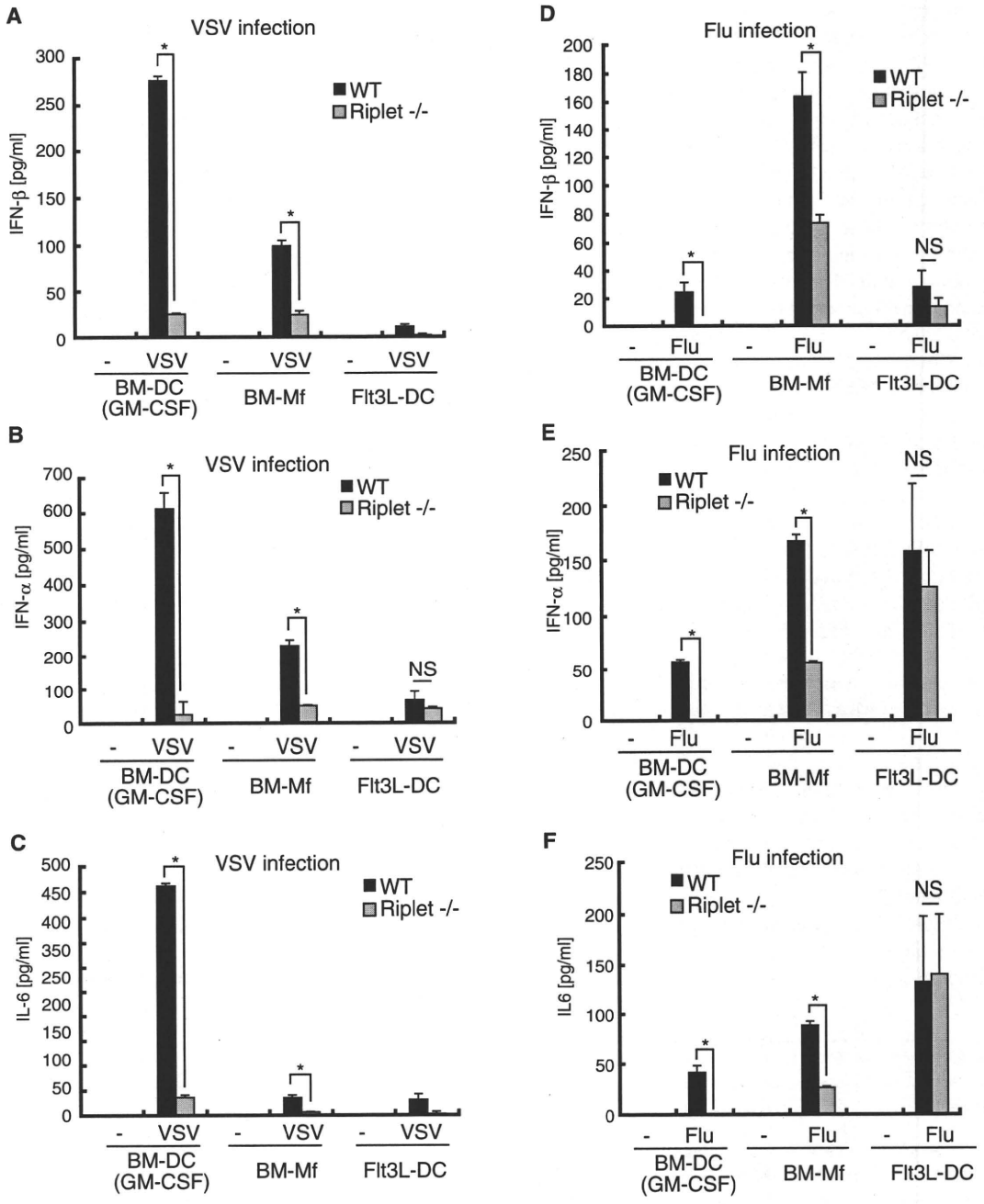

Figure 6. Role of Riplet in Responses to VSV or Flu Infection in Bone Marrow-Derived Cells

GM-DCs, BM-Mf, or Flt3L-DCs were induced from BM-derived cells in the presence of GM-CSF, M-CSF, or Flt3L and infected with VSV or influenza A virus at moi = 1. Twenty-four hours after viral infection, amounts of IFN-β (A and D), -α (B and E), and IL-6 (C and F) in culture supernatants were measured by ELISA. Data are shown as means ±SD and are representative of two independent experiments. *$p < 0.05$ (Student's t test). NS indicates not statistically significant.
See also Figure S4.

by overexpression of Riplet was also abolished by the 5KA mutation. These data support our model. However, we do not exclude the possibility that other Lys residues of RIG-I are ubiquitinated by Riplet, because we have not yet directly detected polyubiquitinated residues of RIG-I CTD by mass spectrometory analysis. Further in vitro studies are required to determine the polyubiquitination sites and to reveal precise RIG-I regulatory mechanisms by Riplet-mediated Lys63-linked polyubiquitation.

In general, E3 ubiquitin ligase targets several types of proteins. Therefore, it is possible that Riplet targets other proteins. Previous work has shown that Riplet binds to the Trk-fused gene (TFG) protein (Suzuki et al., 2001). The TFG protein interacts with TANK and NEMO, which are involved in the NF-κB pathway (Miranda et al., 2006). Although NEMO is involved in IPS-1-mediated signaling, RIG-I CARDs- or MDA5-mediated signaling was normal in *Riplet*$^{-/-}$ MEFs. Therefore, interaction between Riplet

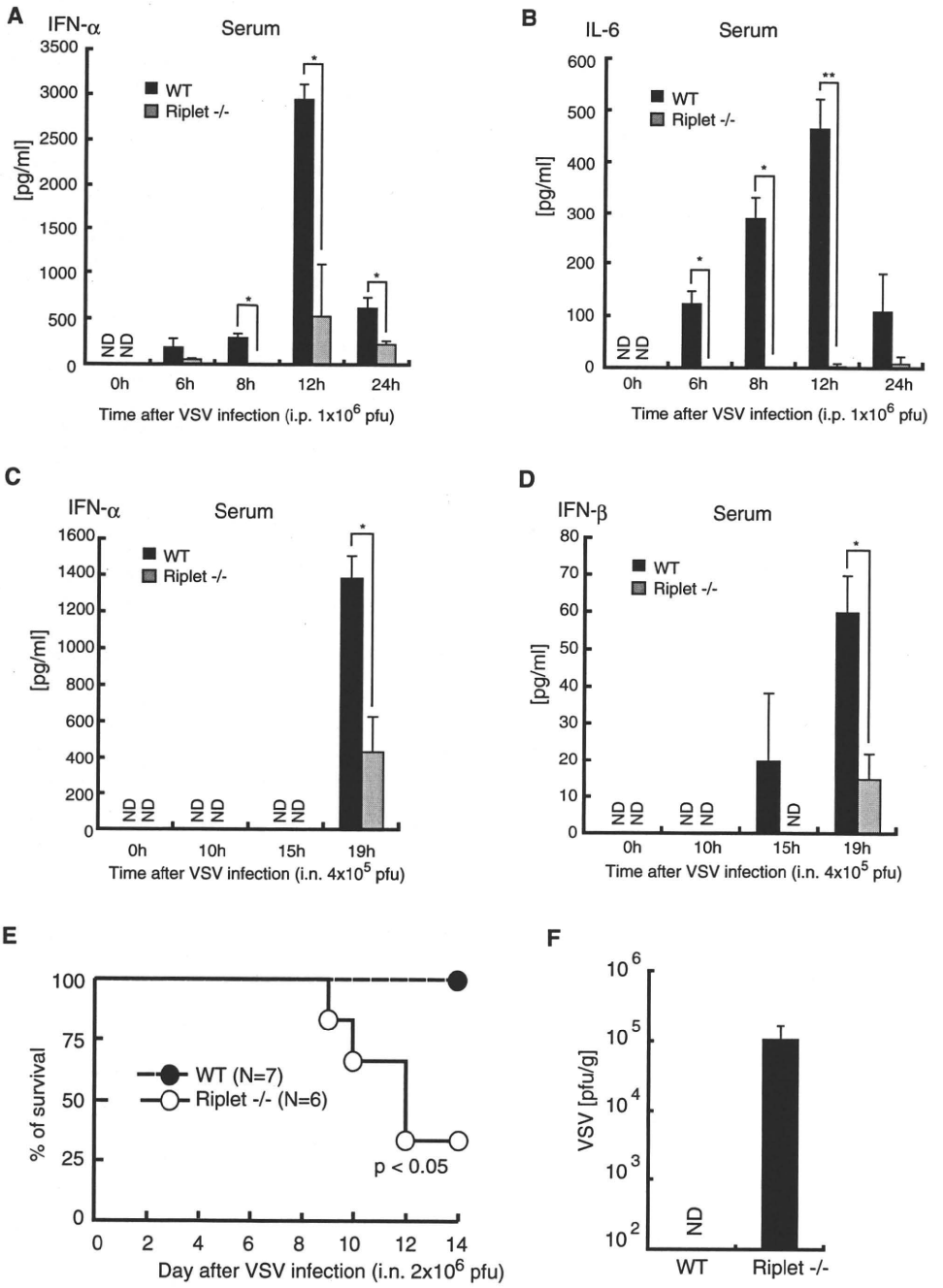

Figure 7. Role of Riplet in Antiviral Responses In Vivo

(A and B) Wild-type or *Riplet*$^{-/-}$ mice were injected intraperitoneally with 1 × 10^6 pfu of VSV. Amounts of IFN-α (A) and IL-6 (B) in mouse serum were measured by ELISA. Data are shown as mean ±SD of samples obtained from three wild-type and three Riplet −/− mice at each time point. *$p < 0.05$ (Student's t test). "ND" indicates not detected.

