune response (1). Live vaccines usually contain microbe-specific Ags and PAMPs. Since DNA *per se* has an adjuvancy, DNA vaccine also includes PAMPs. Nevertheless, potential vaccines have not been established for some viral infections. For example, HCV, HIV and influenza infections have their own problems. Low titers of antiviral antibody and CTL induction may be dissolved by developing efficient adjuvants by inducing appropriate effectors. In this stand, an effective strategy for tackling the issue of low immune response against vaccines has yet to be proposed with obstinacy infectious diseases. Even the fundamental immune aberrance present in the focal nests of infection has not been grasped by researchers in some virus-induced persistent infections.

There is almost no information concerning the molecular mechanisms by which mDCs drivie these effector cells. Each DC subset seems to correspond to a specific effector, although the selection strategy how DCs induce various effectors is not clear in most instances. However, it is known from mouse models that splenic CD8+ DCs induce Treg (7) and NK cells (8) in the mouse spleen, and lamina propria pDCs in the mouse enteric canal promotes IgA production (9). In addition, CD70+/CD11c+ DCs induce Th17 cells by the ATP of enterobacteria (10), and bone-marrow (BM)DCs markedly activate NK cells via the TICAM-1 pathway (11). It is known that plasmacytoid dendritic cells (pDCs) induce tremendous amounts of IFN-α in response to CpG DNA through TLR9. Although what molecular background supports this pDC phenotype has long been unknown, pDC-specific events should regulate the activation of IRF-7 (12, also see the review of T. Kaisho in this issue). Further examples of DC subsets that preferentially function with specific effectors will likely be demonstrated through continued investigation.

For future studies, it is necessary to determine the potential of peptide-conjugating materials including Ags and inflammation-inducing reagents. A number of reports have suggested that adjuvants can greatly increase the efficiency rate of treatment, although there are no criteria to fairly evaluate the function of adjuvants in vaccine recipients or patients. The method for stimulating DCs needs to be carefully selected as systemic administration of inflammation-inducing material can also lead to the acceleration or exacerbation of infection at the same time. In this case, the route and molecule that selectively raises the degree of DC maturation without severe malicious inflammation should be clarified. The design of DC maturation can be manipulated without helping flare inflammation. In the future, we hope that through continued research, patients will have access to convenient and highly effective prophylactic immunotherapy.

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*Review Article*

# Pattern recognition receptors of innate immunity and their application to tumor immunotherapy

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**Dendritic cells (DC) begin maturation in response to complex stimuli consisting of antigens and pattern molecules (PAMP) for the activation of the immune system. Immune adjuvant usually contains PAMP. Infection represents one event that is capable of inducing such a complex set of stimuli. Recently, DC were subdivided into a number of subsets with distinct cell-surface markers, with each subset displaying unique differential maturation in response to pattern molecules to induce various types of effector cells. In the present study, we review how pattern recognition molecules and adaptors in each DC subset drive immune effector cells and their effect in the stimulated DC. Although tumor cells harbor tumor-associated antigens, they usually lack PAMP. Hence, we outline the properties of exogenously-added PAMP in the modulation of raising tumor immunity. In addition, we describe the mechanism by which DC-dependent natural killer activation is triggered for the induction of antitumor immunity. (*Cancer Sci* 2010; 101: 313–320)**

Adjuvants are typically administered with target antigens in order to enhance the host immune response. Freund complete adjuvant (FCA), Freund incomplete adjuvant (FIA), and hydrated alumina (alum) are representative adjuvants that are used as antigen conjugates to potentiate immune responses and antibody production in animals. Although the mechanism by which these reagents enhance immunity was not completely understood, it appeared that the addition of adjuvants to antigens potentially induced immunity by "making it dirty".[1] However, more recently the agonistic features of adjuvants for pattern-recognition receptors (PRR) have been highlighted based on elucidation of the ligand properties of Toll-like receptors (TLR) and TLR-mediated dendritic cell (DC) maturation. The accumulated evidence on TLR-dependent DC maturation has solidified the current understanding that DC TLR confer the direction of the effector driving on the DC that present antigens. We hold that antigens determine the object toward which immune cells are proliferated, whereas adjuvants determine what effectors will be selected for immunological output.[2] The fundamental concepts of the immune system should be re-evaluated through the understanding of TLR-mediated DC immune responses, which will also revolutionize the concepts related to antitumor immunity.

