Luciferase Reporter assay systems (Promega) and was shown as the means ± S.D. of three experiments.

2.7. Native PAGE, SDS-PAGE, Western blotting, and immunoprecipitation assay

Cells were solubilized in the lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% NP-40, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF, and 1 mM Na_3VO_4) on ice for 30 min and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatants were separated by SDS-PAGE, and the gel was transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with Tris-buffered saline (TBS) pH 8.0 containing 5% skim milk, immunoblotted with specific antibodies, and visualized with the appropriate horseradish peroxidase-conjugated secondary antibodies using the ELC plus Western Blotting Detection System (Amersham Pharmacia). For detection of IRF3-dimerization, whole cell extracts were subjected to 7.5% polyacrylamide gel Native (Daiichi Pure Chemicals). For immunoprecipitation, cells were lysed in the Triton X-100 lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF, and 1 mM Na_3VO_4) and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatants were incubated with anti-Myc antibody and protein G-Sepharose (Amersham Pharmacia) for overnight at 4 °C. The immunoprecipitates were collected by centrifugation, washed 4 times in the lysis buffer, and then analyzed by SDS-PAGE.

2.8. Confocal microscopy

HeLa cells (2.5×10^4 cells/well) were plated on a micro cover glass (Matsunami Glass) in 12-well plate. The following day, cells were transfected with the indicated plasmids using FuGENE HD (Roche). The total amounts of DNA were kept constant by adding empty vector. After 24 h, cells were fixed in acetone and blocked in PBS containing 1% BSA and then labeled with the indicated primary antibodies for 1 h at room temperature. Alexa Fluor 488- or Alexa Fluor 594-conjugtaed secondary antibodies were used for the visualizing proteins detected by the primary antibodies. For nucleus staining, cells were treated with DAPI in PBS. After all staining procedures were finished, micro cover glasses were mounted onto a slide glass using PBS containing 2.3% DABCO and 50% glycerol. Cells were visualized at 63× magnification under an LSM510 META microscope (Zeiss).

2.9. Statistical analysis

The statistical significance was analyzed using Student's *t*-test. *p* values < 0.05 were considered significant.

3. Results

3.1. Laboratory adapted strain ED induces IFN-β mRNA in A549/CD150 cells

We tested whether MV induced the expression of IFN-β mRNA in infected A549/CD150 cells and found that laboratory-adapted strain ED induced IFN-β mRNA expression, whereas IFN-β mRNA was virtually undetectable in wild-type strain MS-infected cells (Fig. 1A). To confirm the efficiency of virus infection, we measured MV-H mRNA levels by RT-PCR (Fig. 1B). The MV-H mRNA level in MS-infected cells was comparable to that found in ED-infected cells. Our previous report showed that DI RNA in MV isolates is a crucial determinant for high IFN induction (Shingai et al., 2007). However, no amplifiable 5′ copy-back DI RNA was detected in the MV culture supernatants (Fig. 1C), suggesting that the ED and MS strains used

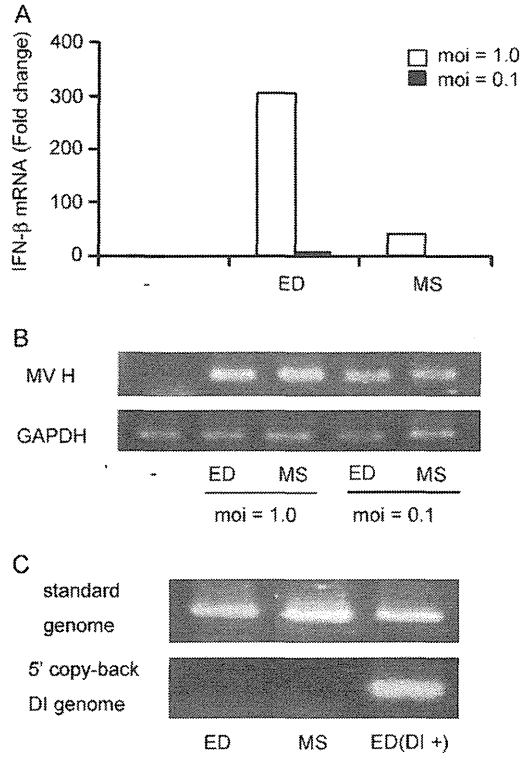

Fig. 1. ED strain induces IFN-β mRNA expression in the infected cells. (A) A549/CD150 cells were infected with mock, ED (vaccine strain) or MS (wild-type strain) at MOI = 0.1 or 1. After 12 h, RNA samples were collected and mRNAs of IFN-β and β-actin were measured by real-time PCR. The value for IFN-β mRNA expression was normalized to that of β-actin mRNA. Fold induction against control medium is shown. (B) MV-H mRNA level was determined by RT-PCR. (C) RT-PCR amplification of 5′ copy-back DI RNA from MV culture supernatants. RT-PCR was performed using standard genome-specific primers or DI-specific primers.

in this study, do not contain 5′ copy-back DI RNA. Thus, in this DI RNA-negative ED strain, a factor other than DI RNA is implicated in the induction of IFN-β mRNA.

3.2. The ED-V protein barely suppresses MDA5-induced IFN-β promoter activity

To explain the differential type I IFN-inducing abilities of ED versus wild-type strains, we transfected cDNAs encoding MV proteins into A549/CD150 cells, established in our laboratory (Tanabe et al., 2003). In these pilot studies, we found that expression of MV V protein suppresses IFN-β promoter activation, as reported by other groups (Nakatsu et al., 2008; Ohno et al., 2004; Takeuchi et al., 2003). We then focused on the function of the V and C proteins of various MV strains. The V and C proteins of MV are not essential products (Radecke and Billeter, 1996) but play important roles in MV virulence (Patterson et al., 2000). The V protein has been shown to inhibit IFN induction via binding to MDA5 (Childs et al., 2007, 2009). On the other hand, the C protein does not block the IFN-inducing pathway, but affects infectivity by acting as a regulator of viral RNA synthesis (Nakatsu et al., 2008). When A549/CD150 cells were stimulated with polyI:C or transfected with RIG-I or MDA5, efficient IFN-β promoter activation was detected using a reporter assay (Fig. 2A–C). Using this assay, we examined the effects of the transfected V and/or C proteins on IFN-β promoter activity. PolyI:C-induced IFN-β promoter activation was inhibited by the V protein expressed by wild-type strains, MS and IC-B, and an attenuated NV strain, which possesses DI RNA (Shingai et al., 2007). The ED-V pro-

