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Virulence-dependent induction of interleukin-10-producing-tolerogenic dendritic cells by *Mycobacterium tuberculosis* impedes optimal T helper type 1 proliferation

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Summary

Mycobacterium tuberculosis inhibits optimal T helper type 1 (Th1) responses during infection. However, the precise mechanisms by which virulent _M. tuberculosis_ limits Th1 responses remain unclear. Here, we infected dendritic cells (DCs) with the virulent _M. tuberculosis_ strain H37Rv or the attenuated strain H37Ra to investigate the phenotypic and functional alterations in DCs and resultant T-cell responses. H37Rv-infected DCs suppressed Th1 responses more strongly than H37Ra-infected DCs. Interestingly, H37Rv, but not H37Ra, impaired DC surface molecule expression (CD80, CD86 and MHC class II) due to prominent interleukin-10 (IL-10) production while augmenting the expression of tolerogenic molecules including PD-L1, CD103, Tim-3 and indoleamine 2,3-dioxygenase on DCs in a multiplicity-of-infection (MOI) -dependent manner. These results indicate that virulent _M. tuberculosis_ drives immature DCs toward a tolerogenic phenotype. Notably, the tolerogenic phenotype of H37Rv-infected DCs was blocked in DCs generated from IL-10$^{-/-}$ mice or DCs treated with an IL-10-neutralizing monoclonal antibody, leading to restoration of Th1 polarization. These findings suggest that IL-10 induces a tolerogenic DC phenotype. Interestingly, p38 mitogen-activated protein kinase (MAPK) activation predominantly mediates IL-10 production; hence, H37Rv tends to induce a tolerogenic DC phenotype through expression of tolerogenic molecules in the p38 MAPK–IL-10 axis. Therefore, suppressing the tolerogenic cascade in DCs is a novel strategy for stimulating optimal protective T-cell responses against _M. tuberculosis_ infection.

Keywords: interleukin-10; p38 mitogen-activated protein kinase; Th1-type T-cell proliferation; tolerogenic dendritic cells; virulence.

Introduction

Mycobacterium tuberculosis is a successful human bacterial pathogen that causes tuberculosis (TB), one of the major causes of human death by infection.[1] Despite the great time and effort spent researching *M. tuberculosis*, elucidation of its pathogenesis remains ongoing. A robust T helper type 1 (Th1) response has widely been considered essential for providing immunity against TB.[2] However, *M. tuberculosis* possesses diverse immune evasion mechanisms that can overcome host defences during infection. Once targeted cells of the host such as

Abbreviations: BMDCs, bone marrow-derived DCs; DCs, dendritic cells; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; IL, interleukin; JNK, Jun-N-terminal kinase; mAbs, monoclonal antibodies; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; PE, phycoerythrin; PerCP, Peridinin chlorophyll protein; TB, tuberculosis; TGF-β, transforming growth factor-β; Th1, T helper type 1; TNF-α, tumour necrosis factor-α; WT, wild-type

macrophages and dendritic cells (DCs) are infected by *M. tuberculosis*, these cells attempt to kill the *M. tuberculosis* via cellular processes such as autophagy and phagosome–lysosome fusion. However, if the host cells fail to control *M. tuberculosis* growth, the bacteria survive and manipulate host cells for immune evasion.[3,4] Although the cellular mechanisms shared between immune defence and evasion are relatively well-studied, the phenotypic and functional alterations of *M. tuberculosis*-infected immune cells are not yet clearly understood.

The genetic diversity of *M. tuberculosis* results in variation in virulence that induces marked differences in pathogenesis,[5] including bacterial counts, and in immune responses, such as lung inflammation, chemokine and cytokine profiles,[6] and cell death processes.[7] In addition, the outcome of protective T-cell immunity clearly depends on the *M. tuberculosis* strain. Hence, differences in virulence among *M. tuberculosis* strains may initially affect the function and phenotype of antigen-presenting cells, such as DCs. For example, the virulent strain H37Rv, but not the attenuated strain H37Ra, induces necrosis by disrupting the mitochondrial inner membrane,[7] and H37Rv up-regulates Th2-type cytokines such as interleukin-5 (IL-5), IL-10 and IL-13 compared with H37Ra in macrophages *in vitro*.[6] As another example, the virulent strain S7 from South India suppresses the Th1 response more than the less virulent strain S10.[8] Hence, investigating different cell death processes, cytokine secretion profiles, and effector expression levels in DCs in the presence of different bacterial strains of varying virulence is essential to determine the optimal Th1-type T-cell response during the early phase of infection and to understand how virulent *M. tuberculosis* subverts host protective immunity.

Dendritic cells are the most important antigen-presenting cells for defending *M. tuberculosis* infection because they prime and educate T cells in draining lymph nodes. Consequently, these T cells migrate to infected tissues to combat *M. tuberculosis*. Hence, the generation of protective Th1-type T cells against *M. tuberculosis* infection depends on the speed or mechanism by which different DCs translate and deliver information about the infection to T cells. In other words, the initial encounter between DCs and *M. tuberculosis* is the first critical step that determines the type and velocity of the T-cell response. Hence, understanding the nature of DC maturation and phenotypic changes in response to *M. tuberculosis* strains of varying virulence is of great interest for the development of an effective TB vaccine and new immunological interventions to control *M. tuberculosis* infection.

It is well documented that DCs differentiate into mature phenotype in response to various inflammatory stimuli, such as microbial products or cytokines, and then prime naïve T cells to proliferate and engage in an effective T-cell immune response. In general, mature DCs are characterized according to phenotypic and functional changes, such as enhanced expression of MHC class I and II molecules, co-stimulatory molecules (CD80, CD86, and CD40) and a variety of innate cytokines. With respect to *M. tuberculosis* infection, DCs perform important functions in containing and killing *M. tuberculosis* by inducing T-cell responses.[3,9]

After DCs take up *M. tuberculosis*, they migrate to secondary lymphoid organs to prime T cells. Activated T cells proliferate and migrate to the site of infection. Once a sufficient number of effector T cells accumulates at the infection site, *M. tuberculosis* growth becomes hindered. However, T-cell responses to *M. tuberculosis* infection are relatively delayed and impaired compared with T-cell responses to infection with other intracellular pathogens.[10] The mechanism underlying the delay in T-cell responses to *M. tuberculosis* infection is currently uncertain, but several studies reported that IL-10 is one factor that is involved in this delay.[11,12] The levels of immunoregulatory cytokines, such as IL-5, IL-10 and IL-13, are increased in monocytes and monocyte-derived macrophages in the presence of a virulent strain of *M. tuberculosis* compared with those in an attenuated strain.[6,13] Many studies have reported that innate and adaptive cells are associated with pathogenesis during *M. tuberculosis* infection.[14] However, the interaction between the DC phenotype according to *M. tuberculosis* virulence and the T-cell response has not been thoroughly studied.

One mechanism by which virulent *M. tuberculosis* might subvert T-cell responses could be alteration of DC function and phenotype. Hence, in this study, we hypothesized that a highly virulent *M. tuberculosis* strain would drive immature DCs towards a tolerogenic phenotype, thereby preventing an optimal Th1 response. To address this hypothesis, we infected DCs with the two most thoroughly studied laboratory-adapted reference strains *M. tuberculosis* H37Rv and H37Ra, which are virulent and attenuated strains, respectively, to gain insight into DC responses and consequent changes in T-cell polarization. In addition, we dissected the precise mechanism by which virulent *M. tuberculosis* regulates the phenotype and function of DCs. These findings will help us to understand how TB manipulates the host immune system toward pathogen-favouring conditions by interacting with DCs.

Materials and methods

Experimental animals and ethics statement

Specific pathogen-free female 6- to 8-week-old wild-type (WT) C57BL/6 (H-2Kb and I-Ab), IL-$10^{-/-}$ transgenic C57BL/6, and OT-II ovalbumin peptide 323–339 ($OVA_{323\text{-}339}$) specific T-cell receptor transgenic C57BL/6 mice were purchased from the Jackson Laboratory (Bar

Harbor, ME). The experimental protocols used in this study were reviewed and approved by the Ethics Committee and Institutional Animal Care and Use Committee (Permit Number: 2014-0055) of the Laboratory Animal Research Centre at Yonsei University College of Medicine (Seoul, Korea).

Antibodies and reagents

Recombinant mouse granulocyte–macrophage colony-stimulating factor (GM-CSF) was purchased from Creagene, Inc. (Seoul, Korea), and an FITC-Annexin V/propidium iodide (PI) kit was purchased from BD Biosciences (San Diego, CA). LIVE/DEAD® Fixable Dead Cell Stain Kits were purchased from Molecular Probes (Carlsbad, CA). The OT-II peptide ($OVA_{323\text{-}339}$, ISQAVHAAHAEINEAGR) was synthesized by AbFrontier (Seoul, Korea). The anti-phosphorylated (p-) extracellular signal-regulated kinase (ERK) 1/2, anti-p-Jun N-terminal kinase (JNK), anti-p-p38, anti-p-Akt (Ser473), and anti-p-GSK-3β (Ser9) monoclonal antibodies (mAbs) were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Inhibitors of ERK (U0126), JNK (JNK inhibitor II), and p38 (SB203580) were purchased from Calbiochem (San Diego, CA), and Wortmannin was purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Horseradish peroxidase (HRP) -conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG were obtained from Calbiochem. Phycoerythrin (PE) -conjugated mAbs against CD80, MHC-II, PD-L1 (CD274), FoxP3 and GATA-3; PE-Cy7-conjugated mAbs against CD11c and T-bet and CD25; the Peridinin chlorophyll protein (PerCP) -Cy5.5 or FITC-conjugated mAbs against CD4; PerCP-Cy5.5-conjugated indoleamine 2,3-dioxygenase (IDO); allophycocyanin-conjugated mAbs against CD49b, CD86, CD103 and RORγt; and PerCP-eFluor710-conjugated mAbs against CD223 (LAG-3) were purchased from eBioscience (San Diego, CA). Tumour necrosis factor-α (TNF-α), IL-5, IL-10, IL-12p70, IL-17A, transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ) ELISA kits were obtained from eBioscience. Anti-mouse CD3e (145-2C11 and anti-mouse CD28 (37.51) were also purchased from eBioscience. The neutralizing anti-PD-L1 and anti-IL-10 mAbs (10F.9G2 and JESS-2A5, respectively) and their respective isotype controls rat IgG2b (LTF-2) and rat IgG1 (HRPN) were purchased from BioXCell (West Lebanon, NH).