The two major arms of the innate immune signaling pathway, the MyD88 and toll-interleukin 1 receptor domain (TIR)-containing adoptor molecule (TICAM-1) pathways (Fig. 1), have been identified through the investigation of TLR signaling.[3] Although MyD88 is dominant in mammals living on land, most aquatic vertebrates preferentially use TICAM-1 for TLR signaling.[4] TLR employing MyD88 adaptors usually recognize bacterial patterns, whereas TLR taking TICAM-1 recognize virus products, including nucleic acids. In addition to these PRR, the retinoic acid-inducible protein I (RIG-I)-like receptor and nucleotide-binding oligomerization domain-containing protein (NOD)-like receptor (NLR) systems are located in the cytoplasm[5,6] and are inherent in most animals.[7] PRR systems are also distributed across the cell membrane and cytoplasm. The mineral oil component of FIA, crystallized uric acid, and alum are able to activate the NLR-inflammasome pathway,[5] which yields interleukin (IL)-1β and IL-18. These cytokines in turn stimulate their respective receptors to activate the MyD88 pathway in myeloid DC (mDC).[8] The activation of the MyD88 pathway in mDC is a common feature in bacterial stimulation.

The MyD88 pathway of plasmacytoid DC (pDC) is unique, as TLR7 and TLR9 predominantly activate interferon-regulatory factor (IRF)-7 and induce interferon (IFN)-α.[9] Human mDC lack TLR7 and TLR9 and the IFN-inducing MyD88 pathway, although mouse mDC harbor the TLR7 and TLR9 MyD88 pathway, which are inducible by RNA and CpG DNA respectively.[10] In contrast, TICAM-1 links the type I IFN-inducing pathways in the mDC of both humans and mice,[11] while TLR3 represents the sensor of dsRNA of viral origin.[12] In addition, viral products, double-stranded (ds) RNA, and 5'-triphosphate RNA stimulate the intracytoplasmic helicases melanoma-differentiation-associated gene 5 (MDA5) and RIG-I, which in turn activate the IRF-3- and IRF-7-activating kinases (TANK-binding kinase (TBK1)/I kappa B kinase (IKK)ε.[5,11] The adaptor of this pathway is IPS-1,[5] and it is therefore known as the interferon-beta promoter stimulator 1 (IPS-1) pathway. The IPS-1 pathway shares the downstream signaling components, including the kinases, with the TICAM-1 pathway to activate the IFN-inducing pathway.[13] Thus, the representative inflammatory responses in pattern recognition are rooted in the properties of the adaptors in the case of TLR, MyD88, and TICAM-1. In DC, these pathways play a significant role in differential maturation.

## Bacterial and viral pattern molecules revisited

It is known that FCA contains heat-killed mycobacteria (the causative agent of tuberculosis), which functions as a ligand of TLR.[14] These are MyD88-dependent properties and the features of the DC maturation profiles with these TLR ligands have been examined (Table 1). Although the toxicity of the TLR agonists is not removed, their role in triggering antitumor immunity, including cytokine- and effector-inducing abilities, are being examined with respect to their practical use for patients with cancer. Alum (aluminum hydroxide) acts as an NLR agonist involving the secondary activation of MyD88[15] and is currently used as a standard adjuvant in humans. However, a sufficient immune potential may not be accomplished with a

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single stimulation of the NLR system. The adjuvant BCG– cell wall skeleton (CWS), which contains mycolic acid, arabinogalactan, and peptidoglycan (PGN), has been used for patients with cancer, and a good prognosis was reported after BCG–CWS treatment.[16] This adjuvant contains muramyl dipeptide (MDP) as a center for the activation of TLR2 and TLR4 and also involves MyD88 activation.[17] The DC maturation profile induced by BCG-CWS is comparable to that induced by Pam2 peptides that activates TLR2 (4) BCG–CWS does not contain DNA, which excludes the possibility of activating TLR9. Only rare examples of fatal shock and interstitial pneumonia have been reported with BCG–CWS that stimulates TLR2 and TLR4.[18]

In contrast, viral products, including dsRNA (and its analog polyI:C), and the lipopolysaccharide (LPS) of Gram-negative bacteria were identified as TLR ligands with TICAM-1 agonistic function.[3] dsRNA and LPS stimulate TLR3 and TLR4, respectively, both of which link the adaptor TICAM-1.[3,11] As they activate nuclear factor (NF)-κB and IRF-3, cytokine storm (hypercytokinemia) or endotoxin-like shock tends to occur *in vivo*.[19] It is therefore mandatory to reduce their toxic properties before they are applied to human patients. Importantly, polyI:CLC (TLR3-complexed poly inosinic: polycytidylic (IC) with carboxymethylcellulose and poly-L-lysine to improve resistance to ribonucleases (i.e. TLR3),[20] and monophosphoryl lipid A (i.e. TLR4)[21] have been considered promising candidates for immunotherapy. These TLR agonists mainly stimulate the TICAM-1 pathway without the robust activation of the MyD88 pathway[20,21] and rarely induce side-effects, such as cytokine storms, skin festering, and the symptoms of inflammation during preclinical trials. It is important that the differential view of the MyD88 and TICAM-1 adjuvants in terms of their DC maturation and effector-driving properties be examined. The development of TLR agonists with properties superior to those of alum can be expected to be revealed through these studies. In this review, the molecular mechanisms of effector activation by DC TLR are outlined and discussed.

