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FOXO1 Regulates Dendritic Cell Activity through ICAM-1 and CCR7

Guangyu Dong,* Yu Wang,*† Wenmei Xiao,*† Sandra Pacios Pujado,* Fanxing Xu,*§ Chen Tian,* E Xiao,*§ Yongwon Choi,§ and Dana T. Graves*

The transcription factor FOXO1 regulates cell function and is expressed in dendritic cells (DCs). We investigated the role of FOXO1 in activating DCs to stimulate a lymphocyte response to bacteria. We show that bacteria induce FOXO1 nuclear localization through the MAPK pathway and demonstrate that FOXO1 is needed for DC activation of lymphocytes in vivo. This occurs through FOXO1 regulation of DC phagocytosis, chemotaxis, and DC–lymphocyte binding. FOXO1 induces DC activity by regulating ICAM-1 and CCR7. FOXO1 binds to the CCR7 and ICAM-1 promoters, stimulates CCR7 and ICAM-1 transcriptional activity, and regulates their expression. This is functionally important because transfection of DCs from FOXO1-deleted CD11c.Cre+FOXO1L/L mice with an ICAM-1–expressing plasmid rescues the negative effect of FOXO1 deletion on DC bacterial phagocytosis and chemotaxis. Rescue with both CCR7 and ICAM-1 reverses impaired DC homing to lymph nodes in vivo when FOXO1 is deleted. Moreover, Ab production following injection of bacteria is significantly reduced with lineage-specific FOXO1 ablation. Thus, FOXO1 coordinates upregulation of DC activity through key downstream target genes that are needed for DCs to stimulate T and B lymphocytes and generate an Ab defense to bacteria.

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Abbreviations used in this article: BMDC, bone marrow–derived DC; ChIP, chromatin immunoprecipitation; CMTMR, 5- and 6-(4-chloromethyl)benzoyl-amino-tetra-methylrhodamine; DC, dendritic cell.

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CD11c.Cre mice and OT-II mice were purchased from The Jackson Laboratory. FOXO11/− mice were generously provided by Dr. Ronald DePinho (University of Texas MD Anderson Cancer Center, Houston, TX) (18). FOXO11/−Cre mice were bred with CD11cCre mice to generate FOXO1-deleted mice (CD11c.Cre+FOXO11/−) and control littermates (CD11c.Cre−FOXO11/−) (19). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Materials and Methods

Mice

CD11c.Cre mice and OT-II mice were purchased from The Jackson Laboratory. FOXO11/− mice were generously provided by Dr. Ronald DePinho (University of Texas MD Anderson Cancer Center, Houston, TX) (18). FOXO11/−Cre mice were bred with CD11cCre mice to generate FOXO1-deleted mice (CD11c.Cre+FOXO11/−) and control littermates (CD11c.Cre−FOXO11/−) (19). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

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Mice were challenged by i.p. injection of lightly fixed Porphyromonas gingivalis (ATCC #33277, 10^5 CFU once weekly) or vehicle alone (PBS) and euthanized 1 wk after the last injection. Alternatively, they were inoculated orally with P. gingivalis/Fusobacterium nucleatum (ATCC #25586), as previously described (20), three times weekly for 2 wk and euthanized 6 wk after the last oral inoculation. Ab (IgG1 or IgG2a) against P. gingivalis was measured by ELISA, as previously described, and the concentration was determined by reference to a standard curve (20).

Dendritic cells

In vitro generation of bone marrow–derived DCs (BMDCs) was carried out as described (19). Nonadherent cells were harvested on day 8 and identified by typing DC markers (>90%). DCs also were isolated from mouse spleens (21) and enriched by CD11c MicroBeads (Miltenyi Biotech), followed by FACS sorting (FACSaria; BD Bioscience). Typically, DC purity was >95% as CD11c^+MHCII^+B220^+NK1.1^- cells. T cells were gated as CD90.2^-B220^- and B cells were gated as CD90.2^+B220^- and were >95% enriched. DC2.4 cells were kindly provided by Dr. Kenneth Rock (University of Massachusetts Medical Center, Worcester, MA) (22). Raw 264.7 cells were cultured in complete RPMI 1640 (eBioscience) for 4 d to differentiate to DCs.

Migration and DC-homing assays

Chemotaxis was measured in LPS pretreated DCs in polycarbonate filter, 5-μm pore size Transwell chambers (Corning), with or without cCCL19 or cCCL21 (both from PeproTech), for 3 h at 37°C. DCs that had migrated to the bottom chamber were counted by fluorescence microscopy.

