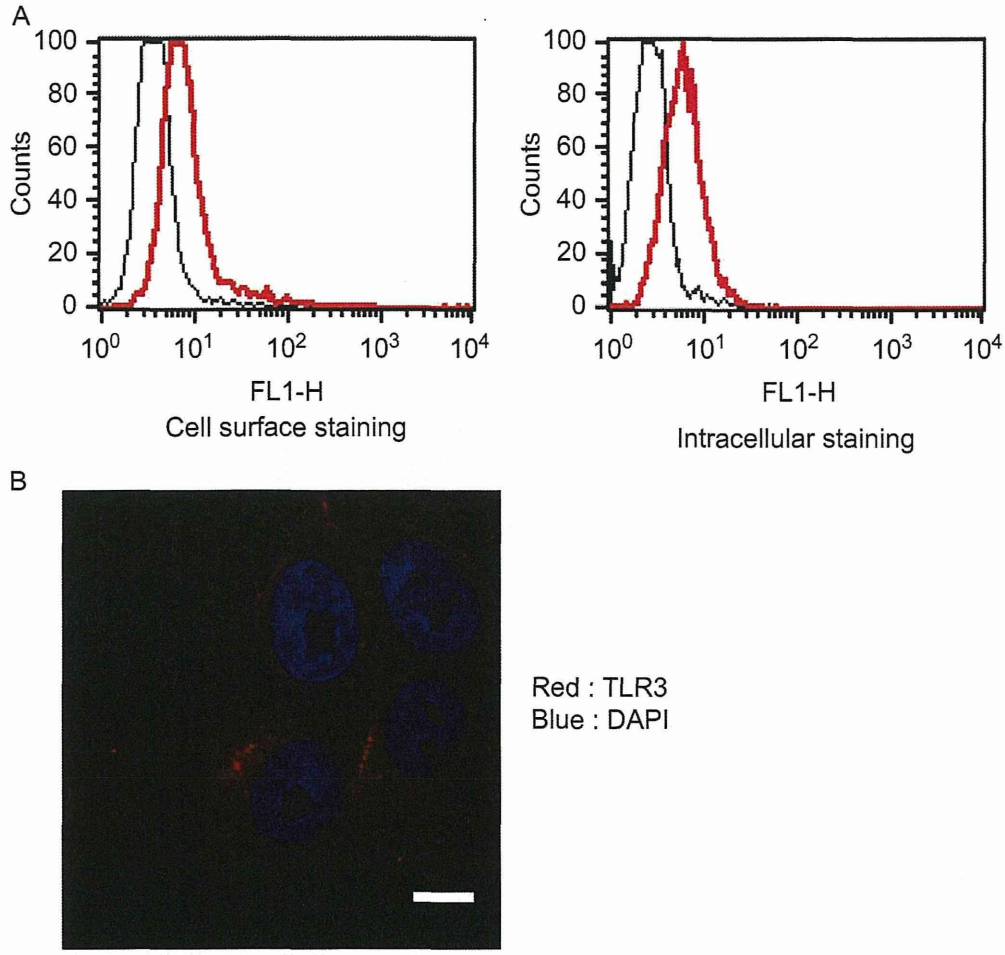

Figure 10.1 Expression of human TLR3 in HeLa cells. (A) TLR3 is expressed on the cell surface and inside the cells in HeLa cells. Cell-surface (left) staining and intracellular (right) staining were performed using the TLR3.7 mAb and analyzed by flow cytometry. The black line indicates control mouse IgG staining. The red line indicates TLR3.7 staining. (B) TLR3 is localized to intracellular vesicles in HeLa cells. Cells were fixed and permeabilized, and endogenous TLR3 was stained with the TLR3.7 mAb. The red signal indicates endogenous TLR3 and the blue signal indicates DAPI staining. Scale bar, 10 μm.

