International Immunology, Vol. 29, No. 3, pp. 121–131
doi:10.1093/intimm/dxx013
Advance Access publication 23 March 2017

CD40–CD40L cross-talk drives fascin expression in dendritic cells for efficient antigen presentation to CD4+ T cells

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Received 20 September 2016, *editorial decision* 14 March 2017; *accepted* 17 March 2017

Abstract

Fascin is an actin-bundling protein that, among immune cells, is restricted to expression in dendritic cells (DCs). Previous reports have suggested that fascin plays an important role in governing DC antigen presentation to CD4+ T cells. However, no report has clearly linked the receptor–ligand engagement that can direct downstream regulation of fascin expression. In this study, bone marrow-derived DCs from wild-type versus CD40-knockout C57BL/6 mice were used to elucidate the mechanisms of fascin expression and activity upon CD40–CD40 ligand (CD40L) engagement. These investigations now show that CD40 engagement governs fascin expression in DCs to promote CD4+ T-cell cytokine production. Absence of CD40 signaling resulted in diminished fascin expression in DCs and was associated with impaired CD4+ T-cell responses. Furthermore, the study found that loss of CD40–CD40L engagement resulted in reduced DC–T-cell contacts. Rescue by ectopic fascin expression in CD40-deficient DCs was able to re-establish sustained contacts with T cells and restore cytokine production. Taken together, these results show that cross-talk through CD40–CD40L signaling drives elevated fascin expression in DCs to support acquisition of full T-cell responses.

Keywords: actin bundling, co-receptor, co-stimulation, cytoskeleton, immune synapse

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that function as principal mediators of immunity (1). DCs have the capacity to migrate to lymph nodes and present captured antigens in the form of peptides bound to MHC class molecules (2). Recognition of peptide-bound MHC class molecules (pMHC) by the TCR leads to activation of adaptive immune responses (1, 3). Appropriate engagement of DCs and T cells leads to long-term and sustained contacts governed by a combination of MHC class, co-stimulatory, adhesion and cytoskeletal molecules. This formation provides a framework for DCs to impart context-specific information in priming cognate T-cell responses (4, 5).

At the contact plane, the actin cytoskeleton in T cells is induced to undergo retrograde actin flow upon TCR engagement with cognate pMHC presented by DCs (6–11). This retrograde actin flow provides a radial symmetric all-stop mechanism for T-cell arrest on the APCs (12, 13). Adhesion and co-stimulatory molecule engagement between DCs and T cells further supports sustained contact and drives intracellular signaling activities (14–17). Although the mechanism(s) that allows for retention of TCR microclusters against retrograde actin flow is not well understood, reports have suggested that the DC cytoskeleton may provide opposing mechanical force through the immunological synapse (IS) to coordinately control TCR signaling activities (18–20).

One appealing candidate to modulate the DC cytoskeleton is fascin, a protein that organizes actin filaments into tightly packed parallel bundles (21–23). The actin-bundling activity of fascin provides tensile structural support to the cytoskeleton (21, 24, 25). An increase in fascin concentration results in conversion of actin networks into rigid states strongly resistant to mechanical forces (25). Among immune cells, fascin expression is restricted to bone marrow-derived mature DCs (mDCs) (26, 27); very low expression is found in bone marrow-derived immature DC (iDC) populations (28). Neither granulocytes, T cells nor B cells express fascin (27). Further reports have shown that fascin expression in mDCs is required for full activation and acquisition of effector functions in responder T cells (26, 29, 30). In addition to expression, reports have also shown that fascin polarizes to the site of DC–T-cell contact

upon cognate pMHC–TCR engagement (31). The signals that regulate fascin function in DCs are unknown, but it has been suggested that CD40 may be involved (32, 33).

CD40 is expressed on mDCs upon stimulation by TLR-agonists and/or pro-inflammatory cytokines. CD40 ligand (CD40L; CD154) is expressed on activated $CD4^+$ T cells after engagement of the TCR with cognate pMHC and co-stimulatory molecules (34, 35). CD40–CD40L engagement occurs after initial TCR–pMHC engagement (36). An intact DC–T-cell engagement allows CD40L expression and accumulation at the IS (36, 37). This cross-talk effectively *licenses* DCs to maximally drive T-cell responses and differentiation into effector versus memory subsets (36, 38). Suitably, CD40 *licensing* has been suggested to lead to cytoskeletal re-orientation in promotion of MHC class II clustering at the IS (33, 39). This present study aimed to elucidate CD40 cross-talk signaling and actin-bundling activities of fascin in DCs as a means to govern $CD4^+$ T-cell responses.

Methods

Animals

Wild-type (WT; 6–12 weeks old, C57BL6/J) and CD40-deficient ($CD40^{-/-}$) mice were used to generate bone marrow-derived DCs (40). Ovalbumin transgenic for MHC class II (OT-II) mice (6–10 weeks old) were used as a source of $CD4^+$ T cells. These T cells recognize the ovalbumin peptide region 323–339 ($OVA_{323–339}$) (41). All mice were purchased from Jackson Laboratories and housed under approved IACUC guidelines at Howard University.

Generation of DCs and isolation of $CD4^+$ T cells

Femur and tibia bones harvested from mice were used to isolate bone marrow cells. Total bone marrow cells were then washed and cultured in IMDM medium supplemented with pen/strep, L-glutamine and 20 ng ml^{-1} of GM-CSF for 7 days, following approaches described by Inaba *et al.* (42). Cells were replenished with fresh IMDM complete medium on day 3 (of the 7-day DC-generation process). By day 6, there was a large proportion of bone marrow-derived DCs. For maturation of DCs, 250 ng ml^{-1} of LPS was added to the culture on day 6 for 24 h. Live versus dead cell viability assays (Thermo Fisher Scientific, Waltham, MA, USA) were performed using the Countess II (Thermo Fisher Scientific) and flow cytometry. Magnetically activated cell sorting (Miltenyi Biotec, Cologne, Germany) approaches were employed for isolating $CD11c^+$ cells; purification was >95%. For isolation of $CD4^+$-naive T cells, lymph nodes and spleen were harvested from OT-II transgenic mice. Tissues were disassociated into a single cell suspension; red blood cell lysis buffer was used to remove erythrocytes. Next, antibodies to CD8 (clone 53–6.7) and MHC class II (clone M5/114.15.2), followed by anti-rat IgG microbeads, were used to negatively separate $CD4^+$ T cells. The approach resulted in >90% purity, as assessed by flow cytometry.

Antibodies and bioreagents

Fascin antibody clone 55K2 (EMD Millipore, Billerica, MA, USA and ECM Biosciences, Versailles, KY, USA) and the CD40 antibody clones FGK45.5 (Miltenyi and BioLegend, San Diego, CA, USA) and 1C10 (BioLegend) were used in the studies. Additionally, fluorochrome-labeled and unconjugated CD11c (clone N418), MHC class II (clone M5/114.15.2), CD86 (clone GL1) and CD40 (clone 3/23) antibodies purchased from BioLegend were used for immunophenotyping. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Thermo Fisher Scientific. To evaluate T-cell activation and cytokine phenotypes, fluorochrome-conjugated antibodies to CD4 (clone RM4-4), CD25, CD69, CD62L and IFN-γ (clone XMG1.2) were utilized; all were purchased from BioLegend. Purified CD154 (CD40L) antibody clones MR1 (BioLegend) and 208109 (R&D Systems, Minneapolis, MN, USA) were used for neutralization studies. Secondary antibodies conjugated to fluorochromes or HRP were used for western blot and intracellular flow cytometric analysis; anti-mouse, -rabbit or -goat conjugated to fluorochromes were purchased from Thermo Fisher Scientific. The mouse TLR1–9 agonist kit was purchased from Invivogen (San Diego, CA, USA); TLR 1/2 PamC3CSK4, TLR2 Heat-Killed *Listeria monocytogenes*, TLR3 Poly I:C, TLR4 LPS, TLR5 Flagellin, TLR6/2 diacylated lipoprotein, TLR7 ssRNA and TLR9 CpG ODN1826 were each used to stimulate bone marrow-derived DCs.