(C and D) Wild-type and *Riplet*$^{-/-}$ mice were infected intranasally with 4 × 10^5 pfu of VSV. Amounts of IFN-α (C) and IFN-β (D) in mouse serum were measured by ELISA.

(E) Wild-type and *Riplet*$^{-/-}$ mice were infected intranasally with 2 × 10^6 pfu of VSV and mice mortality was observed for 14 days (*$p < 0.05$ between wild-type and *Riplet*$^{-/-}$ mice, log rank test).

(F) Wild-type and *Riplet*$^{-/-}$ mice were infected intranasally with 2 × 10^6 pfu of VSV, and sacrificed for their tissues on day 7 after infection. Titers in brain were determined by the plaque assay. Viral titers in brains of wild-type mice were below 100 pfu/g, and thus not detected (ND). Data are shown as means ±SD (n = 3). See also Figure S5.

and TFG protein is not required for RIG-I-mediated signaling. However, since TFG is involved in tumorigenesis (Miranda et al., 2006), Riplet may be involved in human tumorigenesis.

Several viral proteins inhibit RIG-I-mediated signaling. For example, Flu NS1 inhibits TRIM25 and HCV NS3/4A cleaves IPS-1 (Meylan et al., 2005; Gack et al., 2009). Therefore, Riplet may be inhibited by viral proteins. Indeed, our pilot study indicated that the Riplet protein is disrupted in human hepatocyte cell lines carrying a full-length HCV replicon. RIG-I is involved in innate immune responses against various viruses. In this study, we showed that Riplet is required for innate immune responses against VSV, Flu, and SeV. Therefore, Riplet is also expected to be involved in innate immune responses against other viruses that are recognized by RIG-I.

EXPERIMENTAL PROCEDURES

Generation of Riplet-Deficient Mice

The Riplet gene was amplified by PCR using genomic DNA extracted from ESCs by PCR. The targeting vector was constructed by replacing the second and third exons with a neomycine-resistance gene cassette (Neo), and a herpes simplex virus thymidine kinase (HSV-TK) driven by PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into 129/Sv mice-derived ESCs, G418 and gancyclovir doubly resistant colonies were selected and screened by PCR. The targeted cell line was injected in C57BL/6 blastocysts, resulting in the birth of male chimeric mice. These mice were then crossed with 129/Sv mice to obtain heterozygous mutants. The heterozygous mutants were intercrossed to obtain homozygous *Riplet*$^{-/-}$ mice.

Cells, Viruses, and Reagents

Wild-type and *Riplet*$^{-/-}$ MEFs were prepared from day 12.5–13.5 embryos. *Riplet*$^{-/-}$ MEFs were immortalized with large T antigen and named R3T cell line. BM cells were prepared from 5- to 10-week-old mice. VSV Indiana strain was provided by A. Takada (Hokkaido University). VSV was amplified using Vero cells and the viral titer was determined by the plaque assay. Flu (PR8 strain) and SeV (HVJ strain) was provided by Y. Sakoda (Hokkaido University). HSV-1 strain was provided by K. Kondo (The JIKEI University). Anti-mouse IRF3 antibody was purchased from Zymed. Anti-phospho-STAT1 antibody was purchased from Cell Signaling and anti-STAT1 antibody from Santa Cruz. Salomon sperm dsDNA was purchased from Invitrogen. To determine the viral titer in the brain, the mice were sacrificed, and the brain was aseptically removed and frozen at −80°C. The brain was homogenized in 1 ml of PBS on ice, and the titer was determined by plaque assay.

Preparation of Viral Double-Stranded RNA

cDNA of the HCV 3′UTR region was amplified from total RNA of the HCV genotype 1b full-length replicon using primers HCV-F1 and HCV-R1, and then cloned in the pGEM-T Easy Vector. The primer set sequences were HCV-F1, CTCCAGGTGAGATCAATAGG; and HCV-R1, CGTGACTAGGGCTAAGATGG. RNA was synthesized using T7 and SP6 RNA polymerases. Template DNA was digested by DNase I, and RNA was purified using TRIZOL (Invitrogen) according to manufacturer's instructions.

Quantitative PCR

For qPCR, total RNA was extracted with TRIZOL (Invitrogen) and 0.5 μg of RNA was reverse-transcribed using the High Capacity cDNA Transcription Kit (ABI) with random primers according to the manufacturer's instructions. qPCR was performed using the Step One Real-Time PCR system (ABI). Primer sequences used for qPCR are listed in Table S1.

Measurement of Cytokines

In brief, 5 × 10^5 cells in a 24-well plate were either infected with VSV or Flu, stimulated with LPS, or transfected with HCV 3′UTR dsRNA or poly(I:C). Twenty-four hours after infection, stimulation, or transfection, culture supernatants were collected and analyzed for IFN-α, -β, and IL-6 production by ELISA. Cytokine levels were measured in mouse serum obtained from the mouse tail vein. ELISA kits for mouse IFN-α and -β were purchased from PBL Biomedical Laboratories. ELSA kit for mouse IL-6 was purchased from Invitrogen.

Preparation of Dendritic Cells and Macrophages

BM cells were prepared from the femur and tibia. The cells were cultured in RPMI1640 medium supplemented with 10% FCS, 100 μM 2-Me, and 100 ng/ml human Flt3 ligand (Pepro Tech), and 10 ng/ml murine GM-CSF or culture supernatant NIH 3T3 expressing M-CSF. After 6 days, cells were collected and used as Flt3L-DC, GM-DC, or BM-Mf. In the case of GM-DC or BM-Mf, the medium was changed every 2 days.

Native PAGE Analysis

Approximately 1 × 10^6 MEFs were infected with VSV at moi = 1 for 9 hr and then lysed. Cell lysates in native PAGE sample buffer (62.5 mM Tris-HCl [pH 6.8], 15% glycerol, and BPB) were separated using native PAGE and then immunoblotted with anti-murine IRF3 antibody (Zymed).

Luciferase Assay

Expression plasmids for mouse RIG-I N-terminal CARDs, full-length RIG-I, or full-length MDA5 were constructed in pEF-BOS. The cDNA fragment encoding the ORF of RIG-I or MDA5 was amplified by RT-PCR using total RNA prepared from MEFs. The Riplet dRING mutant protein lacks 1–69 aa region. Wild-type and mutant (Riplet dRING) Riplet-expression vectors were described previously (Oshiumi et al., 2009). Wild-type or *Riplet*$^{-/-}$ MEFs were transiently transfected in 24-well plates with reporter constructs containing the IFN-β promoter and Renilla luciferase (internal control) together with the empty vector (control), RIG-I CARDs, full-length RIG-I, or MDA5 expression vectors. Twenty-four hours after transfection, cells were lysed and subjected to the luciferase assay using the Dual-Luciferase Reporter Assay system (Promega).

Statistical Analyses

Statistical significance of differences between groups was determined by the Student's t test, and survival curves were analyzed by the log rank test using Prism 4 for Macintosh software (GraphPad Sofware, Inc.). Chi-square goodness-of-fit tests and Student's t tests were performed using MS-Excel software and a chi-square distribution table.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article at doi:10.1016/j.chom.2010.11.008.

ACKNOWLEDGMENTS

We thank Dr. John P. Atkinson (Washington University) and Dr. Ralph Steinman (Rockefeller University) for critical discussions, Yoko Esaki and Kiyo Kawata for technical support in generating Riplet KO mice, N. Irie for a pilot study of B-DNA stimulation assay, and Sakoda Y. for technical instructions for the experiments using Flu and SeV. This work was supported in part by the Mitsubishi Foundation; Mochida Foundation; Akiyama Life Science Foundation; and Grants-in-Aid from Ministry of Education, Science, and Culture and Ministry of Health, Labor, and Welfare of Japan.