For DC homing, BMDCs from FOXO1-deleted mice or control mice were purified with MicroBeads and pretreated with LPS, as described above. After labeling with either 5 μM CFSE (eBioscience) or 10 μM 5- and 6-(4-chloromethyl)benzoyl-amino-tetramethylrhodamine (CMTMR; (PeproTech) for 4 d to differentiate to DCs.

Phagocytosis

Bacteria were labeled with CFSE, as described (24). The labeled bacteria were incubated with BMDCs at a multiplicity of infection of 10:1 (bacteria/cells) for 6 h. The cells were fixed, stained by F-actin (Texas Red-X phalloidin; Life Technologies), and mounted in Fluoroshield (Sigma-Aldrich). Colocalization of bacteria and F-actin was assessed by deconvolution fluorescent microscopy in 2.5-μm sections.

Lymphocyte proliferation and Ab production

Negative-purified naive CD4^+ T cells from OT-II mice were labeled with 5 μM CFSE and cocultured with OVA257-264 peptide (AnaSpec) and BMDCs for 3 d. The number of CFSE-labeled T cell divisions was measured by flow cytometry. Resting B cells from C57BL/6 mice were purified by CD43 (Ly-48) MicroBeads (Miltenyi Biotech). DCs (2 × 10^5) were cocultured with 10^5 resting B cells for 5 d in complete RPMI 1640 plus 5 μg/ml anti-CD40 (BioLegend), and IgG in the supernatant was measured by ELISA (eBioscience). For B cell proliferation, CFSE-labeled resting B cells were cocultured with BMDCs plus anti-CD40 Ab (BioLegend) for 5 d.

Fluorescence microscopy

BMDCs were fixed in methanol for 10 min at −20°C and incubated with Abs against CCR7 (R&D Systems), FOXO1, ICAM-1, as well as matched nonimmune Ab (all from Santa Cruz Biotechnology). Images were captured with fluorescence microscopy and analyzed with Nikon NIS-Elements software.

Western blot

BMDCs from CD11cCre^+FOXO1^+/L and CD11cCre^+FOXO1^−/L mice were analyzed by Western blots, as described previously (25). Briefly, BMDCs were lysed with lysis buffer (Thermo Fisher Scientific) containing protease inhibitor mixture and phosphatase inhibitor mixture (Thermo Fisher Scientific). Protein concentration was measured using a protein assay with BSA as a standard (Bio-Rad Laboratories). A total of 50 μg cell lysate was resolved using SDS-PAGE (Bio-Rad Laboratories) and transferred onto a polyvinylidene difluoride membrane (Thermo Fisher Scientific). The membranes were incubated with primary Abs against FOXO1 (Santa Cruz Biotechnology) and β-actin (Sigma-Aldrich) after blocking with 5% BSA. The samples were incubated with HRP-labeled anti-rabbit IgG or anti-mouse IgG, and immunoreactive bands were detected with ECL Western blotting reagents (Thermo Fisher Scientific).

Treatment by inhibitors

DC2.4 cells were pretreated by Akt inhibitors BML-257 (Santa Cruz Biotechnology) or triciribine or ERK1/2 inhibitor PD098059, JNK inhibitor 1, or P38 inhibitor SB203580 from EMD Millipore for 1 h and then stimulated with light-fixed P. gingivalis, at a multiplicity of infection of 10:1, and with inhibitor overnight, followed by immunofluorescence with Ab to FOXO1 and DAPI counterstaining. Nuclear localization was determined by FOXO1–DAPI colocalization.

Transient transfection and luciferase reporter assay

Cells were transfected with FOXO1 construct, pcDNA empty vector, or pcDNA expressing GFP, as we described previously (26), by electroporation (Neon Transfection System; Life Technologies). Cells were stimulated with 1 μg/ml LPS overnight. For luciferase reporter assays, cells were cotransfected with a reporter construct containing the 2031-bp ICAM-1 promoter and intron-1 (kindly provided by Dr. Chinnaswamy Tiruppathi, University of Illinois at Chicago, Chicago, IL) (28). Luciferase activity was measured using the dual Luciferase Assay System (Promega) and reported as firefly/Renilla luciferase ratio.

