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INAM-expressing stable BaF3 cell lines (INAM/BaF3) did not reveal a function as an NK cell–activating ligand. NK cell cytotoxicity is directed against Rae-1α/BaF3 cells but not against INAM/BaF3 cells (Fig. 5). Therefore, INAM does not represent a typical NK cell–activating ligand. For NK activation, INAM on BMDC appears to require other molecules that are expressed in BMDC but not in BaF3.

INAM has four transmembrane regions, similar to the cell adhesion tetraspanins, which may support cell–cell contact (Levy and Shoham, 2005). Tetraspanins provide a scaffold that facilitates complex formation with associated proteins. INAM on BMDC and NK cells may use cell–cell interaction to assemble in a synaptic formation to activate NK cells. Because the protein constituents of the tetraspanin complexes are cell specific, we are interested in finding partners for INAM that might participate in efficient BMDC–NK interaction. TLR-inducible cell–cell contact may occur through INAM in an immune cell–specific manner. Gene disruption of this INAM will facilitate clarifying this issue. The identification of INAM defines a novel pathway in mDC–NK reciprocal interaction. This study will lead to further research on the molecules that form complexes on BMDC and NK cells to facilitate BMDC–NK interaction.

MATERIALS AND METHODS

Mice. All mice were backcrossed with C57BL/6 mice more then seven times before use. TICAM-1$^{-/-}$ (Akazawa et al., 2007a) and IPS-1$^{-/-}$ mice were generated in our laboratory. IRF-3$^{-/-}$ (Sato et al., 2000) and IRF-7$^{-/-}$ mice (Honda et al., 2005) were provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). All mice were maintained under specific pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine. Animal experiments protocols and guidelines were approved by the Animal Safety Center, Hokkaido University, Japan.

Cells. The B16D8 cell line was established in our laboratory as a subline of B16 melanoma (Tanaka et al., 1988). This subline was characterized by its low or virtually no metastatic properties when injected s.c. into syngeneic C57BL/6 mice. B16D8 was cultured in RPMI 1640/10% FCS. The mouse B cell line BaF3 was obtained from American Type Culture Collection and cultured in RPMI 1640/10% FCS/2 μM 2ME/5 ng/ml IL-3. Mouse NK cells (DX5^{+} cell) were positively isolated with MACS Beads (Miltenyi Biotec). Mouse BMDCs were prepared as previously reported (Akazawa et al., 2007a).

For purification of cells from spleen or LN, these tissues were treated with 400 IU MandleU/ml collagenase D (Roche) at 37°C for 25 min in HBSS (Sigma-Aldrich). Then EDTA was added, and the cell suspension was incubated for an additional 5 min at 37°C. After removal of RBC with ACK lysis buffer, splenocytes and LN cells were stained with CD45-FITC, CD3ε-PE, CD19-PE, DX5-PE, CD11b-FITC (eBioscience), and CD11c-FITC (BioLegend) and sorted by a FACSAria II (BD). The purity of sorted cells were >96%.

Construction and expression. Mouse INAM cDNA (A630077B13Rik) was obtained from RIKEN and placed into expression vector pEFBOS and pLenti-IRES-hrGFP, both of which provide the specialized components needed for expression of a recombinant C-terminal FLAG fusion (Akazawa et al., 2007a). For construction of shRNA-expressing lentivirus vector, The ClaI–XhoI fragment of pLenti6-blockit-dest (Invitrogen) was inserted into pLenti-IRES-hrGFP at the site of ClaI and XhoI. This vector was named pLenti-dest-IRES-hrGFP (pLDIG). INAM sequence 5′-CTTCTCTCCG-GTTAGTTATCT-3′ was targeted for INAM knockdown (shINAM/pLDIG) and 5′-AGTCTGACATACTTATACTTA-3′ was used for negative control (shCont/pLDIG). We used a gene-expression kit, Lentiviral system (Invitrogen), as previously described (Akazawa et al., 2007a). Four plasmids (one of the pLenti vectors, pLP1, pLP2, and pLP/VSVG) were transfected into 293 FT packaging cells, and the viral particles for transfection were prepared according to the manufacturer's protocol. The 100× concentrated virus particles were produced after centrifugation of 8,000 *g* at 4°C for 16 h. Lentivirus produced by pLenti-IRES-hrGFP and pLDIBG could be tittered by GFP expression using flow cytometry. Because the lentivirus vector pLenti-IRES-hrGFP has the IRES-GFP region, we prepared negative control virus by pLenti-IRES-hrGFP without construct. Infection efficiency for BMDC was high with the control vector compared with the INAM-expressing lentivector (Fig. S6 A).

Real-time PCR. BMDCs were harvested after 4 h of stimulation by 100 ng/ml LPS, 50 μg/ml polyI:C, 1 μg/ml Pam_3CSK_4 (Pam3), 100 nM mycoplasmal macrophage-activating lipopeptide-2 (Malp-2), 10 μg/ml CpG, and 2,000 IU/ml IFN-α (Ebihara et al., 2007). Mouse tissues (heart, stomach, small intestine, large intestine, lung, brain, muscle, liver, kidney, thymus, and spleen) were collected from C57BL/6. Splenocytes were stained with CD3-PE, CD19-PE, DX5-PE, CD11b-PE, CD11c-FITC, and PDCA1-PE (eBioscience) and sorted by FACSAria (BD). Purity was >98% in each population. For RNA extraction, we used the RNeasy kit (Invitrogen). After removal of genomic DNA by treatment with DNase, randomly primed cDNA strands were generated with Moloney mouse leukemia virus reverse transcription (Promega). RNA expression was quantified by quantitative RT-PCR with gene-specific primers (IL-15 forward, 5′-TTAACTGAGGCTGGCATTCATG-3′; IL-15 reverse, 5′-ACCTACACTGACACAGCCCAAA-3′; INAM forward, 5′-CAACTGCAATGCCACGCTA-3′; INAM reverse, 5′-TCCAACC-GAACACCTGAGACT-3′; β-actin forward, 5′-TTTGCAGCTCCTTC-GTTGC-3′; β-actin reverse, 5′-TCGTCATCCATGGCGAACT-3′; HPRT forward, 5′-GTTGGATACAGGCCAGACTTTGTTG-3′; and HPRT reverse, 5′-GAAGGGTAGGCTGGCCTATAGGCT-3′) and values were normalized to the expression of β-actin mRNA or HPRT mRNA.

Other primers for PCR were designed using Primer Express software (Applied Biosystems) for another experiment. The following primers were used for PCR: β-actin forward, 5′-CCTGGCACCCAGCACAAT-3′ and reverse, 5′-GCCGATCCACACGGAGTACT-3′; granzyme B forward, 5′-TCCTGCTACTGCTGACCTTGTC-3′ and reverse, 5′-ATGATCTC-CCCTGCCTTTGTC-3′; IFN-α4 forward, 5′-CTGCTGGCTGTGAG-GACATACT-3′ and reverse, 5′-AGGCACAGAGGCTGTGTTTCTT-3′; TRAIL (Tnfsf10) forward, 5′-CTTCACCAACGAGATGAAGCAG-3′ and reverse, 5′-TCCGTCTTTGAGAAGCAAGCTA-3′; and IL-12p40 (Il12b), forward, 5′-AATGTCTGCGTGCAAGCTCA-3′ and reverse, 5′-ATGCCCACTTGCTGCATGA-3′.

Anti-INAM pAb. C-terminal INAM (cINAM; 191–314 aa) was subcloned between the NdeI and SalI sites of pColdI vector (Takara Bio Inc.). 6× His-tagged cINAM protein was expressed in BL21 by manufacturer's methods. The cells were sonicated in 20 mM Tris-HCl, 150 mM NaCL, 1 mM PMSF, and 7 M Urea, pH 7.4, on ice. Expression products of cINAM were purified using the HisTrap HP kit (GE Healthcare). The extracted proteins were refolded by step-wise dialysis against decreasing amounts of urea. Rabbit anti-cINAM polyclonal Ab was produced with the cINAM proteins by standard protocol. IgG was purified by precipitation with 33% ammonium sulfate, dialyzed against PBS.

Surface labeling with biotin. Biotinylation of cell surface proteins was performed according to the reported method (Tsuji et al., 2001). In brief, ~10^8 cells were suspended in 1 ml Hepes-buffered saline (HBS), pH 8.5, and incubated with 10 ml of 10 mg/ml NHS-sulfobiotin (Vector Laboratories) for 1 h at room temperature. Cells were washed in HBS three times and then solubilized with lysis buffer containing 1% NP-40, pH 7.4. The cell lysate was immunoprecipitated with avidin-labeled Abs as described previously (Tsuji et al., 2001).

Immunoblot analysis. Lysates were harvested 24 h after transfection of Flag-tagged INAM/pEFBOS into 293FT cells and treated with *N*-glycosidase F

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(PNGaseF; New England Biolabs, Inc.) by the manufacturer's method in some experiments. Protein samples were separated on SDS-PAGE and immunoblotted by anti-Flag M2 Ab (Sigma-Aldrich). In some experiments, we used highly purified rabbit anti-mouse INAM polyclonal Ab for immunoblotting. The anti-INAM IgG was further purified with protein A–Sepharose and absorbed with BL21 bacterial lysate (where the INAM immunogen was produced) that contained no INAM peptide.

Confocal microscopy. BMDCs and NK cells were infected with control or INAM-expressing lentivirus as described previously (Akazawa et al., 2007a). 24 h later, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with PBS containing 0.5% saponin for 30 min at room temperature. Fixed cells were stained with anti-FLAG mAb and Alexa Fluor 568–conjugated secondary Ab. Stable Ba/F3 transfectants expressing INAM were treated with Cytofix/Cytoperm (BD) according to the manufacturer. Then cells were stained with PE-phalloidin and rabbit anti-INAM pAb followed by Alexa Fluor 488–conjugated secondary Ab. Cells were analyzed on a confocal microscope (LSM 510 META; Carl Zeiss, Inc.) for the detection of INAM.

BMDC–NK interaction. BMDCs were co-cultured with freshly isolated NK cells (BMDC/NK = ~1:2–1:5) with or without 10 μg/ml polyI:C for 24 h (Akazawa et al., 2007a). In some experiments, function of BMDCs and NK cells was modified by lentivirus vector before BMDC/NK co-culture. IRF-3$^{-/-}$ BMDCs were transfected by control lentivirus and INAM-expressing lentivirus (INAM/pLenti-IRES-hrGFP) and incubated with 6 μg/ml polybrene for 24 h before co-culture. WT BMDCs were transfected with shRNA-expressing lentivirus (shCont/pLDIG or shINAM/pLDIG) and incubated with 6 μg/ml polybrene for 48 h before co-culture. Freshly isolated NK cells were transfected with control lentivirus and INAM-expressing lentivirus (INAM/pLenti-IRES-hrGFP) and cultured with 6 μg/ml polybrene in the presence of 500 IU/ml IL-2 for 72 h before co-culture. Activation of NK cells was assessed by concentration of IFN-γ (ELISA; GE Healthcare) in the medium and by NK cytotoxicity against B16D8. Cytotoxicity was determined by standard ^{51}Cr release assay as described previously (Akazawa et al., 2007a).