Enzyme-linked immunosorbent assays

For measuring cytokines produced by DCs, supernatant was harvested 24 h after stimulation of day 6 DCs with TLR-agonists and assayed for TNF-α, IL-1β and IL-12p70 (BD Biosciences) by ELISA. Supernatant was harvested 72 h after initial T-cell stimulation by DCs prior to assaying for IL-2 (BD Biosciences), IL-12p70 (BD Biosciences), IFN-γ (BD Biosciences), IL-4 (BioLegend) or IL-17 (BioLegend) by ELISA according to manufacturer recommended protocol(s).

Antigen presentation by OVA-pulsed DCs to $CD4^+$ T cells

Day 7 mature bone marrow-derived WT or $CD40^{-/-}$ DCs were pulsed with 1.0 or 0.1 μg ml^{-1} of $OVA_{323–339}$ peptide for 5 h at 37°C. DCs were then washed twice and re-suspended in DMEM medium supplemented with L-glutamine, non-essential amino acids, HEPES and pen/strep. For conjugated studies, WT or $CD40^{-/-}$ DCs were added at a 1:3 ratio with naive $CD4^+$ T cells purified from OT-II mice, respectively; $CD4^+$ T cells were confirmed naive by the phenotype: $CD69^{neg}$, $CD25^{neg}$ and $CD62L^+$. For proliferation studies, $CD4^+$ T cells were first labeled with 2.5 μM of CFSE (Thermo Fisher Scientific) for 10 min in PBS, followed by extensive washing in DMEM containing 50% serum. T cells were then cultured with OVA peptide-pulsed WT or $CD40^{-/-}$ bone marrow-derived DCs for 3–4 days and analyzed for proliferation by flow cytometry. Additionally, supernatant from the cultures was harvested for ELISA.

Neutralization assays

$CD4^+$ T cells were isolated from spleens of OT-II mice prior to activation by αCD3/CD28 microbeads (Mouse T-Activator Dynabeads; Thermo Fisher Scientific). After isolation, cells were then treated with 3 or 10 μg ml^{-1} of neutralizing antibodies to CD154 (αCD154; clone MR1 from BioLegend or clone 208109 from R&D Systems). Respective isotype antibodies were used as controls. In some experiments, the T cells were

pre-incubated with neutralizing antibodies prior to culture with WT DCs. Next, $CD4^+$ T cells were added with DC at a 3:1 ratio, respectively, with an additional 10 μg ml^{-1} of respective neutralizing antibodies present throughout length of the co-culture for 24 h.

Western blot analysis

NP-40 lysis buffer (Amresco, Solon, OH, USA) was used to prepare lysates by mixing with cells for 30 min prior to high-speed centrifugation. Lysates were run through hand-casted 10% gels using vertical gel electrophoresis. The protein content was transferred from gels to nitrocellulose blots using the PowerBlotter (Thermo Fisher Scientific) prior to probing for fascin, CD11c, CD4, MHC class II and/or GAPDH. After primary staining, secondary antibodies conjugated to fluorochromes were used to visualize bands on the Odyssey Fc imaging system (Licor, Lincoln, NE, USA). The Odysey Fc imaging software (Image Studio 4.0) was used to generate quantitative datasets based on fluorescence intensity ratios between captured channels.

Flow cytometric analysis of DC–T-cell conjugates

Supernatant from WT or $CD40^{-/-}$ bone marrow-derived DCs cultured with $CD4^+$ T cells was removed and the cells immediately fixed in 3% paraformaldehyde in PBS for 1 h at 4°C. For intracellular antibody labeling, cells were then permeabilized with 0.2% saponin in PBS for 1 h prior to addition of primary antibodies (diluted in 0.2% saponin in PBS). Cells were incubated with antibodies to CD4, CD86 and fascin overnight at 4°C prior to washing four times in 0.2% saponin in PBS. For primary unconjugated antibodies, secondary-tagged fluorochrome-labeled antibodies were prepared and used for staining. These secondary antibodies were diluted to 1:2000 working concentrations, with ~10 μl added per 2×10^5 cells. Cells were allowed to incubate for 2 h or overnight at 4°C prior to extensive washing. Samples were then acquired by flow cytometric analyzers. Datasets were analyzed utilizing FlowJo v10 software (FlowJo LLC, Ashland, OR, USA).

Lentiviral vector generation and transduction into DCs

A pLenti6/V5-DEST Gateway vector (Thermo Fisher Scientific) carrying an empty plasmid or fascin was used to generate high-titer lentiviruses. The pLenti6/V5-DEST-FASCIN was a gift from Lynda Chin (Addgene plasmid #31207) (43). Plasmids were transfected into 293FT cells following the manufacturer recommended protocol. Briefly, 1×10^7 293FT cells were transfected with 3 μg of pLenti-Fascin (pFascin) vector and 9 μg Virapower mix (Thermo Fisher Scientific) with 36 μl of lipofectamine 2000 (Thermo Fisher Scientific) in 3 ml of Opti-MEM (Thermo Fisher Scientific). Medium was replaced with DMEM at the 24-h time point and supernatant was collected post 72 h from initial transfection. Viruses were frozen at –80°C until use. For transduction, day 2 bone marrow cells (undergoing the 7-day DC differentiation process) were cultured with respective lentiviruses. After 48 h, unbound virus was removed by discarding supernatant and replacing with fresh medium supplemented with GM-CSF (to continue the 7-day DC-generation process). The transduction efficiency was evaluated by both western blot and flow cytometric analyses on day 7.

In vivo assays

WT or $CD40^{-/-}$ mice, matched at 10 weeks of age, were used for *in vivo* studies. Two groups of mice were injected intraperitoneally (ip) with a total of 100 μg of LPS in 200 μl of PBS; one control group received 200 μl PBS only. After 24 h, one group of the LPS-injected mice was treated with 200 μg of agonist CD40 antibody (αCD40) in 200 μl of PBS; the other group of LPS-treated and control group each received 200 μg of IgG isotype control antibody. Studies resulted in WT and $CD40^{-/-}$ mice with PBS + IgG, LPS + IgG and LPS + αCD40 antibody. After an additional 24 h (or a total of 48 h), mice were sacrificed. Spleens were harvested and cells stained for flow cytometric analyses.

Statistical analysis

All data are presented as mean ± SD. Comparison of two values between groups was made using two-tailed Student's *t*-tests. Comparisons of more than two means were made using a one-way ANOVA test. Differences were considered significant at $P < 0.05$. All analyses were made using Prism v6.07 software (GraphPad, La Jolla, CA, USA). In all presented datasets, $^*P < 0.05$, $^{**}P < 0.01$ and ns = not significant.