Mycobacterium tuberculosis strains and culture conditions

The *M. tuberculosis* H37Rv (ATCC 27294) and H37Ra (ATCC 25177) strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cultures and single-cell suspensions of each strain were prepared as previously described.[15] The seed lots of each strain were kept in small aliquots at −80° until use. After confirmation of the predominant presence of single cells in the final preparation based on acid-fast staining, the number of colony-forming units (CFUs) per 1 ml of each seed lot on a 7H10 agar plate was measured using a viable cell counting assay, and the cells were used for the subsequent experiments.

Generation and culture of bone marrow-derived DCs (BMDCs)

Generation and culture of DCs from murine bone marrow cells were performed in the presence of GM-CSF as previously described.[16] Briefly, whole bone marrow cells isolated from C57BL/6 mice were lysed with red blood cell lysing buffer and washed with RPMI-1640 medium. Then, the cells were plated in six-well culture plates (10^6 cells/ml) and incubated at 37° in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 100 U/ml penicillin/streptomycin (Lonza, Basel, Switzerland), 10% fetal bovine serum (Lonza), 50 μM mercaptoethanol (Lonza), 0·1 mM non-essential amino acids (Lonza), and 20 ng/ml GM-CSF. On day 8, the purity of CD11c-positive non-adherent cells was confirmed to be > 90%, and this cell population was used for subsequent experiments.

Comparison of cytotoxicity between M. tuberculosis strains and determination of MOIs not causing cytotoxicity to DCs

At 8 days in culture, DCs (2×10^6 cells) were infected with H37Ra or H37Rv at a MOI of 0·5, 1 or 5. After 4 hr, amikacin (Sigma-Aldrich) was applied at 20 μg/ml to kill any extracellular bacteria. After an additional 18 hr, the DCs were harvested, washed with PBS, and stained using an Annexin V-PI Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's protocol. Furthermore, the levels of lactate dehydrogenase (LDH) in the supernatants of *M. tuberculosis*-infected DCs were assessed to evaluate DC cytotoxicity. To measure the viability of the DCs at prolonged time-points (48, 72 and 96 hr post-*M. tuberculosis* infection), the *M. tuberculosis*-infected DCs were stained using LIVE/DEAD® Fixable Dead Cell Stain Kits according to the manufacturer's protocol and analysed using flow cytometry.

Comparison of M. tuberculosis uptake by DCs

To determine whether DCs uptake a similar number of the different *M. tuberculosis* strains, both *M. tuberculosis* strains were labelled with the fluorescent Violet Proliferation Dye 450 (VPD450; BD Biosciences) according to the manufacturer's instructions. Briefly, the *M. tuberculosis*

strains (2×10^8 CFU/ml) were washed with PBS, diluted in 1 ml of PBS supplemented with 10 μM of VPD450 and incubated in a 37° water bath for 15–20 min. Finally, the VPD450-labelled *M. tuberculosis* strains were washed twice in PBS, and the DCs were infected at an MOI of 1 bacterium per cell and analysed at various time-points. After each time-point, the DCs were harvested, the non-internalized bacteria were washed off with PBS, and 0·2% trypan blue was added to the cells. *M. tuberculosis* inside DCs were measured using flow cytometry.

Evaluation of cell surface molecule expression on DCs using flow cytometry

On day 8 in culture, the BMDCs were harvested and washed once. Then, 2×10^6 DCs/ml were infected with *M. tuberculosis* for 4 hr. Afterwards, Amikacin was applied at 20 μg/ml. After an additional 18 hr, the harvested cells were blocked with 10% (volume/volume) normal goat serum for 15 min at 4°, followed by staining with PE-conjugated antibodies against I-Ab (MHC-II), CD80, or PD-L1, allophycocyanin-conjugated antibodies against CD86, CD103 or Tim-3, and a PE-Cy7-conjugated antibody against CD11c for 30 min at 4°. Finally, the stained cells were analysed using a BD FACSverse flow cytometer (Becton Dickinson, San Jose, CA) and FLOWJO software (Tree Star, Inc., Ashland, OR).

ELISA

Supernatants were collected 24 hr after infection and were stored at −70° until assessment of cytokine production. The levels of TNF-α, TGF-β, IL-5, IL-10, IL-12p70, IL-17A and IFN-γ were measured using sandwich ELISA kits (eBioscience) and commercially available pairs of antibodies and standards according to the manufacturer's protocol.

Immunoblotting analyses and treatment of DCs with pharmacological inhibitors of GSK-3β and mitogen-activated protein kinase pathway members

After infection with *M. tuberculosis*, DCs were lysed in 200 μl of lysis buffer containing 50 mM Tris–HCl (pH 7·5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 30 mM Na_4PO_7, 1 mM PMSF, 2 μg/ml aprotinin, and 1 mM pervanadate. Whole-cell lysate samples were resolved on SDS–polyacrylamide gels and then transferred to polyvinylidene fluoride membrane for 2 hr at 80 V. The membranes were blocked in 5% skim milk and incubated with an antibody overnight, followed by incubation with a corresponding HRP-conjugated secondary antibody for 1 hr at room temperature. For detection of target protein epitopes, polyclonal antibodies against p-ERK 1/2, p-JNK 1/2, p-p38, p-Akt (Ser473), and p-GSK-3β (Ser9) (dilutions of 1 : 2000, 1 : 2000, 1 : 2000, 1 : 2000, and 1 : 500, respectively) were used. Specific protein bands recognized by the antibodies were visualized using the ECL Advance Western Blotting Detection kit (GE Healthcare, Little Chalfont, UK). Two hours before *M. tuberculosis* infection, inhibitors of ERK (U0126), JNK (JNK 2 inhibitor), p38 (SB203580), or GSK-3β (Wortmannin) were applied. After 4 hr of *M. tuberculosis* infection, DCs were treated with 20 μg/ml amikacin, and after an additional 18 hr, the supernatants were harvested and analysed using ELISA kits.

T-cell proliferation assay

$OVA_{323\text{-}339}$-specific $CD4^+$ T cells were obtained from the splenocytes of OT-II mice a MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). These T cells were stained with 1·25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen). One hour before co-culture with T cells, DCs (2×10^6 cells/well) were treated with $OVA_{323\text{-}339}$. Then, the DCs were co-cultured with CFSE-stained $CD4^+$ T cells ($2{\cdot}5 \times 10^5$ cells/well) at a DC : T-cell ratio of 1 : 5. At 3 days in co-culture, the T cells were stained with PerCP-Cy5.5-conjugated anti-CD4, PE-Cy7-conjugated anti-T-bet, PE-conjugated anti-GATA-3 and anti-RORγt mAbs and analysed through flow cytometry. Supernatants were also harvested, and the IFN-γ, IL-5 and IL-17A levels were measured via ELISA.

T-cell suppression by M. tuberculosis-infected tolerogenic DCs

To investigate whether *M. tuberculosis* H37Rv-infected tolerogenic DCs are actively involved in T-cell suppression, we conducted a T-cell suppression assay using previously described protocols with slight modifications.[17–19] Briefly, BMDCs were harvested and infected with *M. tuberculosis* as described above. After 24 hr, the BMDCs (3×10^4 cells/well) were washed and co-cultured for 72 hr with $CD4^+$ T cells ($1{\cdot}5 \times 10^5$ cells/well) isolated from the spleen using a MACS system. $CD4^+$ T cells were labelled with 1·25 μM CFSE and stimulated with immobilized anti-CD3/28. T-cell proliferation was analysed via flow cytometry.

Statistical analyses

Statistical analyses were conducted using GRAPHPAD PRISM V5·0 (GraphPad Software, San Diego, CA). Differences between two groups were analysed using an unpaired Student's *t*-test. One-way analyses of variance followed by Tukey's multiple comparison tests were used to analyse data with more than two groups. All values are expressed as the means (± SD). Statistical significance was determined as $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ or $***P < 0{\cdot}001$.

Results

Virulent *M. tuberculosis* strain-infected DCs impede T-cell proliferation

As previously reported, an impaired and delayed T-cell response is a unique hallmark of *M. tuberculosis* infection,[20] and DCs infected with *M. tuberculosis* impede T-cell proliferation.[21] To determine the effect of *M. tuberculosis* infection in DCs on the T-cell response, we first optimized the MOI of *M. tuberculosis* that would not cause DC death and could therefore be used in subsequent experiments for flow cytometry analysis (see Supplementary material, Fig. S1a, b) and measurements of LDH release (see Supplementary material, Fig. S1c). Next, we confirmed that DCs were infected with both *M. tuberculosis* strains to a similar extent at an MOI of 1 bacterium per cell (see Supplementary material, Fig. S1d). Furthermore, at an MOI of 1 bacterium per cell, no differences in cell viability of the DCs infected with either *M. tuberculosis* strain were observed until 96 hr after infection (see Supplementary material, Fig. S1e). We next hypothesized that the differences in the virulence of different *M. tuberculosis* strains result from variations in the ability of DCs infected with different strains of *M. tuberculosis* to activate T cells. To verify the effect of *M. tuberculosis* strains of differing virulence on the T-cell expansion ability of infected DCs, we performed a syngeneic mixed lymphocyte reaction assay using $CD4^+$ T cells from OT-II transgenic mice. $OVA_{323\text{-}339}$-specific transgenic $CD4^+$ T cells were labelled with CFSE and co-cultured with *M. tuberculosis*-infected DCs at a 5 : 1 ratio for 3 days (Fig. 1). Interestingly, virulent *M. tuberculosis* strain H37Rv-infected DCs showed a weaker ability to induce naive $CD4^+$ T-cell proliferation than attenuated H37Ra-infected DCs or non-infected $OVA_{323\text{-}339}$-pulsed DCs (Fig. 1a,b). Next, to verify the types of T-cell responses induced by *M. tuberculosis*-infected DCs, the expression of transcription factors and cytokines related to Th1 (T-bet and IFN-γ) and Th2 responses (GATA-3 and IL-5) and Th17 (RORγt and IL-17A) in co-cultured $CD4^+$ T cells was analysed via flow cytometry (Fig. 1c,d) and ELISA (Fig. 1e). Compared with H37Rv-infected DCs, H37Ra-infected DCs induced stronger T-bet and IFN-γ expression in co-cultured T cells. RORγt expression in T cells as well as IL-17A secretion were higher in T cells subjected to *M. tuberculosis*-infected DCs, but there was no difference between the two strains. T cells co-cultured with non-infected $OVA_{323\text{-}339}$-pulsed DCs showed the highest expression levels of GATA-3, but the overall expression levels of GATA-3 and IL-5 were not intense in every sample (Fig. 1d,e). No detectable expression of $CD25^+$ $FoxP3^+$ Treg and $CD4^+$ $LAG\text{-}3^+$ $CD49b^+$ $CD25^-$ $Foxp3^-$ type 1 regulatory T cell (Tr1 cells) as well as no secretion of IL-10 and TGF-β from T cells co-cultured with *M. tuberculosis*-infected DCs were observed (data not shown). These results suggest that virulent *M. tuberculosis*-infected DCs were not able to induce an effective $CD4^+$ T-cell response.