Ex vivo NK activation. Mice were i.p. injected with 250 μg polyI:C. After 24 h, spleen cells were harvested and then NK cells (DX5$^+$ cells) were positively isolated with the MACS system (Miltenyi Biotec). The DX5$^+$ NK cells were suspended in RPMI1640 with 10% FCS and mixed with ^{51}Cr-labeled B16D8 cells at indicated E/T ratios. After 4 h, supernatants were harvested and ^{51}Cr release was measured. Specific lysis was calculated by (specific release − spontaneous release)/(max release − spontaneous release). In some experiments, blood was drawn from the eyes of mice 8 h after polyI:C administration for cytokine measurement.

Test for in vivo NK activation in LN. 5 × 10^5 WT BMDCs incubated with or without 10 μg/ml polyI:C for 24 h or 5 × 10^5 IRF-3$^{-/-}$ BMDCs infected with control virus or INAM-expressing lentivirus and allowed to stand for 24 h were injected into the footpads of WT C57BL/6 mice. 48 h later, cells in their inguinal LN were harvested, stained with PE-DX5, and sorted by FACSAria II. RNA was extracted from the DX5-positive cells with TRIzol.

DC therapy. DC therapy against mice with B16D8 tumor burden was described previously (Akazawa et al., 2007a). C57BL/6 mice (n = 3) were shaved at the flank and injected s.c. with 6 × 10^5 syngeneic B16D8 melanoma cells (indicated as day 0). For DC therapy, BMDCs were prepared by transfecting control lentivirus or INAM-expressing lentivirus (INAM/pLenti-IRES-hrGFP) and cultured for 24 h. At the time point indicated in the figures, 10^6 BMDCs were injected s.c. near the tumor. To deplete NK cells in vivo, mice were i.p. injected with hybridoma ascites of anti-NK1.1 mAb (PK136; Akazawa et al., 2007a). Tumor volumes were measured using a caliper every 1 or 2 d. Tumor volume was calculated using the formula: tumor volume (cm^3) = (long diameter) × (short diameter) × (short diameter) × 0.4.

Statistical analysis. Statistical analyses were made with the Student's t test. The p-value of significant differences is reported.

Online supplemental material. TICAM-1–inducible genes encoding putative membrane proteins relevant for this study are summarized in Table S1. Fig. S1 shows KO mice results suggesting that both IPS-1 and TICAM-1 in BMDC participate in polyI:C-driven NK activation. Data presented in Fig. S2 characterizes the in vivo polyI:C response of INAM in LN cells. Figs. S3 and S4 demonstrate the properties of surface-expressed INAM analyzed by immunoprecipitation/blotting and confocal microscopy, respectively. Fig. S5 mentions the cytokine expression and maturation profiles of INAM-overexpressing BMDC. Fig. S6 shows the effect of gene silencing of INAM on the polyI:C-mediated cytokine-inducing profile in BMDC. Two pieces of data presented in Fig. S7 confirm the presence of the INAM protein in INAM lentivirus-transduced BMDCs and NK cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091573/DC1.

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REFERENCES

Akazawa, T., T. Ebihara, M. Okuno, Y. Okuda, M. Shingai, K. Tsujimura, T. Takahashi, M. Ikawa, M. Okabe, N. Inoue, et al. 2007a. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proc. Natl. Acad. Sci. USA.* 104:252–257. doi:10.1073/pnas.0605978104

Akazawa, T., M. Shingai, M. Sasai, T. Ebihara, N. Inoue, M. Matsumoto, and T. Seya. 2007b. Tumor immunotherapy using bone marrow-derived dendritic cells overexpressing Toll-like receptor adaptors. *FEBS Lett.* 581:3334–3340. doi:10.1016/j.febslet.2007.06.019

Azuma, M., R. Sawahata, Y. Akao, T. Ebihara, S. Yamazaki, M. Matsumoto, M. Hashimoto, K. Fukase, Y. Fujimoto, and T. Seya. 2010. The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation. *PLoS One.* 5:e12550. doi:10.1371/journal.pone.0012550

Bertram, L., B.M. Schjeide, B. Hooli, K. Mullin, M. Hiltunen, H. Soininen, M. Ingelsson, L. Lannfelt, D. Blacker, and R.E. Tanzi. 2008. No association between CALHM1 and Alzheimer's disease risk. *Cell.* 135:993–994, author reply :994–996. doi:10.1016/j.cell.2008.11.030

Brandt, C.S., M. Baratin, E.C. Yi, J. Kennedy, Z. Gao, B. Fox, B. Haldeman, C.D. Ostrander, T. Kaifu, C. Chabannon, et al. 2009. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J. Exp. Med.* 206:1495–1503. doi:10.1084/jem.20090681

Brilot, F., T. Strowig, S.M. Roberts, F. Arrey, and C. Münz. 2007. NK cell survival mediated through the regulatory synapse with human DCs requires IL-15Ralpha. *J. Clin. Invest.* 117:3316–3329. doi:10.1172/JCI31751

Cerwenka, A., and L.L. Lanier. 2001. Natural killer cells, viruses and cancer. *Nat. Rev. Immunol.* 1:41–49. doi:10.1038/35095564

Cerwenka, A., A.B. Bakker, T. McClanahan, J. Wagner, J. Wu, J.H. Phillips, and L.L. Lanier. 2000. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity.* 12:721–727. doi:10.1016/S1074-7613(00)80222-8

Cerwenka, A., J.L. Baron, and L.L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc. Natl. Acad. Sci. USA.* 98:11521–11526. doi:10.1073/pnas.201238598

Dreses-Werringloer, U., J.C. Lambert, V. Vingtdeux, H. Zhao, H. Vais, A. Siebert, A. Jain, J. Koppel, A. Rovelet-Lecrux, D. Hannequin, et al. 2008. A polymorphism in CALHM1 influences Ca2+ homeostasis,

Published November 8, 2010

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Abeta levels, and Alzheimer's disease risk. *Cell.* 133:1149–1161. doi:10.1016/j.cell.2008.05.048

Ebihara, T., H. Masuda, T. Akazawa, M. Shingai, H. Kikuta, T. Ariga, M. Matsumoto, and T. Seya. 2007. Induction of NKG2D ligands on human dendritic cells by TLR ligand stimulation and RNA virus infection. *Int. Immunol.* 19:1145–1155. doi:10.1093/intimm/dxm073

Fernandez, N.C., A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, T. Tursz, E. Maraskovsky, and L. Zitvogel. 1999. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat. Med.* 5:405–411. doi:10.1038/7403

Fitzgerald, K.A., S.M. McWhirter, K.L. Faia, D.C. Rowe, E. Latz, D.T. Golenbock, A.J. Coyle, S.M. Liao, and T. Maniatis. 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* 4:491–496. doi:10.1038/ni921

Gerosa, F., B. Baldani-Guerra, C. Nisii, V. Marchesini, G. Carra, and G. Trinchieri. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. *J. Exp. Med.* 195:327–333. doi:10.1084/jem.20010938

Hamerman, J.A., K. Ogasawara, and L.L. Lanier. 2004. Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. *J. Immunol.* 172:2001–2005.

Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature.* 434:772–777. doi:10.1038/nature03464

Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdörfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168:4531–4537.

Huntington, N.D., N. Legrand, N.L. Alves, B. Jaron, K. Weijer, A. Plet, E. Corcuff, E. Mortier, Y. Jacques, H. Spits, and J.P. Di Santo. 2009. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *J. Exp. Med.* 206:25–34. doi:10.1084/jem.20082013

Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5:987–995. doi:10.1038/ni1112

Kalinski, P., R.B. Mailliard, A. Giermasz, H.J. Zeh, P. Basse, D.L. Bartlett, J.M. Kirkwood, M.T. Lotze, and R.B. Herberman. 2005. Natural killer-dendritic cell cross-talk in cancer immunotherapy. *Expert Opin. Biol. Ther.* 5:1303–1315. doi:10.1517/14712598.5.10.1303

Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K.J. Ishii, et al. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature.* 441:101–105. doi:10.1038/nature04734

Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K.J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* 6:981–988. doi:10.1038/ni1243

Kubin, M.Z., D.L. Parshley, W. Din, J.Y. Waugh, T. Davis-Smith, C.A. Smith, B.M. Macduff, R.J. Armitage, W. Chin, L. Cassiano, et al. 1999. Molecular cloning and biological characterization of NK cell activation-inducing ligand, a counterstructure for CD48. *Eur. J. Immunol.* 29:3466–3477. doi:10.1002/(SICI)1521-4141(199911)29:11<3466::AID-IMMU3466>3.0.CO;2-9

Lee, A.E., L.A. Rogers, J.M. Longcroft, and R.E. Jeffery. 1990. Reduction of metastasis in a murine mammary tumour model by heparin and polyinosinic-polycytidylic acid. *Clin. Exp. Metastasis.* 8:165–171. doi:10.1007/BF00117789

Levy, S., and T. Shoham. 2005. The tetraspanin web modulates immune-signalling complexes. *Nat. Rev. Immunol.* 5:136–148. doi:10.1038/nri1548

Lucas, M., W. Schachterle, K. Oberle, P. Aichele, and A. Diefenbach. 2007. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity.* 26:503–517. doi:10.1016/j.immuni.2007.03.006

Masuda, H., Y. Saeki, M. Nomura, K. Shida, M. Matsumoto, M. Ui, L.L. Lanier, and T. Seya. 2002. High levels of RAE-1 isoforms on mouse tumor cell lines assessed by anti-"pan" RAE-1 antibody confer tumor susceptibility to NK cells. *Biochem. Biophys. Res. Commun.* 290:140–145. doi:10.1006/bbrc.2001.6165

Matsumoto, M., and T. Seya. 2008. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv. Drug Deliv. Rev.* 60:805–812. doi:10.1016/j.addr.2007.11.005

McCartney, S., W. Vermi, S. Gilfillan, M. Cella, T.L. Murphy, R.D. Schreiber, K.M. Murphy, and M. Colonna. 2009. Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. *J. Exp. Med.* 206:2967–2976. doi:10.1084/jem.20091181

Medzhitov, R., and C.A. Janeway Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell.* 91:295–298. doi:10.1016/S0092-8674(00)80412-2

Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature.* 437:1167–1172. doi:10.1038/nature04193

Miyake, T., Y. Kumagai, H. Kato, Z. Guo, K. Matsushita, T. Satoh, T. Kawagoe, H. Kumar, M.H. Jang, T. Kawai, et al. 2009. Poly I:C-induced activation of NK cells by CD8 alpha+ dendritic cells via the IPS-1 and TRIF-dependent pathways. *J. Immunol.* 183:2522–2528. doi:10.4049/jimmunol.0901500

Mukai, M., F. Imamura, M. Ayaki, K. Shinkai, T. Iwasaki, K. Murakami-Murofushi, H. Murofushi, S. Kobayashi, T. Yamamoto, H. Nakamura, and H. Akedo. 1999. Inhibition of tumor invasion and metastasis by a novel lysophosphatidic acid (cyclic LPA). *Int. J. Cancer.* 81:918–922. doi:10.1002/(SICI)1097-0215(19990611)81:6<918::AID-IJC13>3.0.CO;2-E

Newman, K.C., and E.M. Riley. 2007. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat. Rev. Immunol.* 7:279–291. doi:10.1038/nri2057

Nomura, M., Z. Zou, T. Joh, Y. Takihara, Y. Matsuda, and K. Shimada. 1996. Genomic structures and characterization of Rae1 family members encoding GPI-anchored cell surface proteins and expressed predominantly in embryonic mouse brain. *J. Biochem.* 120:987–995.