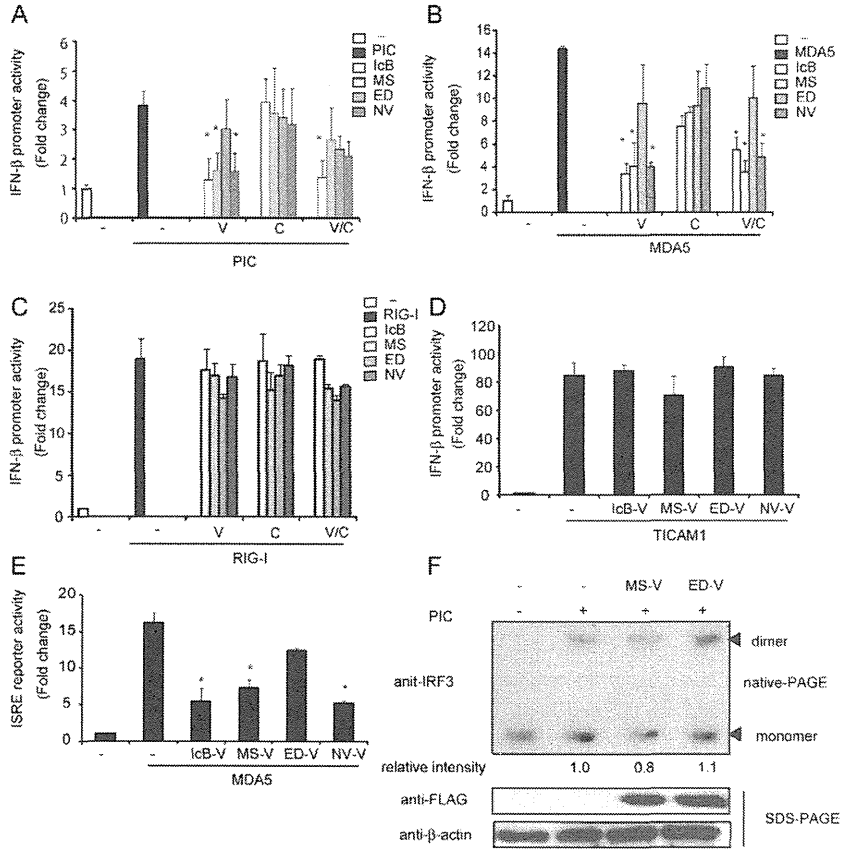

Fig. 2. Strain-to-strain difference in V protein function for MDA5-induced IFN-β promoter activation. (A) A549 cells in 24-well plates were transfected with pCMV10-MV-V (100 ng) and pCMV10-MV-C (100 ng) together with the IFN-β promoter reporter (100 ng) and phRL-TK (50 ng). Twenty-four hours after transfection, the cells were stimulated with 50 μg/ml polyI:C for 6 h, and then the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. A549 cells in 24-well plates were transfected with pEF-BOS FLAG-MDA5 (100 ng, B), pEF-BOS RIG-I (100 ng, C), pEF-BOS TICAM1 (100 ng, D), pCMV10-MV-V (100 ng) and pCMV10-MV-C (100 ng) together with the IFN-β promoter reporter (100 ng) and phRL-TK (50 ng). Twenty-four hours after transfection, the luciferase reporter activity was measured. (E) A549 cells in 24-well plates were transfected with pCMV10-MV-V (100 ng) and pCMV10-MV-C (100 ng) together with the ISRE luciferase gene (100 ng) and phRL-TK (50 ng). Twenty-four hours after transfection, the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. $^*p < 0.05$. (F) HeLa cells transfected with pCMV10 FLAG-MV-V (100 ng). After 24 h, cells were stimulated with 10 μg/ml polyI:C for 1 h and then lysed with native-PAGE lysis buffer or SDS-PAGE lysis buffer. For native-PAGE, the cell lysates were subjected to native-PAGE and immunoblotted with anti-IRF-3 antibody. For SDS-PAGE the cell lysates were subjected to SDS-PAGE and immunoblotted with anti-FLAG antibody or anti-β-actin (internal control). The band intensity was quantified by NIH Image J and relative band intensity was shown. The results were reproducible in three additional experiments.

tein barely suppressed polyI:C-induced IFN-β promoter activation (Fig. 2A). None of the C proteins analyzed affected IFN-β promoter activation.

PolyI:C is regarded as an analog of viral dsRNA and activates TLR3 in the endosomes and RIG-I/MDA5 in the cytoplasm. TLR3 recruits TICAM-1 while RIG-I and MDA5 recruit IPS-1 as adaptors. The two pathways converged upon NAP1, which assembles IKKε and TBK1 to activate IRF-3 and promote induction of IFN-β (Sasai et al., 2006b). Production of a trace amount of IFN-β results in amplified production of type I IFN via the IFNAR pathway, as controlled by the ISRE promoter (Takaoka and Yanai, 2006). To reveal the target pathway inhibited by the V protein of wild-type MV, we examined whether the wild-type MV V proteins block IFN-β induction in cells containing overexpressed MDA5, RIG-I or TICAM-1 (Fig. 2B–D). The V proteins of strains MS and IC-B inhibited MDA5-induced IFN-β and ISRE promoter activation but barely affected RIG-I and TICAM-1-induced IFN-β induction (Fig. 2B–E). It is notable that in our setting, V proteins of various MV strains did not suppress RIG-I-mediated activation of IFN-β promoter (Fig. 2C). These data suggested that the V proteins of wild-type strains suppress the MDA5 pathway for type I IFN induction while the C proteins barely affect MDA5-, RIG-I- and TICAM-1-dependent IFN-β transcription. Under these conditions, only the V protein of strain ED abrogates the inhibitory function of MDA5 in both IFN-β and ISRE reporters.

IRF-3 activation in the cytoplasm occurs via C-terminal phosphorylation of IRF-3 by the TBK1/NAP1/IKKε complex. These modifications promote IRF-3 homodimerization and the subsequent nuclear import of these molecules (Medzhitov, 2007; Platanias, 2005). In our studies for detection of IRF-3 dimer formation, although the V protein of the wild-type strain suppressed polyI:C-induced IRF-3 dimerization, the ED-V protein hardly inhibited polyI:C-induced IRF-3 dimerization (Fig. 2F). These data suggested that the V protein of wild-type strains inhibited polyI:C-induced IFN-β induction via the suppression of MDA5-meditaed IRF-3 activation. To exclude the possibility that the MV-V protein causes MDA5 degradation, we confirmed the MDA5 protein level by Western blotting (Fig. 3). The MDA5 protein levels in the MS-V or ED-V transfected cells were comparable to those found in untreated cells.