ICAM-1 and CCR7 transfection and rescue experiments

BMDCs were transfected with pcDNA-GFP construct (26) by electroporation. In addition, DCs from FOXO1-deleted mice were transfected with CCR7 (OnGene Technologies, Rockville, MD) and/or ICAM-1 (Addgene, Cambridge MA) expression vectors or matched pcMV or pCMV8 empty vector alone. After 24 h, transfected cells were stained and FACS sorted by gating of GFP^+ cells (eBioscience). Purity was >95%, as determined by flow cytometry. After cell sorting, DCs were tested in vitro in bacterial phagocytosis, DC–T cell binding, and Transwell migration assays, as described above. They also were tested in vivo in lymph node–homing experiments. In this case, the cells obtained after sorting were labeled with fluorescent probes, violet dye eFluor 450 (eBioscience), or CMTMR and applied by retro-orbital injection. Homing to cervical lymph nodes was carried out as described above.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP-IT Kit (Active Motif). Cells were fixed in 1% formaldehyde, DNA sheared enzymatically, immunoprecipitated with anti-FOXO1 or matched control Ab, and captured with magnetic protein G beads. The precipitated DNA was amplified by SYBR Green real-time PCR using primers ICAM-1 (5'-GACGACACTGTGATACGCTCTT3' and 5'-GAGAGATATCACAC- CTAGTCTTC-3') or CCR7 (5'-GCTAAGATCTCATCCTGCTGTG-3' and 5'-TCCAGGAGGAGACCTGGTAAAT-3').

Statistical analysis

Experiments were carried out a minimum of two or three times, with similar results. Statistical significance was determined by ANOVA with the Tukey post hoc test or Student t test, set at p < 0.05 level.

Results

**FOXO1 expression**

To establish that CD11c-driven Cre recombinase deletes FOXO1 in CD11c^+ DCs but not T or B cells, leukocytes were isolated from the spleen and purified by FACS sorting. FOXO1 mRNA levels were reduced 94% in splenic DCs from FOXO1-deleted CD11c. FOXO1 mRNA levels were reduced 94% in splenic DCs from FOXO1-deleted CD11cCre^+FOXO1^−/L mice compared with littermate controls (20) (Fig. 1A).

**Statistical analysis**

Experiments were carried out a minimum of two or three times, with similar results. Statistical significance was determined by ANOVA with the Tukey post hoc test or Student t test, set at p < 0.05 level.
FIGURE 1. FOXO1 expression and nuclear translocation in DCs. (A and B) RNA was isolated from purified splenic DC, T, and B cells or from BMDCs from CD11cCre<sup>+</sup>FOXO1<sup>L/L</sup> and CD11cCre<sup>−</sup>FOXO1<sup>L/L</sup> mice. FOXO1 mRNA levels were measured by RT-PCR and normalized to ribosomal protein L32.

(C) The percentage of splenocytes consisting of T cells, B cells, or DCs from CD11cCre<sup>+</sup>FOXO1<sup>L/L</sup> and CD11cCre<sup>−</sup>FOXO1<sup>L/L</sup> mice was determined by flow cytometry. DCs were assessed as CD11c<sup>+</sup>MHCII<sup>+</sup>, T cells were assessed as CD3<sup>+</sup>ε<sup>+</sup>, and B cells were assessed as B220<sup>+</sup>.

(D–F) Cells were isolated from lymphoid tissues from control mice and FOXO1-deleted CD11cCre<sup>+</sup>FOXO1<sup>L/L</sup> mice. Cells were stained by specific Abs and measured by flow cytometry. DCs were gated as CD11c<sup>+</sup>MHCII<sup>+</sup> (D and G), as well as with additional markers CD4 for CD4<sup>+</sup> DCs (E and H) and CD8 for CD8<sup>+</sup> DCs (F and I), respectively. Results are presented as the total number of cells (D–F) or the percentage (G–I) in the population.