Ohteki, T., H. Tada, K. Ishida, T. Sato, C. Maki, T. Yamada, J. Hamuro, and S. Koyasu. 2006. Essential roles of DC-derived IL-15 as a mediator of inflammatory responses in vivo. *J. Exp. Med.* 203:2329–2338. doi:10.1084/jem.20061297

Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003a. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat. Immunol.* 4:161–167. doi:10.1038/ni886

Oshiumi, H., M. Sasai, K. Shida, T. Fujita, M. Matsumoto, and T. Seya. 2003b. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. *J. Biol. Chem.* 278:49751–49762. doi:10.1074/jbc.M305820200

Sasai, M., M. Shingai, K. Funami, M. Yoneyama, T. Fujita, M. Matsumoto, and T. Seya. 2006. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. *J. Immunol.* 177:8676–8683.

Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity.* 13:539–548. doi:10.1016/S1074-7613(00)00053-4

Seth, R.B., L. Sun, C.K. Ea, and Z.J. Chen. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell.* 122:669–682. doi:10.1016/j.cell.2005.08.012

Seya, T., and M. Matsumoto. 2009. The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. *Cancer Immunol. Immunother.* 58:1175–1184. doi:10.1007/s00262-008-0652-9

Sivori, S., M. Falco, M. Della Chiesa, S. Carlomagno, M. Vitale, L. Moretta, and A. Moretta. 2004. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc. Natl. Acad. Sci. USA.* 101:10116–10121. doi:10.1073/pnas.0403744101

Tanaka, H., Y. Mori, H. Ishii, and H. Akedo. 1988. Enhancement of metastatic capacity of fibroblast-tumor cell interaction in mice. *Cancer Res.* 48:1456–1459.

Tsuji, S., J. Uehori, M. Matsumoto, Y. Suzuki, A. Matsuhisa, K. Toyoshima, and T. Seya. 2001. Human intelectin is a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall. *J. Biol. Chem.* 276:23456–23463. doi:10.1074/jbc.M103162200

Vivier, E., E. Tomasello, M. Baratin, T. Walzer, and S. Ugolini. 2008. Functions of natural killer cells. *Nat. Immunol.* 9:503–510. doi:10.1038/ni1582

Xu, L.G., Y.Y. Wang, K.J. Han, L.Y. Li, Z. Zhai, and H.B. Shu. 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol. Cell.* 19:727–740. doi:10.1016/j.molcel.2005.08.014

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003a. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science.* 301:640–643. doi:10.1126/science.1087262

Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003b. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol.* 4:1144–1150. doi:10.1038/ni986

Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5:730–737. doi:10.1038/ni1087

Zanoni, I., M. Foti, P. Ricciardi-Castagnoli, and F. Granucci. 2005. TLR-dependent activation stimuli associated with Th1 responses confer NK cell stimulatory capacity to mouse dendritic cells. *J. Immunol.* 175:286–292.

Zou, Z., M. Nomura, Y. Takihara, T. Yasunaga, and K. Shimada. 1996. Isolation and characterization of retinoic acid-inducible cDNA clones in F9 cells: a novel cDNA family encodes cell surface proteins sharing partial homology with MHC class I molecules. *J. Biochem.* 119:319–328.

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SUPPLEMENTAL MATERIAL

Ebihara et al., http://www.jem.org/cgi/content/full/jem.20091573/DC1

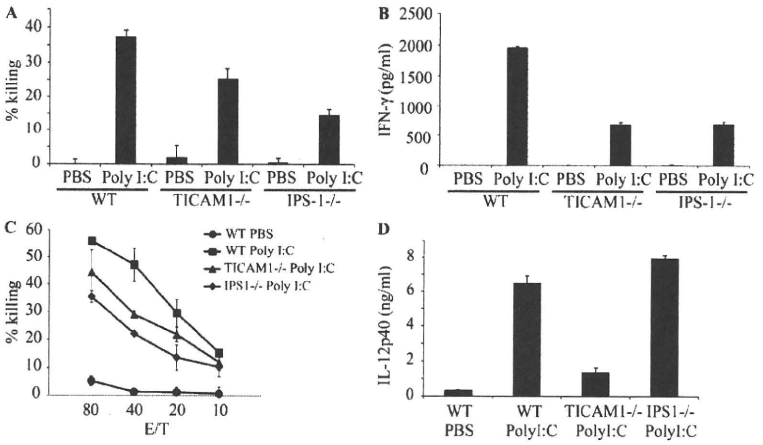

Figure S1. KO mice results suggest that both IPS-1 and TICAM-1 in BMDC participate in polyI:C-driven NK activation. (A and B) IPS-1 and TICAM-1 in BMDC participate in polyI:C-driven NK activation. 2.5 × 10^5 BMDCs prepared from WT, TICAM1$^{-/-}$, and IPS1$^{-/-}$ mice were incubated with 5 × 10^5 NK cells in the presence or absence (PBS) of 50 µg/ml polyI:C for 24 h. Then, the supernatants were harvested for IFN-γ ELISA (B). To determine NK cytotoxicity, ^{51}Cr-labeled B16D8 cells were added to the culture and, 4 h later, released ^{51}Cr was measured (A). One representative of three similar experiments is shown. (C) Both IPS-1 and TICAM-1 participate in in vivo polyI:C-induced NK activation. WT, IPS-1$^{-/-}$, and TICAM-1$^{-/-}$ mice were i.p. injected with 250 µg polyI:C. After 24 h, NK cells were harvested by DX5-MACS beads from spleen and used as effector cells in a cytotoxic assay with ^{51}Cr-labeled B16D8 targets. Cytotoxic activity of NK cells was measured under the indicated E/T ratios 4 h after the E/T mixing. One representative of the three similar experiments is shown. (D) Increasing serum level of IL-12p40 is dependent on TICAM-1. 250 µg polyI:C was i.p. injected into a series of mice as in B. 8 h after injection of polyI:C, blood serum was collected to determine the levels of IL-12p40 by ELISA. Although it is not depicted, IL-12p70 was not detected in these samples by ELISA. Data in A–D represent mean ± SD.

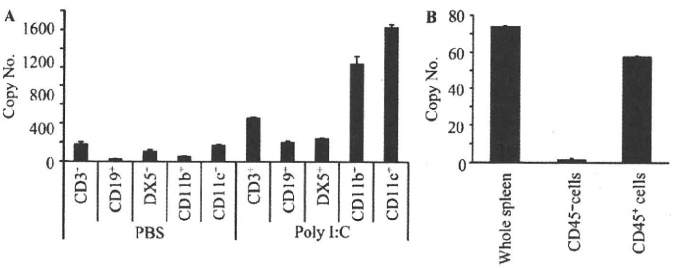

Figure S2. In vivo polyI:C response of INAM in LN cells. (A) Up-regulation of INAM expression in LN cells by polyI:C injection. WT C57BL/6 mice were i.p. injected with 100 µg polyI:C or control buffer. After 24 h, inguinal, axillary, and mesenteric LN were harvested. Cell populations with indicated markers were separated by FACS sorting, and the INAM mRNA level of each population was determined by real-time PCR. (B) CD45$^+$ cells express INAM. Splenocytes were separated into CD45$^-$ and CD45$^+$ cells after the polyI:C injection as in A. The INAM mRNA levels of the two populations were determined by real-time PCR. Representative data from one of three experiments are shown. Data in A and B represent mean ± SD.

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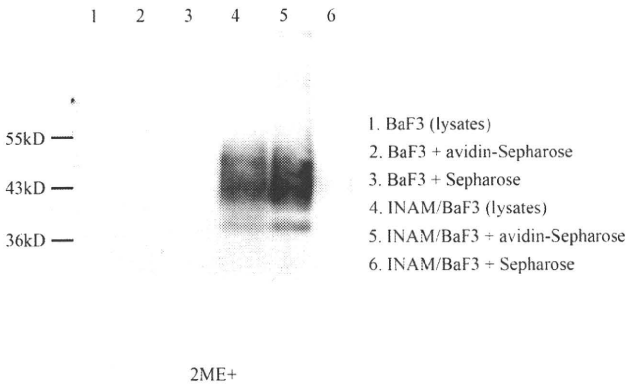

Figure S3. INAM is expressed on cell surface. Membrane proteins of Flag-tagged INAM-expressing BaF3 (INAM/BaF3) and control BaF3 were biotinylated and solubilized. Biotinylated proteins were immunoprecipitated by Avidin-Sepharose or control Sepharose. After electrophoresis on SDS-PAGE, INAM was detected by anti-Flag M2 mAb.

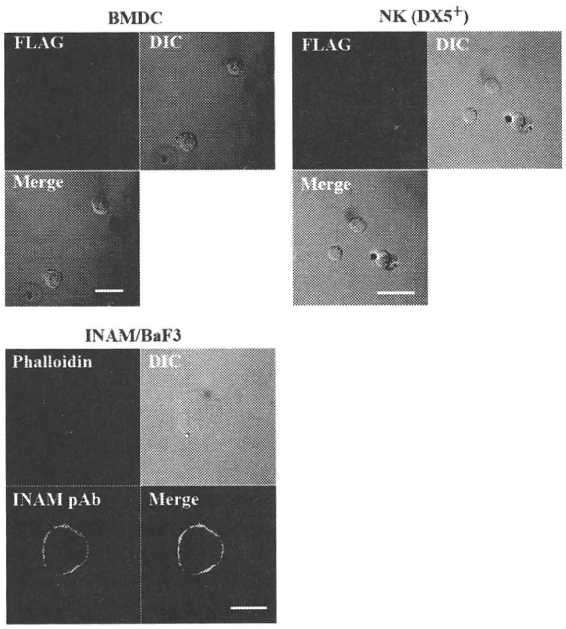

Figure S4. Confocal analysis of surface-expressed INAM. WT BMDC (left) or NK cells (right) were infected with INAM-expressing vector and stained with anti-FLAG mAb (Alexa Fluor 568). Stable Ba/F3 transfectants expressing INAM (bottom) were permeabilized and stained with phalloidin and anti-INAM pAb, followed by Alexa Fluor 488–conjugated secondary antibody. Cells were analyzed on a confocal laser-scanning microscope (LSM 510 META). Bars, 20 μm.