3.3. 272C is responsible for suppression of MDA5-induced IFN-β promoter activity

To reveal the molecular mechanism that determines whether MV V protein inhibits MDA5-induced IFN-β promoter activity, we compared the amino acid sequence of the ED V protein with that of

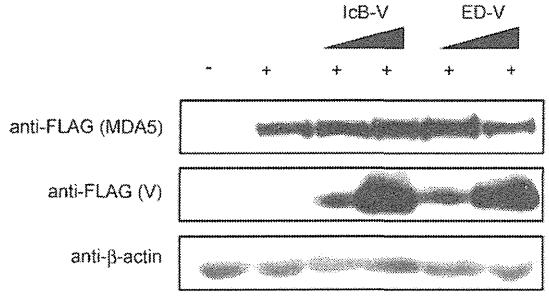

Fig. 3. Forced expression of V protein did not affect the expression level of MDA5 protein. HEK 293FT cells were transfected with pEF-BOS FLAG-MDA5 (100 ng) and pCMV10 FLAG-MV-V (10 ng, 100 ng). After 24 h, cells were lysed and subjected to Western blotting with anti-FLAG antibody and anti-β-actin antibody (internal control).

wild-type V proteins. As shown in Fig. 4, we identified 7 amino acid substitutions (51R, 83P, 97P, 110H, 225G, 272R and 291H) in the ED V protein. These conversions are ED strain V-specific, since the authentic V sequence is conserved in other strains. We then constructed R51K, P83S, P97S, H110Y, G225E, R272C and H291Y mutants of ED V protein and examined the effects of these mutants on MDA5-induced IFN-β promoter activity (Fig. 5A). As shown in Fig. 5A, only R272C mutant of ED V protein suppressed MDA5-activated IFN-β promoter. Next, we examined whether the V protein inhibited polyI:C-induced IRF-3 nuclear translocation. Although WT ED V protein did not inhibit polyI:C-induced IRF-3 nuclear translocation, overexpression of R272C mutant suppressed IRF-3 nuclear translocation (Fig. 5B and C). R51K, P83S, P97S, H110Y, G225E and H291Y mutants did not affect IRF-3 nuclear translocation. Since previous reports have shown that the V proteins of paramyxoviruses interacted with MDA5 to inhibit MDA5 activity and suppress IFN-β induction (Childs et al., 2007, 2009), we examined the interaction between MDA5 and the V proteins by immunoprecipitation. As expected, only R272C mutant interacted with MDA5, whereas WT ED V and the other mutants did not bind MDA5 (Fig. 5D). These data suggest that the arginine at position 272 in ED V protein is responsible for insuppressible activity of MDA5-induced IFN-β promoter activation. The cysteine residue at position 272 of V protein is conserved among paramyxoviruses. To clarify that 272C is important for suppressive activity of WT V protein, we examined effects of IC-B V C272R mutant on MDA5-induced IFN-β promoter activity. As shown in Fig. 6A, although IC-B V protein suppressed IFN-β promoter activity, C272R mutant was not able to inhibit IFN-β promoter activation. Similarly, C272R mutant did not suppressed poly I:C-induced IRF-3 nuclear translocation and interact with MDA5 (Fig. 6B and C). These data infer that the 272C residue of V protein is crucial for interacting with MDA5 and suppressing IRF-3 activation, which reasons that ED V strains fail to interact with MDA5.

4. Discussion

In this study, we demonstrated that the V protein of MV strain ED neither interacted with MDA5 nor suppressed MDA5-induced IRF-3 activation. A C272R mutation in the cysteine-rich region of wild-type V protein rendered the V protein IFN-insuppressible and the R272C conversion in ED strains conferred an IFN-suppressive function on the V protein. The V protein targets MDA5 and V proteins possessing the 272C residue co-precipitate with MDA5 by immunoprecipitation. Only V proteins possessing the 272C residue accelerate nuclear translocation of IRF-3. Based on the results of our reporter assay, V protein does not affect TICAM-1- or RIG-I-induced IFN-β promoter activation. Hence, the 272C residue is crucial for the V protein to block MDA5 function and MDA5 is the molecule which V protein targets for inhibition of the initial induction of IFN-β. For this reason, the ED strain used in this study allowed infected cells to induce IFN-β mRNA even in the absence of DI RNA. A previous report showed that the V protein of Sendai virus binds MDA5 via the cysteine-rich region which is conserved among paramyxoviruses (Childs et al., 2009). Accordingly, we found that the MV V protein interacted with MDA5 via the cysteine-rich region.

Fig. 4. Comparison of the ED V protein amino acid sequences with various MV strain. Several point mutations were found in the ED-V protein. Underline shows the conserved-Cys-rich region. Arrow heads show mutations in ED V protein.