(J and K) BMDCs from CD11cCre<sup>+</sup>FOXO1<sup>L/L</sup> and CD11cCre<sup>−</sup>FOXO1<sup>L/L</sup> mice were cultured in vitro and analyzed by immunofluorescence with an Ab to FOXO1; nuclei were counterstained with DAPI (Supplemental Fig. 2A). (J) Quantitation of FOXO1 expression was determined by fluorescence intensity. In some cases, cells were stimulated with LPS or <i>P. gingivalis</i> (Pg) prior to immunofluorescence. Nuclear localization after Pg or LPS stimulation is evident in the merged image (Supplemental Fig. 2B). (K) Quantitation of FOXO1 translocation was determined by FOXO1 nuclear localization. (L) BMDCs from (Figure legend continues)
CD11c.Cre^FOXO1^L/L mice but not control mice (p < 0.05) (Fig. 1B, 1L). There was no deletion of FOXO3 (Supplemental Fig. 1). CD11c-driven Cre recombinase deletion of FOXO1 did not affect the overall production of DCs, T cells, or B cells (Fig. 1C). Additional studies showed that FOXO1 deletion did not alter the number of DCs or the percentage of DCs in the spleen, lymph node, and bone marrow (Fig. 1D, 1G). DC FOXO1 deletion also did not change the DC subsets, having no effect on the number or percentage of CD4^+ DCs (Fig. 1E, 1H) or CD8^+ DCs (Fig. 1F, 1I). FOXO1 protein levels were decreased 96% in BMDCs from FOXO1-deleted experimental mice compared with littermate control mice (Fig. 1J, Supplemental Fig. 2A). Stimulation with LPS or bacteria (P. gingivalis) induced a 61 or 78% (p < 0.05) increase in FOXO1 nuclear localization, indicative of FOXO1 activation (Fig. 1K, Supplemental Fig. 2B). Moreover, bacteria-induced activation of FOXO1 was dependent on MAPK, because inhibitors of p38, JNK, and ERK reduced FOXO1 nuclear localization by ∼34–58% (p < 0.05) when tested individually and by 42–73% (p < 0.05) when tested in combinations. The effect of combined JNK+p38 inhibitors or ERK+JNK+p38 inhibitors on FOXO1 nuclear localization was greater than each alone (p < 0.05). Inhibition of Akt enhanced nuclear localization of FOXO1 by ∼49–56% (p < 0.05) (Fig. 1M).

**Effect of activation of lymphocytes**

The impact of FOXO1 deletion on DCs was investigated further by examining whether it directly affected DC activation of lymphocytes in vivo or in vitro. Cytokine production by lymphocytes from the draining cervical lymph nodes was measured before and after oral inoculation of bacteria. Bacterial inoculation in vivo stimulated an ∼3-fold increase in IFN-γ and IL-13 production in CD4^+ T cells from the draining cervical lymph nodes in wild-type mice. This increase was reduced by 50–65% (p < 0.05) in mice that had lineage-specific FOXO1 deletion (p < 0.05) (Fig. 2A, 2B). Thus, FOXO1 ablation in DCs reduced the activation of CD4^+ T cells in vivo. T cell proliferation also was assessed in CD4^+ T cells from OT-II mice to examine whether FOXO1 deletion reduced DC activation of lymphocytes in vitro. DCs from experimental and control mice were incubated with OVA peptide Ag along with OT-II naive CD4^+ T cells. Wild-type DCs stimulated a 2-fold greater increase in T cell proliferation compared with DCs from FOXO1-deleted experimental mice (p < 0.05) (Fig. 2C, 2D).

DC binding to CD4^+ T cells (Fig. 2E–G) is an important parameter of DC stimulation of the immune response (30). The percentage of DCs that bound to T cells from control mice was 2-fold higher than DCs from experimental mice with DC FOXO1 deletion (p < 0.05) (Fig. 2F). Similarly, the number of T cells bound per DC from control mice was 2.5-fold higher than the number bound to DCs from FOXO1-deleted mice (p < 0.05) (Fig. 2G). Both results demonstrate that FOXO1 deletion in DCs affects DC binding to T cells.

DCs regulate B cells through production of BAFF and APRIL. The production of BAFF in DCs from control mice was 2.1-fold higher than from FOXO1-deleted mice (p < 0.05) (Fig. 2H). Similarly, the production of APRIL in DCs from control mice was 3.4-fold higher than from FOXO1-deleted mice (p < 0.05) (Fig. 2I). To examine the ability of DCs to stimulate B cells, DC-induced B cell proliferation was measured. B cell proliferation was 2.3-fold higher when B cells were incubated with control DCs compared with DCs from FOXO1-deleted mice, demonstrating that FOXO1 plays an important role in the ability of DCs to stimulate B cells (p < 0.05) (Fig. 2J).

**Effect of FOXO1 on DC homing to lymph nodes, migration, bacterial phagocytosis, and generation of an Ab response**

Because DC homing to lymph nodes is an important aspect in activating the adaptive immune response to bacterial infection, we determined whether FOXO1 affected DC homing. DCs from control and FOXO1-deleted mice were labeled with the fluorescent probes CFSE and CMTMR to distinguish them. DCs from both sources were transferred simultaneously by retro-orbital injection into wild-type C57BL/6 mice, and DC homing to cervical lymph nodes was evaluated 24 h later by flow cytometry (Fig. 3A). Lineage-specific deletion of FOXO1 reduced homing to cervical lymph nodes by 78% (p < 0.05). This is unlikely to be due to differences in survival because FOXO1 does not significantly affect short-term DC survival (Fig. 3B).