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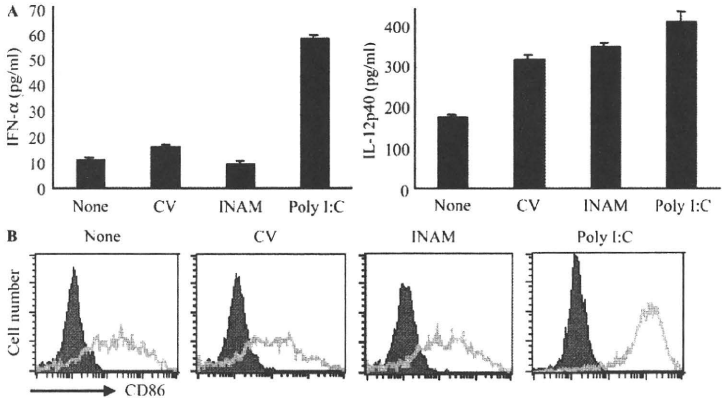

Figure S5. INAM-overexpressing BMDC did not induce cytokine responses and maturation. WT BMDCs were transfected with control lentivirus (CV) or INAM-expressing lentivirus (INAM-virus) and cultured for 24 h. (A) ELISA of IFN-α and IL-12p40 in the culture supernatants. Data shown are means ± SD of triplicate samples from one experiment representative of three. (B) Flow cytometry for CD86 in the transfected BMDC. Polyl:C stimulation (10 μg/ml) was used for positive control.

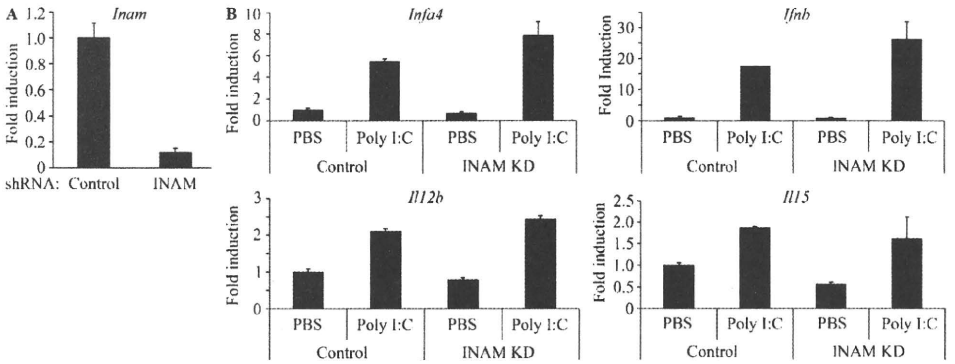

Figure S6. The effect of gene silencing of INAM on the polyI:C-mediated cytokine inducing profile in BMDC. (A) Gene silencing of INAM in BMDC. 5 × 10^5 WT BMDCs were infected with INAM shRNA-generating lentivirus or control lentivirus. After 36 h, the levels of INAM mRNA expression were assessed by real time PCR. Data show one of three similar experiments. (B) Effect of BMDC INAM on cytokine expression. INAM in 5 × 10^5 WT BMDCs was silenced as in A. Then, control or INAM-silenced BMDC were stimulated with 10 μg/ml polyI:C for 8 h. RNA was harvested from BMDC with RNeasy and the levels of indicated mRNA were determined by real-time PCR. Data show one of two similar experimental results. Data in A and B represent mean ± SD.

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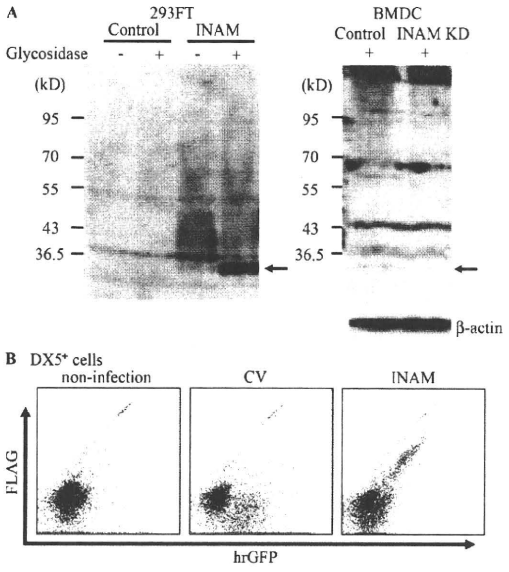

Figure S7. Detection of the INAM protein in DCs and NK cells. (A) Detection of the endogenous INAM protein in BMDC. 5 × 10^6 BMDCs were transduced with INAM-shRNA or control shRNA-expressing lentivirus. 48 h later, these cells were lysed and treated with *N*-glycosidase F for 2 h at 37°C. All cell lysates were subjected to SDS-PAGE and immunoblotted by rabbit anti-INAM pAb. The cell lysates from 293FT cells transfected with pEFBOS or pEFBOS/INAM were used as negative and positive control, respectively. Arrows indicate the band for INAM. Mr markers are shown to the left. One of three similar experiments is shown. (B) DX5+ NK cells express GFP and FLAG, markers for INAM. 5 × 10^5 DX5+ cells were transduced with control or INAM-expressing lentivirus for 48 h. Then, these cells were permeabilized and stained with rabbit anti-FLAG pAb and PE-anti rabbit IgG. Levels of FLAG and hrGFP, reflecting INAM expression, were measured by FACSCalibur. Experiments were performed more than six times with different conditions and representative data are shown.

Table S1. TICAM-1-inducible genes encoding putative membrane or GPI-anchored proteins

Official symbol	Other aliases	UniGene ID	Fold induction (poly I:C stimulation/nonstimulation)			
			WT	MyD88$^{-/-}$	TLR3$^{-/-}$	TICAM-1$^{-/-}$
Aplnr	APJ, Agtrl1, msr/apj	Mm.29368	2.074101377	0.79485698	0.24913528	0.296911294
Fam26f	INAM, A630077B13Rik	Mm.34479	15.57360865	8.048081457	0.939239821	1.221297574
Clec4e	Clecsf9, Mincle	Mm.248327	5.65851862	7.142025946	2.761541794	2.087684899
Ly6i	Ly-6M, AI789751	Mm.358339	5.679941154	26.36364231	0.734513568	1.09611157
Slamf8	Blame, SBBI42	Mm.179812	6.814581008	5.127202394	1.802731559	1.122849288
Tmem171	Gm905, MGC117733	Mm.28264	12.42279971	7.454421156	2.274145126	3.051240138
Pvrl4	1200017F15Rik, Prr4	Mm.263414	5.02297837	4.096701442	1.627391239	1.961829994
Vcam1	CD106	Mm.76649	4.742423155	4.572993249	0.948952117	0.554171652
Tnfsf10	APO-2L, TL2, Trail	Mm.1062	41.9745751	30.22262268	6.007858781	2.631939934

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

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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, αβTCR 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 μg 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 μg 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 μg 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 μg 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

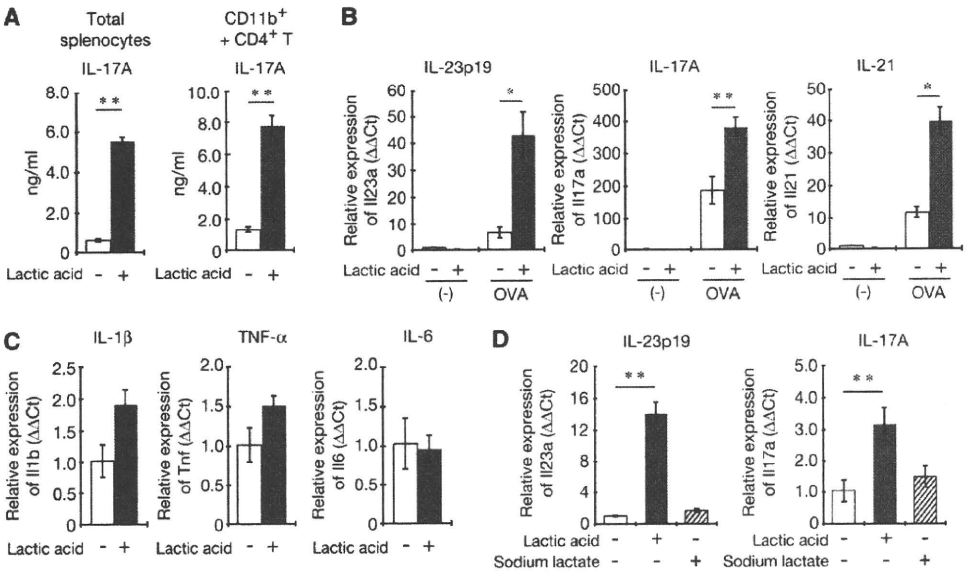

Fig. 1. The effect of lactic acid in a co-culture of fractionated CD11b$^+$ cells and CD4$^+$ T cells. (A) CD11b$^+$ cells and CD4$^+$ T cells derived from wild-type and OT-II splenocytes, respectively, were co-cultured in the presence (gray bars) or absence (white bars) of 15 mM lactic acid together 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.

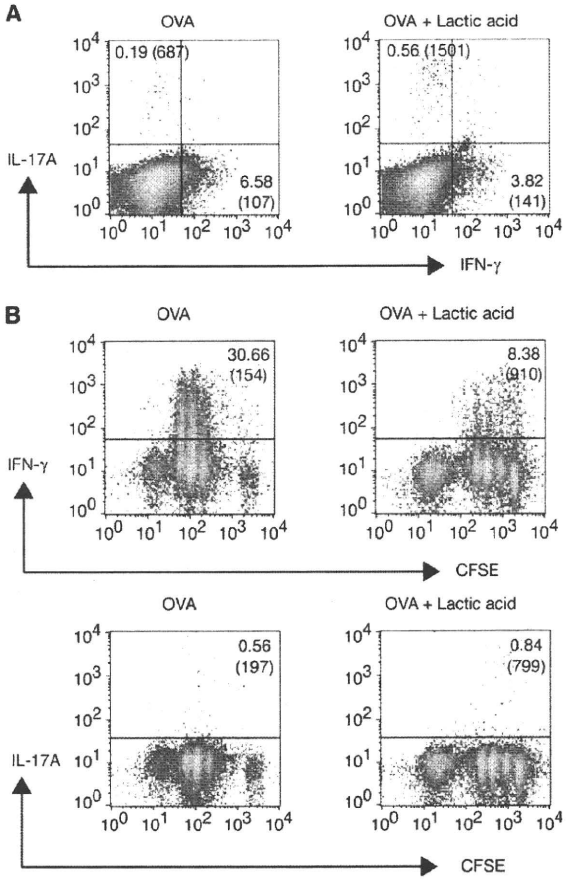

Fig. 2. Effect of lactic acid on the proportion and proliferation of IL-17A- and IFN-γ-producing cells. (A) $CD4^+$ T cells were co-cultured with $CD11b^+$ cells and stimulated with OVA peptide in the presence (right plot) or absence (left plot) of lactic acid for 4.5 days. T cells were then re-stimulated 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.