Results

Fascin is expressed in DCs upon TLR-induced maturation and further up-regulated upon anti-CD40 agonist stimulation

Immature versus mature bone marrow-derived DCs were evaluated for fascin expression. Briefly, bone marrow cells were treated with GM-CSF for 6 days to generate $CD11c^+$ iDCs prior to treatment with or without the TLR-agonist LPS (at 250 ng ml^{-1}) for maturation. mDCs showed increased fascin expression, as has been reported by Ross *et al.* (26). Western blot analysis revealed mDCs increased by 6-fold and mDCs treated with αCD40 increased by 10-fold compared to iDCs (Fig. 1A). Stimulation of iDCs with αCD40 moderately increased fascin because of the low levels of CD40 expression on the surface even in the immature state. Corroboratively, flow cytometric analysis revealed fascin expression is initially increased upon LPS stimulation and further augmented upon addition of αCD40 using CD11c-sorted DCs; staining for CD86 was used to identify the maturation status relative to fascin co-expression in the groups (Fig. 1B).

CD40-deficient mDCs are unable to further up-regulate fascin expression

Next, studies aimed to delineate whether CD40 signaling directly regulates fascin expression. Stimulation by TLR-agonists resulted in similar increased expression of fascin between WT and CD40-deficient ($CD40^{-/-}$) DC groups (Supplementary Figure 1A, available at *International Immunology* Online). These results are consistent with fascin expression being directly linked to TLR-induced maturation of DCs in response to pathogen stimuli (44–46). Next, mature WT versus $CD40^{-/-}$ DCs were treated with 3 or 10 μg

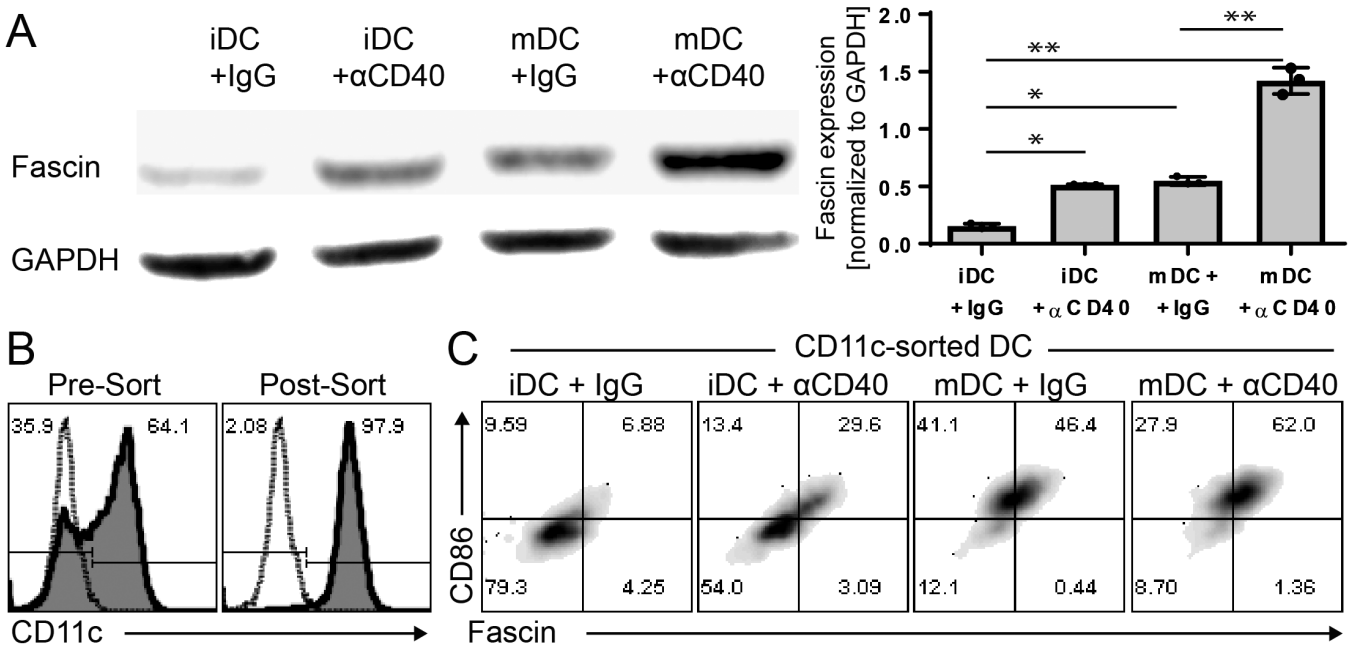

Fig. 1. Increased expression of fascin in WT DCs upon CD40 stimulation. (A) DCs were prepared from WT bone marrow cells by treatment for 7 days with GM-CSF. These *in vitro* generated DCs were left immature and stimulated with 10 μg ml^{-1} of IgG isotype control (iDC + IgG) or agonist CD40 antibody (iDC + αCD40). For maturation, DCs were stimulated with 250 ng ml^{-1} LPS prior to addition of 10 μg ml^{-1} of IgG control (mDC + IgG) or agonist CD40 antibody (mDC + αCD40). DCs were collected 24 h after treatment and lysates were prepared to detect fascin expression by western blot. Fascin levels were normalized to GAPDH loading controls. The bar graph represents mean and SD of three independent studies. Flow cytometric analyses of iDC + IgG, iDC+ αCD40, mDC+ IgG and mDC + αCD40 were performed at the 24-h time point after LPS and/or agonist αCD40 stimulation of sorted CD11c-positive DC subsets by the magnetically activated cell sorting approach. (B) Pre-sorted bone marrow-derived DCs are on the left and post-sorted CD11c-isolated DCs are on the right histogram (dark solid line; filled); isotype control is dashed and unfilled. (C) CD11c-isolated DCs were co-stained with CD86 and fascin; dot plots show CD86 on the y-axis versus fascin on the x-axis. Gates were established using isotype controls. Data are representative of three independent studies. *$P < 0.05$ and **$P < 0.01$ as assessed by two-tailed Student's *t*-test.

ml^{-1} of αCD40. Western blot analysis revealed a significant increase in fascin expression in WT DCs. However, CD40$^{-/-}$ DCs were unable to further up-regulate fascin (Fig. 2A). Flow cytometric analyses of CD11c-sorted DCs revealed fascin expression to be up-regulated upon αCD40 stimulation in WT DCs (with an increase in total fascin from 52.8 to 75.1%), but not in CD40$^{-/-}$ (which remained at 33.4% ± 1.3; Fig. 2B). These results suggest that CD40 engagement is able to increase fascin levels beyond that of initial TLR-agonist engagement.

Cognate engagement by antigen-specific CD4$^+$ T cells drives fascin expression through CD40 in DCs

Antigen presentation by mDCs (bearing cognate peptide) leads to increased expression of CD40L on early activated CD4$^+$, but not CD8$^+$, T cells (34, 47, 48). CD40–CD40L cross-talk signaling then acts to promote survival and acquisition of additional stimulatory functions in DCs. However, it remained to be seen whether fascin was a critical element downstream of this CD40–CD40L axis (as opposed to TCR and/or other co-stimulatory molecules present on the CD4$^+$ T cells). CD4$^+$ OT-II T cells were cultured with WT or CD40$^{-/-}$ peptide-pulsed mDCs. After 24 h of co-culture, total cells were collected and lysates prepared. Results revealed an inability of the CD4$^+$ T cells to increase fascin expression in CD40$^{-/-}$ DC, whereas there was a significant increase in fascin expression in WT DCs (Fig. 3A).

Additionally, studies performed flow cytometric analyses to evaluate fascin expression and determine the percentage of T cells maintaining contact with DCs at the 5-h time point. Culturing at a ratio of ~1:3 of CD11c-sorted mDCs with the CD4$^+$ T cells resulted in less DCs bound to T cells in the CD40$^{-/-}$ (5.41% at 1 μg ml^{-1} and 4.86% in 0.1 μg ml^{-1} of OVA peptide) compared to WT (38.3 and 14.4% at 1.0 and 0.1 μg ml^{-1} of OVA peptide, respectively; Fig. 3B); CD86 was used as marker to identify mDCs and CD4 to identify T cells. Cells cultured in absence of OVA antigen served as internal controls.

