Methotrexate enhances antigen presentation and maturation of tumour antigen-loaded dendritic cells through NLRP3 inflammasome activation: a strategy for dendritic cell-based cancer vaccine

Gao-Na Shi, Min Hu, Chengjuan Chen, Junmin Fu, Shuai Shao, Yu Zhou, Lei Wu and Tiantai Zhang

Abstract

Background: Dendritic cells (DCs) are antigen-presenting cells that play a pivotal role in adaptive cell-mediated immunity by priming and activating T cells against specific tumour and pathogenic antigens. Methotrexate (MTX), a folate derivative, functions as an immunoregulatory agent. However, the possible effect of MTX on tumour antigen-loaded DCs has not yet been investigated.

Methods: We analysed the effect of MTX on the maturation and function of DCs along with tumour cell lysates (TCLs). Using bone marrow-derived DCs, we investigated the effect of MTX combined TCL-loaded DCs on T cells priming and proliferation. We also tested the anti-tumour immune effect on DCs when treated with MTX and/or TCL *in vivo*.

Results: MTX combined with TCL not only enhanced DC maturation and stimulated cytokine release but also promoted CD8$^+$ T cell activation and proliferation. The latter was associated with increased tumour antigen uptake and cross-presentation to T cells. Mechanistically, DC maturation and antigen presentation were partly modulated by NLRP3 inflammasome activation. Furthermore, immunisation of mice with MTX and TCL-pulsed DCs before a tumour challenge significantly delayed tumour onset and retarded its growth. This protective effect was due to priming of IFN-γ releasing CD8$^+$ T cells and enhanced killing of tumour cells by cytotoxic T lymphocytes isolated from these immunised mice.

Conclusion: MTX can function as a potent adjuvant in DC vaccines by increasing antigen presentation and T cell priming. Our findings provide a new strategy for the application of DC-based anti-tumour immunotherapy.

Keywords: antigen presentation, dendritic cells, IFN-γ releasing CD8$^+$ T cells, methotrexate, NLRP3 inflammasome

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Background

As a bridge between innate and adaptive immunity, dendritic cells (DCs) play critical roles in the initiation and regulation of both innate and acquired immunity.[1,2] DCs can enhance or inhibit host inflammatory responses, and their suppression might result in increased propensity to infection.[3] Antigen-loaded mature DCs have been successfully prepared *in vitro* by stimulating human peripheral blood monocytes with appropriate cytokines and growth factors, and exposing them to tumour antigens and stimulating them with adjuvants.[4,5] These cellular "vaccines" can elicit anti-tumour immune responses in cancer

patients, which make them highly promising tools for anti-tumour immunotherapy.[3] The key factor determining the efficacy of any vaccine is the generation of cell-mediated immunity, which in turn depends on antigen cross-presentation by mature DCs, and the optimal priming of antigen-specific CD8^{+} T cells.[6,7] Efficient antigen presentation by DCs requires the DCs to be mature, which can be triggered by various TLR ligands, including LPS, poly (I:C), and CpG.[8–10] These stimuli upregulate the surface co-stimulatory molecules CD80, CD86, and CD40,[11,12] which activate the downstream molecules, NF-κB and MAPK signalling pathways, resulting in the release of pro-inflammatory factors such as IL-12p70, TNF-α, and IL-6.[13–15] In addition, the nucleotide-binding domain like receptor protein 3 (NLRP3), also known as NALP3, can also induce DC maturation.[16] This pathway culminates in the release of the pleiotropic pro-inflammatory cytokine IL-1β,[17] followed by the production of pro-IL-1β and its cleavage into the "mature" form by caspase-1.[18] In the immune system, IL-1β, a potent pro-inflammatory cytokine, has multiple effects.[17] Although, in chronic inflammation, it is considered that IL-1β may promote tumour growth, Ghiringhelli *et al.* found that the production of IL-1β by DCs is pivotal for CD8^{+} T cell polarisation for IFN-γ production, and the function of IL-1β on CD8^{+} T cells may contribute to the anti-tumour immune response.[19] Therefore, it is essential to augment DC activation combined with antigen exposure through suitable signals and antigens.[20,21]

Currently, DC-based anti-tumour vaccination strategies have limited efficacy owing to insufficient antigen presentation and T cell priming.[22,23] This can be explained by the maturation state of DCs during the DC–T cell crosstalk. During transition from immature DCs to mature DCs, DCs require not only the antigen but also other signals to achieve a fully mature and activated state. Therefore, choosing the appropriate materials to promote the maturation of DCs for cancer-based vaccines is challenging.[3] Apart from the maturation status of DCs, another important point has still not received much attention, which is the ability to induce exogenous antigens presented by CD8^{+} T cell on priming. This process is referred to as antigen cross-presentation, which is important in the anti-tumour immune response. Additionally, there are a variety of DC subsets including plasmacytoid DCs, monocyte-derived DCs, Langerhans cells, and interstitial DCs. Clinical trials for the efficacy of these varying subtypes are limited.[24] Distinct DC subsets differ in their cross-presentation efficacies,[6] and likely work in a concerted manner, which makes specific DC targeting *in vivo* challenging. Therefore, it is essential to study the responses elicited by specific DC subsets, and focus on activating the pathways that can enhance antigen presentation by these cells. To this end, several studies conducted in recent years have focused on developing monocyte-derived DC vaccines *ex vivo*, which have demonstrated anti-tumour immune responses.[7–9]

Methotrexate (4-amino-10-methylfolic acid) is a derivative of folate with immunomodulatory, anti-metabolite, and chemotherapeutic properties.[25–28] Bonadonna *et al.* demonstrated its adjuvant effect on breast cancer chemotherapy thereby significantly prolonging survival.[29] Interestingly, Galina *et al.* reported that methotrexate (MTX) could upregulate the ability of DCs to present antigens to antigen-specific T cells through an unusual signal transduction pathway.[30] However, the action of MTX on the uptake and presentation of tumour antigens by DCs and the underlying mechanism are still incompletely understood. We hypothesised that MTX can enhance the anti-tumour immune response of antigen-exposed DCs through a specific signalling pathway. To this end, we tested the effect of MTX on the maturation and activity of bone marrow-derived dendritic cells (BMDCs) exposed to the lysates of B16 tumour cells. MTX enhanced DC maturation, antigen presentation, T cell priming and tumour growth inhibition *in vivo*, partly through the activation of the NLRP3 inflammasome. Our findings reveal a new mechanism of adjuvant-mediated DC activation and provide a strong rationale for using MTX in anti-tumour DC vaccine preparations.

Materials and methods

Agents, Abs and cytokines

Methotrexate hydrate (Cas: 59-05-2) was purchased from J&K Scientific, and MCC950 sodium (Cas: 256373-96-3) from Med Chem Express. All anti-mouse antibodies CD80-FITC (104706), CD86-PE (105008), CD11c-APC (117310), CD40-PE (124610), I-A/I-E-PerCP/Cyanine 5.5 (107626), CD3-APC (100312), CD4-FITC (100510), CD8a-PE (100708) and IFN-γ-APC (505810) were procured from BioLegend. Cell

Activation Cocktail (without Brefeldin A; 423301), PMA (20 ng/mL), ionomycin (1 μg/mL), Brefeldin A solution (1000×; 420601), and MojoSort™ Mouse CD8 T Cell Isolation Kit (480008) were also purchased from BioLegend. Rabbit mAbs against caspase-1 (D7F10; #3866), cleaved caspase-1 (Asp297) (D57A2; #4199), IL-1β (D6D6T; #31202), cleaved IL-1β (Asp116) (E7V2A, #63124), and NLRP3 (D4D8T; #15101) were purchased from Cell Signaling. Recombinant mouse IL-4 and GM-CSF were obtained from PeproTech, LPS (L2880) and lipopolysaccharides from *Escherichia coli* O55:B5 were purchased from Sigma, Imject Alum (77161) was obtained from Thermo Fisher Scientific, nigericin (Cas: 28380-24-7) was purchased from Med Chem Express. To rule out the possibility of endotoxin contamination during MTX preparation, the Toxin Sensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, NJ, USA) was used to determine the LPS content. The level of endotoxin in the MTX preparation was lower than 0.1 endotoxin unit/mL.