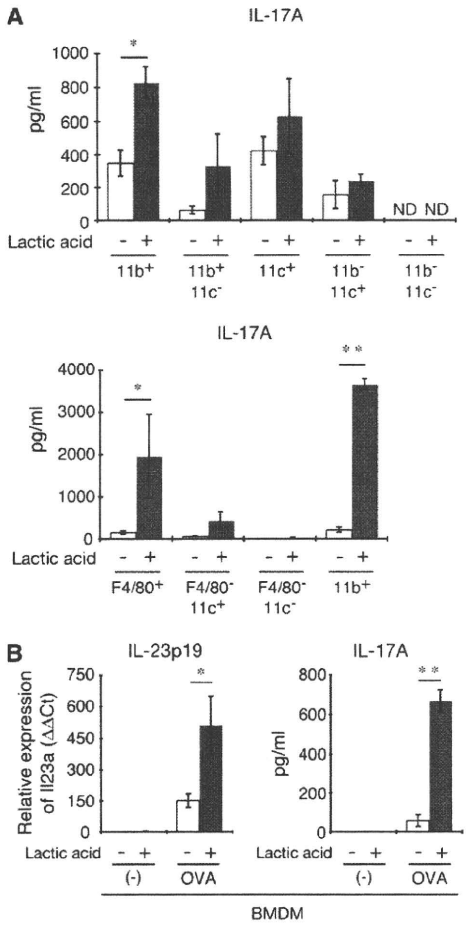

Fig. 3. Monocytes/macrophages are involved in lactic acid-enhanced, antigen-dependent, IL-17A production from $CD4^+$ T cells. (A) Splenic APCs were fractionated by using the cell surface markers CD11b ($11b^+$), CD11c ($11c^+$) and F4/80 ($F4/80^+$), as indicated on the *x*-axis. Each fraction was co-cultured 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

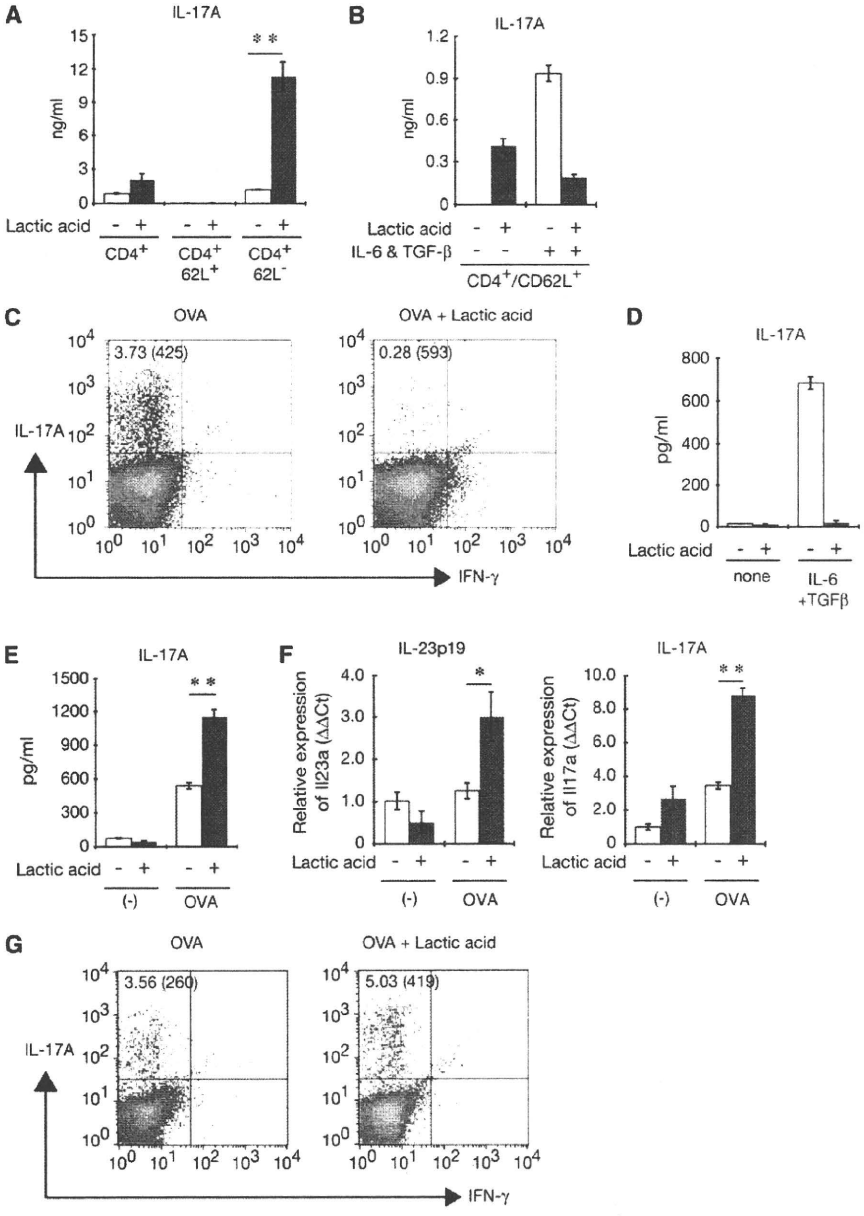

Fig. 4. Lactic acid induces IL-17A production from effector/memory $CD4^+$ T and T_h17 cells but inhibits differentiation of T_h17 from naive T cells. (A) Total $CD4^+$, $CD4^+CD62L^+$ (naive) or $CD4^+CD62L^-$ (effector/memory) T cells derived from OT-II mouse spleens were mixed with $CD11b^+$ cells 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. Effector/memory $CD4^+$ T cells, but not naive $CD4^+$ T cells, produced IL-17A by lactic acid stimulation. (B and C) In the presence of mouse IL-6 and human TGF-β, purified naive $CD4^+CD62L^+$ T cells were co-cultured with OVA peptide-loaded $CD11b^+$ cells and stimulated with or without lactic acid for 4 days for IL-17A production or 4.5 days for T_h17 differentiation. Amounts of IL-17A production are shown in (B). IL-17A-producing $CD4^+$ T cells were stained for CD4, IFN-γ and IL-17A and observed by FACS analysis (C). Plots gated on $CD4^+$ cells are shown. (D) Purified naive T cells were stimulated with plate-coated anti-CD3ε and anti-CD28 antibodies with (right bars) or without (left bars) IL-6 and TGF-β in the presence (gray bars) or absence (white bars) of lactic acid for 4 days. Lactic acid strongly suppressed differentiation into T_h17 cells. (E) Naive $CD4^+$ T cells derived from OT-II mice were differentiated into T_h17 cells *in vitro*. T_h17 cells were mixed with $CD11b^+$ cells at a 1:1 ratio and stimulated with or without OVA peptide in the presence (gray bars) or absence (white bars) of lactic acid for 4 days. (F) T_h17 cells were mixed with $CD11b^+$ cells and stimulated, as described above, for 12 h. The relative expressions of IL-23p19 and IL-17A were determined as described in Fig. 1(B). (G) T_h17 cells were co-cultured with $CD11b^+$ cells for 4.5 days and observed by FACS analysis. Plots gated on $CD4^+$ cells are shown. Numbers in plots indicate percentages (MFI) of $IL\text{-}17A^+$ cells in total cell populations. The data represent mean values ± standard deviation ($n = 3$); $^*P < 0.05$ and $^{**}P < 0.01$.

inhibited IL-17A production by naive T cells co-stimulated with anti-CD3ε and anti-CD28 antibodies (Fig. 4D). These results suggest that lactic acid negatively regulates the differentiation of naive $CD4^+$ T cells into T_h17 cells. However, lactic acid enhanced IL-17A production (Fig. 4E) and the expression of the IL-17A and IL-23p19 transcripts (Fig. 4F) in a co-culture of $CD11b^+$ cells and *in vitro*-differentiated T_h17 cells. In addition, T_h17 cells that were stimulated with OVA and lactic acid showed 1.4-fold (3.56 to 5.03%) and 1.6-fold increases (260 to 419) in the population of T_h17 cells and in the MFI, respectively (Fig. 4G). These data indicate that lactic acid is not involved in T_h17 cell differentiation but that it enhances IL-17A production from effector/memory T cells and *in vitro*-differentiated T_h17 cells but not naive $CD4^+$ T cells.

CD40L–CD40 interactions are involved in the increased production of IL-17A via IL-12/23p40 expression

In the co-culture system, we hypothesize that $CD4^+$ T cells activated by antigen stimulate $CD11b^+$ cells together with lactic acid, leading to the increased production of IL-17A from $CD4^+$ T cells. Activated $CD4^+$ T cells are known to activate nuclear factor-κB (NF-κB) signaling pathways through CD40L–CD40 interactions, leading to the activation of APCs and the efficient induction of various pro-inflammatory cytokines, including IL-12/23p40 (33–35). First, we examined the effect of CD40L–CD40 interactions on the influence of lactic acid. Anti-CD40L blocking antibodies significantly inhibited the increased expression of IL-17A and IL-12/23p40 induced by lactic acid in co-cultures of $CD11b^+$ and OT-II $CD4^+$ T cells (Fig. 5A). Furthermore, anti-IL-12/23p40 blocking antibodies also inhibited the enhanced IL-17A production by lactic acid (Fig. 5B). These results suggest that CD40L–CD40 interactions are essential for the increased expression of IL-12/23p40 that induces IL-17A production. The ligation of CD40 is known to induce the expression of CD40 itself, as well as pro-inflammatory cytokines (36). To clarify whether the enhanced IL-17A production by lactic acid is mediated by the increased expression of CD40, we examined the induction of CD40 expression using FACS and real-time PCR. However, lactic acid did not increase the expression of CD40 protein (Fig. 5C, left) or CD40 transcript (Fig. 5C, right) by $CD11b^+$ cells in this co-culture system. Furthermore, lactic acid did not elicit the nuclear translocation of NF-κB dimers containing either p65 or p52, which are known to be activated by CD40 ligation (33, 37) (Supplementary