Similar results of impaired fascin expression were seen using WT DCs cultured with pre-activated T cells blocking CD40L using neutralizing antibodies. Pre-activated CD4$^+$ T cells cultured with DCs were pre-treated and/or treated during culture with neutralizing antibodies to CD40L. Western blot revealed restrained fascin expression upon cognate engagement of DCs with T cells, suggesting that CD40L engagement by T cells to CD40 presented by T cells supports up-regulation of fascin (Fig. 4A). Corroborative results using flow cytometry also show blocking CD40L on CD4$^+$ T cells reduced fascin expression in DCs from 84.5% ± 1.54 in IgG control to 45.0% ± 1.1 in CD40L neutralizing antibody-treated groups upon engagement with the CD11c-sorted mDCs (Fig. 4B). Taken together, these results show that CD40 engagement is important for further up-regulation of fascin in DCs which supports sustained contacts with T cells.

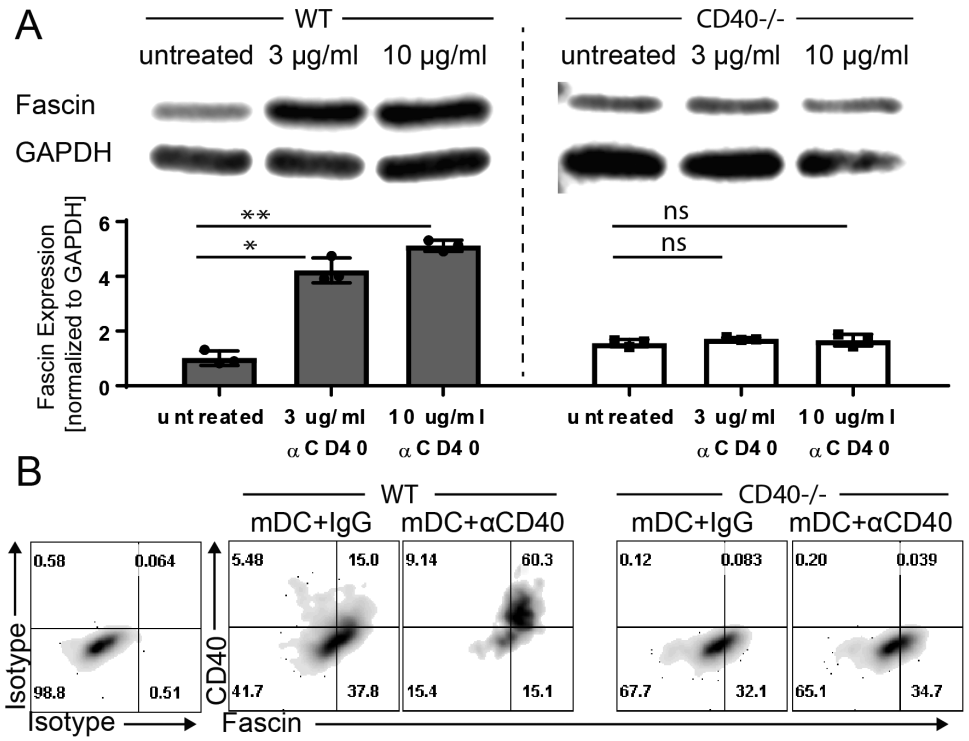

Fig. 2. CD40-deficient DCs are unable to up-regulate fascin. (A) WT versus CD40$^{-/-}$ bone marrow-derived LPS-matured DCs were left untreated or treated with 3 or 10 µg ml^{-1} of agonist αCD40 for 24 h prior to harvesting cells and generating lysates. Western blots were performed to evaluate fascin expression. Graphs represent the mean and SD of three independent studies with fascin levels normalized to GAPDH. (B) WT versus CD40$^{-/-}$ bone marrow-derived LPS-matured CD11c-sorted DCs were stimulated with 10 µg ml^{-1} of isotype control (mDC + IgG) or agonist CD40 antibody (mDC + αCD40) prior to staining for CD40 and fascin for flow cytometric analyses. Dot plots show CD40 on the y-axis versus fascin expression on the x-axis. Gates were established using isotype controls. Data are representative of three independent studies. *$P < 0.05$ and **$P < 0.01$ as assessed by two-tailed Student's *t*-test.

Lentivirus-mediated ectopic expression of fascin in CD40$^{-/-}$ DCs

Rescue studies were next performed to evaluate the role of fascin in the absence of CD40 signaling. CD40$^{-/-}$ DCs were rescued for fascin expression by transduction with lentiviral vectors carrying fascin (pFascin). An empty lentiviral vector (pEmpty) served as control. DCs were transduced on day 2 and subsequently matured with LPS and αCD40 on day 6 for 24 h; IgG isotype antibodies were used as controls. Lentiviral transduction successfully rescued fascin expression in the CD40$^{-/-}$ pFascin group (as measured in CD11c-sorted DCs). Ectopic expression in CD40$^{-/-}$ increased fascin expression to 71.3% ± 1.1 compared to the 36.2% ± 1.2 for the pEmpty control (Fig. 5A). In all studies, ectopic expression of WT DCs with pFascin resulted in a small increase in fascin expression compared to pEmpty controls, but a larger median fluorescence intensity (MFI). Additionally, studies found that ectopic expression using pEmpty or pFascin lentiviral vectors in iDCs did not result in DC maturation/activation, as measured by MHC class II, CD40 or CD86 (data not shown).

Corroborative studies by western blot of bone marrow-derived DCs confirmed success in rescuing fascin expression levels. CD40$^{-/-}$ DCs were transduced with pEmpty or pFascin to evaluate total fascin expression; WT DCs transduced with pEmpty served as an internal control (Fig. 5B). Ectopic expression in CD40$^{-/-}$ versus WT DCs with pFascin did not result in any significant differences in expression of co-stimulatory molecules (i.e. MHC class II, CD80, CD86) or cytokines (i.e. IL-12p70, TNF-α or IL-1β) when compared to pEmpty treated controls (Supplementary Figure 1B and C, available at *International Immunology* Online).

Finally, studies confirmed that rescue of fascin restored the frequency of mDCs bound to CD4$^+$ T-cell conjugates. Briefly, CD11c-sorted WT, CD40$^{-/-}$ pEmpty or CD40$^{-/-}$ pFascin mDCs were cultured with naive OT-II CD4$^+$ T cells at 1.0 or 0.1 µg ml^{-1} of $OVA_{323–339}$ peptide for 5 h. Cells were then evaluated for ability to form conjugates. The CD40$^{-/-}$ pEmpty DCs had lower levels of DCs bound to T cells (as evaluated by CD86 CD4 double-positive subsets; CD86$^+$ CD4$^+$) to that of WT (Fig. 5C). However, over-expression of fascin in the CD40$^{-/-}$ DCs (CD40$^{-/-}$ pFascin) resulted in restoring the percentage of CD86$^+$ CD4$^+$ double-positive conjugates. Even with ectopic fascin expression, the ability to form DC–T-cell conjugates was antigen-specific, as no contacts were seen in the non-antigen-pulsed DCs among all treatment or control groups.