Infection with *M. tuberculosis* promotes a tolerogenic DC phenotype in a virulence-dependent manner

To investigate the reason that virulent *M. tuberculosis* H37Rv-infected DCs ineffectively induced T-cell responses, we measured the expression levels of surface molecules related to T-cell activation on DCs (Fig. 2). The gating strategy for each surface molecule and enzyme is shown in Fig. 2(a). H37Ra-infected DCs displayed increasing expression of CD80 and CD86 as the MOI increased, but H37Rv-infected DCs presented reduced expression of CD80 and CD86 as the MOI increased. H37Rv but not H37Ra repressed the expression of MHC-II as the MOI increased. Moreover, both *M. tuberculosis* strains up-regulated CCR7 compared with its expression in non-infected DCs, and H37Rv-infected DCs exhibited up-regulation of CCR7 in an MOI-dependent manner (Fig. 2b). Next, the expression of inhibitory molecules such as PD-L1, Tim-3, CD103 and IDO was analysed via flow cytometry (Fig. 2c). H37Rv-infected DCs displayed higher expression of PD-L1, Tim-3, CD103 and IDO than H37Ra-infected DCs, and infection at higher MOIs further induced these molecules. These results suggest that the virulent *M. tuberculosis* strain H37Rv induces a more tolerogenic DC phenotype than the attenuated *M. tuberculosis* strain H37Ra.

The virulent *M. tuberculosis* strain induces marked IL-10 production in DCs

Cytokine signalling is a crucial regulator of DC–T-cell interactions. To verify the cytokine profiles of DCs infected with different *M. tuberculosis* strains, the TNF-α, IL-10 and IL-12p70 levels in cell culture supernatants were measured (Fig. 3). After 24 hr of *M. tuberculosis* infection at an MOI of 0·5 or 1, H37Rv-infected DCs secreted two-fold greater levels of TNF-α than H37Ra-infected DCs (Fig. 3a). Remarkably, H37Rv-infected DCs induced three-fold greater secretion of IL-10 than H37Ra-infected DCs; however, IL-12p70 was not detected in cells infected with either *M. tuberculosis* strain. To evaluate the kinetics of each cytokine, DCs were infected with either *M. tuberculosis* strains at an MOI of 1, and the TNF-α, IL-10 and IL-12p70 levels were then measured via ELISA at 2, 4, 8, 12 and 24 hr after infection (Fig. 3b). TNF-α was secreted at high levels at the early time-points, and the secreted level of TNF-α peaked at 8 hr after infection in DCs infected with both

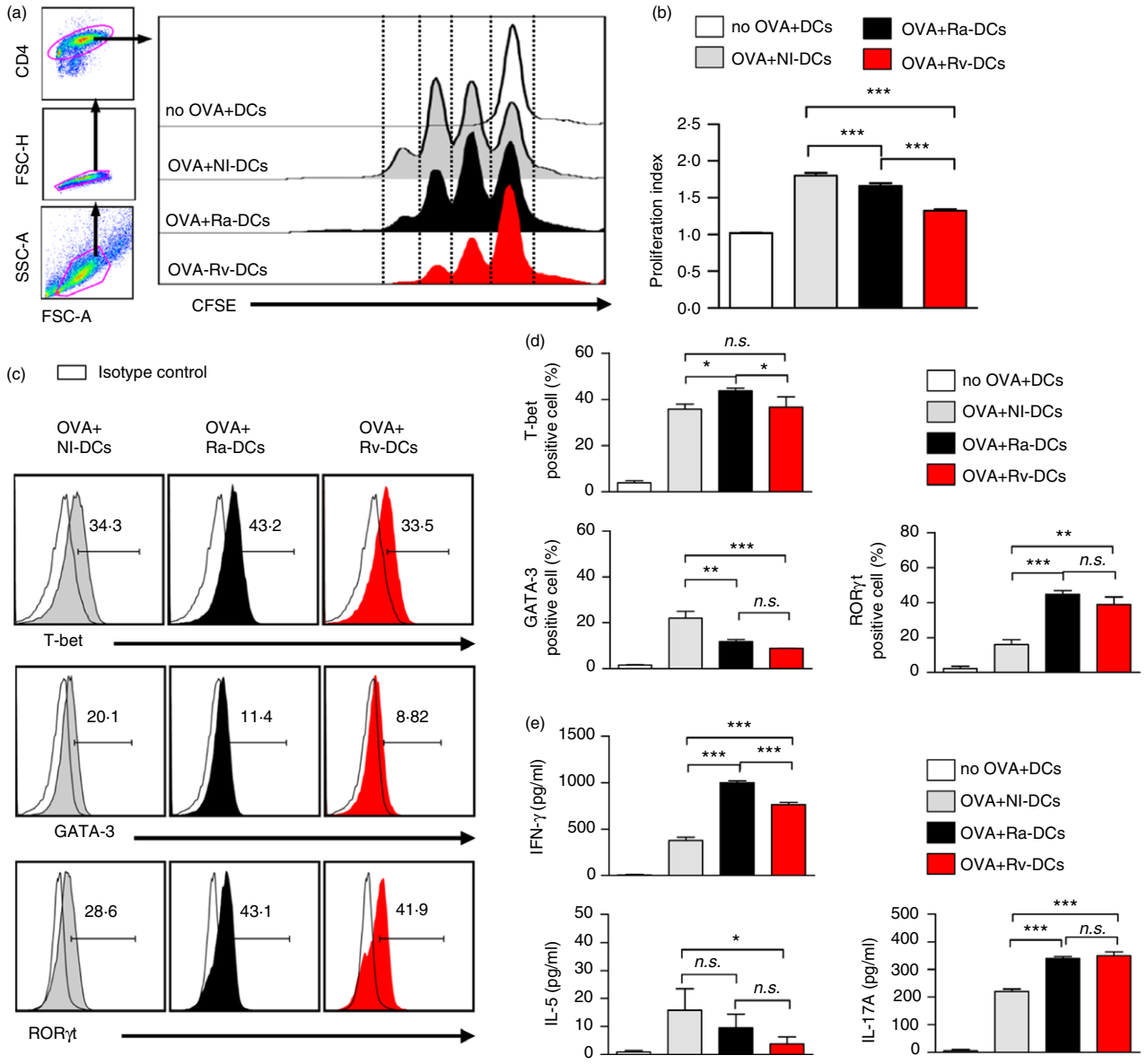

Figure 1. Virulent *Mycobacterium tuberculosis*-infected dendritic cells (DCs) impede effective T-cell proliferation. At 24 hr after infection with *M. tuberculosis* at a multiplicity of infection (MOI) of 1, DCs were pulsed with 0·5 μg/ml chicken ovalbumin peptide ($OVA_{323-339}$) for 1 hr. $OVA_{323-339}$-specific $CD4^+$ T cells from OT-II transgenic mice were isolated using a MACS system and were stained with 1·25 μM CFSE; $OVA_{323-339}$ pulse-treated DCs were co-cultured with isolated T cells for 72 hr. (a, b) Proliferation of T cells was analysed via flow cytometry. The proliferation rate is shown in the histogram and as the proliferation index. (c, d) After 72 hr of co-culture with DCs, T cells were harvested and stained with anti-CD4, anti-T-bet, anti-GATA-3 and anti-RORγt antibodies to analyse the T-cell polarization via flow cytometry. T-bet-, GATA-3- and RORγt-expressing $CD4^+$ T cells are shown in the histogram (c) and bar graphs (d). (e) The interferon-γ (IFN-γ), interleukni-5 (IL-5) and IL-17A secretion levels were determined by ELISA. Transforming growth factor-β (TGF-β), IL-10, regulatory T (Treg) cells ($CD25^+$ $FoxP3^+$) and Tr1 cells ($CD4^+$ $LAG\text{-}3^+$ $CD49b^+$ $CD25^-$ $Foxp3^-$) were not detected (data not shown). All data are expressed as the mean ± SD (n = 3 samples) of one representative experiment of three independent experiments. *n.s*, not significant, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$, one-way analysis of variance. no OVA+NI-DCs: non-pulsed non-infected DCs, OVA+NI-DCs: OVA-pulsed non-infected DCs, OVA+Ra-DCs: OVA-pulsed H37Ra-infected DCs, OVA+Rv-DCs: OVA-pulsed H37Rv-infected DCs. [Colour figure can be viewed at wileyonlinelibrary.com]

M. tuberculosis strains. Interleukin-10 secretion increased rapidly beginning from 4 hr after infection; in particular, H37Rv-infected DCs showed high secreted IL-10 levels, which peaked at 12 hr after infection. IL-12p70 was not detected at any time-point (Fig. 3a,b). Interestingly, the phenotype of DCs infected with H37Rv showed a similar phenotype (Fig. 2) and cytokine profile (Fig. 3) to those of tolerogenic DCs.