To determine whether FOXO1 contributes to DC migration, BMDCs were stimulated with chemokines CCL19 and CCL21 and assessed in a Transwell assay. Both chemokines induced a dose-dependent increase in migration (Fig. 3C, 3D). DC migration stimulated by CCL21 was reduced by up to 75% when FOXO1 was deleted (p < 0.05) (Fig. 3C), and chemotaxis induced by CCL19 was diminished by up to 70% (p < 0.05) (Fig. 3D).

The impact of FOXO1 deletion on DC phagocytosis of bacteria also was examined in BMDCs (Supplemental Fig. 3). The percentage of DCs that phagocytosed bacteria was reduced by almost 50% (p < 0.05) in experimental mice compared with control littermate mice, and the number of bacteria phagocytosed per DC was reduced by ∼65% (p < 0.05) (Fig. 3E, 3F). To examine whether FOXO1 deletion affected the DC-mediated T cell proliferation induced by bacteria, T cell proliferation assays were performed in cocultures of T cells from wild-type mice and DCs from FOXO1-deleted experimental or littermate control mice. Immune cells were stimulated with P. gingivalis bacteria. FOXO1 deletion in DCs reduced T cell proliferation induced by bacteria by 40% (p < 0.05) (Fig. 3G).

During bacterial infection, DCs capture bacterial Ags and present them to lymphocytes to generate an Ab response. We injected P. gingivalis bacteria into control CD11c.Cre^FOXO1^L/L and FOXO1-deleted CD11c.Cre^FOXO1^L/L mice (Fig. 3H, 3I). The production of anti-P. gingivalis IgG1 was 1.8-fold higher in control mice than in FOXO1-deleted mice 3 wk after injection, and it was 2.5-fold higher after 5 wk (p < 0.05) (Fig. 3H). Serum anti-P. gingivalis IgG2a Ab followed a similar pattern; it was >2-fold higher in control mice compared with mice with DC deletion of FOXO1 (p < 0.05) (Fig. 3I). The impact of FOXO1 on DC-stimulated Ab production also was tested in vitro. IgG produced by B cells was 54% less when B cells were stimulated by DCs with FOXO1 deletion compared with wild-type DCs (p < 0.05) (Fig. 3J). Thus, FOXO1 is needed for several aspects of DC function that serve as antecedents to lymphocyte activation and Ab production.

**FOXO1 regulation of factors needed for DC homing and migration**

To examine how FOXO1 regulates DC homing to lymph nodes, we examined chemokine receptor CCR7, which plays an essential role in this process (31). FOXO1 deletion in FOXO1-deleted mice