Ectopic expression of fascin in CD40$^{-/-}$ DCs rescues cytokine profiles, but not CD4$^+$ T-cell early activation or proliferation

CD40 engagement exerts a myriad of *licensing* fates in DCs upon cognate ligation, which include: enhanced cytokine production, increase expression of co-stimulatory and adhesion molecules, and survival, among others. However, these studies questioned to what extent fascin expression plays a role in

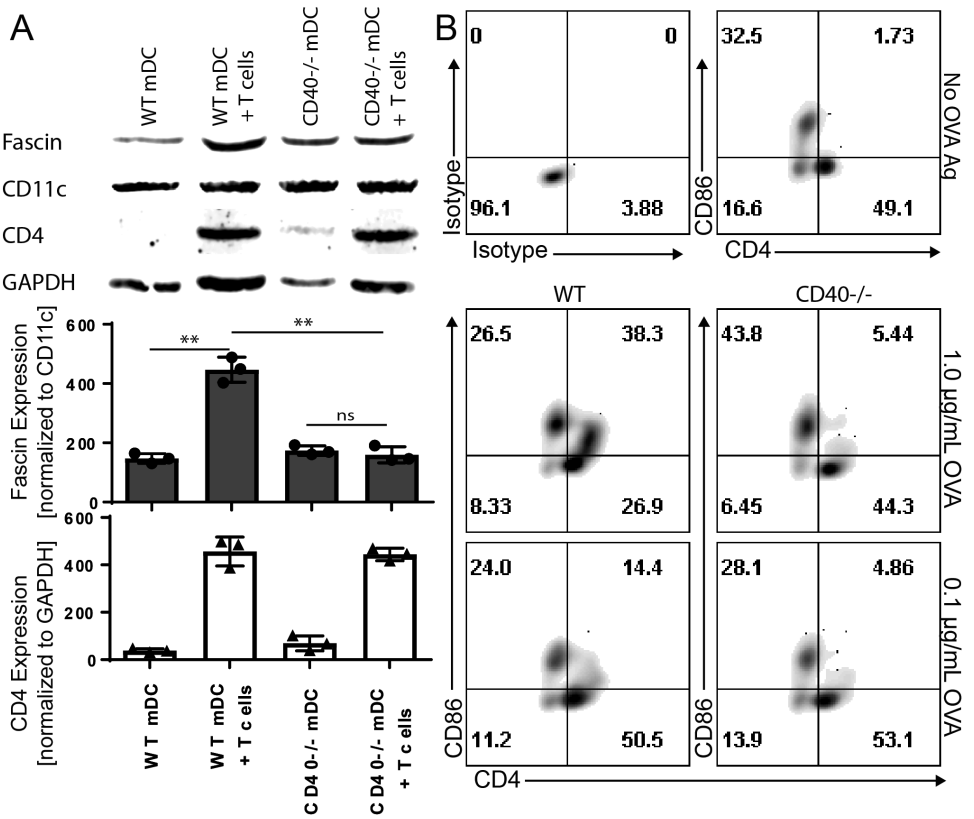

Fig. 3. CD40 engagement with cognate T cells results in increased fascin expression in DCs. (A) Day 7 WT or CD40$^{-/-}$ bone marrow-derived mDCs pulsed with 1.0 µg ml^{-1} of $OVA_{323-339}$ peptide were cultured with naive CD4$^+$ OT-II T cells. After 24 h of DC culture with T cells, at an ~1:3 ratio, respectively, total cells were collected and lysates were immediately prepared for western blot analysis. Blots were probed for fascin, CD11c, CD4 and GAPDH using respective antibodies. Bar graphs for fascin expression are depicted as a ratio normalized to CD11c total expression; CD4 expression is normalized to GAPDH. The graphs represent the mean and SD of three replicate samples; data are representative of three independent studies. (B) WT or CD40$^{-/-}$ bone marrow-derived magnetically activated cell sorting-sorted CD11c$^+$ mDCs pulsed with or without 1.0 or 0.1 µg ml^{-1} $OVA_{323-339}$ peptide antigen were cultured with CD4$^+$ OT-II T cells at a ratio of 1:3. The No OVA Ag group is DCs cultured with CD4$^+$ T cells in the absence of OVA peptide (top right); negative control. After 5 h, the supernatant was carefully removed and cells fixed in 3% paraformaldehyde in PBS for 1 h at 4°C to retain conjugated states. Gently, cells were washed and permeabilized prior to staining for CD4 and CD86. For the dot plot, the CD4$^+$ CD86$^+$ population represents T cells engaged to the DCs as conjugates. Gating strategies took care to exclude dead cells; all gates were established using isotype controls (top left dot plot). Data are representative of three independent studies. $^{**}P < 0.01$ as assessed by two-tailed Student's *t*-test.

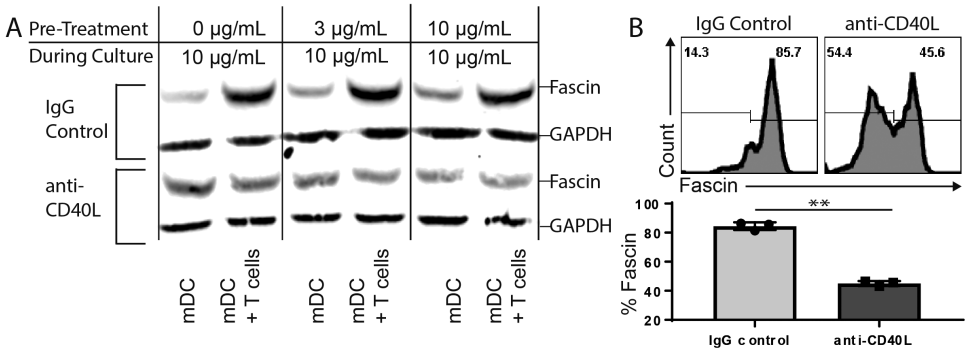

Fig. 4. Blocking CD40L (CD154) on CD4$^+$ T cells cultured with DCs restrains cognate fascin expression. Pre-activated CD4$^+$ T cells were generated by stimulation with agonist αCD3/CD28 microbeads for 24 h in culture prior to culture with WT DCs in the presence of neutralizing antibodies to CD40L/CD154. (A) For the Pre-Treatment group, pre-activated CD4$^+$ T cells were cultured in the presence of 3 or 10 µg ml^{-1} of isotype control (IgG Control) or CD40L neutralizing antibodies (anti-CD40L) for 1 h prior to culture with DCs. For the During Culture treatment group, pre-activated CD4$^+$ T cells and DCs were cultured in the presence of 10 µg ml^{-1} of IgG Control or anti-CD40L antibodies throughout the length of the incubation. After 24 h, cells were harvested and lysates prepared for western blot. Blots were then probed for fascin and GAPDH. (B) CD11c-isolated mDCs and T cells were cultured in the presence of 10 µg ml^{-1} of IgG Control or anti-CD40L antibodies. Next, cells were centrifuged, supernatant removed and cells immediately fixed with 3% paraformaldehyde in PBS for 1 h prior to staining for fascin. Flow cytometric analyses were then performed to evaluate the percentage of fascin expression. $^{**}P < 0.01$ as assessed by two-tailed Student's *t*-test.