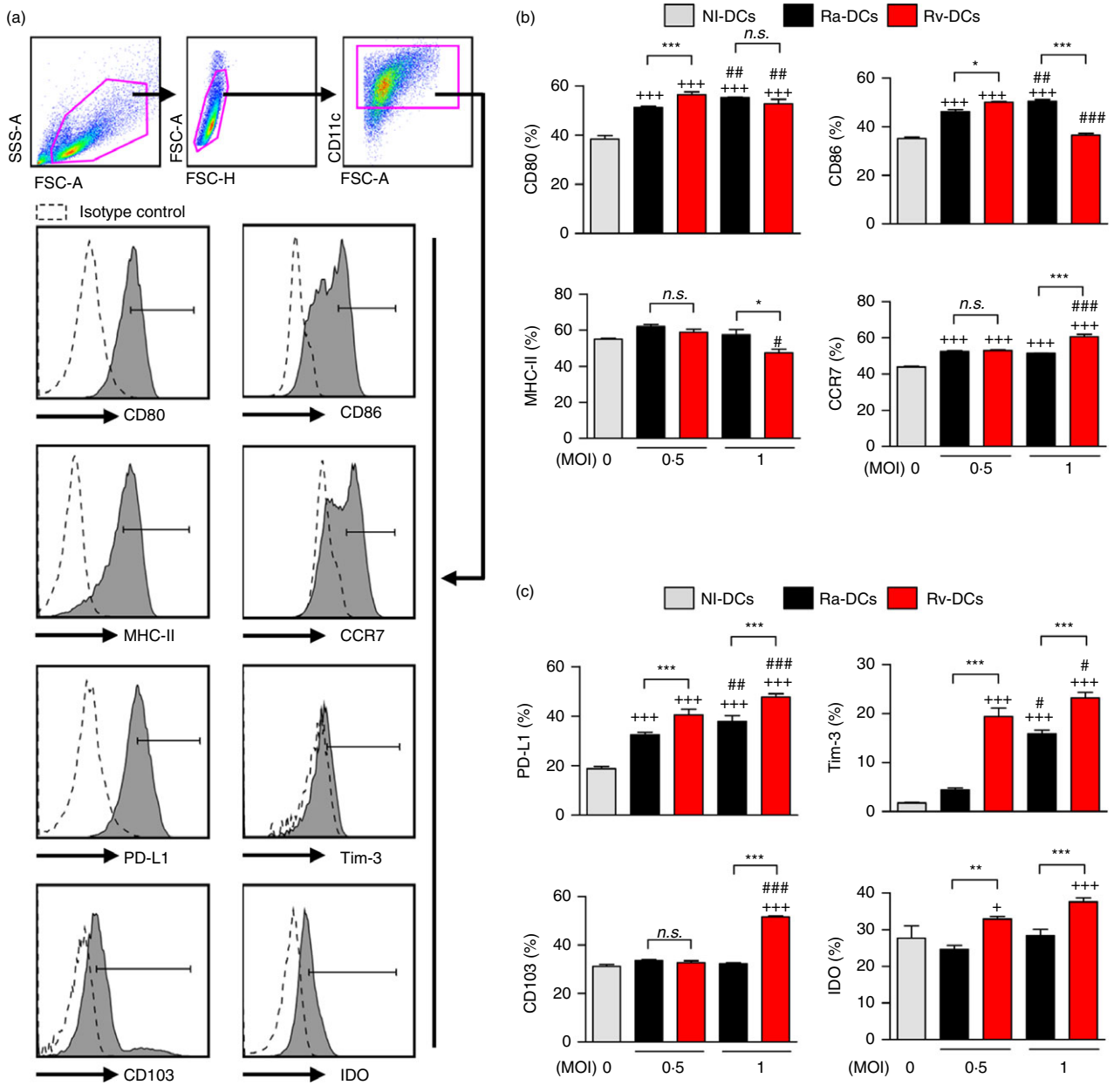

Figure 2. The virulent *Mycobacterium tuberculosis* strain H37Rv induces a tolerogenic dendritic cell (DC) phenotype in a multiplicity of infection (MOI) -dependent manner. DCs were infected with *M. tuberculosis* at an MOI of 0·5 or 1. At 24 hr post-infection, the levels of DC surface molecules and tolerogenic molecules were analysed. The harvested cells were stained with monoclonal antibodies against CD11c, CD80, CD86, MHC class II, CCR7, PD-L1, CD103, Tim-3 and IDO and analysed by flow cytometry. The gating strategies for each molecule are shown in (a), and the expression levels of these molecules are shown in bar graphs (b, c). The significance of the differences in expression between *M. tuberculosis* strains and between MOIs is indicated. All data are expressed as the mean ± SD (n = 3 samples) of one representative experiment of three independent experiments. $+P < 0{\cdot}05$, $+++P < 0{\cdot}001$ between *M. tuberculosis*-infected DCs and non-infected DCs; $\#P < 0{\cdot}05$, $\#\#P < 0{\cdot}01$, $\#\#\#P < 0{\cdot}001$ between MOIs of the same strain; *n.s.*, not significant; $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$ between H37Ra-infected DCs (Ra-DCs) and H37Rv-infected DCs (Rv-DCs). NI-DCs: non-infected DCs. [Colour figure can be viewed at wileyonlinelibrary.com]

The virulent *M. tuberculosis* strain-mediated conditioning of DCs into a tolerogenic phenotype is mediated by the IL-10 cascade

Virulent H37Rv-infected DCs displayed a tolerogenic phenotype that impeded T-cell proliferation and polarization. Among the factors induced by *M. tuberculosis* infection in DCs, IL-10 showed dramatically increased secretion from H37Rv-infected DCs compared with H37Ra-infected DCs beginning from early time-points after infection (Fig. 3). Moreover, previous studies have reported that IL-10

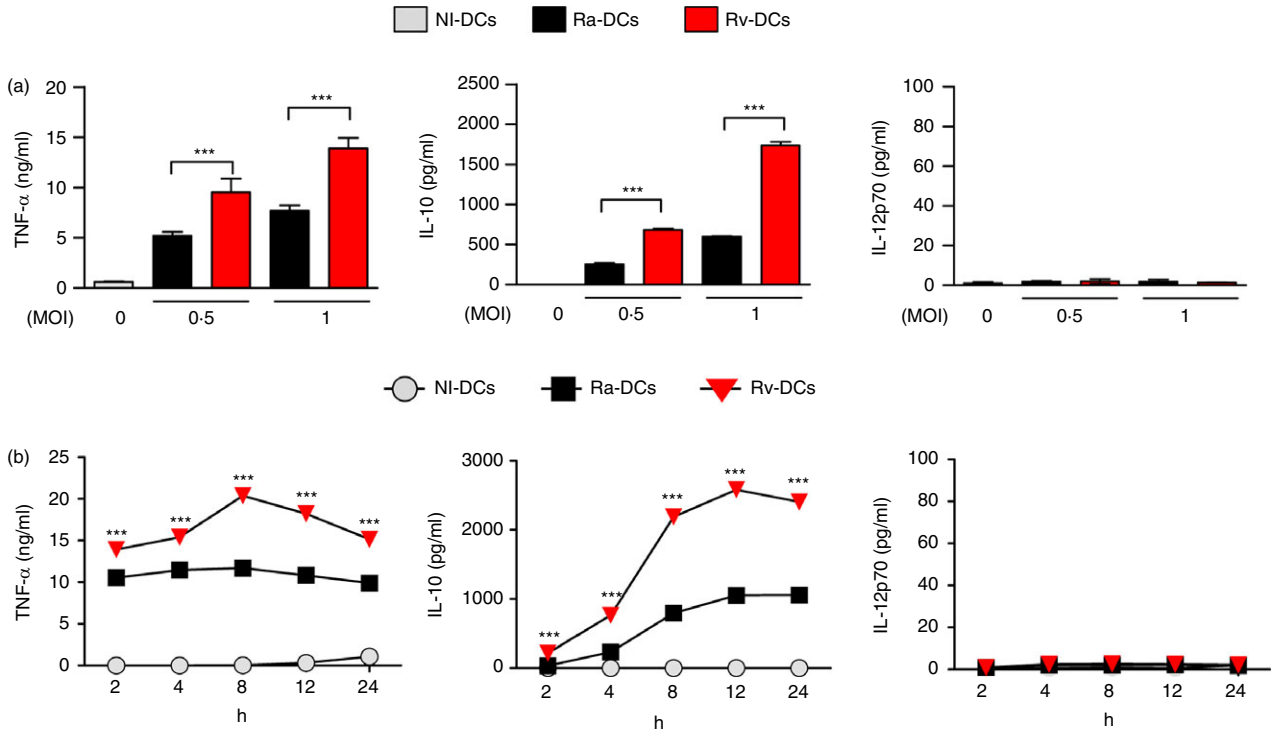

Figure 3. Dendritic cells (DCs) infected with the virulent *Mycobacterium tuberculosis* strain H37Rv induce early and marked production of interleukin-10 (IL-10). (a) The supernatant was collected from DCs infected with *M. tuberculosis* for 24 hr, and the levels of tumour necrosis factor-α (TNF-α), IL-10, and IL-12p70 were determined via ELISA. (b) To analyse cytokine kinetics, DCs were infected with bacilli at a multiplicity of infection (MOI) of 1, and supernatants were harvested at various time-points. The cytokine levels in supernatants were determined by ELISA. All data are expressed as the mean ± SD ($n = 3$ per designated time-point) of one representative experiment of three independent experiments. ***$P < 0{\cdot}001$ between H37Ra-infected DCs (Ra-DCs) and H37Rv-infected DCs (Rv-DCs). NI-DCs: non-infected DCs. [Colour figure can be viewed at wileyonlinelibrary.com]

induces immune tolerance via DCs or tolerogenic DCs *in vitro*.[22,23] Hence, we focused on IL-10 as a modulator of the tolerogenic DC phenotype. To verify the effect of IL-10 on the phenotype of *M. tuberculosis*-infected DCs, H37Rv and H37Ra were infected into DCs at an MOI of 1, followed by treatment of the cells with an anti-IL-10 mAb. At 24 hr after infection, the levels of cytokines (IL-10 and TNF-α) and DC surface markers (MHC-II, CD80, CD86, CD103, Tim-3, and PD-L1) were analysed (Fig. 4). The anti-IL-10 neutralizing antibody functioned properly (Fig. 4a). The *M. tuberculosis* infection-induced impairment in the expression of maturation markers, such as MHC-II, CD80 and CD86 in DCs was ameliorated by treatment with the anti-IL-10 neutralizing antibody (Fig. 4b), whereas the expression of tolerogenic molecules, such as Tim-3, CD103 and PD-L1 were decreased by by the anti-IL-10 neutralizing antibody in DC treatment (Fig. 4c).