Animals and cell lines

Six- to eight-week-old female C57BL/6 mice were purchased from Huafukang (Beijing, China) and housed in a pathogen-free facility under controlled temperature, humidity and a 12 h light/dark cycle, with food and water available *ad libitum*. The animals were acclimatised for at least 1 week before the experiments. All animal experiments were approved by the Animal Care and Use Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (Approval No. 00004601). The *in vitro* experiments for DCs and T cells from animals were also approved by the Animal Care and Use Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (Approval No. 00005662). Mouse B16 melanoma tumour cell lines were kindly provided by Prof. Kong from Tianjin Key Laboratory of Biomaterial Research and cultured according to the manufacturer's guidelines. All cell lines were tested and confirmed to be free of *Mycoplasma* and other rodent pathogens by the China Centre for Type Culture Collection; no other authentication assay was performed.

Isolation and generation of tumour cell lysates

Tumour cell extracts were prepared according to previously described methods.[31,32] Briefly, B16 tumour cell pellets were collected and re-suspended in ice-cold PBS at a cell concentration of 1×10^7/mL. The cell suspension was frozen in liquid nitrogen for 5 min and then thawed at 37°C for another 5 min. This cycle was repeated five times. The resulting lysates were then centrifuged at 3000 rev/min for 15 min to remove cellular debris. The supernatants were collected, and the protein concentration was measured using the BCA method. Suitable dilutions of the lysates were prepared for *in vitro* and *in vivo* experiments.

Bone marrow-derived DC culture and cell analysis

The bone marrow cells were flushed out of the mice femur and tibia in PBS, centrifuged, and re-suspended in erythrocyte lysis buffer. After incubating for 2 min, the cell suspension was filtered through a 70-μm mesh. The single cells were re-suspended in the BMDC culture medium [RPMI 1640 supplemented with 10% FBS, sodium pyruvate, 50 μM 2-mercaptoethanol (Sigma), 10 mM HEPES and penicillin/streptomycin] containing 20 ng/mL recombinant mouse GM-CSF (PeproTech), 10 ng/mL recombinant mouse IL-4 (PeproTech) at a density of 5×10^6 cells/mL, and dispensed in 24-well plates. The medium was exchanged on day 3 to remove the free-floating cells, and fresh medium containing the suitable reagents was added on day 5. The BMDCs were harvested on day 7, and the supernatants were collected for enzyme-linked immunosorbent assay (ELISA).

T cell activation and proliferation assays

T cells were isolated from mouse lymph nodes, then, MojoSort™ Mouse CD8 T Cell Isolation Kit was used to isolate the CD8+ T cells and they were labelled with carboxyfluorescein succinimidyl amino ester (CFSE) (BioLegend) according to the manufacturer' instructions. MTX and/or tumour cell lysate (TCL)-loaded DCs were obtained by incubating the cells with either or both reagents for 48 h. Then the DCs in each treatment group were washed three times in PBS to exclude the MTX or TCL residue. For antigenic stimulation, 2×10^6 CFSE-labelled CD8+ T cells were incubated with control DCs, MTX-DCs, TCL-DCs, and MTX-TCL-DCs for 5 days. The cells were analysed by flow cytometry at the end of the stimulation period, and the supernatants were collected for ELISA.

DC vaccination

Six-week-old female C57BL/6 mice were inoculated subcutaneously with 2×10^6 control, MTX, and/or TCL-treated, or MCC950 + MTX-TCL-treated DCs on their left hind flanks on days −14, −13 and −7. On day 0, 3×10^5 B16 cells in 50 μL PBS were subcutaneously inoculated into the right hind flank. The mice were monitored every 2 days for pain or distress, tumour growth, and body weight. Tumour sizes were measured every 2 days using electronic calipers, and the volume was calculated as length (mm) × width (mm) × width (mm)/2. The mice were euthanised either on day 21 or when the tumours measured more than 20 mm on the longest axis. The spleens were excised and homogenised mechanically into single cells for further analysis.

Cytotoxic T lymphocytes (CTL) response assay

The CTL assay was performed as previously described.[31,33] Briefly, the lymphocytes (effector) obtained from the DC-immunised and tumour-challenged mice were cultured with B16 cells (target) at a ratio of 25:1, 50:1, and 100:1 [effector:target (E:T)]. After 72 h of co-culture, the supernatants were collected, and the amount of lactate dehydrogenase released by the lysed B16 cells was measured colorimetrically using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, G1780, Madison, USA). Cytotoxicity was evaluated in terms of percentage specific lysis $(E_{Spontaneous} - T_{Spontaneous})/(T_{Maximum} - T_{Spontaneous}) \times 100$

Flow cytometry analysis

The murine BMDCs were immunophenotyped by staining with antibodies against CD11c (clone N418), CD86 (clone GC-1), CD40 (clone 3123), MHC II (clone M5/114.15.2), and CD80 (clone 16-10A1), followed by flow cytometry analysis.

For intracellular cytokine staining, spleen cells were re-stimulated with ionomycin (1 μg/mL) and phorbol 12-myristate 13-acetate [PMA]) (20 ng/mL) for 4 h, blocked with Brefeldin A (BioLegend) for 2 h and then stained with anti-CD8a antibody (clone 53-6.7). After surface staining, the cells were permeabilised with Fix/Perm solution and stained with anti-IFN-γ antibody (clone XMG1.2).

Immature BMDCs at day 5 were cultured with TCL-fluorescein isothiocyanate (FITC), MTX plus with TCL-FITC, MCC950, and TCL-FITC plus with or without MTX at 37°C for 24 h. The mature BMDC cells were harvested and stained with antibodies against CD11c (clone N418), followed by flow cytometry analysis.

Flow cytometry analysis was performed with BD FACS verse™, and data were analysed using FlowJo software.

Measurement of secreted factors

Cell culture supernatants were assayed for IL-1β (BioLegend) and IFN-γ (eBiosciences) using ELISA kits according to the manufacturer's instructions. Lactate dehydrogenase (LDH) was measured using the CytoTox-ONETM Homogeneous Membrane Integrity Assay (Promega Corporation, G7891, Madison, USA) according to the manufacturer's instructions. The percentage of LDH release (%) was calculated as $(LDH_{Experimental} - LDH_{Medium})/(LDH_{TritonX-100-treated} - LDH_{Medium}) \times 100$.[34] Caspase-1 levels were determined using the Caspase-Glo® 1 Inflammasome Assay kit (Promega Corporation, G9951, Madison, USA) according to the manufacturer's instructions. Briefly, BMDCs were treated with MTX for 48 h with/without TCL, and lipopolysaccharide (LPS) plus nigericin (Nig) was added as a positive control and 50 μL supernatant per sample was incubated with 50 μL Caspase-Glo 1 reagent for 30 min. Luminescence was measured using a luminometer (BioTek).[35]

Confocal microscopy

TCL was labelled with a FITC tracker (Sigma-Aldrich) as previously described.[31] DCs were incubated with 50 μL FITC-TCL with/without MTX for 48 h, followed by 1 h incubation with lysotracker Red DND-26 (Life Technologies) at 37°C. The cells were then washed with PBS and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature, followed by three more washes with PBS. The labelled cells were counter-stained with 4',6-diamidino-2-phenylindole (Sigma), and observed under a confocal laser scanning microscope (Leica DMi8; Leica).