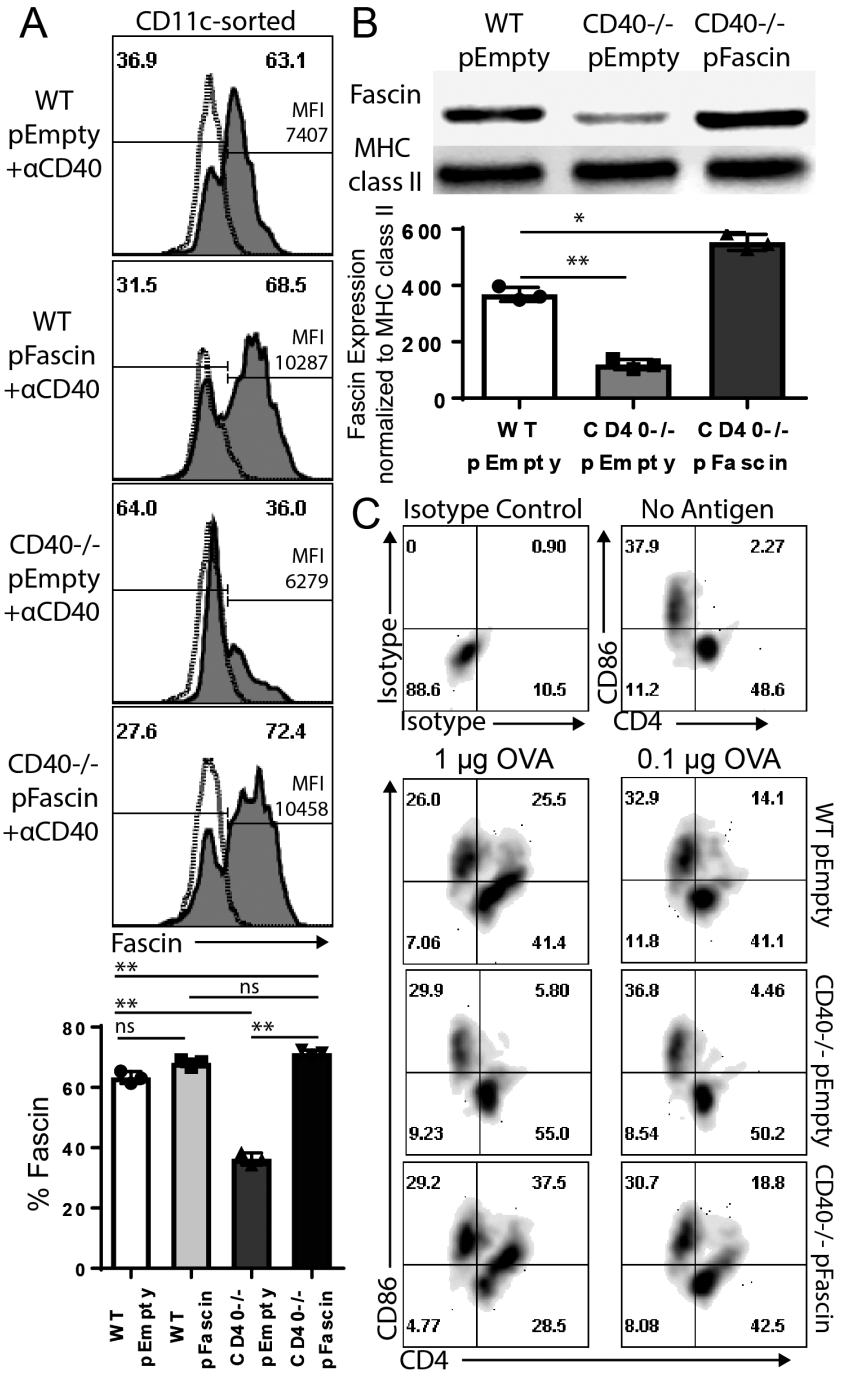

Fig. 5. Rescue of fascin expression in CD40-deficient DCs using lentiviral expression vectors. Bone marrow-derived DCs were prepared from WT or CD40$^{-/-}$ mice. Next, lentivirus as an empty vector (pEmpty) or carrying the fascin gene (pFascin) was transduced into WT or CD40$^{-/-}$ DCs, resulting in WT pEmpty, WT pFascin, CD40$^{-/-}$ pEmpty and CD40$^{-/-}$ pFascin. (A) CD11c-sorted DCs from each group were treated with 10 µg ml^{-1} of agonist CD40 antibody prior to harvesting and staining for fascin. Isotype controls were used to establish gating strategies for flow cytometric analyses. Histogram dot plots display fascin expression among the groups; dashed lines represent isotype control and filled histograms are fascin. The MFIs are displayed on the histograms. The bar graph below depicts mean and SD. (B) For western blot, lysates were prepared from WT pEmpty, CD40$^{-/-}$ pEmpty, and CD40$^{-/-}$ pFascin groups stimulated with 10 µg ml^{-1} agonist CD40 antibody for 24 h prior to probing for fascin and MHC class II. Bar graphs represent the mean and SD of three independent studies with fascin levels normalized to MHC class II. (C) WT pEmpty, CD40$^{-/-}$ pEmpty or CD40$^{-/-}$ pFascin bone marrow-derived mature CD11c-sorted DCs were cultured at a ratio of 1:3 with CD4^{+} OT-II T cells in the presence of 1.0 or 0.1 µg ml^{-1} of $OVA_{323–339}$ peptide. DCs without antigen pulsing served as internal controls. After 5 h, cells were centrifuged, supernatant carefully removed and fixed in 3% paraformaldehyde in PBS for 1 h at 4°C to retain conjugated states. Cells were stained with CD86 and CD4 to evaluate the percentage of bound DC and T-cell subsets. Gating strategies excluded dead cells using a live/dead cell staining kit. Dot plots display WT pEmpty, CD40$^{-/-}$ pEmpty or CD40$^{-/-}$ pFascin DC groups cultured with CD4^{+} T cells. Data are representative of three independent studies. *$P < 0.05$ and **$P < 0.01$ as assessed by two-tailed Student's *t*-test.

the downstream CD40 signaling pathways for promoting T-cell effector responses. Therefore, CD40$^{-/-}$ DCs were transduced with lentiviral vectors expressing fascin (or empty vector-control). WT pEmpty, CD40$^{-/-}$ pEmpty and CD40$^{-/-}$ pFascin OVA peptide-pulsed DCs were then used to prime naive OT-II CD4^{+} T cells. At the 24-h time point, no significant changes in early activation markers CD69, CD25 or CD62L on the responder T cells were identified (Supplementary Figure 2, available at *International Immunology* Online). At the 72-h time point, the absence of CD40 did not have a significant effect on responder T-cell proliferation (Fig. 6A). However, IFN-γ production in proliferating cells was reduced from 72.6% ± 0.7 in WT to 53.2% ± 0.8 in CD40$^{-/-}$ pEmpty primed T cells. Rescue of fascin in CD40$^{-/-}$ DCs restored IFN-γ expression (to 73.3% ± 0.8) in the proliferating cells. These results support literature reports that CD40 *licensing* is attributed to T-cell cytokine responses (49). To corroborate these findings, supernatant collected at 72 h was assessed for IL-2, IFN-γ, IL-4 and IL-17 levels (Fig. 6B). Results revealed that the CD40$^{-/-}$ pEmpty group had lower levels of each cytokine, but that the levels could be rescued upon ectopic fascin expression.