CD11c.Cre^FOXO1^L/L and CD11c.Cre^FOXO1^L/L mice were analyzed by Western blot for FOXO1 expression and actin as a loading control. (M) DC2.4 cells were treated by Akt inhibitors and MAPK inhibitors 1 h before adding bacteria, followed by immunofluorescence staining with an Ab to FOXO1 and DAPI. Quantitation was determined by FOXO1 nuclear translocation. The data are representative of two or three independent experiments of mean ± SEM from triplicate samples. *p < 0.05, DCs from FOXO1-deleted CD11c.Cre^+ mice compared to control DCs from CD11c.Cre^+ mice or treated compared to untreated group. **p < 0.05, DCs treated by single versus combination inhibitors.
FIGURE 2. FOXO1 deletion significantly reduces DC-involved lymphocyte activation. (A and B) FOXO1-deleted CD11cCre+FOXO1L/L and control CD11cCre+FOXO1L/L mice were administered bacteria (P. gingivalis and F. nucleatum) by oral inoculation. The cytokine production of IFN-γ or IL-13 was measured by immunofluorescent flow cytometry from CD4+ T cells obtained from cervical lymph nodes using specific Abs. (C and D) CFSE-labeled naive CD4+ T cells were cocultured with BMDCs and stimulated with OVA. T cell proliferation was determined by calculating the number of CD4+ T cell divisions by flow cytometry. (E-G) Purified naive CD4+ T cells from OT-II mice were incubated with BMDCs stimulated with OVA peptide. DC–T cell binding was visualized by staining with F-actin (Texas Red–X phalloidin) and DAPI. Original magnification ×200. Red arrows indicate DCs bound to T cells; yellow arrows indicate T cells bound to DCs. (H and I) Purified resting B cells were cocultured with BMDCs from FOXO1-deleted and control mice and stimulated with anti-CD40 Ab for 5 d. Intracellular BAFF and APRIL expression in DCs was measured by immunofluorescent flow cytometry following incubation with the specific Ab. (J) B cell proliferation was determined by calculating the number of CFSE B cell divisions by flow cytometry. The data are representative of two or three independent experiments of mean ± SEM from triplicate samples. *p < 0.05, DCs from FOXO1-deleted CD11cCre+ mice versus CD11cCre− mice.
FIGURE 3. FOXO1 deletion significantly reduces DC homing to lymph nodes, migration of DCs, bacterial phagocytosis, and bacteria-induced Ab production. (A) BMDCs from FOXO1-deleted CD11c-FOXO1L/L mice or control CD11c-FOXO1L/L mice were purified and labeled with CFSE or CMTMR. Labeled DCs (1 x 10^6 each) were administered simultaneously by retro-orbital injection into wild-type C57BL/6 mice. After 24 h, donor cell recovery from cervical lymph nodes was analyzed by flow cytometry for CFSE+ or CMTMR+ cells and calculated by the total cell number of cervical lymph nodes. The data are representative of two independent experiments of mean ± SEM from six mice. (B) BMDCs from CD11cCre-FOXO1L/L and CD11cCre-FOXO1L/L mice were cultured or not in vitro with LPS (1 μg/ml) in the BMDC culture medium for 0, 8, 24, and 48 h and analyzed by flow cytometry with DAPI labeling for the dead cells. Live cells (left peak) and dead cells (right peak) as experimental control (right panel). (C and D) DC migration was examined in BMDCs from CD11cCre-FOXO1L/L or control CD11cCre-FOXO1L/L mice stimulated with CCL19 or CCL21 added to the bottom chamber in a Transwell assay. After 3 h, the number of DCs that migrated to the bottom chamber was quantified following DAPI staining and fluorescence microscopy. (E and F) CFSE-labeled P gingivalis was incubated with BMDCs from FOXO1-deleted CD11c.Cre-FOXO1L/L and littermate control CD11c.Cre-FOXO1L/L mice for 6 h at 37°C and stained with Texas Red–labeled F-actin and nuclear counterstain (DAPI). The data are expressed as the percentage of DCs with phagocytosed bacteria (E) or the number of bacteria phagocytosed per DC (F). (G) CFSE-labeled (Figure legend continues)
reduced CCR7 mRNA levels by 60% \( (p < 0.05) \) (Fig. 4A) and protein levels by 87% \( (p < 0.05) \) compared with control DCs (Fig. 4B, 4C). In contrast, FOXO1 overexpression increased CCR7 mRNA levels \( >200 \)-fold in RAW264.7-derived DCs, which was enhanced further by LPS stimulation (Fig. 4D). Transfection with FOXO1 stimulated a 5-fold increase in CCR7 reporter activity, which was increased further with LPS stimulation of RAW264.7-derived DCs compared with empty vector control (Fig. 4E). FOXO1 also increased CCR7 promoter activity 4- and 18-fold by LPS in DC2.4 cells compared with empty vector alone and a CCR7 promoter-luciferase reporter construct. Luciferase activity was measured (Fig. 4F). Luciferase assays were performed in DC2.4 cells cotransfected with FOXO1 or empty vector alone and CCR7 luciferase reporter construct. ChIP assays were performed with RAW264.7-derived DCs treated with LPS or vehicle alone. The data are representative of two or three independent experiments of mean ± SEM from triplicate samples. *\( p < 0.05 \), DCs from FOXO1-deleted CD11cCre\(^+\) mice versus control CD11cCre\(^+\) mice, or DCs from transfection of FOXO1 versus empty vector alone or anti-FOXO1 Ab and control IgG.

FIGURE 4. FOXO1 in DCs regulates the expression of genes that control homing to lymph nodes. (A) Total RNA was isolated from BMDCs from FOXO1-deleted and control mice. CCR7 mRNA levels were measured by real-time PCR normalized to ribosomal protein L32 or 18S. (B and C) The protein levels of CCR7 in BMDCs from FOXO1-deleted and control mice were visualized by immunofluorescence using the specific Ab indicated (green) and nuclear counterstaining with DAPI. Original magnification \( \times 200 \). The quantitation of CCR7 at the protein level was determined by measuring fluorescence intensity. (D) RAW264.7-derived DCs were transfected with FOXO1 or empty vector alone and stimulated overnight with LPS. Real-time PCR was carried out to assess mRNA levels of CCR7. (E) RAW264.7-derived DCs were cotransfected with a FOXO1-expression vector or empty vector alone and a CCR7 promoter-luciferase reporter construct. Luciferase activity was measured. (F) Luciferase assays were performed in DC2.4 cells cotransfected with FOXO1 or empty vector alone and CCR7 luciferase reporter construct. ChIP assays were performed with RAW264.7-derived DCs treated with LPS or vehicle alone. The data are representative of two or three independent experiments of mean ± SEM from triplicate samples. *\( p < 0.05 \), DCs from FOXO1-deleted CD11cCre\(^+\) mice versus control CD11cCre\(^+\) mice, or DCs from transfection of FOXO1 versus empty vector alone or anti-FOXO1 Ab and control IgG.