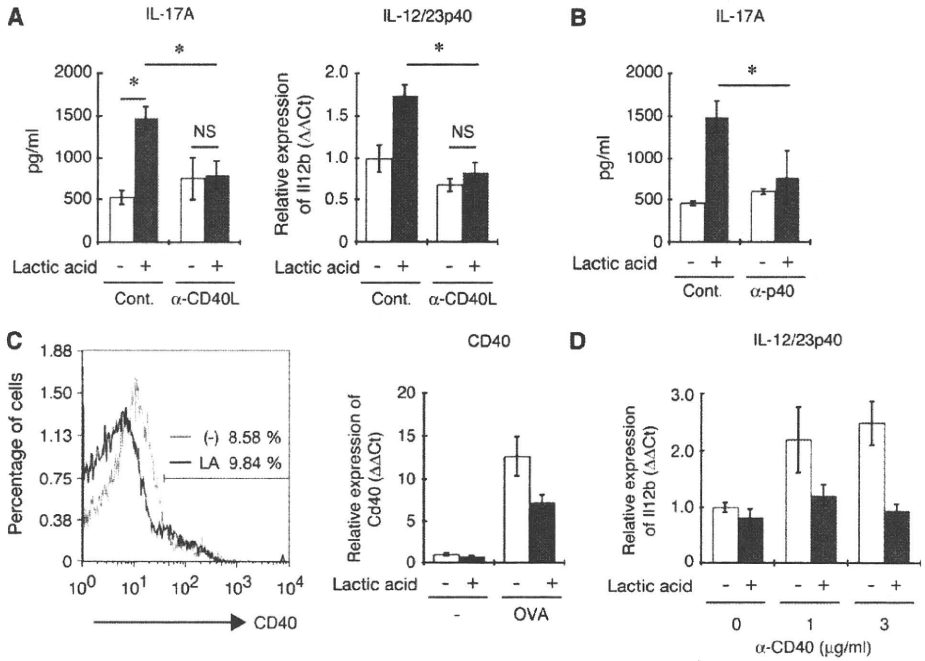

Fig. 5. CD40L–CD40 interactions are involved in the increased production of IL-17A via IL-12/23p40 expression. (A) $CD11b^+$ and OT-II $CD4^+$ T cells were co-cultured at a 1:1 ratio and stimulated with (gray bars) or without (white bars) lactic acid in the presence of anti-CD40L (α-CD40L) or isotype control antibodies (Cont.) for 4 days for IL-17A production or 12 h for the relative expression of IL-12/23p40. Anti-CD40L antibodies suppressed the expression of IL-17A and IL-12/23p40 by lactic acid stimulation; NS, not significant. (B) $CD11b^+$ and OT-II $CD4^+$ T cells were stimulated as described above in the presence of anti-IL-12/23p40 (α-p40) or isotype control antibodies (Cont.) for 4 days. Anti-IL-12/23p40 also suppressed the production of IL-17A. (C) $CD11b^+$ and OT-II $CD4^+$ T cells were stimulated as described above and the expression of CD40 was analyzed by FACS (left) and real-time PCR (right). Cell percentages indicated on the *y*-axis were calculated using FlowJo software (Tree Star Inc., Ashland, OR, USA). $CD40^+$ cell populations in $CD11b^+$ cells stimulated with (9.84%) or without (8.58%) lactic acid (LA) are presented in a representative histogram (left) and relative expression of CD40 transcripts is shown (right). (D) In the presence (gray bars) or absence (white bars) of lactic acid, $CD11b^+$ cells were stimulated for 12 h with anti-CD40 activating antibody (α-CD40) at the indicated concentrations. The relative expression levels of IL-12/23p40 were measured. Stimulation with anti-CD40 antibodies induced the expression of IL-12/23p40, and lactic acid suppressed the induced expression of IL-12/23p40. The data represent mean values ± standard deviation ($n = 3$); $^*P < 0.05$.

Figure 1 is available at *International Immunology* Online). Although the stimulation of CD11b$^+$ cells by anti-CD40 antibodies increased the expression of IL-12/23p40 in a dose-dependent manner, lactic acid suppressed the CD40-dependent induction of IL-12/23p40 (Fig. 5D). These results indicate that, in addition to CD40L–CD40 interactions, further stimulations by activated T cells are required for the increased IL-17A production by lactic acid via IL-12/23p40 expression. In contrast, CD40L–CD40 interactions were not involved in the increased expression of IL-23p19 by lactic acid (Supplementary Figure 2A and B is available at *International Immunology* Online).

The involvement of an IL-23-independent pathway in the increased production of IL-17A

We previously showed that increased production of IL-17A from CD4$^+$ T cells is induced through the enhanced production of IL-23 by lactic acid (21). Because we showed that IL-12/23p40 was essential for enhanced IL-17A production in Fig. 5(C), we predicted that IL-12/23p40 was required to form a heterodimer with IL-23p19. To clarify the involvement of IL-23 in the co-culture of CD11b$^+$ and CD4$^+$ T cells, we first examined the effect of anti-IL-23p19 blocking antibodies on the increased production of IL-17A by lactic acid. Anti-IL-23p19 antibodies reproducibly, but only partially, inhibited the IL-17A production in total splenocytes as previously described (Fig. 6A, left panel) (21). However, in the co-culture of CD11b$^+$ and CD4$^+$ T cells, anti-IL-23p19 antibodies hardly affected IL-17A production (Fig. 6A, right panel). Two other antibodies, goat anti-IL-23p19 and rat monoclonal anti-IL-23 receptor, also failed to suppress the increased IL-17 production (Supplementary Figure 3 is available at *International Immunology* Online). Although the IL-23 that was secreted from J774.1 cells stimulated with TLR ligands and lactic acid was detected by using commercially available ELISA kits (Invitrogen-Biosource cytokines & signaling) (21), IL-23 production was not detectable in the co-culture of CD11b$^+$ and CD4$^+$ T cells, and in total splenocytes, without TLR ligand stimulation (<7.8 pg ml^{-1}, data not shown).

We next examined the effect of IL-12 family cytokines sharing the IL-12/23p40 subunit on this enhancement. In the presence of IL-23, IL-12p70 or the IL-12p40 homodimer, CD11b$^+$ and CD4$^+$ T cells were stimulated by OVA peptide with or without lactic acid (Fig. 6B and C). Stimulation by IL-23 increased IL-17A production in the absence of lactic acid in a dose-dependent manner and lactic acid further enhanced the increased production of IL-17A by IL-23 (Fig. 6B). IL-17A production by lactic acid in the absence of exogenous IL-23 was also significantly higher than after treatment with 0.25 ng ml^{-1} IL-23 ($P < 0.01$). In contrast, the

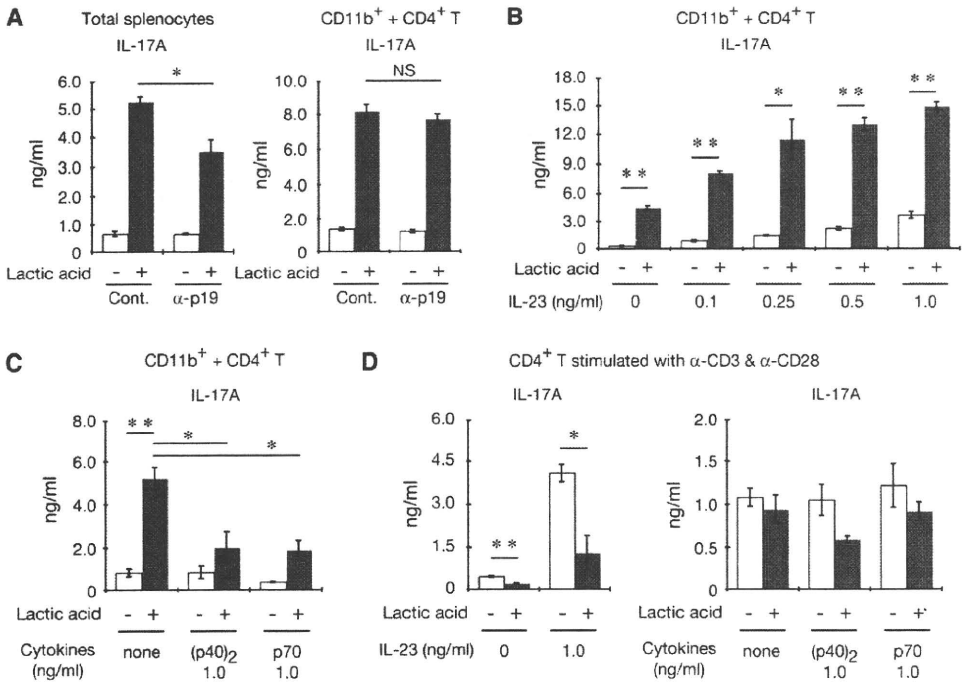

Fig. 6. The activation of IL-23-dependent and -independent pathway by lactic acid. (A) The effect of anti-IL-23 antibodies on the increased production of IL-17A in total OT-II splenocytes (left) or the co-culture of CD11b$^+$ and OT-II CD4$^+$ T cells (right). In the presence of anti-IL-23p19 (α-p19) or control antibodies (Cont.), cells were stimulated with OVA peptide and lactic acid (gray bars) or OVA peptide alone (white bars) for 4 days. (B and C) The effect of exogenous cytokines sharing the IL-12/IL-23p40 subunit on the increased production of IL-17A in the co-culture of CD11b$^+$ and OT-II CD4$^+$ T cells. In the presence of IL-23 (B), IL-12p70 (p70) or the IL-12p40 homodimer [$(p40)_2$] (C) at the indicated concentration, cells were stimulated with OVA peptide and lactic acid (gray bar), or OVA peptide alone (white bar) for 4 days. (D) In the presence of IL-23, IL-12p70 or the IL-12p40 homodimer, OT-II CD4$^+$ T cells alone stimulated with plate-coated anti-CD3ε and anti-CD28 antibodies were treated with lactic acid. At day 4, IL-17A production was measured. The data represent mean values ± standard deviation ($n = 3$); $^*P < 0.05$ and $^{**}P < 0.01$.

IL-12p40 homodimer and IL-12p70 remarkably suppressed the production of IL-17A in the presence of lactic acid (Fig. 6C). However, IL-23 increased IL-17A production from $CD4^+$ T cells activated by plate-coated anti-CD3ε and anti-CD28 antibodies, but lactic acid inhibited its production. The IL-12p40 homodimer and IL-12p70 did not affect IL-17A production from $CD4^+$ T cells activated by plate-coated anti-CD3ε and anti-CD28 antibodies (Fig. 6D). Therefore, in addition to increased IL-23 production, lactic acid may induce the expression of unidentified cytokines that share IL-12/23p40 or other factors involved in the enhanced production of IL-17A.