5. Add 50 μL of fluorescent-conjugated goat anti-mouse IgG (highly absorbed and appropriately diluted with FACS buffer), mix thoroughly, and incubate for 30 min in the dark.
6. Wash cells three times with FACS buffer.
7. Resuspend cells in 0.5 mL of FACS buffer and analyze fluorescence signals using a flow cytometer. If samples will not be analyzed immediately, add 0.5 mL of 1% paraformaldehyde in PBS, mix thoroughly, and keep them in the dark at 4 °C and analyze within 1 week.

2.1.2 Intracellular TLR3

1. Wash cells (~10^5–10^6 cells/sample) three times with DPBS at 4 °C.
2. Add 1 mL of BD FACS Permeabilizing Solution 2 (Becton Dickinson); incubate for 10 min at room temperature.
3. Wash cells three times with FACS buffer.
4. Add 50 μL of anti-human TLR3 mAb (e.g., TLR3.7) or isotype control Ab (10 μg/mL in FACS buffer) together with 1/10 volume of goat serum and incubate for 30 min at room temperature.
5. Wash cells three times with FACS buffer.
6. Add 50 μL of fluorescent-labeled goat anti-mouse IgG (highly absorbed and appropriately diluted with FACS buffer) together with 1/10 volume of goat serum, mix thoroughly, and incubate for 30 min at room temperature in the dark.
7. Wash cells three times with FACS buffer.
8. Resuspend cells in 0.5 mL of FACS buffer and analyze fluorescence signals using a flow cytometer (Fig. 10.1A).

2.2. Immunofluorescent analysis

1. Plate cells (5.0×10^4 cells/well) on micro cover glasses (Matsunami, Tokyo, Japan) in a 24-well plate.
2. On the next day, wash the cells twice with PBS.
3. In the case of cell-surface staining, fix cells with PBS containing 4% paraformaldehyde for 15 min. In the case of intracellular staining, fix cells with PBS containing 4% paraformaldehyde for 30 min and permeabilize with PBS containing 0.2% Triton X-100 for 15 min at room temperature.
4. Wash cells four times with PBS.
5. Incubate cells in blocking buffer (PBS containing 1% BSA and 10% goat serum) for at least 10 min.
6. Add 20 μg/mL anti-TLR3 mAb (TLR3.7) or an isotype control Ab in blocking buffer and incubate for 1 h.
7. Wash cells four times with PBS.
8. Incubate in Alexa-conjugated secondary Ab (Molecular Probes, highly absorbed, 1:1000 dilution) in blocking buffer for 30 min.
9. Wash cells four times with PBS.
10. Mount cover glasses onto slide glass using ProLong Gold antifade reagent with DAPI (Molecular Probes).
11. Analyze fluorescence signals by confocal microscopy (Fig. 10.1B).

In the case of DCs,

1. stain TLR3 as described for FACS sample preparation (intracellular staining 1-5);
2. add Alexa-conjugated goat anti-mouse IgG (Molecular Probes, highly absorbed, 1:1000 dilution) together with 1/10 volume of goat serum, mix thoroughly, and incubate for 30 min at room temperature;
3. wash cells four times with PBS;
4. plate cells onto slide glass by centrifugation using a cytospin;
5. mount cover glasses onto slide glass using ProLong Gold antifade reagent with DAPI (Molecular Probes);
6. analyze fluorescence signals by confocal microscopy.

3. ASSAY FOR TLR3-MEDIATED SIGNALING

TLR3 consists of an extracellular domain containing 23 leucine-rich repeats and N- and C-terminal flanking regions, a transmembrane domain, and an intracellular TIR domain (Bell et al., 2003). Based on structural analyses of the TLR3 ectodomain–dsRNA complex, it has been proposed that 40–50 bp dsRNA is the minimum signaling unit with two TLR3 molecules (Liu et al., 2008). On a cellular basis, *in vitro*-transcribed dsRNAs, >90 bp in length, trigger TLR3 oligomerization, and effectively induce IFN-β and proinflammatory cytokine production in murine myeloid DCs (Jelinek et al., 2011; Leonard et al., 2008).