purified allogeneic CD4\(^+\) T cells were cocultured with purified splenic DCs isolated from control mice and experimental CD11cCre\(^+\)FOXO1\(^{+/+}\) mice and stimulated with formalin-killed P. gingivalis and light-fixed P. gingivalis for 4 d. T cell proliferation was determined by calculating the number of CD4\(^+\) T cell divisions by flow cytometry. (H and I) FOXO1-deleted CD11cCre\(^+\) FOXO1\(^{+/+}\) and littermate control CD11cCre\(^-\)FOXO1\(^{+/+}\) mice were injected i.p. with formalin-killed P. gingivalis once weekly for 3 or 5 wk. The level of P. gingivalis–specific IgG1 or IgG2a was measured by ELISA. (J) Purified resting B cells were cocultured with BMDCs from FOXO1-deleted and control mice and stimulated with anti-CD40 Ab for 5 d. Supernatant was collected, and IgG production was measured by ELISA. The data are representative of two or three independent experiments of mean ± SEM from 10 mice. *\( p < 0.05 \), DCs from FOXO1-deleted CD11cCre\(^+\) mice versus littermate control CD11cCre\(^-\) mice.
compared with empty vector control, and this was enhanced further by LPS stimulation in RAW264.7-derived DCs (Fig. 5E). In DC2.4 cells, FOXO1 overexpression increased ICAM-1 promoter activity 7- and 9-fold by LPS compared with empty vector alone (Fig. 5F). Direct interactions between FOXO1 and the ICAM-1 promoter were demonstrated by ChIP assay. When DCs were stimulated with LPS, FOXO1 binding to the ICAM-1 promoter was increased >5-fold with anti-FOXO1 Ab compared with matched IgG control (Fig. 5G).

Transfection of FOXO1-ablated DCs with ICAM-1- or CCR7-expression plasmids rescues DC phagocytosis, binding to T cells, migration, and homing to lymph nodes

DCs were transfected with vectors that express GFP. In addition, some DCs also were transfected with vectors that express ICAM-1 or CCR7. After GFP+ cells were sorted, immunofluorescence was carried out. The results indicated that deletion of FOXO1 in DCs substantially reduced ICAM-1 and CCR7, which was restored by transfection with ICAM-1 or CCR7 expression vectors (Supplemental Fig. 4). Deletion of FOXO1 significantly reduced DC phagocytosis of bacteria ($p < 0.05$, Fig. 6A). The reduced phagocytosis was increased 54% by transfection with an ICAM-1 expression vector. Similarly, DC binding to T cells was reduced significantly by FOXO1 ablation in DCs from FOXO1-deleted mice and rescued by 56% with ICAM-1 transfection ($p < 0.05$, Fig. 6B). Rescue experiments with a CCR7-expression vector also were performed. The CCR7-expression vector rescued the reduced migration in FOXO1-deleted DC by 66% ($p < 0.05$, Fig. 6C). DC-homing experiments were performed in vivo to test the role of FOXO1 in DCs regulating the expression of genes that control DC–T cell interaction. (A) Total RNA was isolated from BMDCs from FOXO1-deleted and control mice. The mRNA level was measured by real-time PCR normalized to ribosomal protein L32 or 18S. (B) and (C) expression of ICAM-1 at the protein level in BMDCs was examined by immunofluorescence using specific Ab as indicated (green) and nuclear counterstaining with DAPI. Original magnification ×200. Quantitation was determined by fluorescence intensity. (D) Real-time PCR was carried out to assess mRNA levels of ICAM-1 in RAW264.7-derived DCs transfected with FOXO1 or empty vector alone and stimulated overnight with LPS or vehicle alone. (E) Luciferase assays were performed in RAW264.7-derived DCs cotransfected with FOXO1 or empty vector alone and an ICAM-1 promoter-luciferase construct. Cells were stimulated overnight with LPS or vehicle alone. (F) Luciferase assays were performed in DC2.4 cells cotransfected with FOXO1 or empty vector alone and an ICAM-1 promoter-luciferase construct. Cells were stimulated overnight with LPS or vehicle alone. (G) ChIP assays were performed with RAW264.7-derived DCs treated with LPS or vehicle alone. The data are representative of two or three independent experiments of mean ± SEM from triplicate samples. *$p < 0.05$, DCs from FOXO1-deleted CD11cCre+ mice versus control CD11cCre+ mice, or DCs from transfection of FOXO1 versus empty vector alone or anti-FOXO1 Ab and control IgG.
CCR7 and ICAM-1 in mediating the effects of FOXO1 ablation. DC homing to cervical lymph nodes was determined in fluo-
rescently labeled DCs following transfection and retro-orbital in-
jection. Lineage-specific deletion of FOXO1 reduced homing to
cervical lymph nodes by 91%. Homing was increased by 37%
\((p, 0.05)\) by CCR7 transfection in FOXO1-deleted DCs (Fig.
6D) and by 72\% \((p, 0.05)\) with simultaneous transfection of
CCR7 and ICAM-1 plasmids (Fig. 6E, 6F).