Enhanced IL-23p19 and IL-17A expression by lactic acid is independent of MyD88, CARD9 and IL-1β

It is known that the MyD88, CARD9 (caspase-recruitment domain 9) and NALP3 (NACHT-, LRR- and pyrin-domain

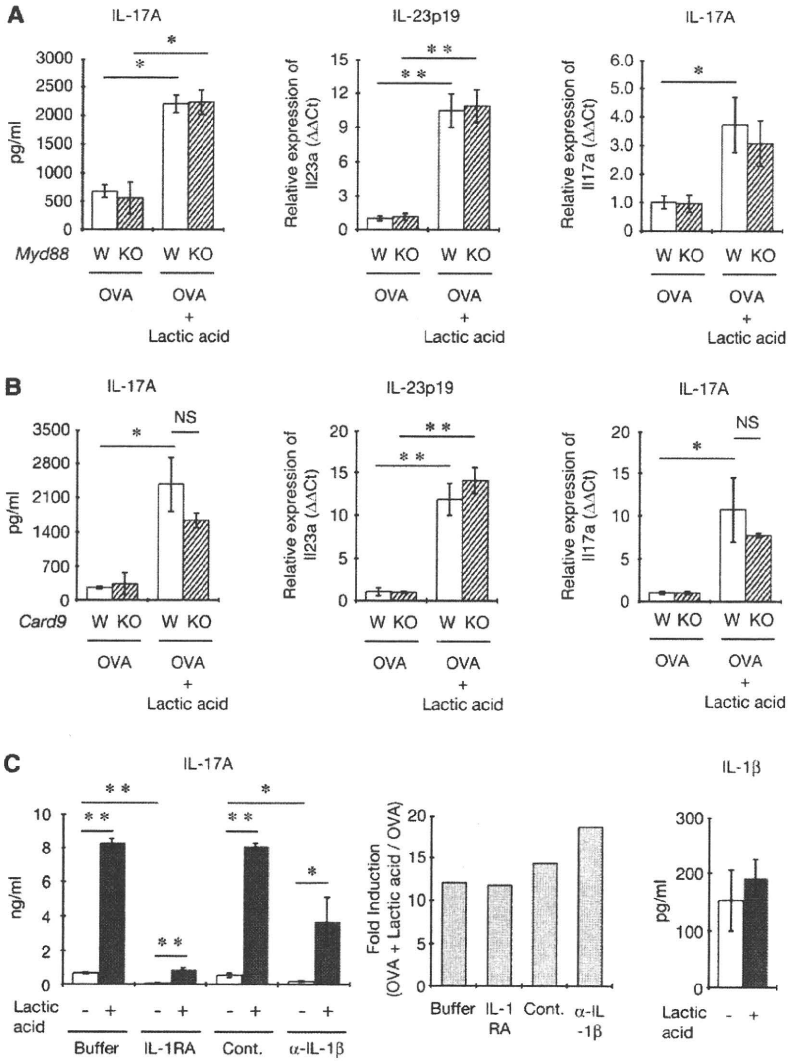

Fig. 7. Lactic acid enhances IL-17A production independently of MyD88, CARD9 and IL-1β. (A) and (B) $CD11b^+$ cells derived from *MyD88* (*Myd88*$^{-/-}$) (A), *Card9* (*Card9*$^{-/-}$) (B) deficient mice (KO, hatched bars) or their littermate controls (W, white bars) were mixed with OT-II $CD4^+$ T cells at a 1:1 ratio and stimulated with OVA peptide in the presence or absence of lactic acid for 4 days for IL-17A production or 12 h for the relative expression of IL-23p19 and IL-17A transcripts. *Myd88*$^{-/-}$ and *Card9*$^{-/-}$ $CD11b^+$ cells showed enhanced expression of IL-23p19 and IL-17A in the presence of lactic acid; NS, not significant. (C) $CD11b^+$ and OT-II $CD4^+$ T cells were stimulated with OVA peptide alone (white bars) or OVA peptide plus lactic acid (gray bars) in the presence of IL-1RA, anti-IL-1β (α-IL-1β) or isotype control (Cont.) antibodies for 4 days (left panel). Buffer (PBS containing 0.1% BSA) indicates control for IL-1RA. Fold induction by lactic acid in left panel was calculated relative to OVA peptide alone (middle panel). Although IL-1RA and anti-IL-1β antibodies suppressed the total amount of IL-17A produced when stimulated with OVA peptide alone or OVA peptide plus lactic acid, enhanced IL-17A production by lactic acid was hardly impaired. IL-1β production, when incubated with buffer, is shown in right panel. The data represent mean values ± standard deviation ($n = 3$); $^*P < 0.05$ and $^{**}P < 0.01$.

containing protein 3)-inflammasome pathways are activated by intrinsic stimuli from damaged cells as well as external pathogens and that they play roles in the induction of inflammation and inflammatory diseases. To determine whether lactic acid is involved in these signaling pathways, we examined CD11b$^+$ cells derived from *Myd88* and *Card9* knockout mice splenocytes. In co-cultures with OT-II CD4$^+$ T cells, enhanced expression of IL-23p19 and IL-17A after lactic acid stimulation was not impaired in CD11b$^+$ cells from *Myd88* (Fig. 7A) and *Card9* (Fig. 7B) knockout mice. The activation of the NALP3-inflammasome pathway by molecules secreted from damaged cells such as ATP (5, 6) mediates the processing of caspase-1 to convert pro-IL-1β into its active form, IL-1β. Secreted IL-1β is involved in the induction of IL-17A expression and the maintenance of T_h17 cells (38–40). To examine the effect of IL-1β on enhanced IL-17A production by lactic acid, we stimulated CD11b$^+$ and CD4$^+$ T cells with OVA peptide and lactic acid in the presence of IL-1RA or anti-IL-1β antibodies (α-IL-1β) (Fig. 7C, left panel). IL-1RA and anti-IL-1β antibodies potently inhibited IL-17A production when stimulated with OVA peptide alone or with OVA peptide plus lactic acid. However, the relative activity induced by lactic acid was still elevated in the presence of IL-1RA (12.1-fold for Buffer and 11.8-fold for IL-1RA) or anti-IL-1β antibodies (14.4-fold for Control and 18.7-fold for anti-IL-1β antibodies) (Fig. 7C, middle panel). Furthermore, even when we observed the remarkable enhancement of IL-17A production, we detected only a slight or no increase in IL-1β transcription and IL-1β secretion by lactic acid (Figs 1C and 7C, right panel). These data suggest that IL-1β is involved in IL-17A production (38–40) but plays no significant role in enhanced IL-17A expression by lactic acid.

Discussion

This study analyzed the induction mechanism of IL-17A by lactic acid without TLR ligands using splenocytes of OT-II transgenic mice. It is postulated that lactic acid enhances the antigen-dependent production of IL-17A via three steps: Step 1, through CD4$^+$ T cell activation by APCs in an antigen-dependent manner; Step 2, through activation of APCs by activated CD4$^+$ T cells and lactic acid; and Step 3, through activation of the IL-17A promoter in CD4$^+$ T cells stimulated by APCs. Using fractionated splenocytes, this study showed that monocytes/macrophages and effector/memory CD4$^+$ T cells are essential for the enhanced activity of antigen-dependent production of IL-17A by lactic acid. Lactic acid suppresses the proliferation of both IL-17A- and IFN-γ-producing CD4$^+$ T cells but maintains the phenotype of IL-17A-producing cells. Furthermore, CD40L–CD40 interaction was found to be essential for co-stimulation of CD11b$^+$ cells together with lactic acid in Step 2, leading to IL-12/23p40 production. IL-12/23p40 production is necessary to up-regulate IL-17A production, but known cytokines containing IL-12/23p40, such as IL-23, IL-12 and the IL-12/23p40 homodimer, were not involved in this up-regulation in the co-culture of CD4$^+$ T and CD11b$^+$ cells. Therefore, a new cytokine containing IL-12/23p40 may be one of the molecules that enhance IL-17A production by effector/memory CD4$^+$ T cells after lactic acid stimulation in Step 3. While IL-1β induction plays an important role in IL-17A up-regulation in both the presence and absence of lactic acid in Step 3, IL-1β was not involved in the enhancement of IL-17A production by lactic acid.

In splenocytes, CD11b$^+$ and F4/80$^+$ cells most significantly stimulated IL-17A production from CD4$^+$ cells by lactic acid. Likewise, BMDMs differentiated by treatment with M-CSF potently up-regulated IL-17A. Therefore, monocytes/macrophages were activated by lactic acid and were involved in amplifying IL-17A expression. Although DCs generally have higher antigen-presenting abilities, these studies demonstrated that CD11b$^-$CD11c$^+$ and F4/80$^-$CD11c$^+$ cells in splenocytes had less stimulatory activity than CD11b$^+$ and F4/80$^+$ cells. BMDCs strongly induced IL-17A production from CD4$^+$ T cells, but lactic acid only slightly enhanced the IL-17A expression mediated by BMDCs (data not shown). These results suggest that lactic acid acts mainly on monocytes/macrophages, despite their weak antigen presenting activity. In support of these findings, it is well known that tumor-associated macrophages often infiltrate tumor microenvironments to support tumor proliferation and progression (41, 42). Therefore, tumor-secreted lactic acid could act on macrophages that induce chronic inflammation in tumors.

In this study, we also clarified the effects of lactic acid on IL-17A-producing cells. Lactic acid enhanced IL-17A production from CD4$^+$CD62L$^-$ effector/memory T cells and T_h17 cells, but not naive CD4$^+$ T cells. Meanwhile, lactic acid strongly inhibited differentiation of T_h17 cells from naive T cells in response to IL-6 and TGF-β. Furthermore, lactic acid increased the proportion of IL-17A-producing cells and the MFI of IL-17A but suppressed the proliferation of both IL-17A- and IFN-γ-producing cells. The Kreutz group also reported that lactic acid suppresses the proliferation of human cytotoxic T cells and the production of IFN-γ and IL-2 in these T cells upon antigen stimulation (24, 25). Therefore, lactic acid acts on monocytes/macrophages to maintain only the cells that are involved in the induction of inflammation, to induce specific pro-inflammatory cytokines such as IL-17A and IL-21 and to negatively regulate the T_h1 and cytotoxic T cells that are involved in anti-tumor immunity.

It has been reported that cell–cell contacts through CD40L–CD40 interactions are necessary for the activation of APCs by CD4$^+$ T cells to induce IL-12/23p40 expression (34, 35). We verified that the CD40L–CD40 interaction is essential for the IL-12/23p40 expression that induces increased IL-17A production by CD4$^+$ T cells. However, lactic acid did not enhance either NF-κB signaling pathways or the expression of IL-12/23p40 elicited by CD40 ligation. Therefore, we predict that lactic acid does not directly modify the CD40 signaling pathway. The expression of IL-12/23p40 was also slightly enhanced in the presence of lactic acid (Fig. 5A) (21), but the enhanced expression of IL-12/23p40 by lactic acid was not always detected even when increased IL-17A production was induced (data not shown). We predicted that IL-23 would induce elevated IL-17A production in response to a lactic acid-induced increase in IL-23p19, as previously described (21). However, anti-IL-23p19 and anti-IL-23 receptor antibodies did not suppress increased

IL-17A production in the co-culture system of $CD4^+$ T and $CD11b^+$ cells. Furthermore, IL-23 production was not detectable in this co-culture system (<7.8 pg ml^{-1}). Lactic acid induced a higher level of IL-17A than 0.25 ng ml^{-1} exogenous IL-23, even when IL-23 induced by lactic acid was not detected. These results indicate the involvement of another IL-12/23p40-containing factor that can induce IL-17A production independent of IL-23. However, the IL-12p40 homodimer and IL-12p70, which also share the IL-12/23p40 subunit, suppressed IL-17A production. Therefore, an additional unidentified cytokine containing IL-12/23p40 may act directly on effector/memory $CD4^+$ T cells to stimulate increased IL-17A production. The IL-12p40 homodimer and IL-12p70 may block the binding of the new IL-12/23p40-containing cytokine to their shared receptor IL-12Rβ1. Lactic acid may induce the enhanced expression of a subunit of the cytokine that contains IL-12/23p40, leading to increased IL-17A production. Alternatively, the new cytokine may act on $CD11b^+$ cells to stimulate the expression of accessory molecules that cooperate with TCR–MHC interactions, but not in increased IL-17A production. The expression of these accessory molecules may be enhanced by lactic acid stimulation. Furthermore, Lactic acid also induced the expression of IL-21 produced by T_h17 cells, which increases their population through an autocrine or paracrine feedback loop (13). Therefore, other factors that mediate the increased expression of IL-21 by stimulation of lactic acid are likely involved in the increased proportion of cells that express IL-17A.