After oligomerization in the endosomes, TLR3 recruits adaptor molecule TICAM-1 into the cytoplasmic TIR domains (Funami et al., 2007). Once TICAM-1 is oligomerized, the transcription factors IRF-3, NF-κB, and AP-1 are activated, which then induce IFN-β and proinflammatory cytokine production. The N-terminal region of TICAM-1, where TRAF2, TRAF6, and TBK1 binding sites exist, is crucial for IRF-3 activation (Sasai et al., 2010; Tatematsu et al., 2010). TRAF3 and NAP-1 participate in the recruitment and activation of the IRF-3 kinase, TBK1 (Fitzgerald et al., 2003; Oganesyan et al., 2006; Sasai et al., 2005). On the other hand, RIP1 associates with TICAM-1 via the RHIM domain in the C-terminal region and, together with TRAF6, mediates NF-κB activation (Meylan et al., 2004; Sato et al., 2003) (Fig. 10.2).

Because TLR3-mediated signaling is initiated from endosomal compartments, uptake and delivery of TLR3 ligands to endosomes is critical for TLR3 activation. Indeed, poly(I:C) is internalized via clathrin-mediated endocytosis (Itoh, Watanabe, Funami, Seya, & Matsumoto, 2008).

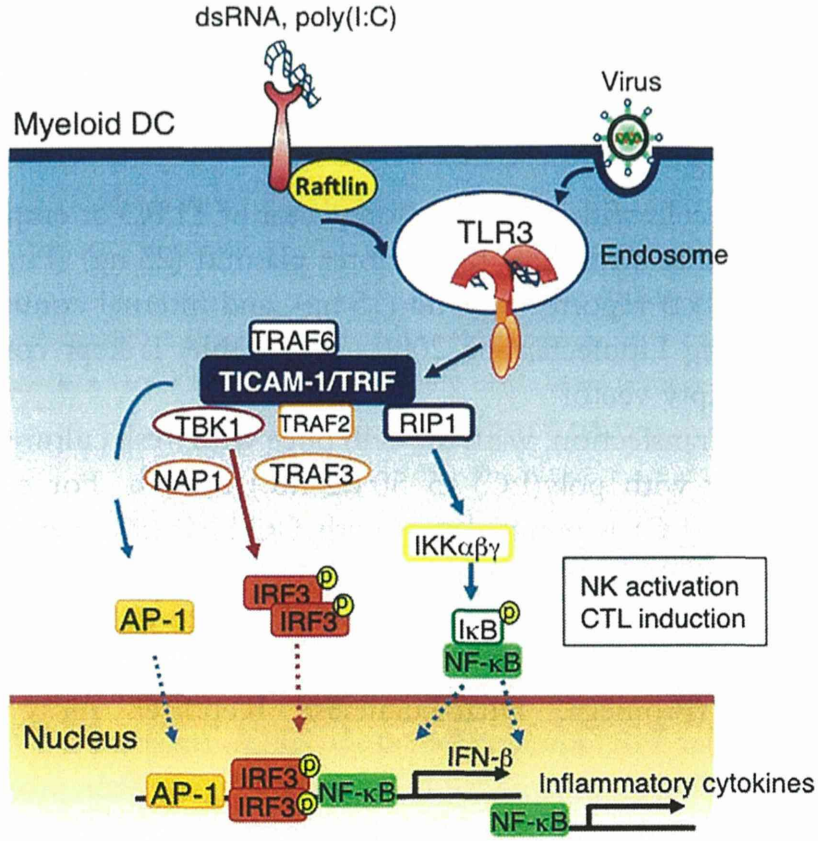

Figure 10.2 TLR3–TICAM-1-mediated signaling in myeloid DCs. An extracellular dsRNA is delivered to early endosomes via clathrin–Raftlin-dependent endocytosis. Once TLR3 is oligomerized with internalized dsRNA, it recruits TICAM-1. After the transient association of TLR3 with TICAM-1 through the TIR domains, TICAM-1 dissociates from TLR3 to form a speckle-like structure containing downstream signaling molecules where TICAM-1-mediated signaling is initiated. The TICAM-1–TBK-1–IRF-3 axis is essential for TLR3-mediated IFN-β production and DC-mediated activation of NK cells and CTLs.