Western blot

Culture supernatants of DCs treated with MTX and TCL for 2 h were collected and precipitated with equal volumes of methanol and 1/4th volume of chloroform. The solutions were vortexed and centrifuged for 15 min at $20,000 \times g$, and the upper

phase was discarded. After adding 600 μL methanol to the interphase, the mixture was centrifuged for 15 min at 20,000 × g, and the resulting protein pellets were dried at 55°C and re-suspended in loading buffer. The cell pellets were homogenised with a cell lysis buffer containing Phenylmethylsulfonyl fluoride (PMSF), complete protease inhibitors and phosphatase inhibitors (Cell Signaling Technology). The lysates were quantified by BCA assay (Thermo Fisher Scientific), and equal amounts of proteins were mixed with the loading buffer. All protein samples were boiled for 5 min, separated using a 12 % SDS-PAGE gel and then transferred onto nitrocellulose membranes. The blots were incubated with primary antibodies against caspase-1, cleaved caspase-1, IL-1β, cleaved IL-1β, NLRP3, and β-actin at the recommended dilutions, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The bands were visualised using enhanced chemiluminescence reagents from Cell Signaling Technology and Abcam.

Statistical analysis

All values are expressed as the mean ± SEM. Each experiment was repeated at least three times. Data were analysed using the conventional Student's *t*-test, one-way/two-way analysis of variance for statistical evaluation. Data were analysed using GraphPad Prism 8 software. *p*-values of less than 0.05 were considered statistically significant.

Results

MTX promotes the transition of immature BMDCs into mature DCs in the presence of TCL

Previous studies have reported the direct immune-stimulatory effects of MTX on DCs *in vitro*.[20] We evaluated the effect of MTX on antigen processing and presentation by DCs, using B16 TCL as the source of tumour antigens. As shown in Figure 1, the presence of both MTX and TCL significantly increased the percentage of CD11c+CD80+ [Figure 1(a) and (e)], CD11c+CD86+ [Figure 1(b) and (f)], CD11c+MHCII+ [Figure 1(c) and (g)], and CD11c+CD40+ [Figure 1(d) and (h)] cells compared with the presence of either MTX or TCL alone, and the maturation state of DCs treated with MTX and TCL was similar to that of the LPS treatment group (positive control). There results suggest that the combination of MTX with TCL shifted immature DCs

phenotypically to mature DCs. Therefore, MTX along with tumour antigens promotes DC maturation and upregulates co-stimulatory molecules. Increasing the number of co-stimulatory molecules on DCs may represent the effect of the Ag-presenting capacity of DCs treated with MTX.

Increased release of cytokines and associated factors from DCs treated with MTX combined TCL

To further investigate the function of DCs prepared by MTX, we tested the cytokines in the supernatant of DCs using ELISA. The concentration of IFN-γ in the group treated with MTX-TCL was significantly higher than those treated alone or in the control group (no treatment with DMSO as a negative control), and there was a similar release of IFN-γ in DCs treated with LPS as a positive control [Figure 2(a)]. Surprisingly, the same result was found in the cytokine IL-1β, and the same result was observed in DCs treated with LPS combined with Alum adjuvant (also as a positive control) [Figure 2(b)]. Interestingly, we then evaluated the concentration of LDH (Promega) and caspase-1 (Promega). LDH concentration in the supernatant of MTX combined TCL treatment group was significantly higher than that of other control groups, which was the same level as the LPS combined Nig double-signals treatment group [Figure 2(c)]. IL-1β release DCs treated with MTX and TCL reflected caspase-1 activation. Caspase-1 activation was confirmed by the detection of caspase-1 (Caspase-Glo 1 Inflammasome Assay chemistry, Promega) in the medium of DCs treated with MTX and TCL but not TCL alone. The level of caspase-1 after treatment with MTX and TCL was similar to that after treatment with LPS combined with Nig [Figure 2(d)].

MTX enhances T cell activation and proliferation by TCL-loaded DCs

Since DC maturation is a prerequisite for effective antigen presentation and the ensuing immune response, we next evaluated the effect of MTX treatment on T cell proliferation. To this end, we established a mixed lymphocyte reaction system with differentially treated DCs and CD8+ T cells. As expected, T cells co-cultured with the tumour antigen and MTX-stimulated BMDCs showed significantly increased proliferation compared with that in those cultured with MTX or TCL-treated or control DCs [Figure 3(a) and (b)]. Furthermore, IFN-γ production by T cells
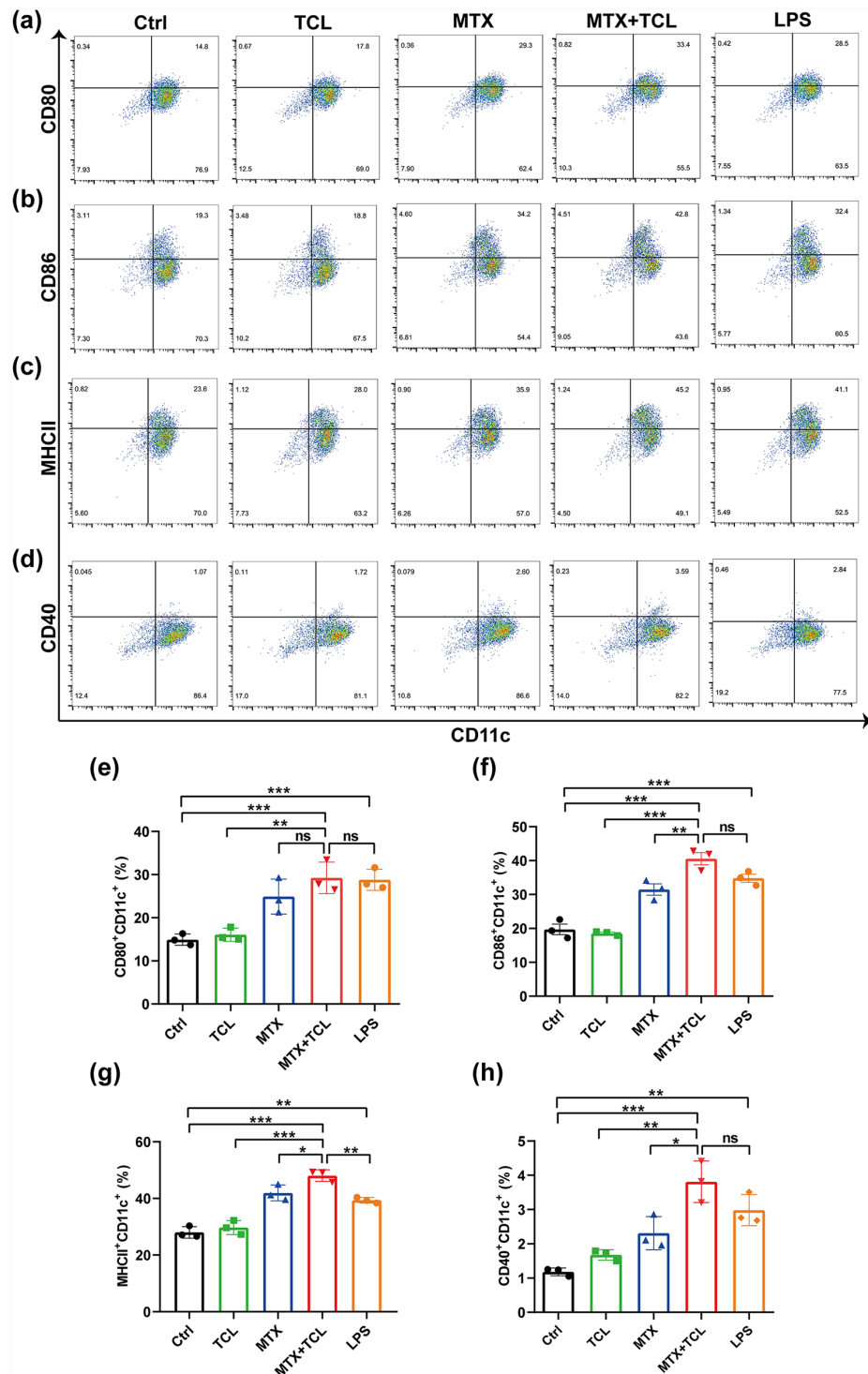
Figure 1. Enhanced maturation status of BMDCs after treatment with MTX *in vitro*. BMDCs were cultured for 5 days and then treated with TCL, MTX, MTX + TCL, and LPS for 48 h, LPS treatment group as the positive control. Cells were then stained with fluorescence-conjugated antibodies and subjected to flow cytometry analysis. Live cells were first gated and then plotted based on CD11c and CD80 expression (a), and percentage of CD11c+CD80+ (e), CD11c and CD86 expression (b), and percentage of CD11c+CD86+ (f), CD11c and MHCII expression (c), and percentage of CD11c+MHCII+ (g), CD11c and CD40 expression (d) and percentage of CD11c+CD40+ DCs (h) in each treatment group.