Received: June 4, 2010
Revised: August 18, 2010
Accepted: November 5, 2010
Published: December 15, 2010

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Review Article

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

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(Received October 7, 2009/Accepted November 10, 2009/Online publication January 4, 2010)

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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doi: 10.1111/j.1349-7006.2009.01442.x

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.

doi: 10.1111/j.1349-7006.2009.01442.x

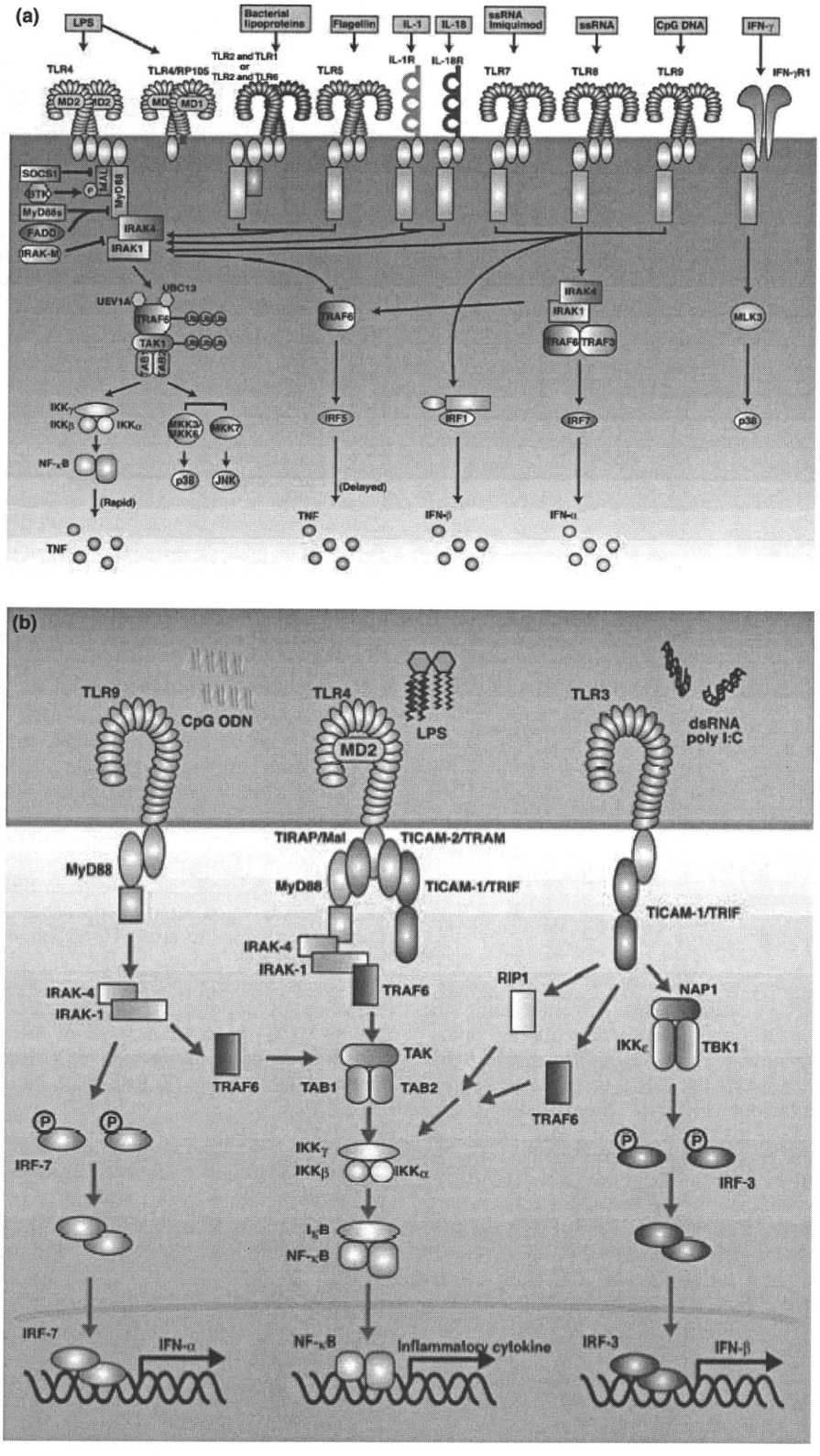

Fig. 1. MyD88 and Toll-interleukin I receptor-domain (TIR)-containing adoptor molecule (TICAM-1) pathways. MyD88 is an adaptor for all Toll-like receptors (TLR), except TLR3 (a). TLR2 and TLR4 recruit MyD88 via the bridging adaptor Toll-interleukin I receptor-domain (TIR)-containing adoptor protein (TIRAP) (MAL) (b). Other TLR directly recruit MyD88. MyD88 activates nuclear factor-kappa β (NF-κB) in most cell types, except plasmacytoid dendritic cells (pDC), which activate the interferan-regulatory factor (IRF-7) transcription factor. MyD88 pathway is involved in the production of pro-inflammatory cytokines in most cells. In contrast, the MyD88 pathway in pDC and the TICAM-1 pathway in myeloid dendritic cells (DC) activate the type interferon (IFN) promoter via IRF-3 or IRF-7 (b). TLR4 can recruit both MyD88 and TICAM-1, whereas other TLR recruit either of them. Each TLR responds to different agonistic stimuli, as shown in Table 1. DC, dendritic cells; IFN, interferon; IRF, interferon-regulatory factor; pDC, plasmacytoid DC; TICAM-1, Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule; TIRAP, Toll-interleukin 1 receptor (TIR) domain-containing adapter protein; TLR, toll like receptor.

Table 1. Human TLR and pattern molecules with MyD88- or Toll-interleukin I receptor domain (TIR)-containing adoptor molecule (TICAM-1)-activating properties

Human TLR		Ligands
TLR1		Pam3
TLR2		Pam2, Pam3, PGN
TLR3		dsRNA
TLR4		LPS, virus fusion units
TLR5		Flagellin
TLR6		Pam2
TLR7		ssRNA
TLR8		ssRNA
TLR9		CpG DNA
TLR10		–
MyD88 activators (Lipoproteins, PGN)		**Reference**
M161Ag (MALP-2)		(62)
TAN33		(63)
OM-174		(64)
BCG-CWS (Azuma lot)		(22)
SMP105		(65)
TICAM-1 activators (RNA, lipid A)		
DI RNA (stem loop)		(66)
Poly(A:U)		(67)
Poly(I:C_{12}U)		(68)
PolyI:C(LC)		(20)
MPLA		(21)
Anti-human TLR monoclonal antibodies		
TLR1	TLR1.136	(58)
TLR2	TLR2.45	(59)
TLR3	TLR3.7	(60)
TLR4	HTA125	(61)
TLR6	TLR6.127	(58)

dsRNA, double-stranded RNA; TLR, Toll-like receptor. SMP105 is a lot of BCG-CWS that activates only TLR2.

In the present study, we used polyI:C for evaluating the TICAM-1 potential in mDC maturation and antitumor immunity.[38] The TICAM-1 pathway allows mDC to activate IRF-1 and IRF-3, which in turn activate the IFN-β promoter, as well as unidentified antitumor factors (Fig. 1). The data imply that cross-priming and the NK-driving signal are also dependent upon TICAM-1, but the transcription factors utilized by TICAM-1 are wholly distinct from those of MyD88. The search for the molecules that participate in the TICAM-1 CTL driving is underway, and a molecule downstream of IRF-1, but not IRF-3, has been shown to be crucial for *in vivo* CTL induction. In contrast, TICAM-1-mediated antitumor NK activation largely relies on the IRF-3-derived NK-activating molecule (INAM), in addition to the reported cytokines IL-15, IFN-α, and IL-12p70.[39]

MyD88 and TICAM-1 activate different signaling platforms for the recruitment of second adaptors.[3] In mDC, TLR2 and TLR4 recruit the combined adaptor Mal/Toll-interleukin I receptor domain-containing adoptor protein (TIRAP)–MyD88 to signal the transcription factor NF-κB.[4] In contrast, TLR3 and TLR4 can utilize TICAM-1 as the adaptor.[3] TLR4 recruits the combined adaptor Toll-IL-IR domain-containing adoptor inducing IFN-beta-related adaptor molecule (TRAM) (TICAM-2)–TICAM-1 while TLR3 directly recruits TICAM-1 for signaling.[3] TLR4 is unique in that it uses both MyD88 and TICAM-1 adaptors (Fig. 1). The classic example in which both routes are activated is during LPS-induced endotoxic shock.[40] Like BCG-CWS and PolyI:C, activation of either one route would be required for a condition of less toxic adjurants. Studies of the TICAM-1 signalosome suggest that upon TLR3 activation, TICAM-1 recruits a variety of molecules as secondary adaptors, including NAK-associated protein I (NAP1),[41] receptor-interacting protein I (RIP1),[42] similar to NAP I TBK adoptor (SINTBAD),[43] adenovirus 5 E1A-binding protein (BS69),[44] and TNF receptor-associated factor (TRAF) family proteins.[45] Whether or not these molecules are associated with antitumor CTL or NK induction remains to be determined.

The mode by which mDC are matured differs in the MyD88 and the TICAM-1 pathways. The TICAM-1 pathway preferentially induces IL-12 and type I IFN in mDC and drives NK activation.[38] Type I IFN induction by MyD88 has been observed only in pDC.[9,10] In contrast, mDC MyD88 strongly induces pro-inflammatory cytokines, such as tumor necrosis factor-α, IL-1β, and IL-6.[9,46] The molecular mechanism that facilitates the cross-presentation ability in mDC is currently unknown.

DC subsets and TLR expression

BMDC (representative of mDC) and pDC can be prepared from mouse bone marrow cells by using granulocyte-macrophage colony-stimulating factor (GM–CSF) and the Flt3 ligand[46] while Langerhans cells can be generated by the addition of transforming growth factor-β to GM–CSF and IL-4.[47] The DC subsets in the spleen and the intestinal tract can be separated using a flow cytometry (FACS) sorter. The characteristics of these mouse DC subsets have been described previously.[36] In humans, monocyte-derived DC can be used as mDC, but their characteristics are somewhat different from mDC prepared in the peripheral blood using the mDC marker plasmocytoid DC antigen (PDCA1). Human peripheral blood pDC can be isolated from whole blood using PDCA4.