Watanabe et al. reported that the cytoplasmic protein Raftlin induced poly (I:C) internalization through an interaction with the clathrin–AP-2 complex in human myeloid DCs and fibroblasts/epithelial cells (Watanabe et al., 2011). CD14 and the scavenger receptor class A were reported to act as the poly(I:C) uptake receptor in mouse macrophages and human bronchial epithelial cells, respectively, but this is apparently not the case in human fibroblasts/DCs. These results suggest that the uptake machinery for TLR3 ligands may differ between mouse and human cells and also by cell type. To assess TLR3-activating ability, direct delivery of ligands into endosomes with cationic liposome, such as DOTAP, is required in addition to extracellular stimulation of TLR3-negative or TLR3-positive cells.

3.1. Reporter assay for IFN-β promoter activation and NF-κB activation

1. Plate HEK293 cells in a 96-well plate and grow them to approximately 80% confluence.
2. Transfect the cells with the expression vector of TLR3 or empty vector (25 ng), together with IFN-β promoter plasmid (25 ng) (Fujita et al., 1998) or NF-κB reporter plasmid (15 ng), and internal control vector (1.25 ng) using Lipofectamine 2000. Total DNA is kept constant by adding an empty vector.
3. At 24 h after transfection, wash the cells once with fresh culture medium and stimulate with poly(I:C) (5–50 μg/mL) for 6 h. For endosomal delivery, poly(I:C) is preincubated with DOTAP (Roche; 0.5 μL for 1.0 μg poly(I:C)).
4. Lyse the cells in Passive Lysis Buffer (50 μL/well).
5. Measure dual luciferase activities according to the manufacturer's instructions (Promega, Dual-Luciferase Reporter Assay System) (Fig. 10.3).

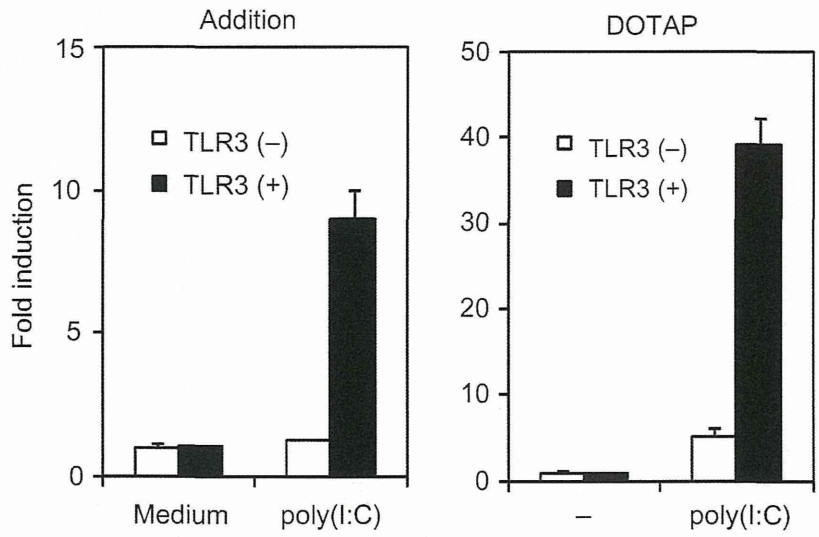

Figure 10.3 Poly(I:C)-induced TLR3-mediated IFN-β promoter activation. HEK293 cells in 96-well plates were transfected with an expression vector for human TLR3 (pEFBOS/hTLR3: filled column) or empty vector (open column) together with the IFN-β reporter plasmid (p125-luc). Then, 24 h after transfection, cells were stimulated with 10 μg/mL poly(I:C) or medium alone (left panel) or stimulated with 1 μg poly(I:C) complexed with DOTAP or DOTAP alone (–) (right panel). After 6 h, luciferase reporter activities were measured and expressed as the fold induction relative to the activity of unstimulated vector-transfected cells.