Data are shown as mean ± SEM of % positive incidence from three independent experiments.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

BMDC, bone marrow-derived dendritic cell; Ctrl, control; DC, dendritic cell; LPS, lipopolysaccharide; MTX, methotrexate; ns, no significant difference; TCL, tumour cell lysate.
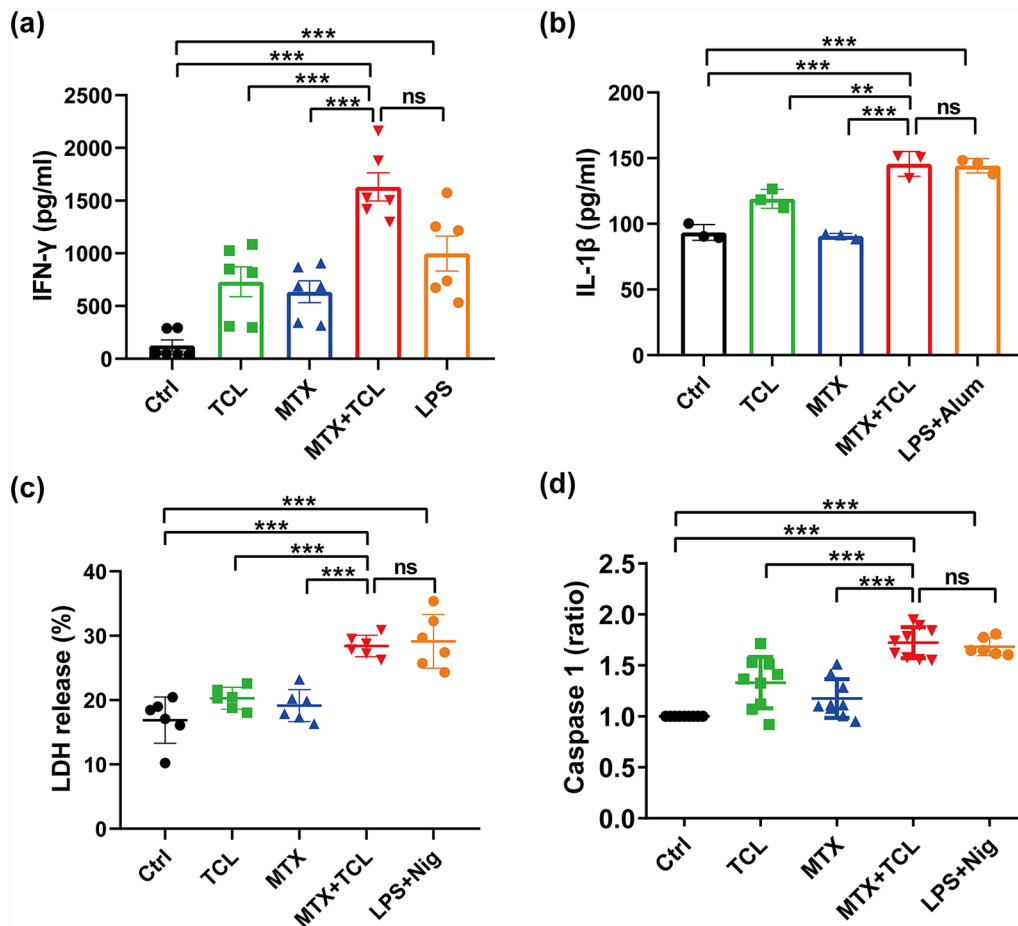
Figure 2. Cytokines releasing from MTX combined TCL treated DCs. (a) IFN-γ released in the supernatant of mature DCs treated in the different treatment groups for 48 h, LPS treatment group as the positive control. (b) Amount of IL-1β produced in the supernatant of mature DCs treated in the different treatment groups for 48 h, LPS combined with Alum treatment group as the positive control. (c) LDH released from DCs treated in the different treatment groups, LPS combined with Nig treatment group as the positive control. Caspase-1 released from DCs treated in the different treatment groups is shown in (d), LPS combined with Nig treatment group as positive control.

Data are shown as the mean ± SEM of three independent experiments.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Ctrl, control; DC, dendritic cell; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTX, methotrexate; Nig, nigericin; ns, no significant difference; TCL, tumour cell lysate.

increased two-fold in the presence of TCL-MTX-stimulated DCs compared with that in the presence of TCL-stimulated DCs [Figure 3(c)]. Taking together the findings observed thus far, MTX increased T cell activation by the tumour antigen-loaded DCs, likely by enhancing antigen presentation and activating the pathways downstream of the co-stimulatory molecules.