The distribution of TLR of the DC subset were examined by using human TLR-specific monoclonal antibodies generated in our laboratory, and the TLR repertoires of monocyte-derived DC and pDC were determined (Table 2). The TLR distribution roughly resembles mouse DC, although a clear result could not be obtained with mouse BMDC and pDC because of a lack of appropriate specific antibodies against mouse TLR.[36] The discrepancy of appropriate TLR7 levels in mouse BMDC and human mDC could be a result of differences in the inducible nature of mouse, but not human, TLR7. It was also shown that human mDC express TLR8, while mouse mDC do not.[36] The

Table 2. TLR expression profiles in human DC subsets

	Freshly isolated			*In vitro*-differentiated	
	Monocytes	mDC*	pDC**	DCs	Macrophages
TLR1	++	+	–	+	++
TLR2	++	++	–	++	++
TLR3	–	++	–	++	+
TLR4	++	+	–	+	+
TLR6	++	+	–	+	+
TLR7	–	–	+	–	–
TLR8	+	+	–	+	+
TLR9	–	–	+	–	–

Positive and negative symbols denote the results of the flow cytometry (FACS) analyses using monoclonal antibodies, except TLR7, TLF8, and TLR9. Results were determined by reverse transcription–polymerase chain reaction. TLR3, TLR7, TLR8, and TLR9 reside in the endosome to recognize nucleotide derivatives. (*) PDCA1+ cells; (**) PDCA4+ cells. PDCA, plasmacytoid dendritic cell antigen; DC, dendritic cell; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; TLR, Toll-like receptor.

doi: 10.1111/j.1349-7006.2009.01442.x

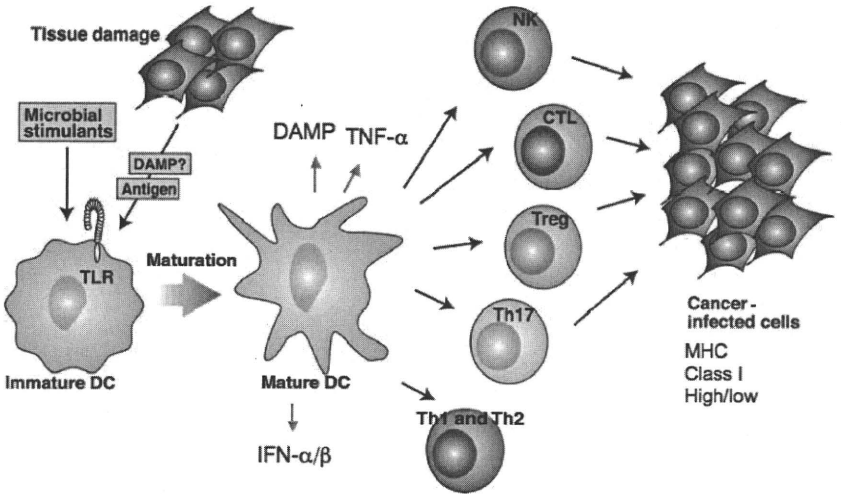

Fig. 2. Selective induction of immune effector lymphocytes by different agonistic stimuli. Each pattern molecule (PAMP) has its own uniqueness in myeloid dendritic cell (mDC) maturation. Differential maturation of mDC results in different effector driving as shown. CD8 T, various CD4 T, and B cells are proliferated by myeloid DC (mDC) with different properties. Tumor regression is a marker for evaluating which lymphocytes are activated in response to pathogen-associated molecular patterns (PAMP).

failure of CpG DNA to raise effective antitumor immunity can be attributable to the low or absent induction of TLR9 in human mDC, unlike the situation in mouse mDC.

DC subsets and effector induction

CTL and NK cells can be induced by mature mDC, with or without the presentation of MHC class I antigens, while CD4 T-cell subsets are induced by the presentation of class II antigens. In addition to CTL and NK cells, the tumor-modulating functions of Th1, Th2, Th17, and Treg were evaluated (Fig. 2). NK activation is a result of the balance between NK-activating and inhibitory ligands on mDC. NK cells can also be activated with cytokines, such as IL-2, IL-15, IFN-α/β, and IL-12.[48] CTL is a result of the activation of the CD8+ T cell by the presentation of class I antigens on mDC. Other effectors are the result of the activation of CD4+ T cells by MHC class II antigen presentation on mDC. A master transcription factor in addition to T-bet, GATA-3, RORgT, and Foxp3 are known to exist for Th1, Th2, Th17, and each Treg on the CD4 lymphocyte side.[49] However, there is little information concerning the mDC properties driving these effector cells.

Each DC subset seems to correspond to a specific effector, although the selection mechanism by which DC induce various effectors is not clear in most instances. However, it is known that CD8+ DC induce Treg[50] and NK cells[51] in the mouse spleen, and lamina propria pDC in the mouse enteric canal promotes immunoglobulin A production.[52] In addition, CD70+/CD11c+ DC induce Th17 cells by the adenosine tri phosphate (ATP) of enterobacteria,[53] and BMDC activate NK cells via the TICAM-1 pathway.[54] Further examples of DC subsets that preferentially function with specific effectors will likely be demonstrated through practical experiments.

Mechanism of DC-mediated antitumor NK activation

It has been reported that BMDC drive antitumor NK activation in a TICAM-1-dependent manner.[38,54] This NK activation does not rely upon a soluble factor, such as a cytokine, but instead was generated by BMDC–NK cell–cell contact.[39] Therefore, there must be an NK-activating molecule that is induced on the BMDC surface in response to TICAM-1 signaling (Fig. 3a). We focused our attention on this key molecule, which is crucial for antitumor NK immunity and found that DC-mediated NK activation occurred normally in IRF-7 −/− BMDC stimulated with polyI:C, but this response was absent in IRF-3 −/− BMDC.[39] Therefore, the putative NK-driving signal in mDC involves transcription factor IRF-3 downstream of the activated TICAM-1. Ultimately, the NK activation molecule was identified using a screening method in which candidate molecules were expressed in IRF-3 −/− BMDC using a lentiviral vector.[39] We named this molecule

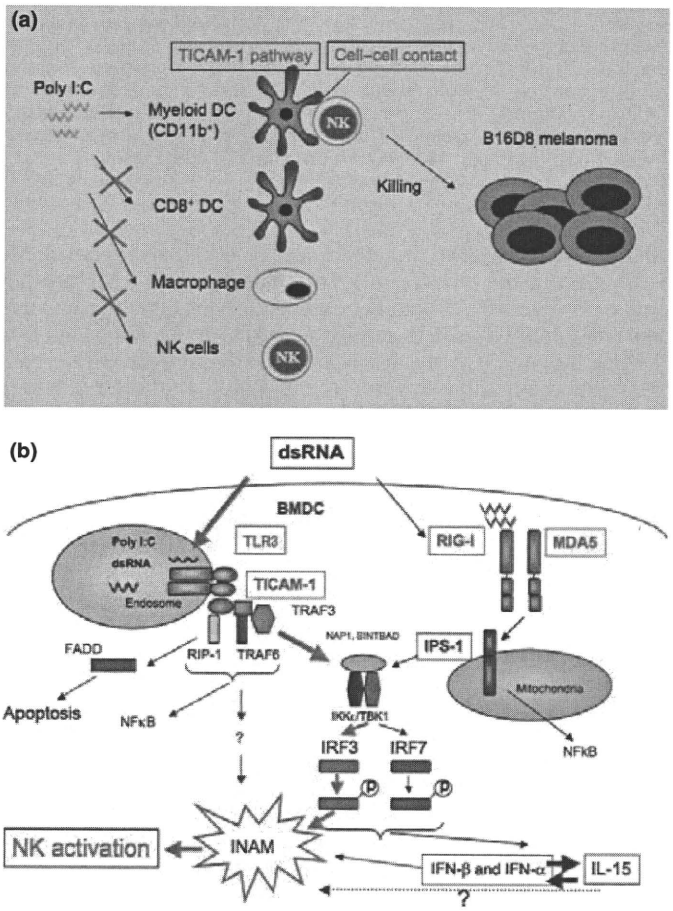

Fig. 3. A molecular mechanism of myeloid dendritic cell (mDC)-mediated natural killer (NK) activation. (a) CD11b+ bone marrow-derived dendritic cells (BMDC) act for natural killer (NK) activation by double-stranded (ds) RNA. NK cells express tumoricidal activity against major histocompatibility complex (MHC) low implant tumors if they are primed by polyI:C plus bone marrow-derived dendritic cells (BMDC), but not other myeloid cells. Dendritic cell–NK cell–cell contact is essential for the induction of polyI:C-mediated antitumor NK cells. (b) Route for mDC maturation for the induction of NK activation.[39]

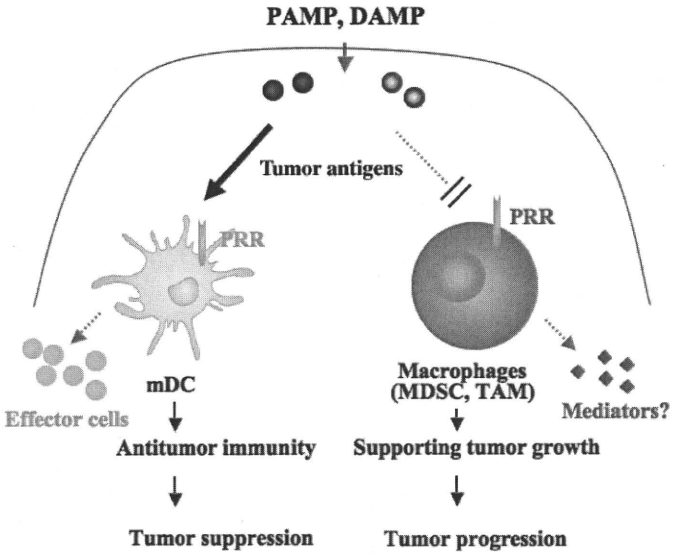

Fig. 4. Diverged functions of myeloid cells in tumor mass. A variety of myeloid subsets reside in tumor masses. Some of the subsets exhibit an immune suppressive feature that facilitates escape of tumor cells from immune effectors. Since pattern molecules (PAMP) act on both myeloid dendritic cells (mDC) and myeloid-derived immune suppressing cells, complicated immune responses occur in tumors. Selective maturation of mDC circumventing the exacerbation of tumor progression by myeloid suppressor cells should be considered as adjuvant therapy of cancer. DAMP, damage-associated molecular patterns; MDSC, myeloid-derived suppressor cells; PRR, pattern-recognition receptors; TAM, tumor-associated macrophages.

