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 Th17 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 Th17 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 Th1 and Th17 cells, Th17 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.

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 (Th17 cells), CD8+ T cells, γδT cells and natural killer T cells (9). Th17 cells differentiate from naive Th1 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 secrete (13). IL-17A production from Th17 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\(_{h}17\) cells (14) and in the maintenance of the T\(_{h}17\) 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 oxidation 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-\(\gamma\). 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 effecter/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, \(\gamma\)T\(\delta\)TCR transgenic (OT-II) mice (26) and Myd88 knockout mice (27) were kindly provided by Dr W. R. Heath (The Walter and Eliza Hall Institute of Medical Research) and Dr Shizuo Akira (Osaka University), respectively. Card9 knockout mice were previously characterized (28). All mice were maintained under specific pathogen-free conditions in the Osaka Medical Center animal facility. All animal experiments were performed in accordance with institutional guidelines and approved by the Animal Care and Use Committee of the Osaka Medical Center.

**Reagents and antibodies**

L-lactic acid was purchased from Sigma–Aldrich (St Louis, MO, USA), sodium lactate from WAKO Pure Chemical (Osaka, Japan), OVA \(_{323-335}\) peptide from Bio Synthesis (Lewisville, TX, USA) and phorbol-12-myristate 13-acetate (PMA) and ionomycin from Merck Biosciences (Darmstadt, Germany). Anti-mouse cytokine antibodies (10 \(\mu\)g ml\(^{-1}\)) were used to neutralize IL-12/23p40 (C17.8; ebioscience, San Diego, CA, USA), IL-23p19 (G23-8; ebioscience) and IL-1\(\beta\) (B122; ebioscience). Rat IgG2a (eBR2a; ebioscience), rat IgG1, and Armenian hamster IgG (eBio299Arm; ebioscience) were used as isotype-matched control antibodies for IL-12/23p40, IL-23p19 and IL-1\(\beta\). RmIL-12p70 (RmIL-12p70) 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), rmlIL-12p40 homodimer (Biolegend, San Diego, CA, USA) and rml-23 (R&D systems, Minneapolis, MN, USA) were used as cytokines containing the IL-12/23p40 subunit. The IL-1 receptor antagonist (IL-1RA, 0.25 \(\mu\)g ml\(^{-1}\); R&D systems) was used to block the effects of IL-1.

**Cell cultures**

Cells isolated from splenocytes of C57BL/6J and OT-II mice were cultured in RPMI1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 \(\mu\)g ml\(^{-1}\) streptomycin. All cells were cultured at 37\(^\circ\)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 Biotech, 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 effecter/memory CD4\(^+\) T cells were purified by negative selection of CD4\(^+\) T cells with a CD4\(^+\) T cell isolation kit (Miltenyi Biotech), followed by positive selection with anti-CD62L microbeads.
acid. On day 5, the differentiated cells were re-stimulated for 3 days before harvesting on day 5 for assays. Bone marrow-derived macrophages (BMDMs) and bone marrow-derived dendritic cells (BMDCs) were induced as described above. To examine intracellular cytokine production, T cells were stimulated with plate-coated anti-CD3 and anti-CD28 antibodies and then stained 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 x 10^5 cells) was mixed with 1 x 10^5 of OT-II CD4^+ T or in vitro-differentiated T17 cells in a round bottom 96-well cell culture microplate. BMDMs and BMDCs (1 x 10^5 cells) were mixed with 5 x 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 x 10^5) were stimulated with plate-coated anti-CD3 and anti-CD28 antibodies in the presence of cytokines containing the IL-12/23p19 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 & Signal, 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.

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 (21), lactate does not activate the IL-23p19 promoter. Therefore, we hypothesized that sodium lactate functions intracellularly in this co-culture system of CD11b^+ and CD4^+ cells, we examined the effects of sodium lactate in...
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 IFN-γ (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 (6.5% to 3.8%). 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 mouse 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.
Lactic acid induces the production 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. 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 of brefeldin A 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<sub>r</sub>17 cells but not naive T cells or T<sub>r</sub>17 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<sub>r</sub>17 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<sub>r</sub>17 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\textsubscript{h}17 cells but inhibits differentiation of T\textsubscript{h}17 from naive T cells. (A) Total CD4\textsuperscript{+}, CD4\textsuperscript{+}CD62L\textsuperscript{+} (naive) or CD4\textsuperscript{+}CD62L\textsuperscript{-} (effector/memory) T cells derived from OT-II mouse spleens were mixed with CD11b\textsuperscript{+} 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\textsuperscript{+} T cells, but not naive CD4\textsuperscript{+} T cells, produced IL-17A by lactic acid stimulation. (B and C) In the presence of mouse IL-6 and human TGF-\textbeta, purified naive CD4\textsuperscript{+}CD62L\textsuperscript{+} T cells were co-cultured with OVA peptide-loaded CD11b\textsuperscript{+} cells and stimulated with or without lactic acid for 4 days for IL-17A production or 4.5 days for T\textsubscript{h}17 differentiation. Amounts of IL-17A production are shown in (B). IL-17A-producing CD4\textsuperscript{+} T cells were stained for CD4, IFN-\gamma and IL-17A and observed by FACS analysis (C). Plots gated on CD4\textsuperscript{+} cells are shown. (D) Purified naive T cells were stimulated with plate-coated anti-CD3 and anti-CD28 antibodies with (right bars) or without (left bars) IL-6 and TGF-\textbeta in the presence (gray bars) or absence (white bars) of lactic acid for 4 days. Lactic acid strongly suppressed differentiation into T\textsubscript{h}17 cells. (E) Naive CD4\textsuperscript{+} T cells derived from OT-II mice were differentiated into T\textsubscript{h}17 cells in vitro. T\textsubscript{h}17 cells were mixed with CD11b\textsuperscript{+} 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\textsubscript{h}17 cells were co-cultured with CD11b\textsuperscript{+} 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\textsubscript{h}17 cells were co-cultured with CD11b\textsuperscript{+} cells for 4.5 days and observed by FACS analysis. Plots gated on CD4\textsuperscript{+} cells are shown. Numbers in plots indicate percentages (MFI) of IL-17A\textsuperscript{+} 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-CD3c and anti-CD28 antibodies (Fig. 4D). These results suggest that lactic acid negatively regulates the differentiation of naive CD4+ T cells into Th17 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 Th17 cells. In addition, Th17 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 Th17 cells and in the MFI, respectively (Fig. 4G). These data indicate that lactic acid is not involved in Th17 cell differentiation but that it enhances IL-17A production from effector/memory T cells and in vitro-differentiated Th17 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](https://academic.oup.com/intimm/article-abstract/23/1/29/661466/1466) by guest on 08 March 2019
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-23 p19 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 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-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 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).

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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...
containing protein 3)-inflammasome pathways are activated by 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 Tئئ7 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). Furthermore, 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 Tئئ7 cells, but not naive CD4+ T cells. Meanwhile, lactic acid strongly inhibited differentiation of Tئئ7 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 Tئئ1 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 Tₘ17 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, Samuel 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 Tₘ17 cell differentiation and maintains the expression of IL-17A in Tₘ17 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 adenylyl 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 Tₘ17 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**