MTX promotes tumour antigen uptake by BMDCs and this effect can be partly blocked by the NLRP3 inhibitor MCC950

We next explored the effect of antigen uptake by immature DCs treated with MTX and its association with the NLRP3 pathway. Immature DCs treated with MTX on day 5 for 24 h resulted in an increase in the percentage of FITC-TCL in the MTX-treated group compared with that in the TCL control group or in the MCC950 plus TCL control group [Figure 4(a) and (b)]. In confocal analysis, we found that MTX can increase the tumour antigen presentation of BMDCs [Figure 4(c) and (d)]. After treatment with the NLRP3 inhibitor MCC950, the effect of antigen presentation was found to be attenuated to some extent compared with that in the TCL control group or in the MCC950 plus TCL control group. These results indicate that tumour antigen uptake may
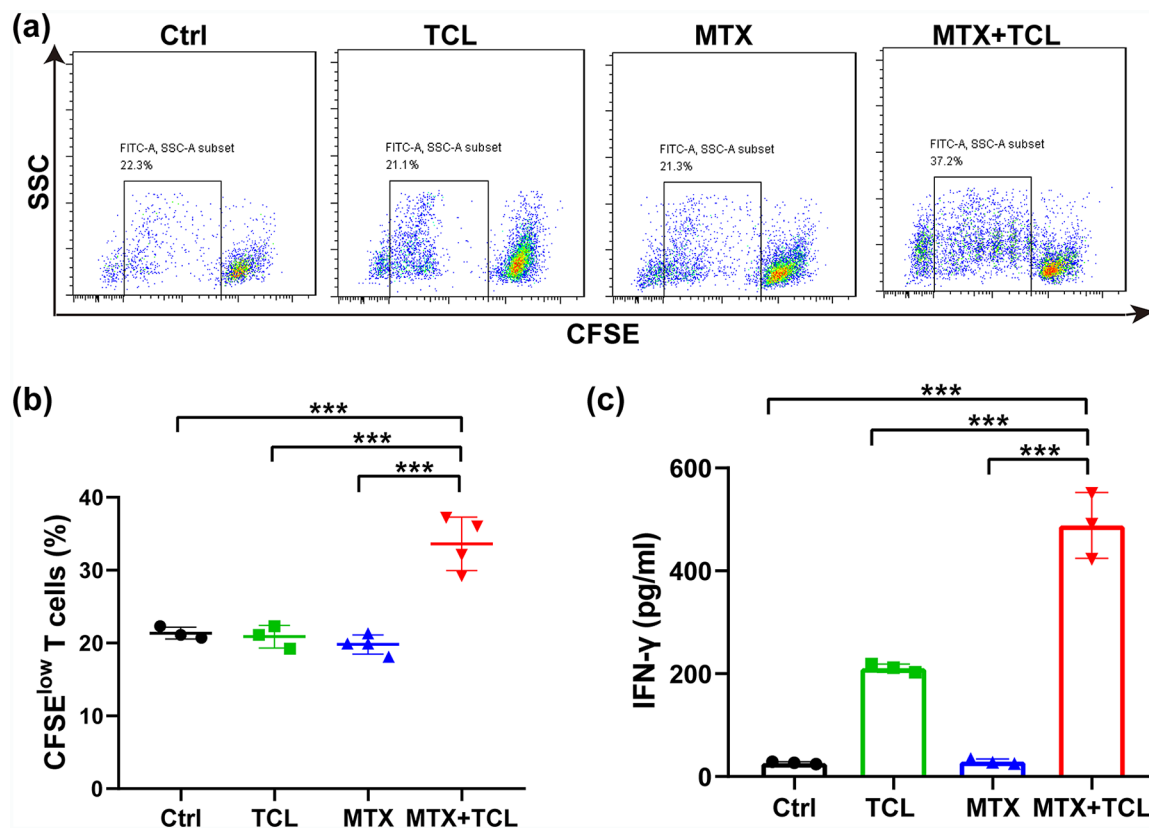
Figure 3. MTX/TCL-treated BMDCs stimulate CD8^{+} T cell proliferation *in vitro*. (a) CD8^{+} T cells were labelled with CFSE and activated using TCL pulsed BMDCs in the presence or absence of MTX. As a control, un-pulsed BMDCs were co-cultured with CD8^{+} T cells. Proliferation rates after 5 days were measured by flow cytometry. (b) Effect of MTX on antigen presentation of DCs and CD8^{+} T cells proliferation is shown as CFSE^low. Data are shown as mean ± SEM of % positive incidence from three independent experiments. ***$p < 0.001$. (c) In the same set of experiments, T cells IFN-γ secretion by CD8^{+} T cells was measured by ELISA in each culture supernatant 5 days after culture initiation and shown as T cell proliferation in response to TCL presentation by BMDCs.
Data are shown as mean ± SEM of three independent experiments.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
BMDC, bone marrow dendritic cell; CFSE, carboxyfluorescein diacetate succinimidyl; ELISA, enzyme-linked immunosorbent assay; MTX, methotrexate; TCL, tumour cell lysate.

be a prerequisite for the activation of the NLRP3 inflammasome.

MTX mediates its effects on TCL-loaded DCs through NLRP3 inflammasome activation

To determine the pathway underlying DC maturation after dual signalling activation, we investigated whether both MTX and TCL could influence NLRP3 inflammasome activation. As reported before, the NLRP3 inflammasome has been implicated in DC maturation, and we analysed the effects of MTX and TCL on the components of this pathway. Western blot analysis showed that MTX and TCL induce the upregulation of the protein expression of NLRP3 and pro-IL-1β. After stimulation with MTX and TCL for 24 h, cleaved caspase-1 p10 (10 kDa) and

mature IL-1β (17 kDa) were detected in the supernatants of DCs. This indicates that the activation of the NLRP3 inflammasome pathway participates in the maturation of DCs. Therefore, the activation of caspase-1 and IL-1β processing is mediated by NLRP3 inflammasome activation (Figure 5). Taken together, the NLRP3 inflammasome is essential for the maturation and antigen presentation of DCs, which is consistent with the finding of a previous study which showed that antigen uptake is a prerequisite for the activation of NLRP3 inflammasome in DCs.[36]

MTX combined with TCL-loaded DC vaccine significantly retarded tumour growth in vivo

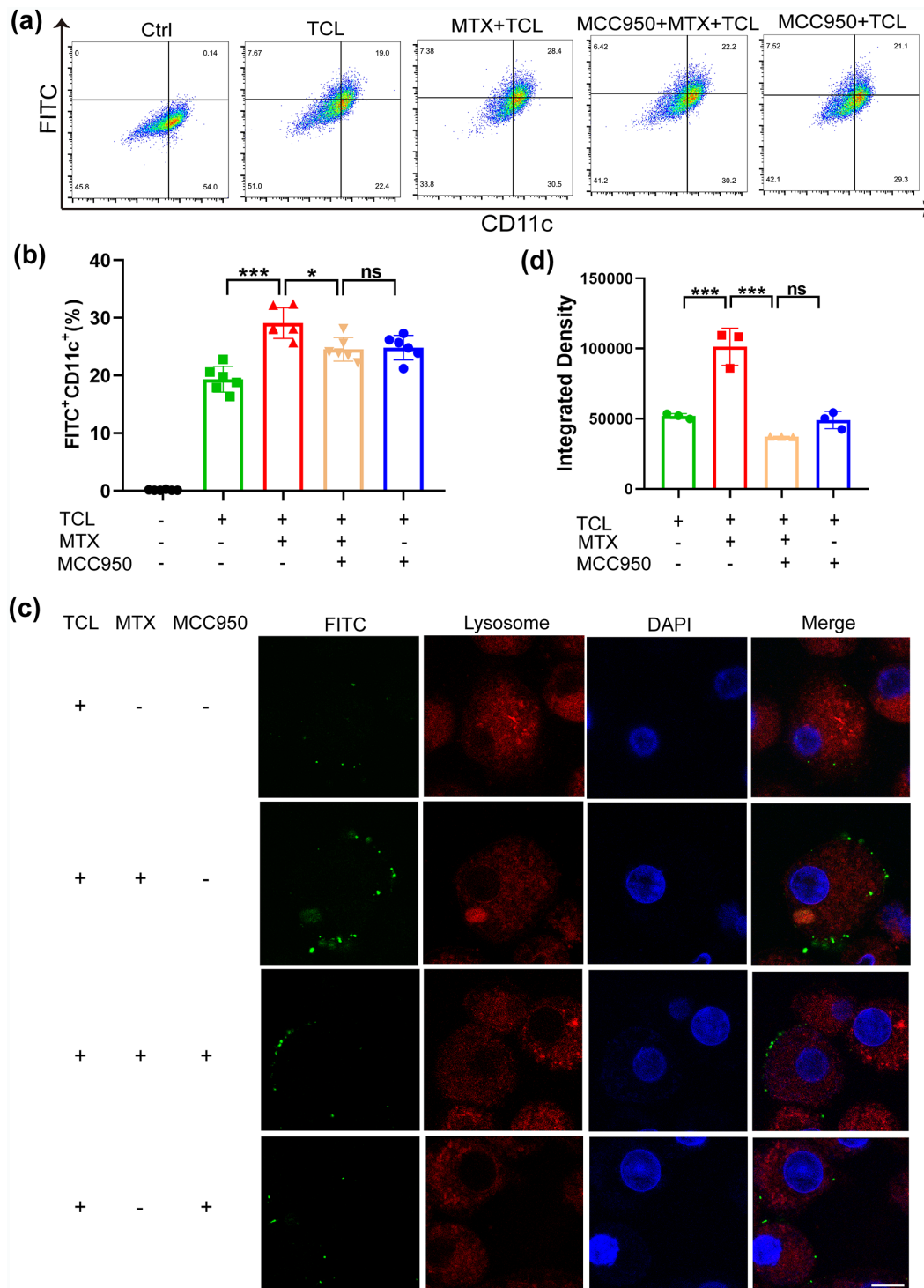The anti-tumour effect of the MTX and TCL-loaded BMDCs was assessed in mice that were