INAM. When INAM was expressed in mDC, it promoted NK activation in the mixture of mDC (expressing INAM) and NK cells; however, INAM did not exhibit an NK-activating function on BaF3 cells. INAM is an NK-activating molecule peculiar to BMDC whose TICAM-1 has been activated, and there have been no reports suggesting the presence of this kind of molecule until recently (Fig. 3b). In BMDC, INAM receives a sugar chain modification by a similar membrane protein to tetraspanin with a molecular weight of 45 kDa. INAM is distributed in the spleen and lymph nodes, and is actually expressed by a variety of lymphocyte subsets present in the lymph nodes. It has been predicted to make a loop card structure on the surface of the cell in two portions based on the amino acid sequence.[39]

It is predicted that INAM is related to the composition of immune synapses in the BMDC–NK contact. When BMDC, which forcibly express INAM, are prepared and adoptively transferred around the tumors of tumor-bearing mice, the tumor is efficiently regressed. These results suggest that INAM is the factor directly responsible for driving antitumor NK activation. Humans have an ortholog of INAM, although its distribution profile appears to be somewhat different than to that of mice.

Points to trigger antitumor immune potential

Effector tumor cell–cell contact is essential for tumor damage by immune effector cells. The material liberated from cancer cells on one side generates the modulators of the PRR of mDC and influences the trigger of effector induction. The host molecules that modulate PRR are the previously-mentioned DAMP.[32] For effective tumor damage, the effector must reach the tumor mass. A suitable strategy is needed for determining the basic factor(s) of the immune response involved in cancer, and can be achieved by using immunomodulatory reagents and gene-disrupted mice with abrogated TLR pathways.

We have analyzed how BMDC acquire effector-driving functions by focusing on the innate immune response. The results suggest that PRR stimuli become a trigger that leads to the alteration of precancerous cells to the malignant form. However, PRR are indispensable to the activation of antitumor immunity. In both cases, myeloid cells are intimately involved in the process of tumor–immune cell interaction. Indeed, BCG has high therapeutic potential for patients with bladder transitional epithelial cancer,[55] but it has less of an effect on a variety of other solid cancers. This discrepancy can be rooted in the fact that myeloid cells interact with tumor cells with ambivalent reaction profiles (Fig. 4). An effective strategy for tackling the issue of immune abnormality has yet to be proposed, and even the fundamental immune aberrance present in the microenvironment of tumors is not generally recognized by researchers. It has been speculated that tumor cells produce cytokines that modulate the inflammatory environment as tumor develops. When tumor is surgically excised, many constitutional accidents are often diminished,[56] which can reflect the fact that tumors develop concomitantly with immune modulation. It has become clear that some modulating factors of the innate immune system, such as DAMP, cause cancer-mediated idiosyncrasies (Fig. 2).

Up until now, the effectiveness of cancer immunotherapy has been primarily evaluated based on tumor regression and the survival prognosis of patients. A representative study involved the evaluation of peptide vaccine therapy for cancer treatment. According to the report by Rosenberg,[57] the peptide vaccine administered to melanoma patients had an effective rating of approximately 2.6%. For future studies, it is necessary to determine the potential of peptide-conjugating materials, including adjuvants and inflammation-inducing reagents.[20] A number of reports have suggested that adjuvants can greatly increase the efficiency rate of treatment, although the criteria is prerequistie to fairly evaluate the function of adjuvants in cancer patients.

The method for stimulating DC needs to be carefully selected, as the systemic administration of inflammation-inducing material can also lead to the acceleration or invasion of developing malignancies at the same time (Fig. 4). The adoptive transfer of adjuvant-treated mDC to patients is a promising choice; however, it might be difficult for this treatment to be adapted by the Japanese health insurance system. The molecular manipulation of a specific PRR in DC that is involved in effector driving can lead to effective treatment with minimal side-effects. In this case, the route and molecule that selectively raises the degree of DC maturation without enhancing MDSC should be clarified. If the inflammatory signals that promote carcinogenesis are properly controlled using adjuvants, the design of DC maturation can be manipulated without helping tumor progression. The search for the functional molecule of antitumor effector induction in mDC will help establish an effective treatment of cancer and facilitate the evaluation of the efficacy of peptide vaccines. In the future, we hope that through continued research, cancer patients will have access to convenient and highly effective immunotherapy.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan by the Akiyama Foundation and Yakulto Foundation. Financial support from the Sapporo Biocluster "Bio-S" Knowledge Cluster Initiative of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) is gratefully acknowledged. We are grateful to our laboratory members for their critical discussions, and Ms R. Hatsugai and Ms H. Sato in our laboratory for secretarial assistance.

doi: 10.1111/j.1349-7006.2009.01442.x

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doi: 10.1111/j.1349-7006.2009.01442.x

Vaccine 28 (2010) 8041–8042

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Innate immunity and vaccine

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ARTICLE INFO

Article history:
Received 23 February 2010
Accepted 26 April 2010
Available online 17 September 2010

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 accumulating 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-cell wall skeleton (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 is undetermined.

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 infec-

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doi:10.1016/j.vaccine.2010.09.023

tions. 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 which potentially augment 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 if the fundamental immune aberrance is present in the focal nests of infection. The microenvironmental situation should be grasped by researchers and be improved by their best. If this is feasible, adjuvant therapy be a good choice for some virus-induced persistent infections.

There is almost no information concerning the molecular mechanisms by which mDCs drive these effector cells. Each DC subset seems to correspond to a specific effector, although the selection strategy about 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 raise 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.

Acknowledgement

We are grateful to Dr. H. Kida (Hokkaido University, Sapporo) for his giving us this opportunity.

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International Immunology, Vol. 23, No. 1, pp. 29–41
doi:10.1093/intimm/dxq455
Advance Access publication 3 December 2010

IL-23-dependent and -independent enhancement pathways of IL-17A production by lactic acid

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Received 12 April 2010, *accepted* 26 October 2010

Abstract

Interleukin-17A (IL-17A) is a cytokine produced by T_h17 cells that plays an important role in inflammatory and autoimmune diseases and cancer. Stimulation with IL-6, transforming growth factor-β , IL-21, IL-1β and IL-23 is required for differentiation of T_h17 cells and the production of IL-17A. Recently, we reported that tumor-derived lactic acid enhances the toll-like receptor (TLR) ligand-mediated expression of IL-23, leading to increased IL-17A production. Tumor cells secrete large amounts of lactic acid due to the up-regulation of glycolysis, which is known as the Warburg effect. Even without TLR ligand stimulation, lactic acid enhanced antigen-dependent IL-17A production from splenocytes in an IL-23-dependent manner. Here, we show that macrophages and effector/memory $CD4^+$ T cells are the primary cell types involved in the ability of lactic acid to boost IL-17A production. Although lactic acid suppressed the proliferation of T_h1 and T_h17 cells, T_h17 cells still secreted large amounts of IL-17A. CD40 ligand–CD40 interactions were involved in the up-regulation of IL-17A by lactic acid through IL-12/23p40 production. A new cytokine containing the IL-12/23p40 subunit, but not IL-23, IL-12 or the IL-12p40 homodimer, is a candidate for involvement in the up-regulation of IL-17A. IL-1β also increased IL-17A expression; however, IL-1β, CARD9 and MyD88 signaling pathways activated by known intrinsic inflammatory mediators were hardly required for the enhanced activity induced by lactic acid. Our results show that lactic acid functions as an intrinsic inflammatory mediator that activates IL-23-dependent and -independent pathways, resulting in the promotion of chronic inflammation in tumor microenvironments.

Keywords: IL-17, IL-23, inflammation, lactic acid, tumor

Introduction

Inflammation is a condition often induced not only by extrinsic pathogens but also by host-derived intrinsic stimulation resulting from pathogenic alteration, autoimmune and metabolic diseases, tumors and pathological cell death (1, 2). Toll-like receptors (TLRs), Nucleotide-binding oligomerization domain-like receptors and C-type lectin receptors are all known to sense pathogen-associated molecular patterns. Recently, it was determined that they also recognize host-derived molecules secreted from damaged cells, such as high-mobility group box 1 (3, 4), ATP (5–7) and Sap130, a component of small nuclear riboproteins (8), and activate the innate immune system, leading to inflammation.