To confirm these results, we generated DCs from bone marrow cells of IL-$10^{-/-}$ mice and obtained similar results. WT-DCs and DCs derived from IL-$10^{-/-}$ mice (IL-$10^{-/-}$-DCs) were infected with H37Rv or H37Ra at an MOI of 1 for 24 hr. Afterwards, the expression of surface molecules such as CD80, CD86, MHC-II, CD103, Tim-3 and PD-L1 was analysed using flow cytometry, and the production of cytokines such as IL-10, IL-12p70 and TNF-α was analysed by ELISA (Fig. 5). The *M. tuberculosis* infection-induced impairment in the expression of maturation markers such as MHC-II, CD80 and CD86 in WT-DCs was ameliorated in IL-$10^{-/-}$-DCs (Fig. 5a), whereas the expression of tolerogenic molecules such as CD103, Tim-3 and PD-L1 was increased in *M. tuberculosis*-infected WT-DCs but decreased in IL-$10^{-/-}$-DCs (Fig. 5b). Secretion level of TNF-α was higher in IL-$10^{-/-}$-DCs by infection with either *M. tuberculosis* strain. Although WT-DCs did not produce IL-12p70, IL-$10^{-/-}$-DCs secreted IL-12p70. Furthermore, H37Rv-infected IL-$10^{-/-}$-DCs produced fourfold higher IL-12p70 levels than H37Ra-infected IL-$10^{-/-}$-DCs (Fig. 5c). The restoration of the expression levels of these molecules in DCs by blocking IL-10 signalling was more significant following virulent H37Rv infection than following attenuated H37Ra infection.

IL-10 secretion from DCs infected with the virulent *M. tuberculosis* strain is mediated by the predominant activation of the p38 mitogen-activated protein kinase and is partially regulated by the ERK 1/2 signalling pathway

It has been well documented that IL-10 production in response to mycobacterial infection is mainly mediated by

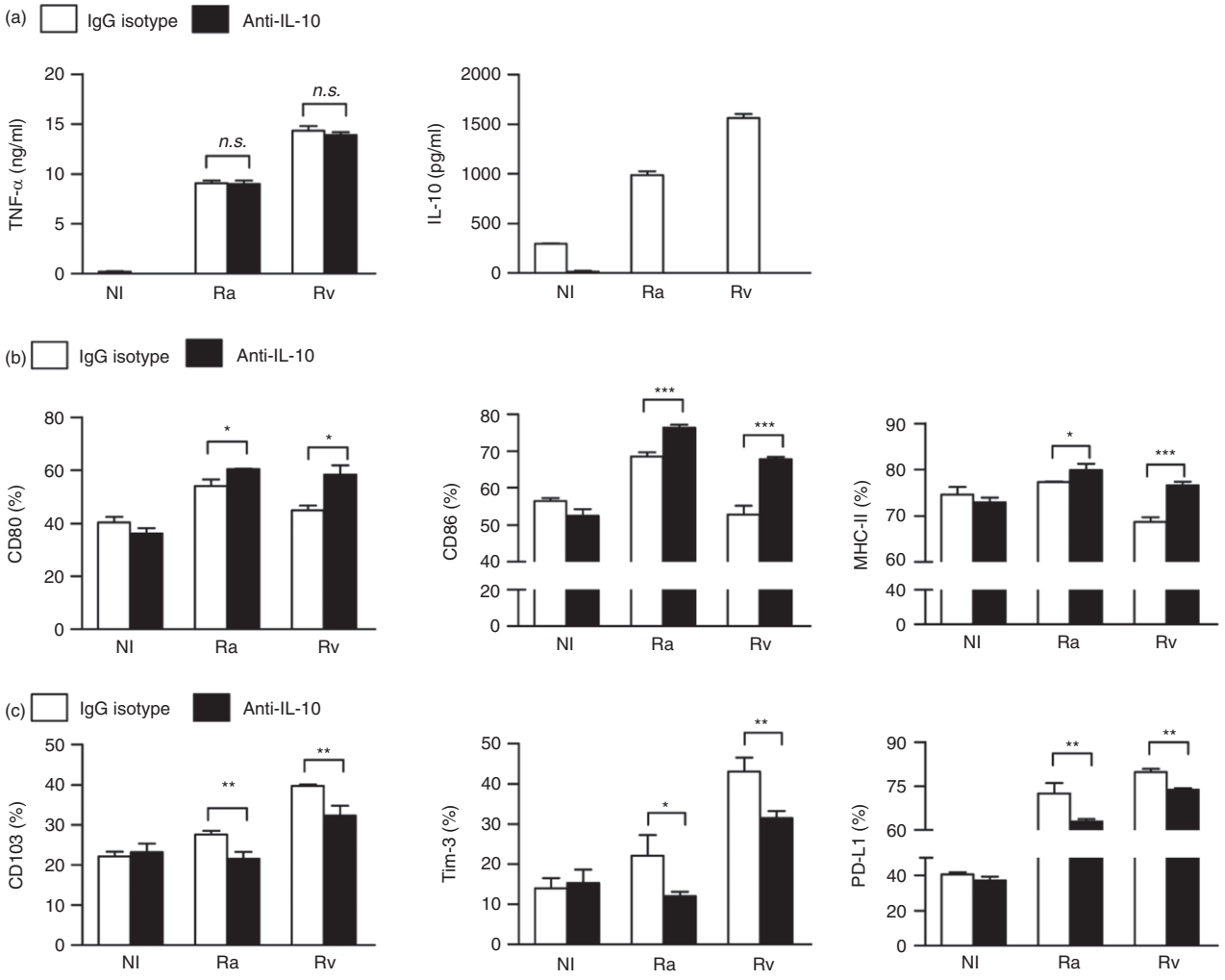

Figure 4. Blocking the interleukin-10 (IL-10) cascade using a neutralizing monoclonal antibody (mAb) restores the activated phenotype of dendritic cells (DCs) despite infection with virulent *Mycobacterium tuberculosis*. Before *M. tuberculosis* infection, an anti-IL-10 neutralizing antibody (Anti-IL-10) or an isotype control antibody (IgG isotype) was applied for 2 hr. Afterwards, the DCs were infected with *M. tuberculosis* (H37Ra or H37Rv strain) at a multiplicity of infection (MOI) of 1. (a) To verify the effects of the anti-IL-10 neutralizing mAb and the isotype control mAb, the levels of IL-10 and tumour necrosis factor-α (TNF-α)in the supernatants were assessed via ELISA. (b and c) The levels of DC surface stimulatory molecules (MHC class II, CD80, CD86) and tolerogenic molecules (CD103, Tim-3, PD-L1) were analysed via flow cytometry. All data are expressed as the mean ± SD (n = 3 samples) of one representative experiment of three independent experiments. *n.s.*, not significant; $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, and $^{***}P < 0{\cdot}001$, between IgG-treated DCs and anti-IL-10 mAb-treated DCs. NI: non-infected DCs, Ra: H37Ra-infected DCs, Rv: H37Rv-infected DCs.

two signalling pathways, the ERK1/2 mitogen-activated protein kinase (MAPK) and GSK-3β, in macrophages.[24,25] To determine the role of different signalling pathways in *M. tuberculosis*-induced IL-10 secretion of BMDCs, we examined the activation of various MAPKs and GSK-3β, all of which are involved in the production from IL-10 in response to *M. tuberculosis* infection (Fig. 6). We observed the most significant phosphorylation of p38 MAPK in H37Rv-infected DCs. In contrast, there were relatively lower levels of ERK1/2 MAPK phosphorylation compared to that of p38 MAPK, and no phosphorylation of JNK in DCs infected with either strain. We also observed that GSK-3β (Ser9) and its upstream, Akt, showed more significant phosphorylation following H37Rv infection than following H37Ra infection (Fig. 6a). As the previous study reported that the phosphorylation of GSK-3β could be regulated by p38 MAPK,[26] we next examined the influence of p38 and ERK1/2 MAPK signalling on GSK-3β inhibitors in *M. tuberculosis*-infected DCs using specific inhibitors. However, the inhibition of p38 and ERK signalling had little influence on GSK-3β phosphorylation (Fig. 6b). We next examined the involvement of the GSK-3β, p38, ERK and JNK MAPK signalling pathways in IL-10 production using inhibitors targeting members of each signalling pathway. Inhibition of p38 signalling exhibited the most significant decrease of IL-10 secretion, and ERK signalling inhibition had a partial effect; however, inhibiting GSK-