Figure 4. MTX promotes antigen cross-presentation activity on immature DCs and the NLRP3 inflammasome inhibitor MCC950 decreases the efficiency to a certain degree. (a) Analysis of the presentation efficiency of TCL-FITC by DCs using flow cytometry. (b) Percentage of CD11c+FITC+ DCs after treatment with TCL-FITC either together with MTX or alone, MCC950-treated TCL-FITC plus with MTX and MCC950 treated TCL-FITC. (c) Confocal analysis of DCs after stimulation under TCL-FITC addition with or without MTX (bar: 12.5 μm). (d) Integrated density of DCs treated with TCL-FITC, TCL-FITC plus with MTX, and MCC950-treated TCL-FITC. Data are shown as mean ± SEM of three independent experiments.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
DAPI, 4',6-diamidino-2-phenylindole; DC, dendritic cell; FITC, fluorescein isothiocyanate; MTX, methotrexate; NLRP3, nucleotide-binding domain-like receptor protein 3; TCL, tumour cell lysate.
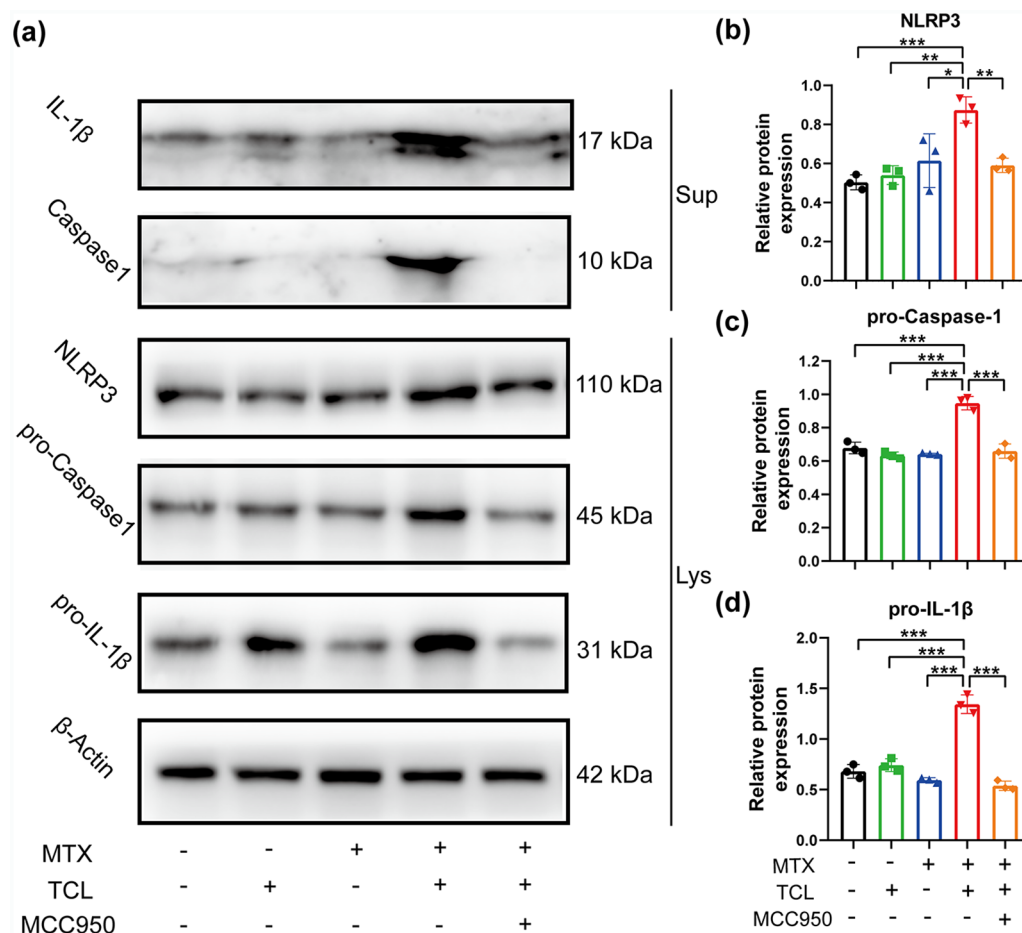
Figure 5. MTX promotes NLRP3 inflammasome activation in murine BMDCs. BMDCs were treated with MTX and TCL for 24 h. (a) Western blot analysis of the indicated proteins in the culture supernatant and cell lysates. (b) Relative protein expression of NLRP3 in the cell lysates. (c) Relative protein expression of pro-caspase-1 in the cell lysates. (d) Relative protein expression of pro-IL-1β in the cell lysates. β-Actin was used as the loading control.

Data are shown as mean ± SEM of three independent experiments.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

BMDC, bone marrow dendritic cell; DC, dendritic cell; Lys, cell lysates; MTX, methotrexate; NLRP3, nucleotide-binding domain-like receptor protein 3; Sup, supernatant; TCL, tumour cell lysate.

challenged with B16 melanoma cells after the respective immunisation. While the non-immunised mice harboured the largest and fastest growing tumours, tumour growth was ameliorated to some extent by immunising the mice with control DCs, or the TCL or MTX-loaded DCs. In contrast, the mice immunised with the dual-treated BMDCs showed delayed tumour onset, the smallest tumours [Figure 6(a) and (b)], and significantly slower tumour growth [Figure 6(c–h)] than the mice in the other groups ($p < 0.01$). Taken together, MTX acts as an effective adjuvant for DC-based tumour vaccines and elicits a strong anti-tumour response.