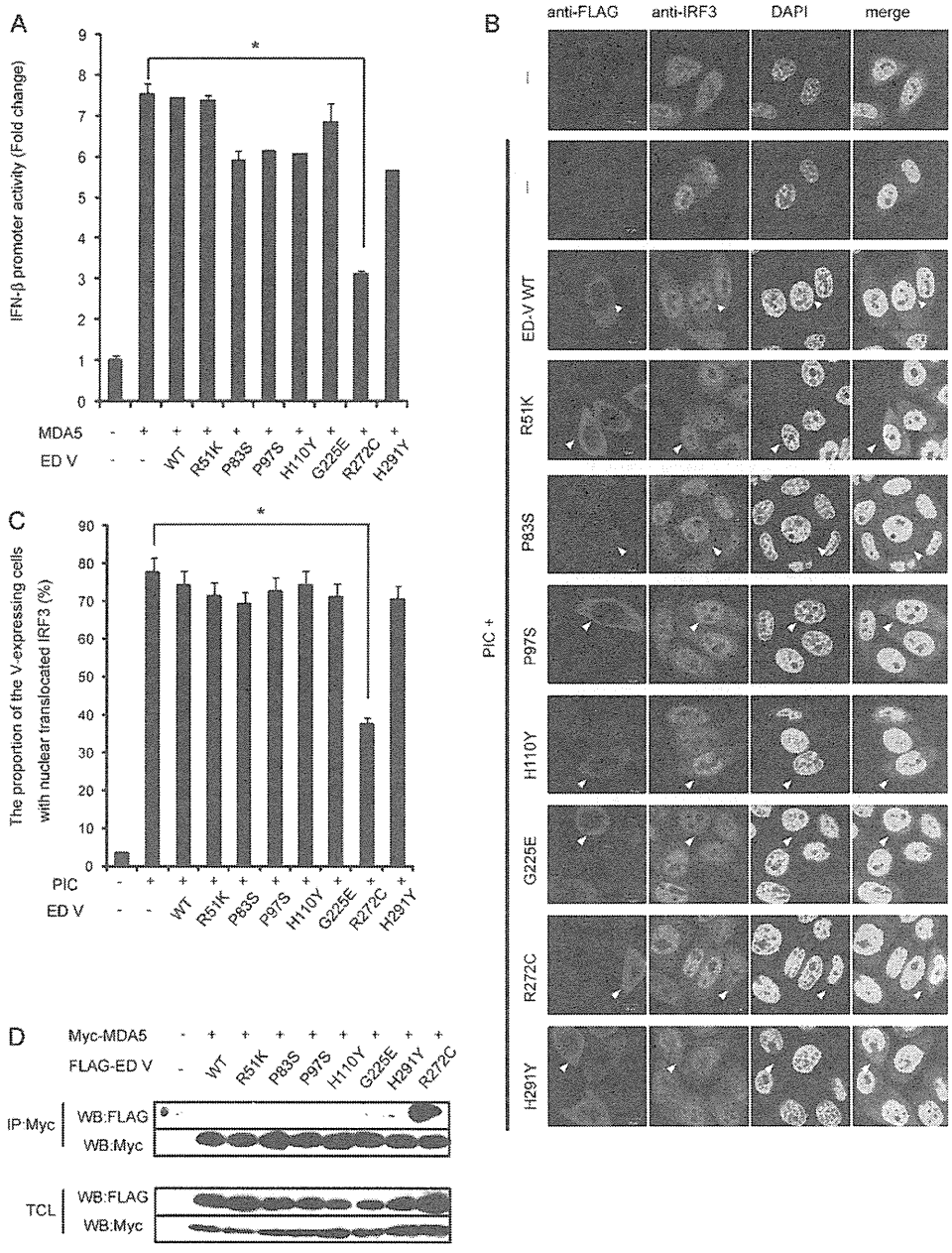

Fig. 5. 272C is a critical residue for suppression of MDA5-induced IFN-β promoter activity. (A) A549 cells in 24-well plates were transfected with pEF-BOS FLAG-MDA5 and pCMV10-MV-V together with the IFN-β promoter reporter and phRL-TK. Twenty-four hours after transfection, the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. $^*p < 0.05$. (B) HeLa cells were transfected with various pCMV10 ED-V plasmids. After 24 h, the cells were stimulated with 10 μg/ml polyI:C for 1 h, fixed and stained with anti-IRF-3 and anti-FLAG antibodies (V protein), and visualized with either Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies. The same slide was also treated with DAPI for the staining of nuclei. Arrow heads show V-expressing cells. (C) The number of the V-expressing cells with nuclear translocated IRF3 (see panel B) were counted. The results are shown by the proportion of the V-expressing cells with nuclear translocated IRF3 ($n = 50$). The average proportions from three independent assays are shown. $^*p < 0.05$. (D) HEK293FT cells were transfected with pcDNA4 Myc-MDA5 and pCMV10 FLAG-MV-V with mutations. After 24 h, the cells were lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc or anti-FLAG antibodies. An aliquot of each total cell lysate (TCL) was immunoblotted with either anti-Myc or anti-FLAG antibodies.

Childs et al. (2009) reported that the V protein of paramyxovirus specifically inhibited activation of the MDA5 pathway, but not the RIG-I pathway, by specifically binding to the helicase domain of MDA5 and hindering MDA5 from recruiting dsRNA. Consistent with their report, the V protein thus blocks sensing dsRNA via MDA5 to disassemble oligomerization of MDA5. These results infer that the IFN-inducible properties of the laboratory-adapted ED strain were largely attributable to the aberrance of the function of the V protein by introduction of the C272R mutation. We only regret that we could not detect the complex of endogenous MDA5 and MV V in this study since resting cells express only a trace amount of MDA5 (Yoneyama et al., 2005).

Ohno et al. (2004) showed that the 110Y and 272C residues of the V protein were responsible for the suppression of IFN-α and IFN receptor signaling using HEK293 transfectants. In contrast, we clarified that C272R mutant but H110Y mutant of ED V protein sup-