IL-17A, which is one of the six IL-17 cytokine family members, plays important roles in inflammatory diseases, autoimmune diseases and cancer, and is secreted from IL-17A-producing $CD4^+$ T cells (T_h17 cells), $CD8^+$ T cells, γδT cells and natural killer T cells (9). T_h17 cells differentiate from naive T_h cells in response to IL-6 and transforming growth factor-β (TGF-β) (10–12) and are amplified through a positive feedback loop involving stimulation by the IL-21 that they secreted (13). IL-17A production from T_h17 cells is induced by IL-1β and IL-23. IL-23, which is a pro-inflammatory heterodimeric cytokine composed of an IL-23-specific p19 subunit and a p40 subunit that is shared with IL-12, is involved in

the terminal differentiation of T_h17 cells (14) and in the maintenance of the T_h17 phenotype (15) and activates memory $CD4^+$ T cells (16). It has been reported that activation of the IL-23/IL-17 pathway is involved in the development of many human autoimmune disorders such as Crohn's disease and rheumatoid arthritis and mouse experimental autoimmune encephalomyelitis (17, 18). In addition, in response to activated signal transducer and activator of transcription 3 (STAT3) signaling, up-regulated IL-23 promotes the incidence and growth of tumors (19, 20).

Recently, we identified lactic acid as a tumor-derived intrinsic inflammatory mediator, which enhances the IL-23/IL-17 inflammatory pathway (21). Normal mammalian cells metabolize glucose to pyruvic acid in the cytoplasm and oxidize it into carbon dioxide and water in the mitochondria to produce ATP in an oxygen-dependent manner. Under hypoxic conditions, cells produce ATP through glycolysis and metabolize pyruvate to lactic acid by lactate dehydrogenase via the oxidization of NADH. However, tumors often produce large amounts of lactic acid by carrying out glycolysis even under aerobic conditions. This phenomenon is known as the 'Warburg effect' (22). High concentrations of lactate in some solid tumors are correlated with higher frequencies of distant metastasis and poor prognosis (23). The Kreutz group also reported that tumor-derived lactic acid modulates the function of human monocyte-derived dendritic cells (DCs) and inhibits the proliferation and cytotoxic activity of human $CD8^+$ T cells (24, 25). We found that lactic acid enhances the activation of the IL-23p19 promoter when monocyte/macrophage cells were stimulated with the TLR2/4 ligand (21). Under stimulated conditions, 10–20 mM lactic acid specifically enhanced transcription of IL-23p19, but not IL-12/23p40, in a dose-dependent manner. Because the effect of lactic acid was regulated by extracellular pH, but low pH itself did not enhance the transcription of IL-23p19, we predicted that lactic acid entered the cytoplasm via monocarboxylate transporters (MCTs) to activate the IL-23p19 promoter. Lactic acid alone did not directly produce IL-23 or activate monocytes/macrophages. However, in splenocytes, upon stimulation with TLR ligands and antigen, lactic acid strongly enhanced the expression of IL-23p19 and IL-17A, but not IFN-γ. This occurred even in the absence of TLR ligands in an antigen-dependent manner. These findings suggest that lactic acid acts not only as a terminal metabolite of anaerobic glycolysis but also as a key player in the immune response. In this study, we analyzed the cells and factors that are involved in the lactic acid-mediated immune responses to further elucidate the actions of lactic acid as an intrinsic inflammatory mediator. Our findings suggest that a new cytokine containing IL-12/23p40 is a candidate for involvement in the enhanced IL-17A production by effector/memory $CD4^+$ T cells after the stimulation with lactic acid.

Methods

Mouse strains

C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Ovalbumin (OVA)-specific, MHC class II-restricted, $\alpha\beta$TCR transgenic (OT-II) mice (26) and *Myd88* knockout mice (27) were kindly provided by Dr W. R. Heath (The Walter and Eliza Hall Institute of Medical Research) and Dr Shizuo Akira (Osaka University), respectively. *Card9* knockout mice were previously characterized (28). All mice were maintained under specific pathogen-free conditions in the Osaka Medical Center animal facility. All animal experiments were performed in accordance with institutional guidelines and approved by the Animal Care and Use Committee of the Osaka Medical Center.

Reagents and antibodies

L-lactic acid was purchased from Sigma–Aldrich (St Louis, MO, USA), sodium lactate from WAKO Pure Chemical (Osaka, Japan), OVA $_{323–339}$ peptide from Bio Synthesis (Lewisville, TX, USA) and phorbol-12-myristate 13-acetate (PMA) and ionomycin from Merck Biosciences (Darmstadt, Germany). Anti-mouse cytokine antibodies (10 $\mu g\ ml^{-1}$) were used to neutralize IL-12/23p40 (C17.8; eBioscience, San Diego, CA, USA), IL-23p19 (G23-8; eBioscience) and IL-1β (B122; eBioscience). Rat IgG_{2a} (eBR2a; eBioscience), rat IgG_1 and Armenian hamster IgG (eBio299Arm; eBioscience) were used as isotype-matched control antibodies for IL-12/23p40, IL-23p19 and IL-1β, respectively. Anti-CD40 activating (HM40-3; eBioscience) and anti-CD40 ligand (CD40L) blocking antibodies (10 $\mu g\ ml^{-1}$, MR1; eBioscience) were used to examine CD40L–CD40 interactions and Armenian hamster IgG (eBio299Arm) was used as a control antibody for CD40L. The expression of CD11b and CD40 was analyzed by staining with FITC-conjugated anti-CD11b (M1/70; eBioscience) and PE-conjugated anti-CD40 antibodies (1C10; eBioscience). RmIL-12p70 (Peprotech, Rocky Hill, NJ, USA), rmIL-12p40 homodimer (Biolegend, San Diego, CA, USA) and rmIL-23 (R&D systems, Minneapolis, MN, USA) were used as cytokines containing the IL-12/23p40 subunit. The IL-1 receptor antagonist (IL-1RA, 0.25 $\mu g\ ml^{-1}$; R&D systems) was used to block the effects of IL-1.

Cell cultures

Cells isolated from splenocytes of C57BL/6J and OT-II mice were cultured in RPMI1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. All cells were cultured at 37°C under a 5% CO_2 atmosphere.

Fractionation of splenocytes

$CD11b^+$ and $CD11c^+$ cells were purified from C57BL/6J splenocytes by positive selection with anti-mouse CD11b and CD11c microbeads (Miltenyi Biotec, Gladbach, Germany), respectively. F4/80$^+$ cells were purified with biotinylated anti-F4/80 antibodies and anti-biotin microbeads. $CD11b^+CD11c^-$ and $CD11b^-CD11c^+$ cells were enriched by negative selection with CD11c or CD11b microbeads, followed by positive selection with anti-mouse CD11b or CD11c antibodies, respectively. $CD11b^-CD11c^-$ cells were isolated by negative selection with CD11b and CD11c microbeads. OT-II naive and effector/memory $CD4^+$ T cells were purified by negative selection of $CD4^+$ T cells with a $CD4^+$ T cell isolation kit (Miltenyi Biotec), followed by positive and negative selection with anti-CD62L microbeads

(Miltenyi Biotec), respectively. The purity of each fraction was measured using the FACScalibur System (BD Biosciences, San Jose, CA, USA). To detect proliferation, purified $CD4^+$ T cells were suspended at 5×10^6 ml^{-1} in Dulbecco's PBS (−), plus 5% FBS and 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Dojindo, Kumamoto, Japan) and then incubated at room temperature for 5 min. The CFSE-labeled cells were incubated with $CD11b^+$ cells.

Generation of bone marrow-derived macrophages and bone marrow-derived dendritic cells

Bone marrow-derived macrophages (BMDMs) and bone marrow-derived dendritic cells (BMDCs) were induced as previously described (29, 30). Briefly, bone marrow cells were obtained from C57BL/6J femurs and cultured in RPMI1640, supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 55 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg ml^{-1} streptomycin, in the presence of 50 ng ml^{-1} mouse macrophage colony stimulating factor (M-CSF) (Peprotech) or 10 ng ml^{-1} mouse granulocyte-macrophage colony stimulating factor (Peprotech). The media were replaced every 2 days before harvesting on day 5 for assays.

Generation of in vitro-differentiated T_h17 cells

Purified OT-II naive $CD4^+$ T cells were stimulated for 4.5 days with plate-coated anti-mouse CD3ε (10 μg ml^{-1}, 145-2C11; eBioscience) and CD28 (10 μg ml^{-1}, 37.51; eBioscience) antibodies or with OVA peptide-loaded $CD11b^+$ cells in the presence of 2 ng ml^{-1} human TGF-β1 (Peprotech) and 20 ng ml^{-1} mouse IL-6 (Peprotech) with or without 15 mM L-lactic acid. On day 5, the differentiated cells were re-stimulated for 5 h with 50 ng ml^{-1} PMA and 750 ng ml^{-1} ionomycin in the presence of brefeldin A. After labeling with PE-conjugated anti-CD4 antibodies (GK1.5; eBioscience), the cells were fixed and permeabilized with a BD Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences) and then stained with FITC-conjugated anti-IFN-γ (XMG1.2; eBioscience) and Allophycocyanin-conjugated anti-IL-17A (eBio17B7; eBioscience) antibodies. The expression of cytokines in the cells was detected by FACS analysis.