MTX with combined TCL-loaded DC vaccine activates IFN-γ producing CD8+ T cells and amplifies the anti-tumour CTL response

Previous studies have shown that mature DCs trigger cell-mediated immunity by activating the IFN-γ producing CD8+ T cells.[37] Consistent with this finding, the MTX and TCL-loaded DCs induced significantly greater expansion of an IFN-γ-producing CD8+ T cell population in the spleens of immunised mice than the other DC vaccines [Figure 7(a) and (b)]. To further evaluate the potential of MTX as an immune adjuvant, we performed *ex vivo* CTL analysis using purified splenic CD8+ T cells from different immunised
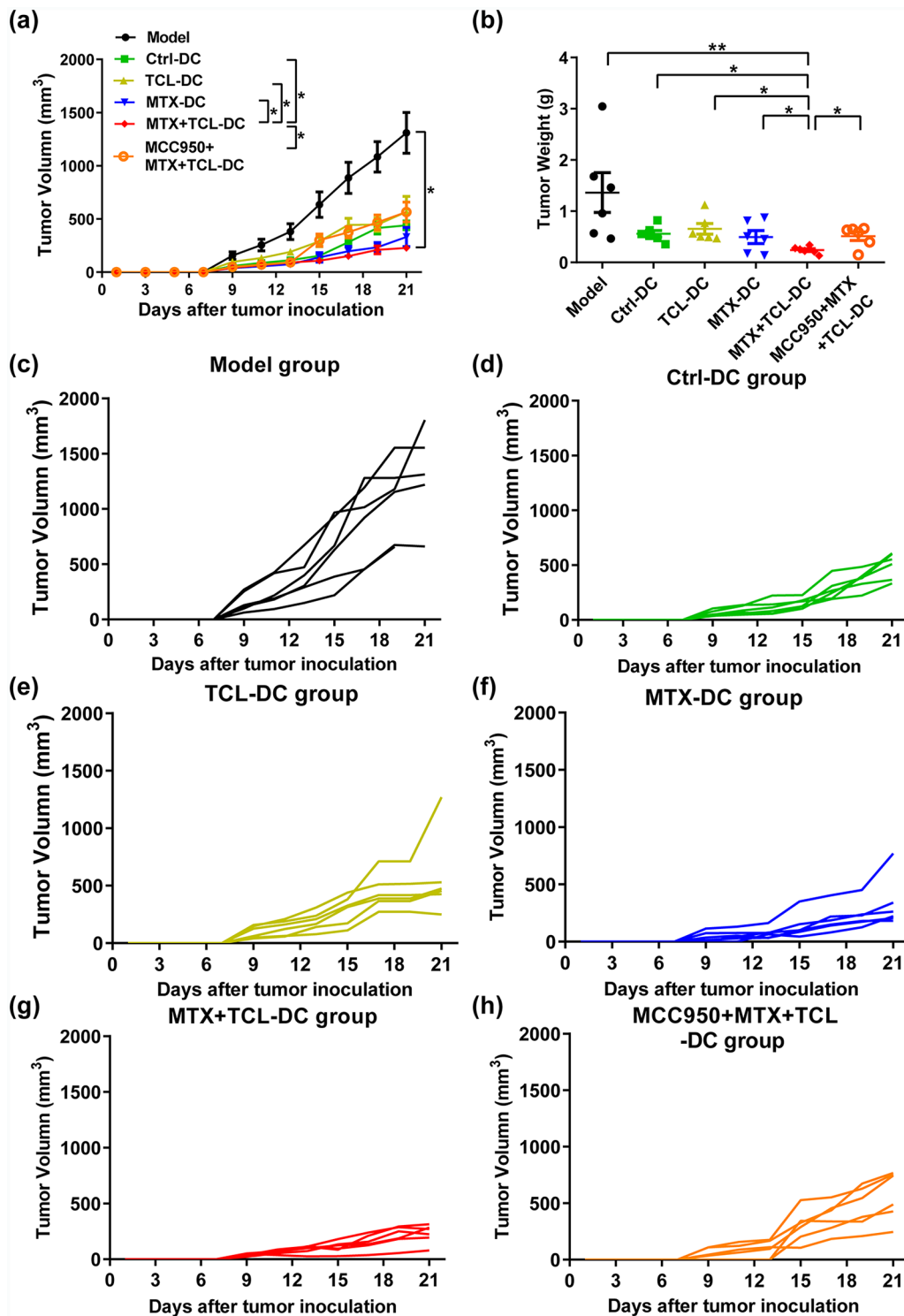
Figure 6. Prophylactic effect of MTX on DC vaccination against B16 cells. MTX plus TCL-loaded DCs show a higher degree of protective effect in tumour growth suppression. (a) B16 tumour volumes in each group. (b) Tumour weight on day 21 in each group. (c) Tumour volume of each mouse in the Model group. (d) Tumour volume of each mouse in the Ctrl-DCs group. (e) Tumour volume of each mouse in the TCL-DCs group. (f) Tumour volume of each mouse in the MTX-DCs group. (g) Tumour volume of each mouse in the MTX plus TCL-DCs group. (h) Tumour volume of each mouse in the MCC950 combined MTX and TCL-treated DCs group. Data are shown as mean ± SEM, $n = 6$.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Ctrl, control; DC, dendritic cell; MTX, methotrexate; TCL, tumour cell lysate.
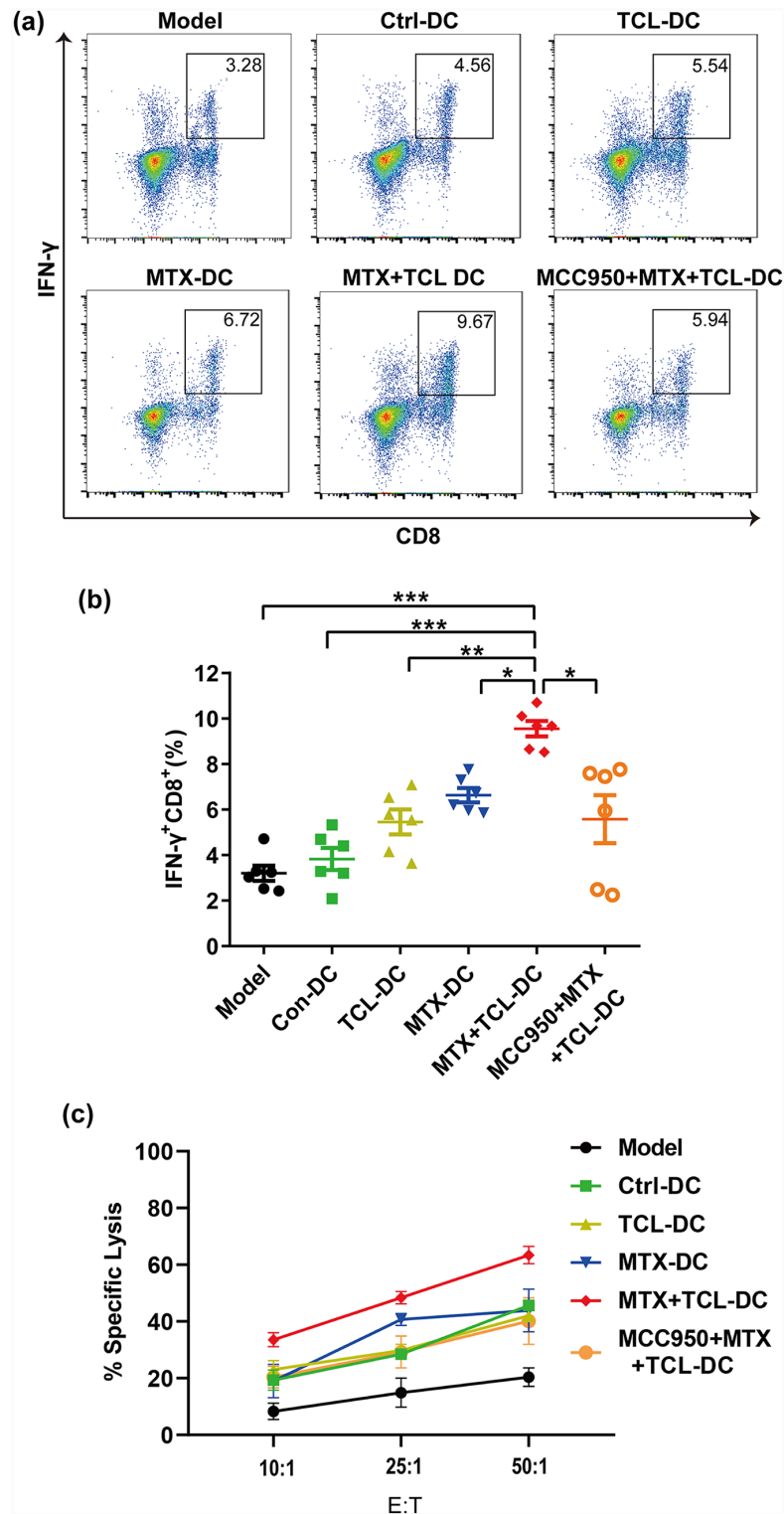
Figure 7. MTX plus TCL-loaded DCs expands IFN-γ-releasing CD8+ T cells and anti-tumour CTL responses. On day 21, the cells in the spleen were isolated and analysed for IFN-γ-secreting CD8+ T cells. Representative flow cytometry analysis results show the percentage of IFN-γ+CD8+ T cells in the spleen. The percentage of IFN-γ+CD8+ T cells in the spleen in each treatment group is summarised in (a) and (b). (c) Lymphocytes were isolated 21 days after B16 tumour inoculation, co-cultured with B16 cells for 72 h at three different effector:target (E:T) ratios.
Data are shown as mean ± SEM, *n* = 6.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Ctrl, control; DC, dendritic cell; MTX, methotrexate; TCL, tumour cell lysate; CTL, cytotoxic T lymphocytes.