3.2. Assay for IRF-3 activation

IRF-3 has an essential role in the TLR3-mediated IFN-β gene transcription. IRF-3 is expressed ubiquitously as an inactive monomer in the cytosol. When cells are stimulated with poly(I:C) or virus infection, serine residues in the C-terminal region of IRF-3 are phosphorylated by the serine/threonine kinases, TBK1 and IKKε, and form homodimers (Sato et al., 2000). Homodimerized IRF-3 translocates from the cytosol into the nucleus and binds to responsive elements for IFN-β gene transcription. Fujita et al. developed a sensitive assay for activated IRF-3 using native PAGE, which clearly detects the inactive and active forms of IRF-3 (Iwamura et al., 2001). Compared with the mobility change on SDS–PAGE used to assess the phosphorylation status of the IRF-3 molecules, the native PAGE assay simply and sensitively detects the functional IRF-3 homodimer.

3.3. Cytokine assay

Cytokine production in response to dsRNA has been examined using human or mouse TLR3-expressing cells. In the case of human cells, the normal embryonic lung fibroblast MRC-5, the cervical epithelial cell line HeLa, or monocyte-derived DCs and macrophages are often used for poly(I:C)-induced cytokine assays. MRC-5 cells produce considerable amounts of IFN-β in response to poly(I:C), which is detectable by ELISA (Matsumoto et al., 2002). In HeLa cells, IFN-β mRNA expression can be detected at 3 h after poly(I:C) stimulation. Human monocyte-derived DCs produce large amounts of IFN-α/β, IL-12p70, and proinflammatory cytokines, TNF-α and IL-6, in response to poly(I:C). The TLR3.7 mAb does not inhibit poly(I:C)-induced cytokine production by DCs, because DCs do not express TLR3 at the cell surface (Matsumoto et al., 2003). In the case of mouse cells, MEFs, bone marrow-derived DCs (BMDCs), or splenic DCs from wild-type or gene-disrupted mice ($TLR3^{-/-}$, $TICAM\text{-}1^{-/-}$, $MDA5^{-/-}$, or $MAVS^{-/-}$) are used. Studies using knockout mice have demonstrated that the TLR3 pathway is primarily involved in IL-12p40 production, while the RLR pathway is important for type I IFN production both *in vitro* and *in vivo*. On the other hand, IL-6 production depends on RLR and TLR3 pathways (Kato et al., 2006).

4. ASSAY FOR DC-MEDIATED NK ACTIVATION

NK cells do not express TLR3, and therefore, dsRNA such as poly (I:C) does not activate NK cells directly *in vitro*. However, when mouse NK cells are cocultured with BMDCs or splenic DCs in the presence of poly(I:C), NK cells are activated through cell–cell contact between DC and NK cells, leading to IFN-γ production. Additionally, activated NK cells are able to kill MHC class I-negative mouse tumor cell lines, including YAC-I and B16 cells (Akazawa et al., 2007). Among splenic DC subsets, $CD8\alpha^+$ DCs, in which the TLR3–TICAM-1 pathway is predominant in response to dsRNA, are mainly involved in NK activation. The TLR3–TICAM-1 pathway in DCs plays important roles in NK activation (Ebihara et al., 2010), while RLR-mediated type I IFNs from nonimmune cells also contribute to NK activation *in vivo* (McCartney et al., 2009).

4.1. Preparation of bone marrow-derived DCs

BMDCs are prepared from the bone marrow of wild-type (C57BL/6) or various gene-disrupted mice by a reported method (Inaba et al., 1992) with minor modifications.