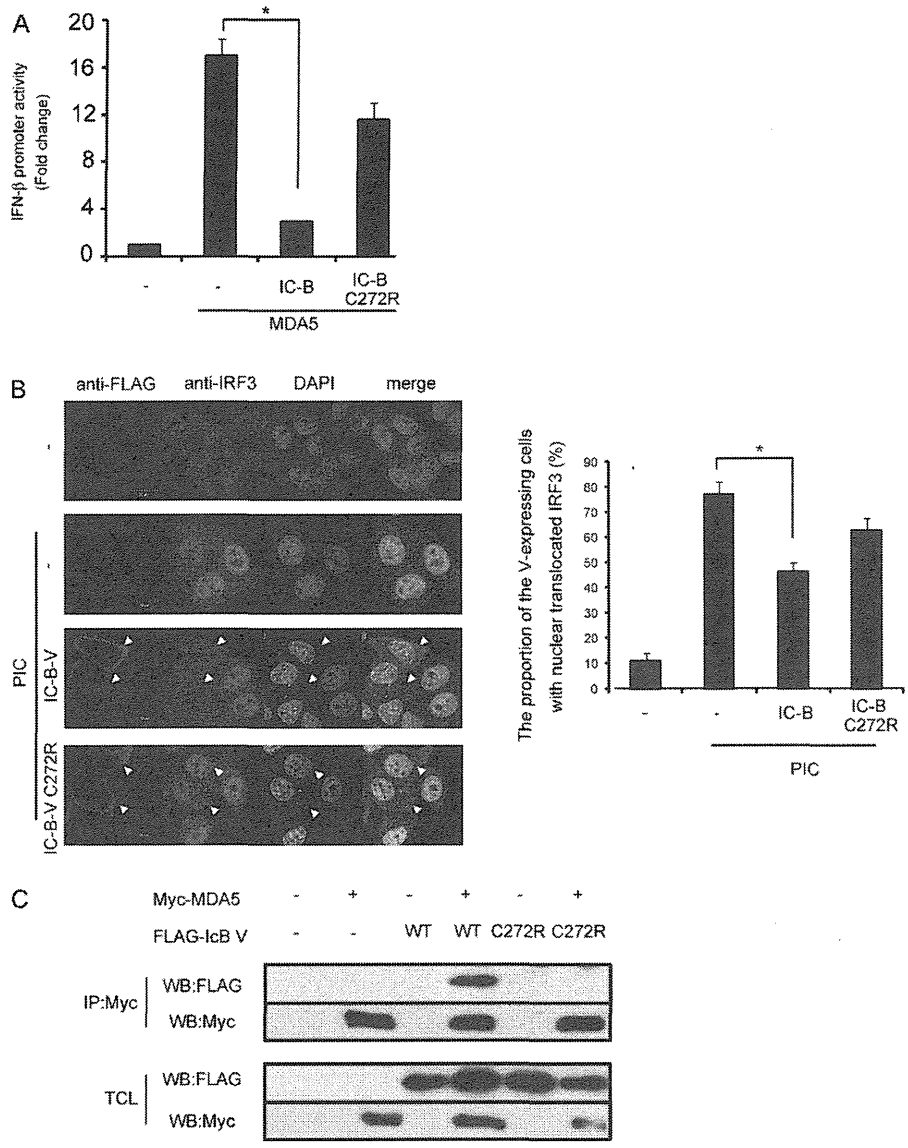

Fig. 6. 272C is important for suppressive activity of WT V protein. (A) A549 cells in 24-well plates were transfected with pEF-BOS FLAG-MDA5 and various pCMV10-IC-B-V plasmids together with the IFN-β promoter reporter and phRL-TK. Twenty-four hours after transfection, the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. *$p < 0.05$. (B) HeLa cells were transfected with various pCMV10 ED-V plasmids. After 24 h, the cells were stimulated with 10 μg/ml polyI:C for 1 h, fixed and stained with anti-IRF-3 and anti-FLAG antibodies (V protein), and visualized with either Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies. The same slide was also treated with DAPI for the staining of nuclei. Arrow heads show V-expressing cells. Right panel shows the proportion of the V-expressing cells with nuclear translocated IRF3. (C) Immunoprecipitation assay in 293T cells. Cells were transfected with pcDNA4 Myc-MDA5 and pCMV10 FLAG-MV-V. After 24 h, the cells were lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc or anti-FLAG antibodies. An aliquot of each total cell lysate (TCL) was immunoblotted with either anti-Myc or anti-FLAG antibodies.

pressed IFN-β promoter activity in the MDA5 pathway. Hence, the tyrosine at position 110 is responsible only for blocking the IFN amplification pathway via IFN-α/β receptor (IFNAR). On the other hand, the cysteine residue at position 272 is important for inhibiting both MDA5-induced IFN-β transactivation and IFNAR amplification loop. The V protein of strain ED is unable to block not only MDA5 but also the IFNAR amplification pathway, thereby ED-based vaccine strains would be able to induce type I IFN. Consistent with this possibility, Ikegame et al. (2010) reported the participation of MDA5 in MV-mediated IFN induction and MV growth promotion using RIG-I-silenced cells and V protein-deficient MV strains. In fact, the V proteins of ED and wild-type strains play no role in blocking the downstream of TBK1 for IFN-β reporter activation (data not shown). However, we wonder if the viruses produce sufficient amounts of long dsRNA (>40 bp in length, enough to be detected by J2 mAb) to be recognized by MDA5 in an early step of infection, i.e. before the production of V protein. Since RIG-I recognizes 5′-3P-ssRNA or short dsRNA, the RIG-I pathway is thought to be predominantly involved in IFN induction in MV-infected cells (Plumet et al., 2007; Shingai et al., 2007). Detailed analysis will be required to elucidate the predominant usage of RIG-I or MDA5 for type I IFN induction in cells infected with a variety of viruses. Why MV blocks MDA5 but not RIG-I activity and which viral products specifically recognize and bind MDA5 are questions that remained to be answered.

The C protein of MV plays an important role in inhibiting the JAK-STAT pathway of IFNAR signaling (Shaffer et al., 2003), and also acts

as a regulator of viral RNA synthesis, thereby indirectly suppressing IFN induction (Nakatsu et al., 2006, 2008; Takeuchi et al., 2005). MV mutants that fail to express the C protein allow infected cells to generate dsRNA (Ikegame et al., 2010), suggesting that the C protein may also function in controlling the generation of long dsRNA. In this study, we observed that the forced expression of C protein did not affect polyI:C-, RIG-I- and MDA5-induced IFN-β reporter activity and there were no significant amino acid changes in this protein among wild-type and vaccine strains (data not shown). C protein appears neither to directly affect the IFN-inducing pathways, nor to be responsible for the IFN-induction of vaccine strains. An interesting issue is the relationship between activation of the MDA5 pathway by MV vaccine strains and the limited production of long dsRNA due to the function of the C protein.

In conclusion, our data suggest that the C272R mutation in the V protein in MV strains is a major cause of insuppressible IFN production in a certain case of MV infection and that the 272C residue of the V protein is responsible for the MDA5-blocking ability of wild-type MV. Although RIG-I recognizes MV products including DI RNA or 5′-3P-ssRNA, the initial response of MDA5 also acts as a cause for amplifying type I IFN production, at least in some vaccine strains.

Conflict of interest

There is no conflict of interest in this study.

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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

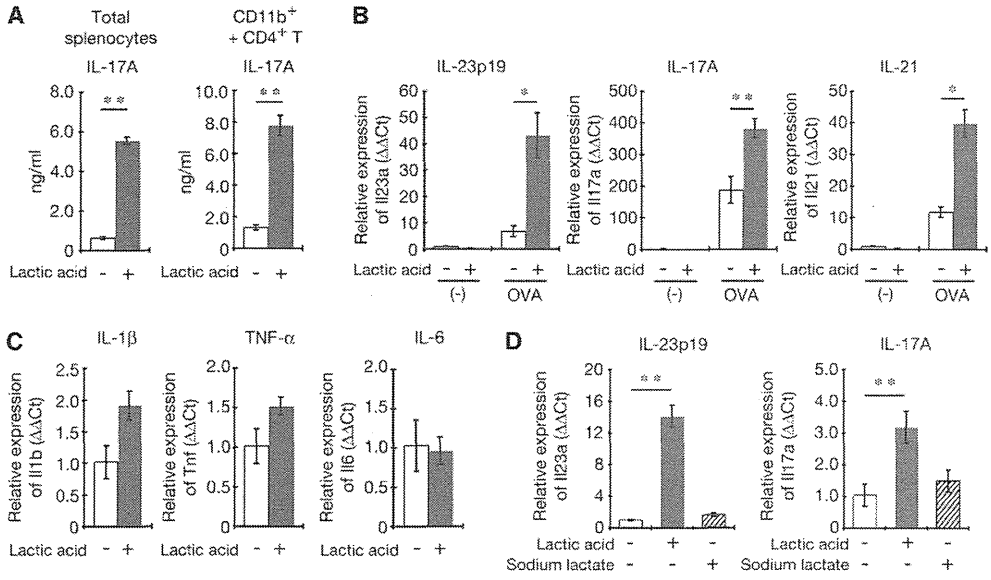

Fig. 1. The effect of lactic acid in a co-culture of fractionated CD11b$^+$ cells and CD4$^+$ T cells. (A) CD11b$^+$ cells and CD4$^+$ T cells derived from wild-type and OT-II splenocytes, respectively, were co-cultured in the presence (gray bars) or absence (white bars) of 15 mM lactic acid together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