## Adjuvants stimulate tumor-associated myeloid cells and DC

We have speculated from *in vitro* studies that immature mDC are matured to antigen-presenting mDC by BCG–CWS, a TLR2 agonist,[22] which also induces a variety of immune effector cells, including CD8+ T cells (CTL)[23] and NK cells.[24] These effector cells can damage tumor cells under high effector target (E/T) ratios *in vitro*.[23,24] Indeed, tumor B16 mutanoma growth is retarded in tumor-bearing mice (C57BL/6) when BCG–CWS-matured mDC or secondary-induced CTL are injected in the area surrounding the tumor. It is the CTL, but not NK, cells that are the main effector responsible for tumor regression *in vivo*.[23] Unexpectedly, however, the immune cells which infiltrate into the tumor largely consist of macrophages and not lymphocytes or mDC in mouse models (Shime H and Seya T, unpublished observation, 2009). The properties of these macrophages remain experimentally undetermined. As the tumor-infiltrating macrophages contain many subsets, and some of them often possess immune suppressing properties,[25] these macrophages could be related to myeloid-derived suppressor cells (MDSC) and act as inflammation inducers to sustain tumor growth. Thus, BCG-CWS-mediated functional modification of these macrophages and their effect on tumor growth in mice remains to be determined. Specific questions also remain concerning adjuvant administration to patients. How myeloid cells mature to DC after they are phagocytozing tumor-associated antigens, how mature mDC are located by effector cells, and how tumors regress in such situations still remain unanswered.

Treg, a regulatory population of CD4 T cells, has an inhibitory activity against antitumor immunity[26] and has been shown to inhibit CD8 CTL tumoricidal activity *in vitro*.[26] Several reports indicate that Treg cells infiltrate into tumors and support tumor progression.[27,28] However, mDC are present at very low levels in the tumor masses where Treg cells invade. Again, the functional modulation of Treg cells in the local tumor environment by adjuvants or mDC is unclearly illustrated.

Recently, several myeloid cell populations have been discovered that are associated with tumor cell progression, including interferon-producing killer DC (IKDC),[29] MDSC,[25,30] and tumor-associated macrophages (TAM).[31] Although the maturation or activation of these myeloid cells is likely crucial for tumor progression, only a few reports have investigated their maturation mechanism and effect on tumors by adjuvant treatment. Early-acting pattern molecules can act on tumor cells to release late-acting substances. In fact, damage-associated molecular patterns (DAMP), such as high-modify group box protein (HMGB1), uric acids, heat-shock protein (HSP), and DNA complexes,[32] are secondary liberated from tumors, and stimulate the TAM. Whether these stimuli alter the tumor-progressing ability of the macrophages should be a point of consideration for adjuvant therapy. The types of TLR present in these myeloid cells and the effect of administered adjuvants are topics that need to be investigated.

Many studies on TLR knockout mice allowed us to describe the properties of mouse bone marrow-derived DC (BMDC) treated with a variety of adjuvants[33] and to show the points for induction of immune effector cells through the adjuvant immunotherapy of cancer. Ambivalent functions between mDC and MDSC in a tumor environment can affect the conformation of antitumor immunity.

## MyD88- and TICAM-1-mediated DC maturation

Soon after the discovery of the TLR,[34] it was shown that TLR agonists have a DC maturation activity.[35] DC maturation is characterized by TLR adaptors, which have common features, including the upregulation of major histocompatibility complex (MHC), costimulators and NK-activating ligands, and the following features which are unique to each adaptor in mDC.[36] MyD88-dependent DC maturation has two modes, with NK activation and CTL induction occurring concomitantly with the activation of NF-κB, followed by the induction of inflammatory cytokines.[37] Using BCG–CWS as an adjuvant for the TLR2 agonist, we examined how the TLR2 agonist acts on mDC and tumor cells.[23] While NK activation by MyD88 is feasible *in vitro*, TLR2 agonists exhibit minimal NK-mediated tumor-suppression activity in tumor-implant mice.[24] The TLR2-dependent antitumor NK activity is abrogated in MyD88–/– mice, suggesting the presence of a NK-activation pathway via MyD88.[24] However, following an *in vitro* analysis, it was revealed that TLR2–MyD88 in NK cells, but not in mDC, is rather dominant in this mode of NK activation, and that activated NK cells barely enter the tumor mass. For this reason, the subcutaneous administration of BCG–CWS marginally retards tumor growth in mice via the activation of NK cells.

In contrast, mDC maturation is accompanied with potent antigen presentation secondary to cross-priming in TLR2-primed mDC.[23] Tumor antigen-specific CTL induction is facilitated in mice with an implant tumor burden, concomitant with the retardation of tumor growth. This CTL induction is MyD88 dependent, since TLR2-mediated cross-priming does not occur in MyD88–/– mDC. Neither CTL induction nor the retardation of tumor growth significantly occurs in MyD88-deficient mice. Thus, MyD88 in mDC preferentially participates in cross-priming and driving CTL *in vivo*. The downstream molecules of MyD88 associated with mDC CTL driving are unknown.



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