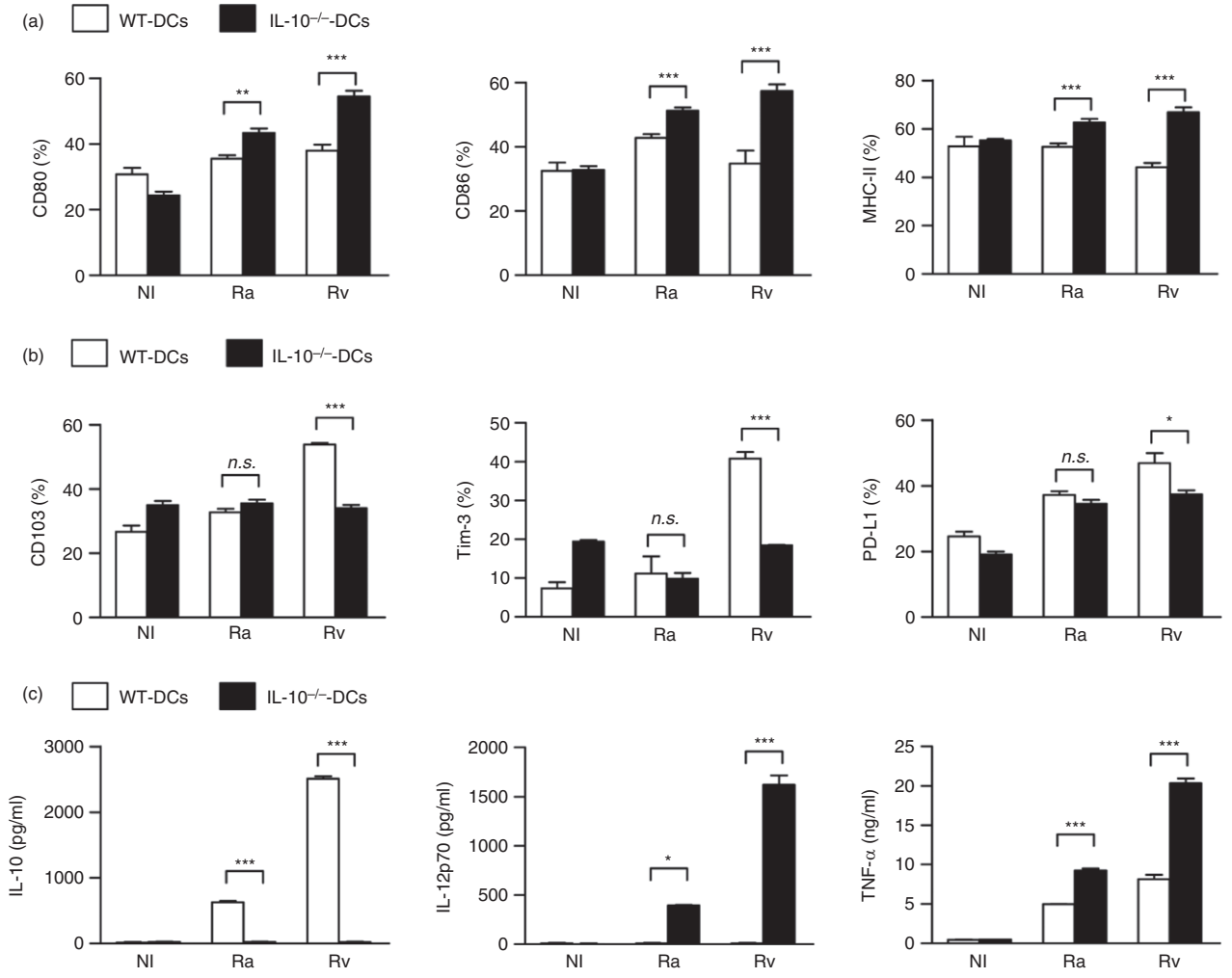

Figure 5. Absence of interleukin-10 (IL-10) from dendritic cells (DCs) restores the phenotypic and functional activation of DCs despite infection with virulent *Mycobacterium tuberculosis*. DCs were derived from wild-type (WT) or IL-$10^{-/-}$ mice. Then, the DCs were infected with *M. tuberculosis* (H37Ra or H37Rv strain) at a multiplicity of infection (MOI) of 1. At 24 hr after infection, the levels of the DC surface molecules CD80, CD86, MHC class II (a) as well as PD-L1, CD103, and Tim-3 (b) were analysed via flow cytometry. (c) The secreted levels of tumour necrosis factor-α (TNF-α), IL-10, and IL-12p70 were determined by ELISA. All data are expressed as the mean ± SD ($n = 3$ samples) of one representative experiment of three independent experiments. *n.s.*, not significant; $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, and $^{***}P < 0{\cdot}001$, between *M. tuberculosis*-infected WT-DCs and *M. tuberculosis*-infected IL-$10^{-/-}$-DCs. NI: non-infected DCs, Ra: H37Ra-infected DCs, Rv: H37Rv-infected DCs.

3β signalling had a minimal effect on IL-10 secretion from *M. tuberculosis*-infected DCs (Fig. 6c). Taken together, our data suggest that IL-10, a cytokine that induces a tolerogenic DC phenotype in response to *M. tuberculosis* infection, is primarily regulated via the p38 MAPK signalling pathway and is partially regulated via the ERK and GSK-3β pathways.

Infection of IL-$10^{-/-}$-DCs with the virulent *M. tuberculosis* strain restores the proliferation ability of T cells

Virulent H37Rv-infected DCs showed limited $CD4^+$ T-cell proliferation, which resulted from a tolerogenic DC phenotype induced by IL-10. Therefore, we analysed the effect of IL-10 on the proliferation ability of T cells in the presence of *M. tuberculosis*-infected DCs. WT-DCs and IL-$10^{-/-}$-DCs were infected or not with virulent H37Rv at an MOI of 1 and then co-cultured with $CD4^+$ T cells from OT-II mice. There was no difference in T-cell proliferation between the two control groups, $OVA_{323\text{-}339}$-treated WT-DCs and $OVA_{323\text{-}339}$-treated IL-$10^{-/-}$ DCs, and T-cell proliferation was markedly recovered in H37Rv-infected IL-$10^{-/-}$-DCs compared with that in H37Rv-infected WT-DCs (Fig. 7a,b). Next, the expression of the master transcription factors of Th1 cells (T-bet) and Th2 cells (GATA-3) was evaluated via flow cytometry (Fig. 7c). The expression of T-bet increased in T cells co-cultured with H37Rv-infected IL-$10^{-/-}$-DCs, but there was no change in GATA-3 expression following H37Rv

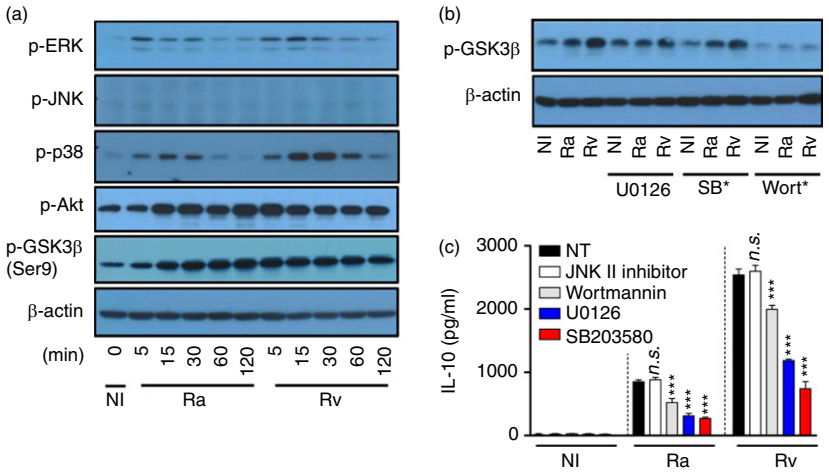

Figure 6. Interleukin-10 (IL-10) production in virulent *Mycobacterium tuberculosis* -infected tolerogenic dendritic cells (DCs) is mediated by the activation of a p38 mitogen-activated protein kinase (MAPK) -dependent signalling pathway. IL-10 secretion from *M. tuberculosis*-infected DCs is primarily regulated by the p38 signalling pathway. (a) DCs were infected at a multiplicity of infection (MOI) of 1 bacterium per cell. DCs were harvested at various time-points (0, 5, 15, 30, 60 and 120 min after infection), and levels of phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated Jun N-terminal kinase (p-JNK), p-p38, p-Akt, p-GSK3β (Ser9), and β-actin were measured via Western blotting. (b) Two hours before infection, U0126 (1 μM), SB203580 (1 μM), or Wortmannin (0·1 μM) was applied to DCs. Then, the DCs were infected at an MOI of 1 bacterium (either H37Ra or H37Rv) per cell. At 15 min after infection, the *M. tuberculosis*-infected cells were harvested, and the protein levels were measured using Western blotting. β-Actin was used as a loading control. One representative Western blot out of two independent experiments is shown. (c) JNK 2 (1 μM), U0126 (1 μM), SB203580 (1 μM), and Wortmannin (0·1 μM) were applied to DCs 2 hr before infection. Then, the DCs were infected at an MOI of 1 bacterium (H37Ra or H37Rv) per cell. After 24 hr, the IL-10 levels in the supernatants were measured through ELISA. The bar graphs show the mean ± SD (n = 3 samples) of one representative experiment of three independent experiments. *n.s.*, not significant; ***$P < 0{\cdot}001$, compared to non-infected DCs (NI). Ra: H37Ra-infected DCs, Rv: H37Rv-infected DCs. SB*: SB203580, Wort*: Wortmannin. [Colour figure can be viewed at wileyonlinelibrary.com]

infection in these cells. IFN-γ expression was also recovered in H37Rv-infected IL-$10^{-/-}$-DCs (Fig. 7d). Moreover, PD-L1 was highly expressed in DCs infected with H37Rv (Fig. 2c). Hence, we also co-cultured anti-PD-L1 blocking antibody-treated DCs with $CD4^+$ T cells from OT-II mice. However, we found no difference in T-cell proliferation ability between co-culture with anti-PD-L1 blocking antibody-treated DCs and with non-treated DCs (see Supplementary material, Fig. S2). These results suggest that IL-10 is one factor that impedes effective T-cell responses by H37Rv-infected DCs.

Discussion

Although numerous studies have examined the functions and alterations of macrophages during *M. tuberculosis* infection, few studies have reported the characteristics of DCs according to the virulence of *M. tuberculosis*. In this study, we investigated how virulent *M. tuberculosis* impairs and delays T-cell responses induced by interactions with DCs. A virulent *M. tuberculosis* strain drove DCs towards a tolerogenic phenotype by impairing DC-activating surface markers (CD80, CD86 and MHC class II) and by augmenting tolerogenic markers, such as PD-L1, CD103, Tim-3 and IDO, on DCs in an MOI-dependent manner, thereby suppressing Th1 responses. Of note, *M. tuberculosis* H37Rv-infected DCs showed markedly increased IL-10 production beginning from early time-points of infection through activation of p38 MAPK signalling pathways. The generation of these tolerogenic DCs by the virulent *M. tuberculosis* strain was dependent on the production of IL-10 by DCs, as neither DCs generated from IL-$10^{-/-}$ mice nor DCs treated with an anti-IL-10 neutralizing antibody generated tolerogenic DCs; consequently, the Th1-type T-cell response was restored in those DCs.