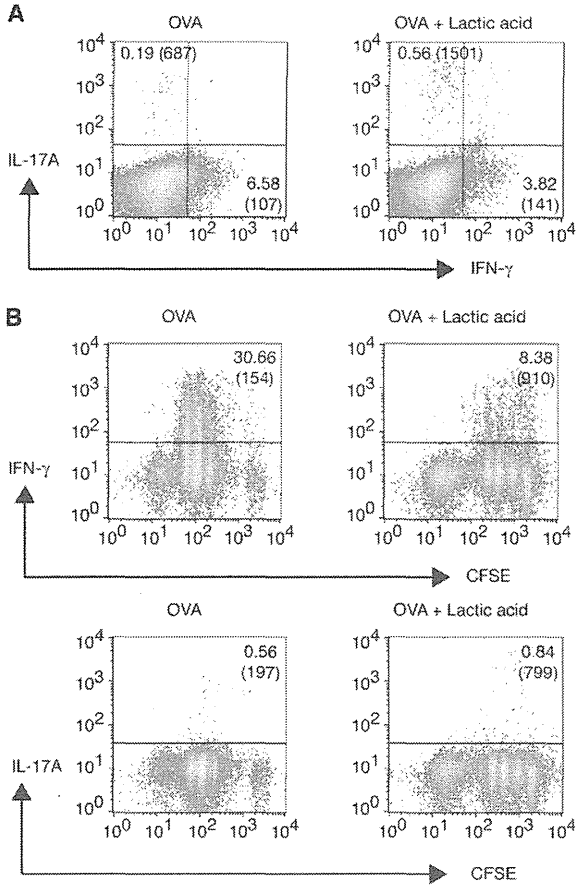

Fig. 2. Effect of lactic acid on the proportion and proliferation of IL-17A- and IFN-γ-producing cells. (A) $CD4^+$ T cells were co-cultured with $CD11b^+$ cells and stimulated with OVA peptide in the presence (right plot) or absence (left plot) of lactic acid for 4.5 days. T cells were then re-stimulated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

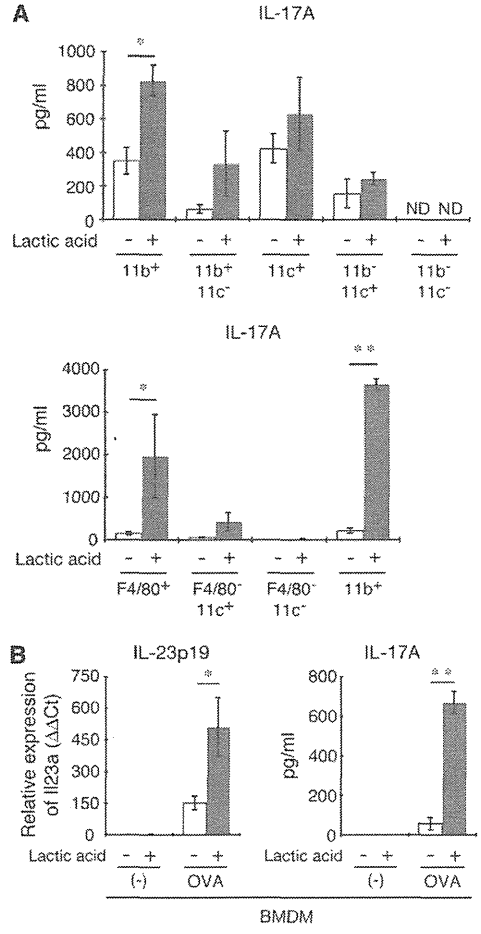

Fig. 3. Monocytes/macrophages are involved in lactic acid-enhanced, antigen-dependent, IL-17A production from $CD4^+$ T cells. (A) Splenic APCs were fractionated by using the cell surface markers CD11b ($11b^+$), CD11c ($11c^+$) and F4/80 ($F4/80^-$), as indicated on the *x*-axis. Each fraction was co-cultured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