Day 0:

1. Sacrifice a mouse and remove the legs.
2. Remove muscle tissues from the femurs and tibias and place the bones in a 60 mm dish with 70% ethanol for 2 min.
3. Wash the bones twice with PBS.
4. Transfer the bones into a fresh dish with RPMI 1640 and cut out both ends of the bones.
5. Flush out bone marrow cells using 5 mL RPMI 1640 with a syringe and 26G needle until the bones turn white.
6. Transfer the cell suspension into a 15 mL tube through a stainless mesh.
7. Wash the dish with 5 mL of medium described in the succeeding text and transfer the cell suspension into the tube through a stainless mesh.
8. Centrifuge at $750 \times g$ for 5 min.
9. Suspend the cell pellets in ACK buffer and incubate for 1 min at room temperature to lyse red cells.
10. Add 9 mL of medium and centrifuge cells at $750 \times g$ for 5 min.
11. Count the cells and resuspend in medium containing 10 ng/mL rmGM-CSF at 1×10^6/mL.
12. Seed cells in a 24-well plate (1 mL/well) and incubate at 37 °C in 5% CO_2.

Day 2:

1. Aspirate the medium and add prewarmed fresh medium containing 10 ng/mL GM-CSF.

Day 4:

1. Wash the adherent cells with culture medium gently and aspirate the supernatant.
2. Add prewarmed medium containing 10 ng/mL GM-CSF.

Day 6 or Day 7:

1. Collect cells into a 50 mL tube after pipetting gently.
2. Centrifuge the cells at 750 × *g* for 5 min.
3. Aspirate the supernatant and count the cells.
4. Resuspend the cells and seed with appropriate conditions for respective experiments.

Medium: RPMI 1640 supplemented with 10% FCS, 10 m*M* HEPES, 55 μ*M* 2-ME, and penicillin/streptomycin.

ACK buffer: 150 m*M* NH_4Cl, 10 m*M* $KHCO_3$, 0.1 m*M* Na_2EDTA (pH 7.4).

4.2. Preparation of NK cells

1. Harvest spleens from C57BL/6 mice and prepare a single-cell suspension by mashing with slide glasses.
2. Pass the cells through a 70 μm cell strainer (BD Falcon) to remove debris.
3. Centrifuge at 750 × *g* for 5 min.
4. Resuspend cell pellet in 1 mL of ACK buffer per 10^8 splenocytes and incubate for 1 min at room temperature.
5. Wash cells twice with 10 mL of medium, and centrifuge at 750 × *g* for 5 min.
6. Isolate NK cells using CD49b MicroBeads (Miltenyi Biotec, 130-052-501) according to manufacturer's protocol.

4.3. Assay for NK activation (IFN-γ production, killing assay)

1. Coculture 5×10^5 NK cells and 2.5×10^5 BMDC in 500 μL of medium per well in a 24-well plate.
2. Stimulate with 10 μg/mL poly(I:C).
3. After 24 h, collect supernatants for evaluating IFN-γ production by ELISA and collect cells for ^{51}Cr release assay.
4. Wash the cells twice with medium and centrifuge at 750×g for 5 min.
5. Count only NK cells.

6. Add 100 μL of DC–NK mixture to 100 μL of target cells at an E/T ratio of 5:1 to 100:1 in a 96-well round-bottom plate. As controls, add 100 μL of medium or 10% NP-40 to the target cells for measuring spontaneous release or total release of chromium, respectively. Target cell preparation is described in the succeeding text.
7. After 4 h, centrifuge plates at 1300 g for 2 min.
8. Collect 150 μL of the supernatant and measure radiation with a gamma counter.
9. Specific cytotoxicity is determined by the following formula: specific cytotoxicity (%) = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

4.3.1 Target cell preparation

1. B16D8, YAC-1, and RMA-S cells are generally used as NK target cells.
2. Suspend 2×10^5 target cells in 180 μL medium and add 50 μCi of $Na_2^{51}CrO_4$.
3. Incubate for 1 h at 37 °C.
4. Wash cells twice with 1 mL of medium, and centrifuge at 1300 × *g* for 2 min.
5. Add 1 mL of medium and keep at 4 °C for 15 min.
6. Centrifuge at 1300 × *g* for 2 min.
7. Suspend cells in medium and plate $0.2-1.0 \times 10^4/100$ μL target cells in a 96-well round-bottom plate.