The immunological environment in lung lesions at the early stage of infection can be regulated by virulent *M. tuberculosis* strains via the manipulation of innate immune cells and of the cytokine balance. Hence, impairment and delay of T-cell responses are generally believed to be a unique hallmark of early-stage infection with virulent *M. tuberculosis*.[20] To eliminate intracellular pathogens such as *M. tuberculosis*, it is essential to initiate an optimal T-cell response, especially an antigen-specific Th1 response characterized by IFN-γ activity. Our previous study showed that the frequency of $CD11b^{high}$ DCs was significantly increased by infection with either *M. tuberculosis* H37Rv or H37Ra at early time-points,[15] although the frequency of this DC population in response to infection was not significantly different between the two strains at 14 days post-infection. However, an *ex vivo* experiment

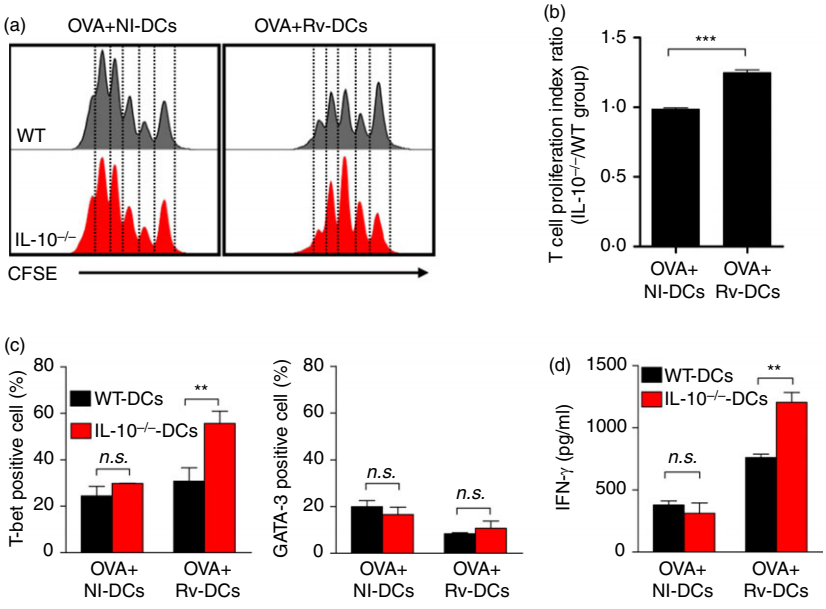

Figure 7. Absence of interleukin-10 (IL-10) from dendritic cells (DCs) restores the capacity of virulent *Mycobacterium tuberculosis*-induced tolerogenic DCs to stimulate T helper type 1 (Th1) T-cell proliferation. T-cell proliferation was recovered and the Th1 response was augmented in IL-$10^{-/-}$-DCs. DCs derived from wild-type (WT) or IL-$10^{-/-}$ mice were co-cultured with $CD4^+$ T cells from the spleens of OT-II mice. (a, b) T cells were labelled with CSFE, and the proliferation of T cells in the presence of DCs from WT or IL-$10^{-/-}$ mice was measured via flow cytometry. The proliferation index of T cells co-cultured with IL-$10^{-/-}$-DCs was divided by the proliferation index of T cells co-cultured with WT-DCs to calculate the proliferation index ratio. (c) T cells were co-cultured with H37Rv-infected WT-DCs or IL-$10^{-/-}$-DCs, followed by immunostaining for T-bet and GATA-3. (d) The levels of interferon-γ (IFN-γ) in supernatants were measured via ELISA. All data are expressed as the mean ± SD ($n = 3$ samples) of one representative experiment of three independent experiments. *n.s.*, not significant; $^{**}P < 0{\cdot}01$ or $^{***}P < 0{\cdot}001$, between T cells co-cultured with WT-DCs and T cells co-cultured with IL-$10^{-/-}$-DCs. OVA+NI-DCs: OVA-pulsed non-infected DCs, OVA+Rv-DCs: OVA-pulsed H37Rv-infected DCs. [Colour figure can be viewed at wileyonlinelibrary.com]

showed that infiltrated $CD11b^{high}$ DCs after virulent *M. tuberculosis* infection suppressed the Th1 response compared with naive $CD11b^{high}$ DCs. These previous results agree with our current results (Fig. 1), indicating that virulent *M. tuberculosis* can suppress the protective Th1 response by driving DCs towards a tolerogenic phenotype.

Many studies reported that *M. tuberculosis* or its products impair DC maturation. For example, *M. tuberculosis* infection inhibit the maturation of human monocyte-derived DCs; *M. tuberculosis*-infected DCs exhibit an impaired ability to induce T cell proliferation;[21] *M. tuberculosis* antigen heat-shock protein 70 impairs the maturation of murine BMDCs;[27] and ManLAM, a glycolipid of *M. tuberculosis*, inhibits lipopolysaccharide-induced DC activation.[28] As another example, *M. tuberculosis* regulates granuloma formation, dissemination, bacterial growth and the host immune response by producing virulence factors such as ESX-1 secretion associated proteins. Carlsson *et al.* [29] demonstrated that the ESX-1 secretion system is required for granuloma persistence and survival in immune cells. Importantly, this system is present in virulent strains (*M. tuberculosis* H37Rv) but is largely absent from attenuated mycobacterial strains (H37Ra).[30,31] These results agree with our data to a certain extent, in terms of inhibition of T-cell proliferation by *M. tuberculosis* H37Rv-infected DCs. Furthermore, our data demonstrated that H37Rv infection of DCs led to the development of tolerogenic DCs because there was no observed secondary effect of IL-10 and TGF-β produced from T cells co-cultured with *M. tuberculosis*-infected DCs on the inhibition of T-cell proliferation (data not shown). In addition, DCs infected with the virulent *M. tuberculosis* strain were actively involved in the suppression of T-cell proliferation in the presence of polyclonal stimulation with anti-CD3e and anti-CD28 in a population-dependent manner (see Supplementary material, Fig. S3) Regarding the role of IL-10 in *M. tuberculosis* infection, several studies have been reported with controversial results. Some groups reported no difference in bacterial load or resistance to *M. tuberculosis* between IL-$10^{-/-}$ and WT C57BL/6 mice,[32–34] even though IL-$10^{-/-}$ mice showed enhanced IFN-γ expression.[34] In contrast, a decreased bacterial load together with an enhanced IFN-γ response in the lungs was detected in IL-$10^{-/-}$ mice compared with WT mice.[11,35] In another previous study, pre-treatment of mice with an anti-IL-10 antibody resulted in an increase in the abundance of Ag85B-specific P25 T-cell

receptor-transgenic $CD4^+$ Th1 cells in the *M. tuberculosis*-infected lung on day 21 post-infection, whereas neutralization of TGF-β, another important suppressive cytokine in the lung, showed no effects on Th1 induction.[4] As revealed in this study, marked IL-10 production in DCs in response to virulent *M. tuberculosis* H37Rv infection is the most important factor that alters the phenotype of DCs towards a tolerogenic state and, consequently, suppresses the Th1 response (Figs 4, 5 and 7). In addition, pulmonary DCs were reported to suppress the Th1 response via IL-10 production.[36] Hence, a virulent *M. tuberculosis* strain may exploit innately programmed suppressive mechanisms in the lungs to suppress the Th1 response at the early stage of infection.

Diverse signalling pathways have been known to regulate the production of IL-10. It was reported that ERK MAPK signalling is one major signalling pathway inducing IL-10 production in *M. tuberculosis*-infected macrophages.[37,38] However, this study indicated that blocking the p38 pathway had a greater effect on IL-10 production than blocking the ERK signalling pathway (Fig. 6c). This difference may have resulted from differences between cell types. According to previous studies, macrophages and DCs showed different degrees of ERK phosphorylation and IL-10 production in response to stimulation by Toll-like receptors 9 and 4.[39] In addition, IL-10 was produced by neutrophils[40] and human lung epithelial cells[41] through p38 MAPK signalling pathway activity in response to bacillus Calmette–Guérin. These results indicated that the signalling pathways involved in IL-10 production may differ among innate immune cells in response to specific stimuli.

The importance of IL-10 in DCs for the induction of immune tolerance has largely been demonstrated. For example, DCs genetically modified to express IL-10 exert suppressive effects in models of alloreactivity and autoimmunity.[42,43] In this regard, Jarnicki *et al.*[44] demonstrated that inhibition of p38 MAPK-dependent IL-10 secretion from DCs led to stronger Th1 responses and weaker regulatory T-cell responses. These findings indicate that the tolerogenic DC phenotype generated in response to H37Rv can be overcome by inhibiting p38 signalling in DCs. Additionally, p38 may serve as an important therapeutic target, and p38 signalling is a potential mechanism for enhancing the efficacy of the Th1 response via vaccine or immunological interventions.

Current TB pathogenesis paradigms are changing in consideration of pathogen diversity because greater virulence has been identified in *M. tuberculosis* clinical isolates than was previously anticipated.[45] Hence, a further understanding of the characteristics of immune cells depending on *M. tuberculosis* virulence may facilitate the rational design of more effective therapeutic interventions, including immunotherapeutic vaccines. In conclusion, the findings of our study suggest that the virulent *M. tuberculosis* strain H37Rv impeded effective T-cell responses by inducing marked IL-10 release from infected DCs via the dominant activation of the p38 MAPK signalling pathway and partial regulation of the ERK MAPK signalling pathway. Hence, virulent *M. tuberculosis* may drive the production of pathogen-favourable tolerogenic DCs to diminish protective Th1 responses and may contribute to the inability of the host to eradicate the infection. Further functional analysis of the direct interplay between the components of *M. tuberculosis* that induce IL-10 production and alter DC maturation and the functionality of T cells may help us to understand how virulent *M. tuberculosis* evolved immunologically as the most highly adapted human pathogen.

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Disclosures

The authors declare no conflicts of interest.

References

1 Dye C, Williams BG, Espinal MA, Raviglione MC. Erasing the world's slow stain: strategies to beat multidrug-resistant tuberculosis. *Science* 2002; **295**:2042–6.

2 Chen K, Kolls JK. T cell-mediated host immune defenses in the lung. *Annu Rev Immunol* 2013; **31**:605–33.

3 Tian T, Woodworth J, Skold M, Behar SM. *In vivo* depletion of $CD11c^+$ cells delays the $CD4^+$ T cell response to *Mycobacterium tuberculosis* and exacerbates the outcome of infection. *J Immunol* 2005; **175**:3268–72.

4 Yahagi A, Umemura M, Tamura T, Kariyone A, Begum MD, Kawakami K *et al.* Suppressed induction of mycobacterial antigen-specific Th1-type $CD4^+$ T cells in the lung after pulmonary mycobacterial infection. *Int Immunol* 2010; **22**:307–18.