In vivo stimulation with agonist αCD40 drives fascin expression in WT, but not in CD40-deficient, splenic DCs

For *in vivo* relevance, WT and CD40$^{-/-}$ mice were challenged with αCD40 agonist to assess expression of fascin in spleen DCs. Briefly, mice were injected ip with LPS; control

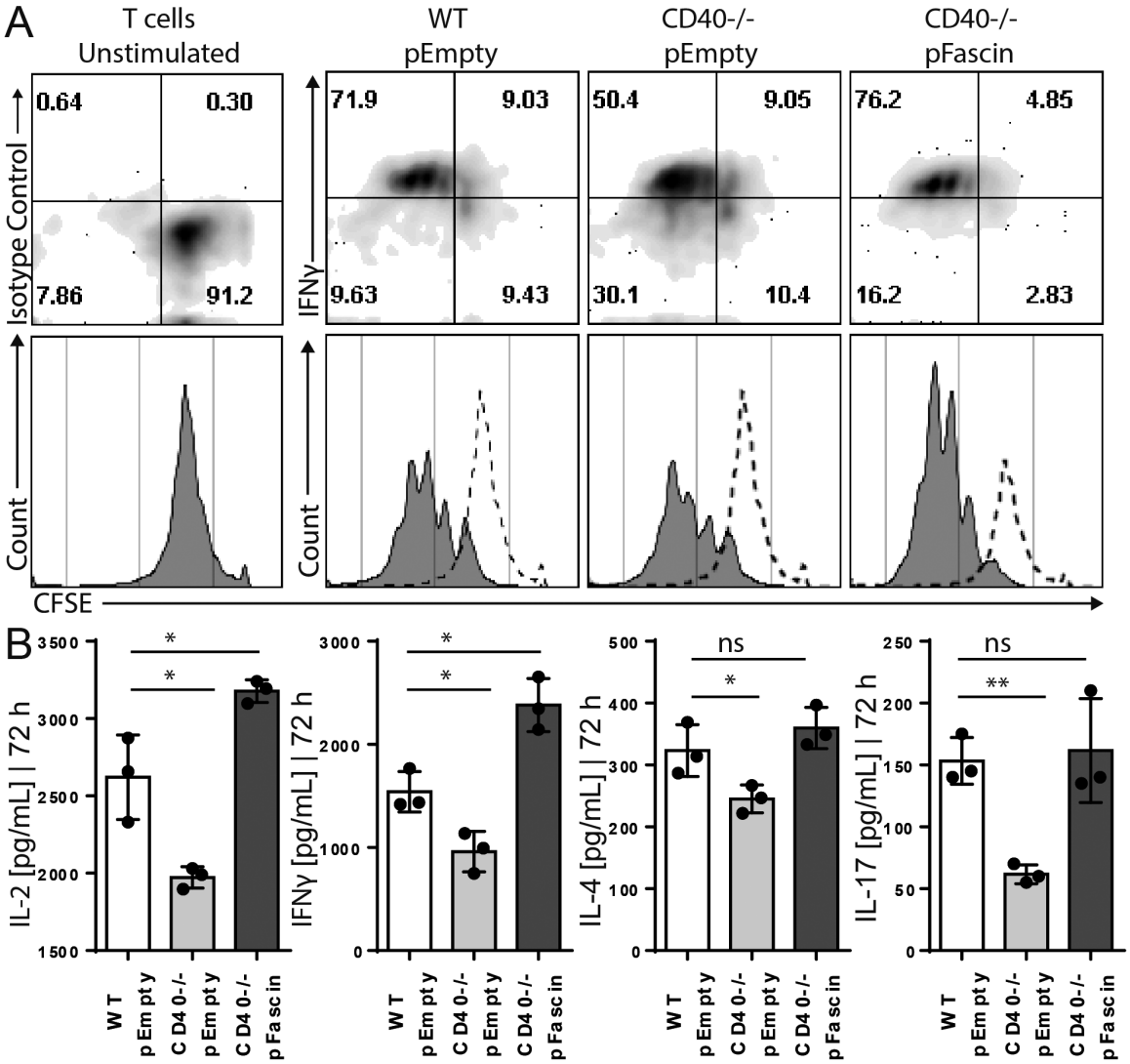

Fig. 6. Fascin over-expression in CD40-deficient DCs rescues T-cell cytokine production. WT transduced with empty lentiviral vector (WT pEmpty), CD40$^{-/-}$ transduced with empty lentiviral vector (CD40$^{-/-}$ pEmpty) or CD40$^{-/-}$ transduced with vector containing fascin (CD40$^{-/-}$ pFascin) DCs were pulsed with $OVA_{323-339}$ peptides prior to culturing with CFSE-labeled CD4^{+} OT-II T cells. (A) After 72 h, cells were harvested, fixed, permeabilized and stained to detect intracellular IFN-γ in proliferating cell subsets. Cells were gated on the CD4^{+} population. Unstimulated CFSE-labeled T cells stained with isotype controls were used to establish gates (as shown in the left panels). The top row displays dot plots of IFN-γ versus CFSE in WT pEmpty, CD40$^{-/-}$ pEmpty and CD40$^{-/-}$ pFascin groups. The bottom row shows histogram plots of CFSE to visualize proliferation peaks; the unstimulated CFSE-labeled T-cell control group is presented as an overlay for each group (unfilled dashed line). (B) At 72 h, supernatant was collected and measured for IL-2, IFN-γ, IL-4 and IL-17 by ELISA. All datasets are representative of triplicate wells in three independent experiments. *$P < 0.05$ and **$P < 0.01$ as assessed by two-tailed Student's *t*-test.

mice received saline. After 24 h, one cohort of LPS-injected mice were injected ip with αCD40; the other cohort received isotype control antibody. Twenty-four hours later (or at the 48-h time point), mice were sacrificed and spleens isolated. Splenocytes were then stained for MHC class II, CD11c and fascin prior to flow cytometric analysis. Studies revealed that αCD40 treatment yielded a significant increase in fascin in the CD11c^{+} MHC class II^{+} Fascin^{+} populations from WT groups (from 23.6 to 55.7%), but no increase in the CD40$^{-/-}$ cohorts (from 22.3 to 26.0%; Fig. 7).

Discussion

Prior works have identified the role of CD40 *licensing* to enhance both humoral and cell-mediated immune responses (50). In the context of antigen presentation, reports have shown that CD40-dependent enhanced antigen presentation occurs at the contact plane via clustering of CD40L to optimally promote cross-talk signaling (51). However, studies of CD40 signaling to regulate the actin cytoskeleton in DCs are limited. Given that fascin has been suggested as a key actin regulator in DCs that promotes T-cell responses (26, 29), these investigations aimed to evaluate the CD40–CD40L cross-talk role in modulating fascin-actin cytoskeletal dynamics in DCs as a mechanism to promote antigen presentation.

Using CD40-deficient DCs (CD40$^{-/-}$), studies found that CD40 orchestrates fascin expression in DCs, which subsequently modulates cognate T-cell cytokine production. As CD40$^{-/-}$ DCs were unable to further up-regulate fascin expression upon treatment with CD40 agonist antibody or by cognate responder CD4^{+} T cells (bearing the CD40L), this would suggest that further up-regulation of fascin triggered an additional role in supporting immune responses. Furthermore, it suggests the importance of CD40–CD40L axis in driving fascin, as other stimulatory cues present on T cells were unable to increase its expression. These investigations now show that reduced DC engagement to T cells in the CD40$^{-/-}$ groups correlated directly with impaired fascin expression.