5. ASSAY FOR DC-MEDIATED CTL ACTIVATION

Myeloid DCs, especially mouse $CD8\alpha^+$ DCs and human $CD141^+$ DCs, are the best professional antigen-presenting cells that can cross present exogenous antigens to $CD8^+$ T lymphocytes (Shen & Lock, 2006). Using TLR3-deficient mice, Schultz et al. showed that TLR3 played an important role in cross priming (Schulz et al., 2005). Immunization with virally infected cells, which contain dsRNA or cells containing poly(I:C), both carrying ovalbumin antigen, induced ovalbumin-specific $CD8^+$ T lymphocyte responses, which were largely dependent on TLR3-expressing DCs. Additionally, Jongbloed et al. reported that $CD141^+$ DCs were able to cross present viral antigens from human cytomegalovirus-infected necrotic fibroblasts (Jongbloed et al., 2010). Physiologically, endosomal TLR3 in

a DC subset specialized for antigen presentation encounters viral dsRNAs when apoptotic or necrotic virus-infected cells are phagocytosed and signals for cross presentation of viral antigens.

Cross priming is also important for induction of CTLs against tumor cells. Azuma et al. recently demonstrated that antitumor CTL induced by a tumor antigen and poly(I:C) depended on the TLR3–TICAM-1 pathway in mouse splenic CD8α$^+$ DCs (Azuma, Ebihara, Oshiumi, Matsumoto, & Seya, 2012). They showed that IRF-3/7 were essential but MAVS and type I IFNs were minimally involved in poly(I:C)-mediated CTL proliferation. Here, we describe the *in vitro* assay for DC-mediated CTL induction (Datta et al., 2003):

1. Plate 5×10^5/500 μL medium BMDC in 24-well plate.
2. Stimulate cells with 10 μg/mL poly(I:C) and incubate for 18 h at 37 °C.
3. Add soluble ovalbumin (Sigma) to a final concentration of 100 ng/mL.
4. After 4 h, collect cells and wash twice with medium. Centrifuge at 750 × *g* for 5 min.
5. Coculture 1×10^5 BMDC and 1×10^5 CFSE-labeled OT-1 T lymphocytes in 200 μL/well in a 96-well round-bottom plate. The CFSE labeling protocol is described in the succeeding text.
6. After 60 h, collect cells and stain with anti-CD8α and anti-TCR-Vα2 Abs.
7. CFSE diminution is evaluated by flow cytometry. It is recommended to count 20,000 cells gated on CD8$^+$ TCR-Vα2$^+$ cells.

5.1. CFSE labeling for OT-1 T lymphocytes

1. OT-1 CD8$^+$ T lymphocytes are positively isolated by CD8(Ly-2) microbeads (Miltenyi Biotech, 130-049-401) according to the manufacturer's protocol.
2. Suspend cells in PBS to a concentration of 4×10^7 cells/mL.
3. Mix the cells with same volume of 2 μ*M* CFSE solution and keep it for 10 min at room temperature.
4. Add an equal volume of FCS and keep it for 1 min at room temperature.
5. Wash three times with medium. Centrifuge at 750 × *g* for 5 min.
6. Plate 1×10^5/100 μL CFSE-labeled OT-1 T lymphocytes in a 96-well round-bottom plate.

Medium: RPMI 1640 supplemented with 10% FCS, 10 m*M* HEPES, 55 μ*M*, 2-ME, and penicillin/streptomycin.