Cytokine production assay

Each fraction of splenic antigen-presenting cells (APCs) (1×10^5 cells) was mixed with 1×10^5 of OT-II $CD4^+$ T or *in vitro*-differentiated T_h17 cells in a round bottom 96-well cell culture microplate. BMDMs and BMDCs (1×10^5 cells) were mixed with 5×10^5 of OT-II $CD4^+$ T cells in a flat bottom 96-well cell culture microplate. Cells were stimulated for 4 days with 200 ng ml^{-1} OVA peptide in the presence or absence of 15 mM L-lactic acid. OT-II $CD4^+$ T cells (1×10^5) were stimulated with plate-coated anti-CD3ε and anti-CD28 antibodies in the presence of cytokines containing the IL-12/23p40 subunit with or without lactic acid. Cytokine levels in the culture supernatants were measured using IL-1β, IL-12/23p40 and IL-23 (Invitrogen-Biosource Cytokines & Signaling, Camarillo, CA, USA) and IL-17A (R&D systems) ELISA kits. To examine intracellular cytokine production, T cells were harvested on day 5, re-stimulated and then assayed as described above.

Real-time reverse transcription–PCR

$CD11b^+$ (1×10^5 cells) were co-cultured with 1×10^5 of OT-II $CD4^+$ T cells for 12 h with 200 ng ml^{-1} OVA peptide in the presence or absence of 15 mM L-lactic acid. Total RNA purification was performed as previously described (21). cDNA was synthesized at 37°C for 15 min using the PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). Real-time PCR was performed using the TaqMan gene expression master mix, the TaqMan gene expression assay system (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems 7500 real-time PCR system. The following TaqMan probes and primer sets were used: *Il1b*, Mm00434228_m1; *Il6*, Mm99999064_m1; *Il12b*, Mm99999067_m1; *Il17a*, Mm00439619_m1; *Il21*, Mm00517640_ml; *Il23a*, Mm00518984_m1; *Cd40*, Mm00441895_m1 and 18S ribosomal RNA (rRNA), 4352930E. The relative expression of each cytokine gene was normalized to that of the 18S rRNA and measured using the $\Delta\Delta Ct$ method (31).

Statistical analyses

For the measurement of cytokines using real-time PCR, the experiments were performed in triplicate and the data presented as the mean values ± standard deviation. Statistical significance was measured using the Student's *t*-test. Representative data from at least two independent experiments are shown in each figure.

Results

Lactic acid induces an increase in IL-17A production in a co-culture of $CD11b^+$ and $CD4^+$ T cells

We previously demonstrated in splenocytes of OT-II mice that lactic acid induces the OVA peptide-dependent activation of the IL-23/IL-17 pathway, even in the absence of the TLR ligand (21). To elucidate the roles of lactic acid in the enhanced activation of the IL-23/IL-17 pathway in this system, $CD11b^+$ cells were fractionated from C57BL/6 splenocytes using anti-CD11b antibody-conjugated magnetic beads as APCs and then were co-cultured with $CD4^+$ T cells purified from OT-II mice splenocytes in the presence of OVA peptide and lactic acid (Fig. 1A). OT-II mouse $CD4^+$ T cells produced high levels of IL-17A when stimulated with lactic acid in a co-culture with $CD11b^+$ cells. Lactic acid also enhanced transcription of IL-23p19, IL-17A and IL-21 in this co-culture system (Fig. 1B). However, IL-23 production was below the detection limit of a commercially available ELISA kit (<7.8 pg ml^{-1}, data not shown). The transcripts of the inflammatory cytokines IL-1β and tumor necrosis factor (TNF)-α were slightly, but not significantly, increased by lactic acid (Fig. 1C, left and middle). In addition, we did not observe up-regulation of IL-6 transcripts, which were reported to be induced by co-stimulation with the TLR ligand and lactic acid (21) (Fig. 1C, right). We previously demonstrated that sodium lactate does not activate the IL-23p19 promoter in J774.1 cells (21). Because the lactate anion is co-transported with a proton into cells via MCTs (32), it is not transported into cells under a neutralized condition. To elucidate whether lactic acid also functions intracellularly in this co-culture system of $CD11b^+$ and $CD4^+$ cells, we examined the effects of sodium lactate in

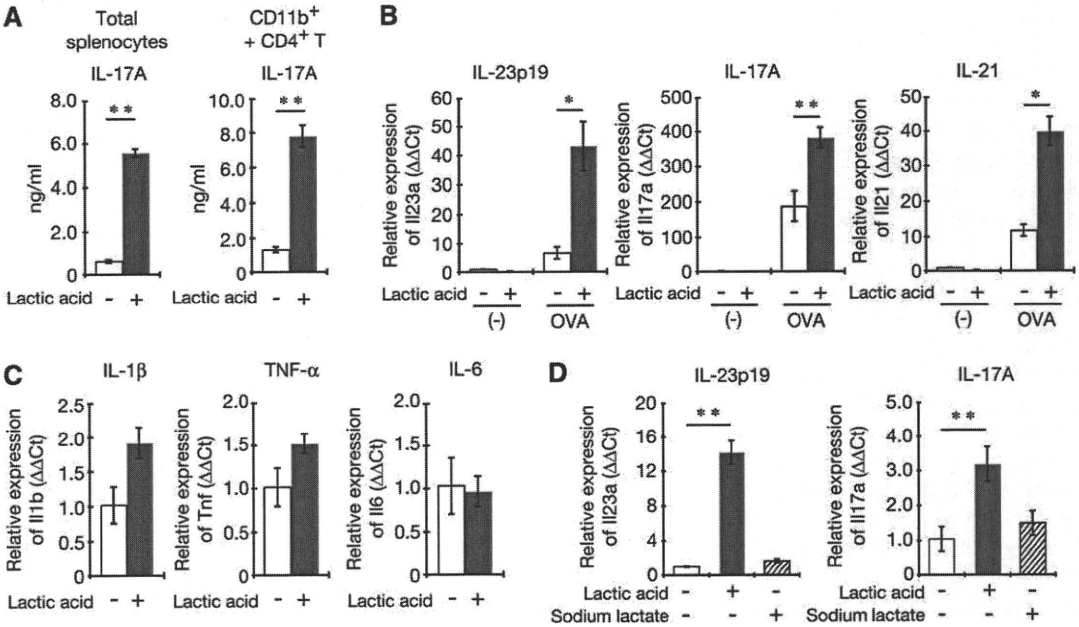

Fig. 1. The effect of lactic acid in a co-culture of fractionated CD11b$^+$ cells and CD4$^+$ T cells. (A) CD11b$^+$ cells and CD4$^+$ T cells derived from wild-type and OT-II splenocytes, respectively, were co-cultured in the presence (gray bars) or absence (white bars) of 15 mM lactic acid together with OVA peptide for 4 days (right panel). IL-17A production was measured by ELISA. At the same time, total splenocytes derived from the OT-II mouse were also stimulated with lactic acid and OVA peptide (left panel). (B) Splenic CD11b$^+$ cells and OT-II CD4$^+$ T cells were stimulated with or without OVA peptide, in the presence (gray bars) or absence (white bars) of lactic acid, for 12 h. The relative expressions of IL-23p19, IL-17A and IL-21 transcripts were measured using real-time PCR and normalized to 18S rRNA. (C) The effect of lactic acid on the expression of IL-6, IL-1β and TNF-α transcripts. The relative expressions of IL-6, IL-1β and TNF-α were measured as described above. (D) Splenic CD11b$^+$ cells and OT-II CD4$^+$ T cells were stimulated with OVA peptide in the presence of 15 mM lactic acid (gray bars) or sodium lactate (hatched bars) for 12 h. The relative expressions of IL-23p19 (left panel) and IL-17A transcripts (right panel) are shown. The data represent mean values ± standard deviation (n = 3); *P < 0.05 and **P < 0.01.

this system. The expression of IL-23p19 and IL-17A transcripts was not enhanced by the addition of sodium lactate (Fig. 1D). Therefore, this finding suggested that lactic acid also functions intracellularly in the co-culture system.

Lactic acid increases the proportion of IL-17A-producing cells but inhibits the proliferation of CD4$^+$ T cells

To clarify whether lactic acid induces the proliferation of IL-17A-producing cells in the co-culture system, we examined whether lactic acid increases the proportion of IL-17A-producing cells in this system. We co-cultured OT-II CD4$^+$ T cells with CD11b$^+$ cells in the presence of lactic acid and OVA peptide for 4.5 days and analyzed the intracellular expression of IL-17A and IFN-γ. Lactic acid increased both the proportion of IL-17A-producing cells and the mean fluorescence intensity (MFI) of IL-17A (0.19 to 0.56%, and 687 to 1501, respectively) (Fig. 2A). In contrast, the MFI of cells expressing IFN-γ hardly changed after stimulation with lactic acid (107 to 141) but the proportion decreased (6.58 to 3.82%). Because the proportion of IL-17A-producing cells increased, we next examined whether lactic acid stimulates the proliferation of IL-17A-producing cells. CD4$^+$ T cells, labeled with CFSE, were co-cultured with CD11b$^+$ cells for 4 days (Fig. 2B). When stimulated with lactic acid, both IFN-γ-producing cells (upper plots, MFI: 154 to 910) and IL-17A-producing cells (lower plots, MFI: 197 to 799) showed high CFSE fluorescence intensities as compared with cells treated with OVA alone, indicating that their proliferation had been dampened. These results suggested that lactic acid suppresses the proliferation of IL-17A-producing cells but maintains the phenotype of IL-17A-producing cells and induces IL-17A production via the activation of CD11b$^+$ cells.