To evaluate the independent role of fascin from CD40–CD40L-signaling, studies rescued fascin by over-expressing in CD40$^{-/-}$ DCs. Although the percentage of cells expressing fascin was greater in WT versus CD40$^{-/-}$ DCs (triggered with αCD40 agonist), ectopic expression of fascin in CD40$^{-/-}$ DCs did not largely yield increased MFI levels beyond what was observed in WT DCs. Given the nature of fascin in modulation of the actin cytoskeletal network, it may be that the cell restrains substantial over-expression of the protein. Thereby, an attempt at overproducing the molecule beyond a certain threshold may be cytotoxic or trigger compensatory mechanism(s) to restrain such elevated levels. Although

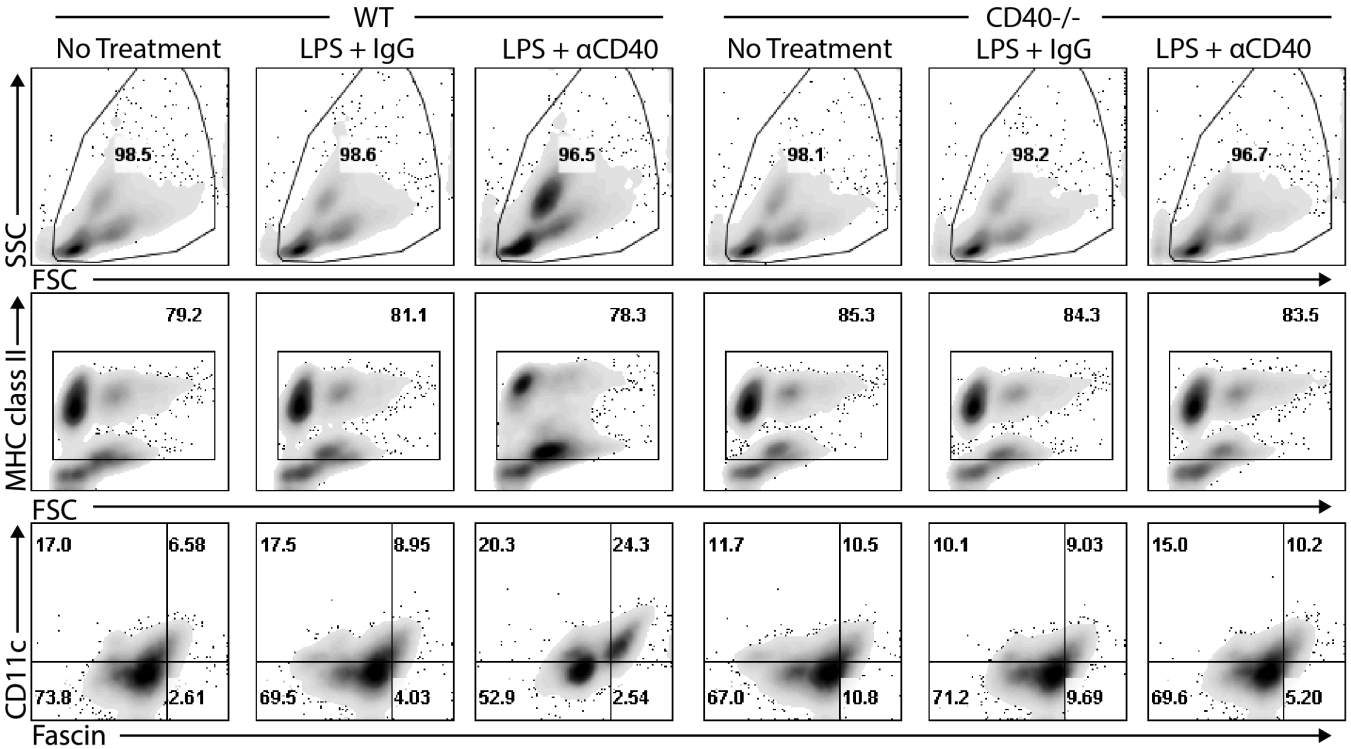

Fig. 7. *In vivo* expression of fascin is depressed in CD40$^{-/-}$ mice. WT or CD40$^{-/-}$ 10-week-old mice were injected ip with 200 μg of LPS. 24 h later, groups were treated with 100 μg of IgG isotype control (LPS + IgG) or αCD40 agonist (LPS + αCD40) antibody through ip injection (in 200 μl of volume); controls were injected with PBS and IgG control antibodies. After a total of 48 h, mice were sacrificed and spleens harvested. Total splenocytes were then stained for CD11c, MHC class II and fascin prior to flow cytometric analysis; isotype controls were used to establish gates. The top row of dot plots show forward scatter channel (FSC) versus side scatter channel (SSC) gating on live cells. The second row shows MHC class II expression versus FSC in the live cell gated group. The MHC class II^{+} gated group was then assessed for CD11c and fascin expression, as shown in the third row of dot plots. All datasets are representative of three independent experiments.

Geyeregger *et al.* (52) also used fascin over-expression studies, their work did not directly show increased levels of fascin in DCs. However, their studies' attempt to over-express fascin in DCs resulted in only 50% survival after 24 h and <20% after 48 h (52). This further supports that DCs are resistant to over-expression of the actin-bundling protein largely beyond endogenous levels. Our results do support their work in that ectopic fascin expression was able to rescue formation of DC–T-cell conjugates. Taken together, these studies demonstrate the role of fascin in DCs as a mechanism for sustained contacts with cognate responder T cells (33, 36).

Although co-stimulatory molecules and cytokine profiles were unchanged by ectopic fascin expression in CD40$^{-/-}$ DCs, the studies clearly found that fascin was able to rescue cytokine production by responder T cells. Ectopic expression of fascin in CD40$^{-/-}$ did not modulate early activation (as measured by CD25, CD69 and CD62L) of CD4^{+} T cells between CD40$^{-/-}$ and WT DCs. This does make sense, as fascin would not yet be up-regulated until T cells are activated to express CD40L (after TCR:pMHC and other co-stimulatory engagement); early activation is largely a CD40-independent process. However, at 72 h, there was a decrease in CD4^{+} T-cell cytokine profiles from the CD40$^{-/-}$. That decreased cytokine expression could be rescued upon ectopic fascin expression in CD40$^{-/-}$ DCs.

Finally, studies investigated *in vivo* challenge responses to αCD40 agonist treatment in WT versus CD40$^{-/-}$ mice. There was not a large increase in fascin expression upon LPS treatment versus controls. However, a significant increase in fascin expression was observed upon addition of CD40 agonist antibody delivery to WT group, whereas no increase in fascin was observed in the CD40$^{-/-}$ mice. This corroborates *in vitro* works and further supports histological examinations of spleen and lymph nodes, whereby fascin expression was correlated with T-cell infiltration (30, 53). Furthermore, it is clear that a large population of CD11c^{+} Fascin^{+} cells are present in the spleen after αCD40 treatment, reaffirming that CD40 engagement is a potent *license* for DC induction of stimulatory capacities to support adaptive immune responses.

Taken together, these studies address the role of CD40 signaling in coordinating DC modulation of fascin. As such, a three-step process is proposed for DCs activating CD4^{+} T cells. First, mDCs displaying pMHC, CD40 and co-stimulatory molecules activate CD4^{+} T cells through TCR/CD28 signaling pathways. The result is early activation events, including that of CD40L expression on CD4^{+} T cells. Second, CD40L expressed by these early activated T cells can then re-engage the same *or* different CD40-bearing DCs. Third, engagement of CD40–CD40L then triggers up-regulation of fascin in DCs to promote sustained DC–T-cell contacts leading to enhanced stimulatory responses in T cells, as measured by cytokine production (33).

Supplementary data

Supplementary data are available at *International Immunology* Online.

Funding

This work was supported by the National Institutes of Health (grant number SC2GM103741 to M.W.L.); Department of Defense (grant number W911NF-14-1-0123 to M.W.L.); National Sciences Foundation (grant number DBI-1428768 to M.W.L. and W. Anderson).

Acknowledgements

The authors would like to thank Lynda Chin for providing the pLenti6/V5-DEST-FASCIN vector. The authors would also like to thank Franklin Ampy and Winston Anderson for assistance in statistical analyses and editing of the manuscript.

Conflict of Interest statement: The authors declare no conflicts of interest.

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