mice, and observed significantly higher CTL activity against B16 cells by the lymphocytes of the MTX combined TCL group than those of the TCL group [Figure 7(c)]. Taken together, MTX significantly enhanced CD8+ T cell expansion by the tumour antigen-loaded DCs and induced a strong CTL response against B16 cells.

Discussion

We report for the first time that MTX can significantly enhance the immunising potential of tumour antigen-loaded DCs by inducing their functional maturation. MTX and TCL antigens mediate this phenotypic and functional switch in DCs through NLRP3 inflammasome activation, which culminates in the production and secretion of IL-1β. To the best of our knowledge, this is the first study to report the adjuvant function of MTX in DC vaccines and provide a mechanistic basis.

In the present study, we found that MTX promotes the activation of DCs with upregulated co-stimulatory molecules and inflammatory cytokines simultaneously, suggesting that MTX may act as an adjuvant in some way. This immunogenic property makes MTX an ideal vaccination platform for cancer. This immune-potentiating effect of MTX on the antigen processing activity of DCs could be mediated by several signalling pathways. The activation of T cells primarily depends on the expression of MHC–peptide complexes and co-stimulatory molecules on DCs.[38] Therefore, MTX upregulated the expression of CD80, CD86, MHCII molecules, and CD40 on DCs. Simultaneously, the levels of the inflammatory cytokines IL-1β and IFN-γ also increased in the MTX-treated TCL-loaded DCs group. In a previous study, the production of IL-1β by DCs and the action of IL-1β on CD8+ T cells may contribute to relevant anti-tumour immune responses.[19]

Various pathways participate in the maturation of DCs. For example, receptor signalling on the surface of antigen-presenting cells (APCs) can be activated by adjuvants then activate transcription factors. Likely, targeting TLRs can activate the NF-κB signalling pathway and promote the maturation of DCs.[39] The maturation of DCs can induce the production of chemokines and cytokines that help direct a special immune response. Inflammasome activation has also been regarded as a mechanism of DC maturation. The activation of inflammasomes can lead to the production of pro-inflammatory cytokines such as IL-1β and IL-18, and the activation of caspase-1. Influence of antigen presentation by DCs can also induce the maturation of DCs, for example, adjuvants that affect the antigen uptake by DCs. In our experiment, we found that the release of IL-1β was enhanced in the MTX and TCL dual treatment group compared with that in the MTX or TCL alone group. Therefore, this drives the mechanisms that participate in the DC antigen presentation pathway. Moreover, the addition of MTX to the TCL-loaded DCs can promote antigen presentation and T cell priming *in vitro*. All these results attracted great interest in terms of the pathways mediating the function of DCs. In further experiments, we noted the production of LDH and caspase-1 in the supernatant of the MTX and TCL dual treatment group. Moreover, the Western Blot results showed the release of IL-1β and caspase-1 in the supernatant of DCs. These results demonstrate the NLRP3 inflammasome activation pathway in MTX and TCL-dual-treated DCs. The NLRP3 inflammasome can be activated by various stimuli, including pathogens and danger signals such as MSU and ATP.[34,40–42] The release of IL-1β can benefit both innate and adoptive immune responses, which are required for priming T cells.[43] IL-1β plays an important role in the activation of APCs, along with the priming, activation, and proliferation of T cells and CTLs.[44] Studies have also shown the involvement of IL-1β in tumour antigen-specific immunity.[19,45] In this way, our focus is on the function of DCs in anti-tumour immunity.

Vaccination with the MTX and TCL-loaded DCs before tumour cell inoculation significantly retarded tumour growth in a mouse model by expanding the population of IFN-γ-releasing CTLs in the spleen. Cytotoxic CD8+ T cells are the key effectors of the immune response against solid tumours, and most anti-cancer immunotherapeutic strategies rely on activating the specific CD8+ T cells, either directly by adoptive transfer, or indirectly by DC vaccination or immune checkpoint blockade.[46] Sipuleucel-T was the first anti-tumour DC vaccine to be developed, and it has been effective in phase III trials against melanoma and prostate cancer cells.[47] However, it has poor overall therapeutic efficacy, prompting researchers to use pro-DC maturation cytokines, optimise the DC culture conditions, and enhance tumour antigen loading.[48] Here, we present MTX as a potent adjuvant that can enhance the anti-tumour efficacy of human DC vaccines.

Together, our results demonstrated the function of MTX combined TCL on bone marrow-derived DCs. We found that MTX can enhance the tumour antigen presentation capacity of DCs by promoting DC maturation and T cell priming. Further studies are needed to determine whether NLRP3 is pivotal in the maturation of DCs and T cell priming. It would be useful to determine the activation of the NLRP3 inflammasome in DCs and its translational application value.

Conclusion

In summary, we demonstrated that MTX and TCL activate the NLRP3 inflammasome pathway for the induction of IL-1β production. MTX can promote TCL uptake by DCs, leading to DC maturation and CD8$^+$ T cell priming (Figure 7). These findings provide novel advances in the knowledge of the immune-regulatory properties of MTX in DCs, which will contribute to a better utilisation of MTX and TCL in DC-based vaccines for translational medicine applications.

Author contributions

GNS and TTZ designed the study. GNS and MH performed the experiments. CJC, JMF and SS assisted with animal experiments. YZ contributed FACS analysis and cell culture experiments. GNS, LW and TTZ interpreted the data. GNS and TTZ wrote the paper. All authors read and approved the final manuscript.

Data Availability

All the data generated or analyzed during this study are included in this published article. The datasets are available from the corresponding author on reasonable request available from the corresponding author on reasonable request.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Ethics approval and consent to participate

All animal experiments were performed in specific pathogen-free facilities accredited by the Animal Care and Use Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College.

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