Lactic acid stimulates macrophages to increase IL-17A production

To examine which type of APCs was involved in increased IL-17A production by lactic acid in this system, splenocytes of C57BL/6 mice were further fractionated using anti-CD11c antibody-conjugated magnetic beads and then were co-cultured with CD4$^+$ T cells derived from OT-II mice splenocytes in the presence of OVA peptide and lactic acid (Fig. 3A, upper panel). IL-17A production was also high in CD11b$^+$CD11c$^-$ cells stimulated with lactic acid but not in CD11c$^+$CD11b$^-$ cells. We also observed enhanced IL-17A expression in the presence of lactic acid in F4/80$^+$, but not F4/80$^-$, cells isolated from splenocytes (Fig. 3A, lower panel). These results indicate that monocytes or macrophages function as APCs in the increased production of IL-17A by lactic acid. Furthermore, in a co-culture of BMDMs that were induced by M-CSF and OT-II mouse CD4$^+$ T cells, lactic acid also intensified the expression of IL-23p19 transcripts (Fig. 3B, left panel) and IL-17A production (Fig. 3B, right panel). BMDCs strongly induced IL-17A secretion, but it was only slightly enhanced by lactic acid (data not shown). Therefore, lactic acid mainly influences the activation of monocytes/macrophages rather than DCs.

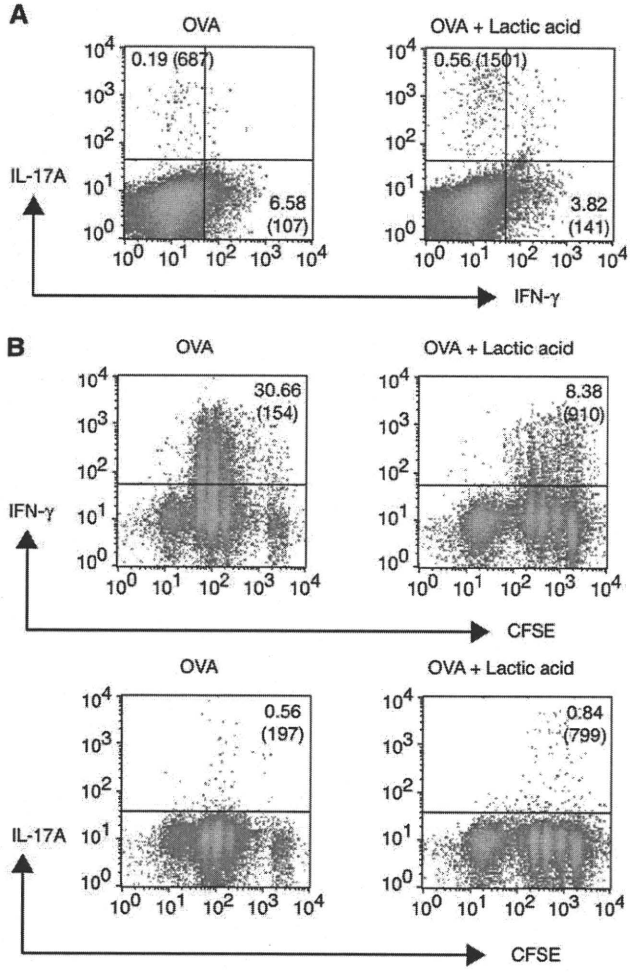

Fig. 2. Effect of lactic acid on the proportion and proliferation of IL-17A- and IFN-γ-producing cells. (A) $CD4^+$ T cells were co-cultured with $CD11b^+$ cells and stimulated with OVA peptide in the presence (right plot) or absence (left plot) of lactic acid for 4.5 days. T cells were then re-stimulated with PMA and ionomycin in the presence of brefeldin A for 5 h and then stained for CD4, IFN-γ and IL-17A. Plots gated on $CD4^+$ cells are shown. Numbers in plots indicate percentages (MFI) of $IL\text{-}17A^+$ or $IFN\text{-}\gamma^+$ cell populations. (B) Effect of lactic acid on the proliferation of IFN-γ- or IL-17A-producing cells. CFSE-labeled $CD4^+$ T cells were co-cultured with $CD11b^+$ cells and stimulated as described above. The cells were stained with CD4 and IFN-γ (upper plots) or IL-17A (lower plots) fluorescent antibodies. Numbers in plots indicate percentages ($CFSE^+$ MFI) of $CFSE^+$ cells in $IFN\text{-}\gamma^+$ or $IL\text{-}17A^+$ populations.

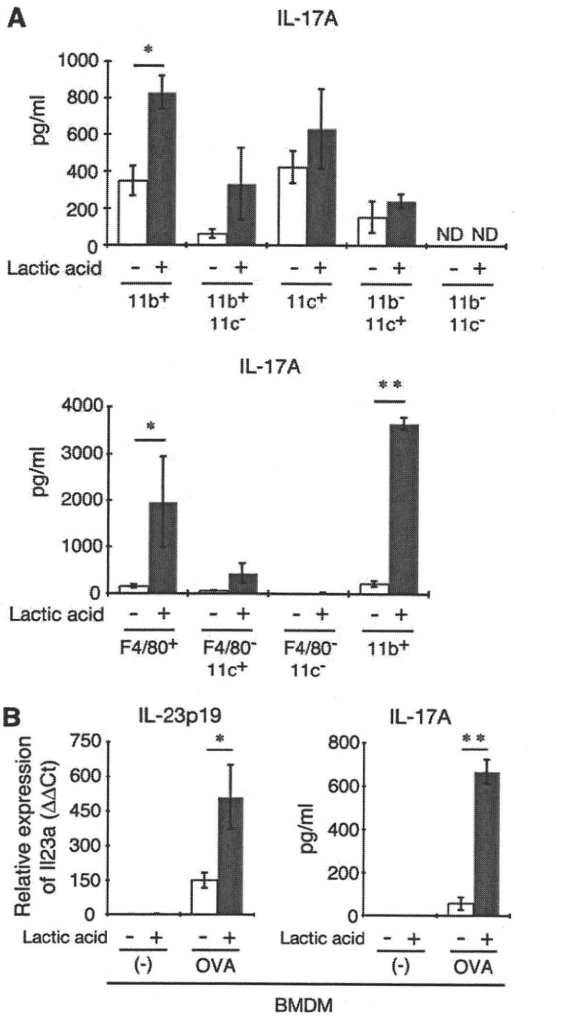

Fig. 3. Monocytes/macrophages are involved in lactic acid-enhanced, antigen-dependent, IL-17A production from $CD4^+$ T cells. (A) Splenic APCs were fractionated by using the cell surface markers CD11b ($11b^+$), CD11c ($11c^+$) and F4/80 ($F4/80^+$), as indicated on the *x*-axis. Each fraction was co-cultured with OT-II $CD4^+$ T cells at a 1:1 ratio and stimulated with OVA peptide in the presence (gray bars) or absence (white bars) of lactic acid for 4 days. $CD11b^+$ and $F4/80^+$ cells positively enhanced IL-17A production more strongly than $CD11c^+$ cells; ND, not detected. (B) BMDMs and OT-II $CD4^+$ T cells were co-cultured at a 1:5 ratio and stimulated with or without OVA peptide in the presence (gray bars) or absence (white bars) of lactic acid for 12 h for the relative expression of IL-23p19 transcripts or 4 days for IL-17A production. Macrophages also induced the enhanced expression of IL-23p19 and IL-17A by lactic acid. The data represent mean values ± standard deviation ($n = 3$); $^*P < 0.05$ and $^{**}P < 0.01$.

Lactic acid induces the production of IL-17A from effector/memory T cells and T_h17 cells but not naive T cells or T_h17 cell differentiation

To determine which type of $CD4^+$ T cell is activated with antigen and lactic acid, and produces IL-17A, we fractionated OT-II $CD4^+$ T cells into $CD4^+CD62L^+$ cells containing naive T cells and $CD4^+CD62L^-$ cells containing mainly effector/memory T cells. When co-cultured with $CD11b^+$ splenocytes in the presence of OVA peptide and lactic acid for 4 days, naive T cells did not produce IL-17A (Fig. 4A). However, in co-cultures with $CD11b^+$ cells stimulated with lactic acid and OVA peptide, $CD62L^-$ effector/memory $CD4^+$ T cells displayed elevated production of IL-17A. In contrast, the production of IFN-γ was nearly unchanged by lactic acid stimulation (data not shown).

In the presence of IL-6 and TGF-β, naive T cells differentiate into T_h17 cells by co-stimulation with anti-CD3ε and anti-CD28 antibodies (10–12). Naive $CD4^+$ T cells were treated with IL-6 and TGF-β in co-cultures with $CD11b^+$ cells stimulated with lactic acid and OVA peptide. IL-6 and TGF-β induced IL-17A production (Fig. 4B) and differentiation into T_h17 cells (Fig. 4C, left) in the absence of lactic acid. However, lactic acid severely inhibited the effects of IL-6 and TGF-β stimulation (Fig. 4B and C, right). Lactic acid also