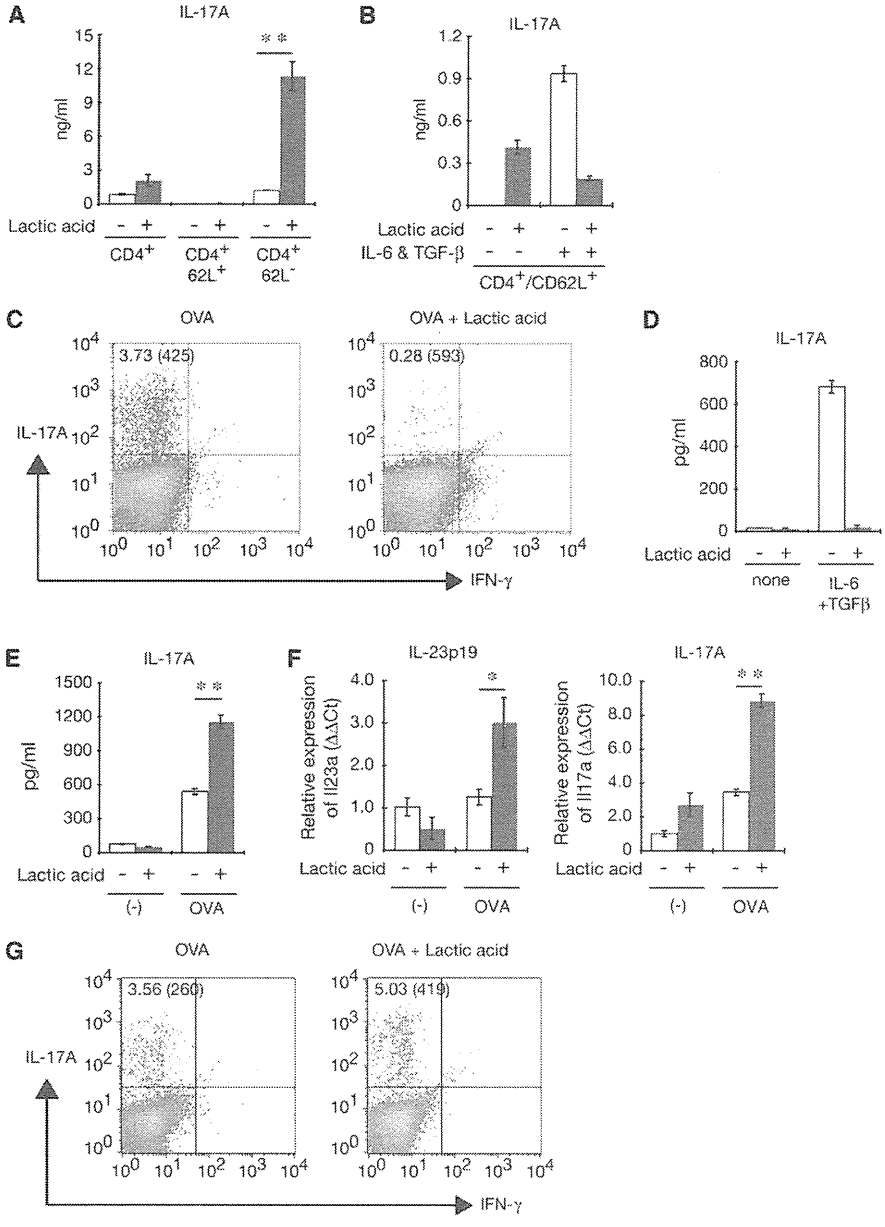

Fig. 4. Lactic acid induces IL-17A production from effector/memory $CD4^+$ T and T_h17 cells but inhibits differentiation of T_h17 from naive T cells. (A) Total $CD4^+$, $CD4^+CD62L^+$ (naive) or $CD4^+CD62L^-$ (effector/memory) T cells derived from OT-II mouse spleens were mixed with $CD11b^+$ cells 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

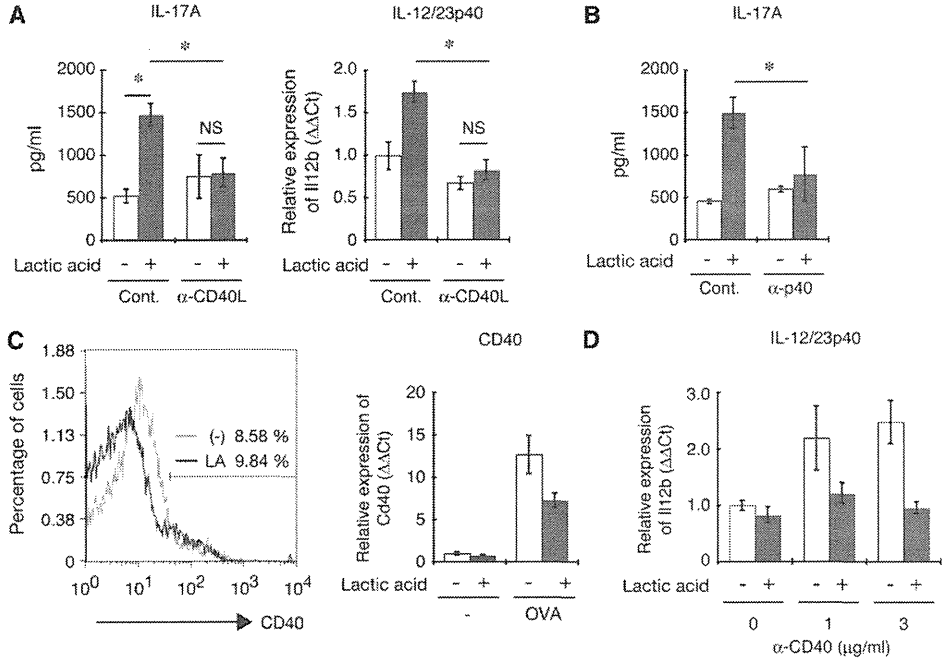

Fig. 5. CD40L–CD40 interactions are involved in the increased production of IL-17A via IL-12/23p40 expression. (A) $CD11b^+$ and OT-II $CD4^+$ T cells were co-cultured at a 1:1 ratio and stimulated with (gray bars) or without (white bars) lactic acid in the presence of anti-CD40L (α-CD40L) or isotype 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

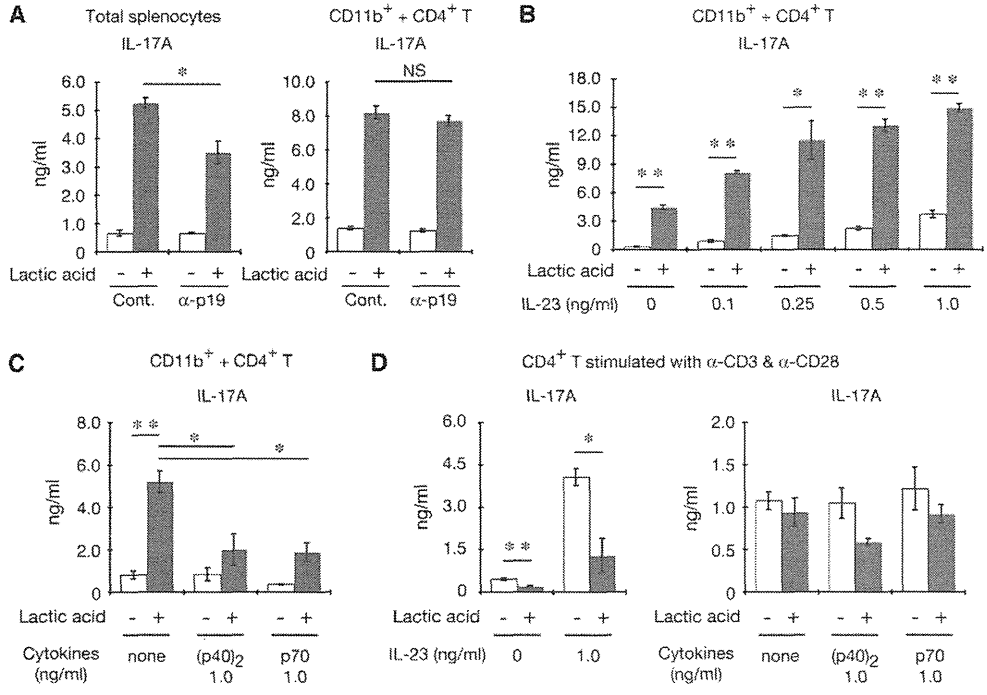

Fig. 6. The activation of IL-23-dependent and -independent pathway by lactic acid. (A) The effect of anti-IL-23 antibodies on the increased production of IL-17A in total OT-II splenocytes (left) or the co-culture of CD11b$^+$ and OT-II CD4$^+$ T cells (right). In the presence of anti-IL-23p19 (α-p19) 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