It is known that several signaling pathways are activated upon stimulation with intrinsic inflammatory ligands. We analyzed whether lactic acid is involved in the activation of three of these known pathways: MyD88, CARD9 and NALP3. The results indicated that the elevated activity induced by lactic acid was independent of the MyD88 and CARD9 signaling pathways. Recently, Samuvel *et al.* (43) reported that lactate enhances TLR4 signaling via MD-2 expression in human U937 histiocyte cells. However, MyD88, which is an adaptor molecule of TLR4, was not involved in the higher expression of IL-23p19 and IL-17A in our system. In addition, lactic acid did not enhance the NF-κB signaling pathway in J774.1 cells co-stimulated with TLR ligands (21). Although it has been reported that the Syk-CARD9 pathway is involved in the differentiation of IL-17A-producing $CD4^+$ cells (44), the enhancement of IL-17A production by lactic acid was unaffected in a co-culture with $CD11b^+$ cells derived from *Card9* knockout mice. To test whether lactic acid participates in the NALP3-inflammasome pathway, we examined the expression of IL-1β. IL-1β was produced after stimulation with the OVA peptide alone and the addition of lactic acid only slightly enhanced or left unchanged its levels of expression. The IL-1 signaling pathway regulates T_h17 cell differentiation and maintains the expression of IL-17A in T_h17 cells (40). The inhibition of IL-1 signaling by IL-1RA or anti-IL-1β antibodies strongly suppressed IL-17A production, stimulated either with the OVA peptide plus lactic acid or with the peptide alone (Fig. 7C). However, the production of IL-17A was still significantly enhanced by lactic acid even in the presence of either IL-1RA or anti-IL-1β antibodies. Therefore, this result suggests that IL-1β stimulation is important for the strong induction of IL-17A but that it acts independently of the lactic acid signaling pathway.

Recently, it has been reported that GPR81, a G-protein coupled receptor (GPCR) expressed in adipocytes, is a sensor for lactate that mediates an anti-lipolytic effect (45–47). Surprisingly, the half-maximal effective concentration for L-lactate to activate GPR81 is remarkably high (~5 mM) compared with values for ligands of other typical GPCRs, which are in the nanomolar range. This value is almost equivalent to that required to induce activity in our system. However, for a number of reasons, we predict that other molecules would sense lactic acid in macrophages and that these molecules induce the increase of IL-23p19 expression and IL-17A production. First, GPR81 is specifically expressed in adipose tissue but not the spleen (46). Second, neutralized lactate and sodium lactate did not enhance the promoter activity of the human IL-23p19 gene (21) or the expression of IL-23p19 and IL-17A in a co-culture of $CD11b^+$ and $CD4^+$ T cells, whereas the lactate anion acts on GPR81 under neutral conditions (46). Third, although GPR81 negatively controls adenylate cyclase activity, lactic acid did not modulate the enhancer activity of cyclic adenosine 3',5'-monophosphate - responsive elements stimulated by the TLR2 ligand in J774.1 cells (data not shown). Therefore, we predict that lactic acid is co-transported with protons into cells via MCTs and recognized by an intracellular molecule that activates monocytes, leading to increased IL-17A production from $CD4^+$ T cells. In this study, lactic acid was shown to act mainly on monocytes/macrophages rather than DCs. Therefore, we speculate that the intracellular lactic acid sensor is specifically expressed in monocytes/macrophages. Because we observed the enhancement of IL-23p19 promoter activity in J774.1 cells but not in RAW264 cells, the lactic acid sensor may be deficient in RAW264 cells. In contrast, the Kreutz group recently reported that lactic acid inhibits glycolytic flux and export of lactate, resulting in suppression of TNF secretion from monocytes (48). Furthermore, inhibition of hexokinase by 2-deoxyglucose also suppressed the secretion of TNF. Thus, the expression of IL-23p19 and other molecules induced by lactic acid may also depend on blocking glycolytic flux. Future studies will help to further elucidate the unique molecular mechanisms controlled by lactic acid in the IL-23-dependent and -independent pathways to increase IL-17A production.

It is known that tumor cells secrete a variety of factors including cytokines, chemokines and damaged cellular components to induce local inflammation around tumors (1, 41, 42). The increase of lactic acid production in tumors is a common feature due to the Warburg effect (22, 49). We and another group reported that lactic acid suppresses the proliferation of T cells and the production of IFN-γ, which mediates anti-tumor activities (24, 25), but enhances the IL-23-dependent and -independent IL-17 pathways that mediate chronic inflammation in our studies (21). Furthermore, in human carcinomas and mouse tumor models, higher proportions of T_h17 cells are recruited to the tumor microenvironment than the tumor-draining lymph nodes and peripheral blood (50). Therefore, lactic acid may be an important pro-inflammatory mediator that supports tumor progression. In fact, it has been reported that high concentrations of lactate

in solid tumors, such as cervical carcinoma and head and neck cancers, are associated with higher frequencies of distant metastasis and poor prognosis (23). Studies have also shown that the inhibition of lactic acid production in tumors reduces tumor growth (51). Therefore, modulation of the lactic acid signaling pathway may become an attractive target for treating many solid tumors. Agents that target this pathway could suppress chronic inflammation and instead induce anti-tumor immunity, as well as inhibit tumor growth.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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References

1 Karin, M., Lawrence, T. and Nizet, V. 2006. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 124:823.
2 Petrilli, V., Dostert, C., Muruve, D. A. and Tschopp, J. 2007. The inflammasome: a danger sensing complex triggering innate immunity. *Curr. Opin. Immunol.* 19:615.
3 Apetoh, L., Ghiringhelli, F., Tesniere, A. *et al.* 2007. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. *Immunol. Rev.* 220:47.
4 Bianchi, M. E. and Manfredi, A. A. 2007. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunol. Rev.* 220:35.
5 Duncan, J. A., Bergstralh, D. T., Wang, Y. *et al.* 2007. Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. *Proc. Natl Acad. Sci. USA* 104:8041.
6 Mariathasan, S., Weiss, D. S., Newton, K. *et al.* 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228.
7 Sutterwala, F. S., Ogura, Y., Szczepanik, M. *et al.* 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 24:317.
8 Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K. and Saito, T. 2008. Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat. Immunol.* 9:1179.
9 Kolls, J. K. and Linden, A. 2004. Interleukin-17 family members and inflammation. *Immunity* 21:467.
10 Park, H., Li, Z., Yang, X. O. *et al.* 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6:1133.
11 Harrington, L. E., Hatton, R. D., Mangan, P. R. *et al.* 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6:1123.
12 Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. and Stockinger, B. 2006. TGF® in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179.
13 Bettelli, E., Korn, T., Oukka, M. and Kuchroo, V. K. 2008. Induction and effector functions of TH17 cells. *Nature* 453:1051.
14 McGeachy, M. J., Chen, Y., Tato, C. M. *et al.* 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells *in vivo*. *Nat. Immunol.* 10:314.
15 Stritesky, G. L., Yeh, N. and Kaplan, M. H. 2008. IL-23 promotes maintenance but not commitment to the Th17 lineage. *J. Immunol.* 181:5948.
16 Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J. and Gurney, A. L. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 278:1910.
17 Korn, T., Bettelli, E., Oukka, M. and Kuchroo, V. K. 2009. IL-17 and Th17 cells. *Annu. Rev. Immunol.* 27:485.
18 Abraham, C. and Cho, J. H. 2009. IL-23 and autoimmunity: new insights into the pathogenesis of inflammatory bowel disease. *Annu. Rev. Med.* 60:97.
19 Langowski, J. L., Zhang, X., Wu, L. *et al.* 2006. IL-23 promotes tumour incidence and growth. *Nature* 442:461.
20 Kortylewski, M., Xin, H., Kujawski, M. *et al.* 2009. Regulation of the IL-23 and IL-12 balance by Stat3 signaling in the tumor microenvironment. *Cancer Cell* 15:114.
21 Shime, H., Yabu, M., Akazawa, T. *et al.* 2008. Tumor-secreted lactic acid promotes IL-23/IL-17 proinflammatory pathway. *J. Immunol.* 180:7175.
22 Kim, J. W. and Dang, C. V. 2006. Cancer's molecular sweet tooth and the Warburg effect. *Cancer Res.* 66:8927.
23 Walenta, S. and Mueller-Klieser, W. F. 2004. Lactate: mirror and motor of tumor malignancy. *Semin. Radiat. Oncol.* 14:267.
24 Gottfried, E., Kunz-Schughart, L. A., Ebner, S. *et al.* 2006. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood* 107:2013.
25 Fischer, K., Hoffmann, P., Voelkl, S. *et al.* 2007. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* 109:3812.
26 Barnden, M. J., Allison, J., Heath, W. R. and Carbone, F. R. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76:34.
27 Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K. and Akira, S. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J. Immunol.* 166:5688.
28 Hara, H., Ishihara, C., Takeuchi, A. *et al.* 2007. The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. *Nat. Immunol.* 8:619.
29 Suzuki, K., Okuno, T., Yamamoto, M. *et al.* 2007. Semaphorin 7A initiates T-cell-mediated inflammatory responses through alpha1-beta1 integrin. *Nature* 446:680.
30 Akazawa, T., Shingai, M., Sasai, M. *et al.* 2007. Tumor immunotherapy using bone marrow-derived dendritic cells overexpressing Toll-like receptor adaptors. *FEBS Lett.* 581:3334.
31 Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402.
32 Halestrap, A. P. and Price, N. T. 1999. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J* (343 Pt) 2:281.
33 Speirs, K., Caamano, J., Goldschmidt, M. H., Hunter, C. A. and Scott, P. 2002. NF-kappa B2 is required for optimal CD40-induced IL-12 production but dispensable for Th1 cell differentiation. *J. Immunol.* 168:4406.
34 Kato, T., Hakamada, R., Yamane, H. and Nariuchi, H. 1996. Induction of IL-12 p40 messenger RNA expression and IL-12 production of macrophages via CD40-CD40 ligand interaction. *J. Immunol.* 156:3932.
35 Schulz, O., Edwards, A. D., Schito, M., Aliberti, J., Manickasingham, S., Sher, A. and Reis e Sousa, C. 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells *in vivo* requires a microbial priming signal. *Immunity* 13:453.