5 Lopez B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V *et al.* A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. *Clin Exp Immunol* 2003; **133**:30–7.

6 Freeman S, Post FA, Bekker LG, Harbacheuski R, Steyn LM, Ryffel B *et al.* *Mycobacterium tuberculosis* H37Ra and H37Rv differential growth and cytokine/chemokine induction in murine macrophages *in vitro*. *J Interferon Cytokine Res* 2006; **26**:27–33.

7 Chen M, Gan H, Remold HG. A mechanism of virulence: virulent *Mycobacterium tuberculosis* strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. *J Immunol* 2006; **176**:3707–16.

8 Rajashree P, Supriya P, Das SD. Differential migration of human monocyte-derived dendritic cells after infection with prevalent clinical strains of *Mycobacterium tuberculosis*. *Immunobiology* 2008; **213**:567–75.

9 Jiao X, Lo-Man R, Guermonprez P, Fiette L, Deriaud E, Burgaud S *et al.* Dendritic cells are host cells for mycobacteria *in vivo* that trigger innate and acquired immunity. *J Immunol* 2002; **168**:1294–301.

10 Cooper AM. Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol* 2009; **27**:393–422.

11 Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E *et al.* Enhanced protection to *Mycobacterium tuberculosis* infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. *Eur J Immunol* 2010; **40**:2200–10.

12 Jeyanathan M, McCormick S, Lai R, Afkhami S, Shaler CR, Horvath CN *et al.* Pulmonary *M. tuberculosis* infection delays Th1 immunity via immunoadaptor DAP12-regulated IRAK-M and IL-10 expression in antigen-presenting cells. *Mucosal Immunol* 2014; **7**:670–83.

13 Fietta A, Meloni F, Francioli C, Morosini M, Bulgheroni A, Casali L *et al.* Virulence of *Mycobacterium tuberculosis* affects interleukin-8, monocyte chemoattractant protein-1 and interleukin-10 production by human mononuclear phagocytes. *Int J Tissue React* 2001; **23**:113–25.

14 Philips JA, Ernst JD. Tuberculosis pathogenesis and immunity. *Annu Rev Pathol* 2012; **7**:353–84.

15 Kim WS, Kim JS, Cha SB, Han SJ, Kim H, Kwon KW *et al.* Virulence-dependent alterations in the kinetics of immune cells during pulmonary infection by *Mycobacterium tuberculosis*. *PLoS One* 2015; **10**:e0145234.

16 Jang B, Kim HW, Kim JS, Kim WS, Lee BR, Kim S *et al.* Peptidylarginine deiminase inhibition impairs Toll-like receptor agonist-induced functional maturation of dendritic cells, resulting in the loss of T cell-proliferative capacity: a partial mechanism with therapeutic potential in inflammatory settings. *J Leukoc Biol* 2015; **97**: 351–62.

17 Knaul JK, Jorg S, Oberbeck-Mueller D, Heinemann E, Scheuermann L, Brinkmann V *et al.* Lung-residing myeloid-derived suppressors display dual functionality in murine pulmonary tuberculosis. *Am J Respir Crit Care Med* 2014; **190**:1053–66.

18 Tsiganov EN, Verbina EM, Radaeva TV, Sosunov VV, Kosmiadi GA, Nikitina IY *et al.* Gr-1^{dim}CD11b^{+} immature myeloid-derived suppressor cells but not neutrophils are markers of lethal tuberculosis infection in mice. *J Immunol* 2014; **192**:4718–27.

19 Qin A, Cai W, Pan T, Wu K, Yang Q, Wang N *et al.* Expansion of monocytic myeloid-derived suppressor cells dampens T cell function in HIV-1-seropositive individuals. *J Virol* 2013; **87**:1477–90.

20 Urdahl KB, Shafiani S, Ernst JD. Initiation and regulation of T-cell responses in tuberculosis. *Mucosal Immunol* 2011; **4**:288–93.

21 Hanekom WA, Mendillo M, Manca C, Haslett PA, Siddiqui MR, Barry C 3rd *et al.* *Mycobacterium tuberculosis* inhibits maturation of human monocyte-derived dendritic cells *in vitro*. *J Infect Dis* 2003; **188**:257–66.

22 McBride JM, Jung T, de Vries JE, Aversa G. IL-10 alters DC function via modulation of cell surface molecules resulting in impaired T-cell responses. *Cell Immunol* 2002; **215**:162–72.

23 Torres-Aguilar H, Aguilar-Ruiz SR, Gonzalez-Perez G, Munguia R, Bajana S, Meraz-Rios MA *et al.* Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory CD4^{+} T cells. *J Immunol* 2010; **184**:1765–75.

24 Klug K, Ehlers S, Uhlig S, Reiling N. Mitogen-activated protein kinases p38 and ERK1/2 regulated control of *Mycobacterium avium* replication in primary murine macrophages is independent of tumor necrosis factor-α and interleukin-10. *Innate Immun* 2011; **17**:470–85.

25 Chan MM, Cheung BK, Li JC, Chan LL, Lau AS. A role for glycogen synthase kinase-3 in antagonizing mycobacterial immune evasion by negatively regulating IL-10 induction. *J Leukoc Biol* 2009; **86**:283–91.

26 Thornton TM, Pedraza-Alva G, Deng B, Wood CD, Aronshtam A, Clements JL *et al.* Phosphorylation by p38 MAPK as an alternative pathway for GSK3β inactivation. *Science* 2008; **320**:667–70.

27 Motta A, Schmitz C, Rodrigues L, Ribeiro F, Teixeira C, Detanico T *et al.* *Mycobacterium tuberculosis* heat-shock protein 70 impairs maturation of dendritic cells from bone marrow precursors, induces interleukin-10 production and inhibits T-cell proliferation *in vitro*. *Immunology* 2007; **121**:462–72.

28 Geijtenbeek TB, Van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, Appelmelk B *et al.* Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* 2003; **197**:7–17.

29 Carlsson F, Kim J, Dumitru C, Barck KH, Carano RA, Sun M *et al.* Host-detrimental role of Esx-1-mediated inflammasome activation in mycobacterial infection. *PLoS Pathog* 2010; **6**:e1000895.

30 Romagnoli A, Etna MP, Giacomini E, Pardini M, Remoli ME, Corazzari M, *et al.* ESX-1 dependent impairment of autophagic flux by *Mycobacterium tuberculosis* in human dendritic cells. *Autophagy* 2012; **8**:1357–70.

31 Lee JS, Krause R, Schreiber J, Mollenkopf HJ, Kowall J, Stein R *et al.* Mutation in the transcriptional regulator PhoP contributes to avirulence of *Mycobacterium tuberculosis* H37Ra strain. *Cell Host Microbe* 2008; **3**:97–103.

32 North RJ. Mice incapable of making IL-4 or IL-10 display normal resistance to infection with *Mycobacterium tuberculosis*. *Clin Exp Immunol* 1998; **113**:55–8.

33 Jung YJ, Ryan L, LaCourse R, North RJ. Increased interleukin-10 expression is not responsible for failure of T helper 1 immunity to resolve airborne *Mycobacterium tuberculosis* infection in mice. *Immunology* 2003; **109**:295–9.

34 Higgins DM, Sanchez-Campillo J, Rosas-Taraco AG, Lee EJ, Orme IM, Gonzalez-Juarrero M. Lack of IL-10 alters inflammatory and immune responses during pulmonary *Mycobacterium tuberculosis* infection. *Tuberculosis* 2009; **89**:149–57.

35 Roach DR, Martin E, Bean AG, Rennick DM, Briscoe H, Britton WJ. Endogenous inhibition of antimycobacterial immunity by IL-10 varies between mycobacterial species. *Scand J Immunol* 2001; **54**:163–70.

36 Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001; **2**:725–31.

37 Boonstra A, Rajsbaum R, Holman M, Marques R, Asselin-Paturel C, Pereira JP *et al.* Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J Immunol* 2006; **177**:7551–8.

38 Redford PS, Murray PJ, O'Garra A. The role of IL-10 in immune regulation during *M. tuberculosis* infection. *Mucosal Immunol* 2011; **4**:261–70.

39 Kaiser F, Cook D, Papoutsopoulou S, Rajsbaum R, Wu X, Yang HT *et al.* TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. *J Exp Med* 2009; **206**:1863–71.

40 Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R. Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. *Immunity* 2009; **31**:761–71.

41 Mendez-Samperio P, Trejo A, Perez A. *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) stimulates IL-10 production via the PI3K/Akt and p38 MAPK pathways in human lung epithelial cells. *Cell Immunol* 2008; **251**:37–42.

42 Coates PT, Krishnan R, Kireta S, Johnston J, Russ GR. Human myeloid dendritic cells transduced with an adenoviral interleukin-10 gene construct inhibit human skin graft rejection in humanized NOD-scid chimeric mice. *Gene Ther* 2001; **8**:1224–33.

43 Li WM, Liu W, Gao C, Zhou BG, Yang SS, Wang Z *et al.* Antigen-specific tolerance induced by IL-10 gene modified immature dendritic cells in experimental autoimmune myocarditis in rats. *Chin Med J (Engl)* 2006; **119**:1646–52.

44 Jarnicki AG, Conroy H, Brereton C, Donnelly G, Toomey D, Walsh K *et al.* Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signaling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapeutics. *J Immunol* 2008; **180**:3797–806.

45 Reiling N, Homolka S, Walter K, Brandenburg J, Niwinski L, Ernst M *et al.* Clade-specific virulence patterns of *Mycobacterium tuberculosis* complex strains in human primary macrophages and aerogenically infected mice. *MBio* 2013; **4**:e00250–13.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The virulent *Mycobacterium tuberculosis* strain H37Rv displayed greater cytotoxicity to DCs than the attenuated *M. tuberculosis* strain H37Ra.

Figure S2. Blocking PD-L1 does not affect the tolerogenic function of H37Rv-infected dendritic cells.

Figure S3. *Mycobacterium tuberculosis*-infected dendritic cells (DCs) exert a suppressive effect on T-cell proliferation. CD4^{+} T cells were isolated from the spleen using a MACS system and labelled with 1·25 μM CFSE.