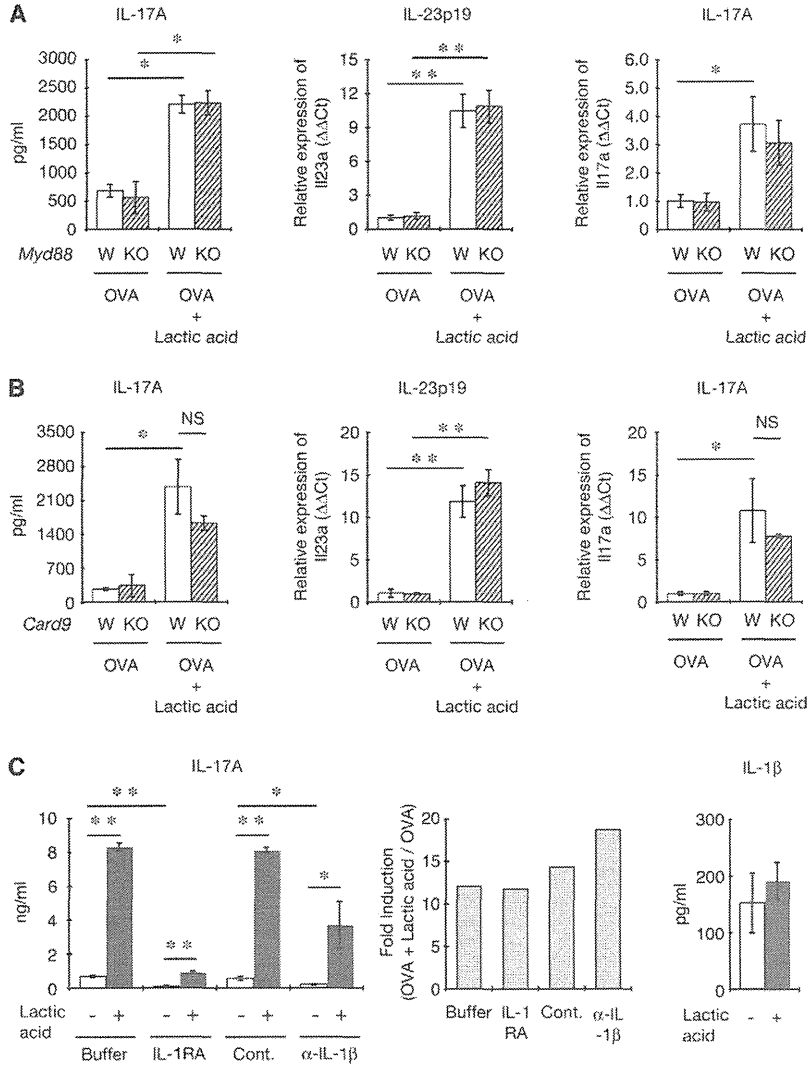

Fig. 7. Lactic acid enhances IL-17A production independently of MyD88, CARD9 and IL-1β. (A) and (B) $CD11b^+$ cells derived from *MyD88* (*Myd88*$^{-/-}$) (A), *Card9* (*Card9*$^{-/-}$) (B) deficient mice (KO, hatched bars) or their littermate controls (W, white bars) were mixed with OT-II $CD4^+$ T 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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Long-term persistent GBV-B infection and development of a chronic and progressive hepatitis C-like disease in marmosets

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It has been shown that infection of GB virus B (GBV-B), which is closely related to hepatitis C virus, develops acute self-resolving hepatitis in tamarins. In this study we sought to examine longitudinally the dynamics of viral and immunological status following GBV-B infection of marmosets and tamarins. Surprisingly, two of four marmosets but not tamarins experimentally challenged with GBV-B developed long-term chronic infection with fluctuated viremia, recurrent increase of alanine aminotransferase and plateaued titers of the antiviral antibodies, which was comparable to chronic hepatitis C in humans. Moreover, one of the chronically infected marmosets developed an acute exacerbation of chronic hepatitis as revealed by biochemical, histological, and immunopathological analyses. Of note, periodical analyses of the viral genomes in these marmosets indicated frequent and selective non-synonymous mutations, suggesting efficient evasion of the virus from antiviral immune pressure. These results demonstrated for the first time that GBV-B could induce chronic hepatitis C-like disease in marmosets and that the outcome of the viral infection and disease progression may depend on the differences between species and individuals.

Keywords: GBV-B, HCV, marmoset, tamarin, hepatitis C

INTRODUCTION

Among the known viruses, GB virus B (GBV-B) is closely related to hepatitis C virus (HCV), with 25–30% homology at the amino acid level, and is tentatively classified in *Hepacivirus* genus of *Flavivirus* family (Muerhoff et al., 1995; Simons et al., 1995; Ohba et al., 1996). Due to limited epidemiological analyses, the natural host(s) and prevalence of GBV-B have remained to be determined.

Hepatitis C virus is a major causative agent for non-A, non-B hepatitis. HCV is globally disseminated and estimated to be carried by more than 170 million people (Chisari, 2005; Lavanchy, 2009). Most HCV-infected individuals develop chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997; Seeff and Hoofnagle, 2002; Rehermann and Nascimbeni, 2005). Since standard therapy with PEGylated interferon and ribavirin is effective for only about 50% of patients, it is crucial to develop more effective therapeutics (Feld and Hoofnagle, 2005; Melnikova, 2008). The only validated animal model for HCV infection is the chimpanzees. This model has been valuable for determining important aspects of this disease, including the relationship between the virus and the antiviral immune responses of the host and the process of viral pathogenesis (Bukh, 2004; Akari et al., 2009; Boonstra et al., 2009). However, chimpanzees are endangered and present ethical complications and the availability of these experimental animals is severely restricted.

When tamarins (members of the New World monkeys) are infected with GBV-B, they generally develop acute viremia and self-resolving hepatitis as indicated by increases in the levels of serum enzymes such as alanine aminotransferase (ALT) (Bukh et al., 1999; Beames et al., 2000; Beames et al., 2001; Sbardellati et al., 2001; Lanford et al., 2003; Martin et al., 2003; Bright et al., 2004; Jacob et al., 2004; Nam et al., 2004; Kyuregyan et al., 2005; Ishii et al., 2007; Weatherford et al., 2009). Thus, the monkeys have been proposed as a surrogate model of HCV infection of chimpanzee and humans. However, a major hurdle for the development of a monkey-based surrogate model is the difficulties encountered in obtaining chronically infected monkeys that exhibit progression of chronic hepatitis C-like diseases (Martin et al., 2003; Nam et al., 2004; Takikawa et al., 2010).

Abbreviations: ALT, alanine aminotransferase; GBV-B, GB virus B; HCV, hepatitis C virus; HE, hematoxylin and eosin; p.i., post infection.