**Discussion**

FOXO1 is a transcription factor found in many cell types with
various functions. We report that FOXO1 is activated by bacteria
challenge in DCs and promotes DC bacterial phagocytosis, mi-
gration, and homing to lymph nodes by CCR7 and ICAM-1.
Moreover, the ability of DCs to activate Ag-specific immune
responses in CD4\+ T cells and resting B cells is FOXO1 depen-
dent, and FOXO1 is needed to generate an Ab response to bac-
terial challenge. To our knowledge, this is the first study to clarify
that FOXO1 is involved in the DC adaptive immune response via
the regulation of CCR7 and ICAM-1.

FOXO1 nuclear localization is a key step in its induction of
downstream target genes (34). We showed that FOXO1 nuclear
localization was increased in DCs by bacteria or LPS and was
mediated by the MAPK pathway. In contrast, Akt limited FOXO1
nuclear localization, consistent with our previous report that Akt
attenuates FOXO1 activation induced by LPS (19). Bacterial
stimulation of epithelial cells also induces host-response genes in
a FOXO1-dependent manner through the MAPK pathway (35,
36), consistent with the results from this study that FOXO1 par-
ticipates in organizing the host response in DCs.

Cell homing, migration, and phagocytosis are important com-
ponents of DC participation in the adaptive immune response (37).
We found that FOXO1 promoted DC homing, migration, and
phagocytosis by regulating CCR7 and ICAM-1. FOXO1 bound to
the CCR7 and ICAM-1 promoters, induced CCR7 and ICAM-1
transcriptional activity, and regulated the expression of both.
These results are supported by findings in T cells and endothelial
cells that FOXO1 directly binds to the ICAM-1 and CCR7 pro-
moters (17, 38, 39). Moreover, the negative effect of FOXO1
deletion on both phagocytosis and DC binding to T cells was
rescued by transfection with an ICAM-1 construct, and reduced
chemotaxis was rescued by transfection with a CCR7-expression
vector. In addition, reduced homing in FOXO1-deleted DCs was
enhanced significantly by dual transfection of CCR7 and ICAM-1.
The results agree well with the roles of CCR7 in DC homing
and ICAM-1 in cell adhesion and migration (38–46). Thus, FOXO1
coordinates the activity of DCs by direct regulation of key
downstream targets ICAM-1 and CCR7.

DCs play an important role in the adaptive immune response by
activating lymphocytes. Bacterial challenge in FOXO1-deleted
mice in vivo decreased the ability of DCs to stimulate CD4\+ T cells to produce cytokines, such as IFN-\(\gamma\) and IL-13. In addition,
FOXO1 deletion reduced DC binding to CD4+ T cells and reduced stimulation of naive CD4+ T cell proliferation in vitro. That FOXO1 deletion affects the ability of DCs to activate T cells is consistent with reports that defects in DC–T cell binding and impaired phagocytosis by DCs negatively affect T cell responses (47, 48). DCs also regulate B cells. We found that lineage-specific FOXO1 deletion in DCs reduced the expression of BAFF and APRIL and decreased the ability of DCs to induce B cell proliferation and stimulate Ab production. BAFF and APRIL are important mediators of B cell activation (49). BAFF deficiency or inhibition of B cell activation severely impairs the Ab response to bacteria (50–52).

In summary, we describe a novel function for FOXO1 in regulating several aspects of DC activity, including phagocytosis and Ag presentation, DC–T cell binding, migration, and DC homing to lymph nodes, by directly regulating CCR7 and ICAM-1. Taken together, the results indicate that FOXO1 plays an important role in the in vivo and in vitro ability of DCs to activate T and B lymphocytes, which is a critical link between the innate and adaptive immune response and is needed to generate an Ab defense to bacteria (53).

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Disclosures

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