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TLR4 on Follicular Dendritic Cells: An Activation Pathway That Promotes Accessory Activity

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Microbial molecular patterns engage TLRs and activate dendritic cells and other accessory cells. Follicular dendritic cells (FDCs) exist in resting and activated states, but are activated in germinal centers, where they provide accessory function. We reasoned that FDCs might express TLRs and that engagement might activate FDCs by up-regulating molecules important for accessory activity. To test this hypothesis, TLR4 expression on FDCs was studied in situ with immunohistochemistry, followed by flow cytometry and RT-PCR analysis. TLR4 was expressed on FDC reticula in situ, and flow cytometry indicated that TLR4 was expressed on surface membranes and TLR4 message was readily apparent in FDCs by RT-PCR. Injecting mice or treating purified FDCs with LPS up-regulated molecules important for accessory activity including, FDC-FcγRIIB, FDC-ICAM-1, and FDC-VCAM-1. Treatment of purified FDCs with LPS also induced intracellular phospho-IκB-α, indicating NF-κB activation, and that correlated with increased FcγRIIB, ICAM-1, and VCAM-1. FDCs in C3H/HeJ mice were not activated with LPS even when mice were reconstituted with C3H/HeN leukocytes, suggesting that engagement of FDC-TLR4 is necessary for activation. Moreover, activated FDCs exhibited increased accessory activity in anti-OVA recall responses in vitro, and the FDC number could be reduced 4-fold if they were activated. In short, we report expression of TLR4 on FDCs for the first time and that engagement of FDC-TLR4 activated NF-κB, up-regulated expression of molecules important in FDC accessory function, including FcγRIIB, ICAM-1, and VCAM-1, as well as FDC accessory activity in promoting recall IgG responses. The Journal of Immunology, 2007, 179: 4444–4450.
accessory cells, activated FDCs have increased accessory activity, as indicated by increased anti-OVA production in comparison with nonactivated FDCs. An important implication of these studies is that TLR agonists may promote immune responses not only by stimulating dendritic cells (DCs) and enhancing T cell function, but also by stimulating FDCs and promoting B cell function. An understanding of how TLR agonists influence FDC activation may give insight into how adjuvants should be formulated to give optimal humoral immune responses when administering vaccines.

Materials and Methods

Animals

Six- to 8-week-old BALB/c mice were obtained from the National Cancer Institute. The C3H/HeNTac mice were from Taconic Farms, and the C3H/HeJ mice were from The Jackson Laboratory. All mice were housed in standard plastic shoebox cages with filter tops and maintained under specific pathogen-free conditions in accordance with guidelines established by Virginia Commonwealth University Institutional Animal Care and Use Committee.

Abs and other reagents

Sigma-Aldrich (L-7895) cell culture-tested, gel filtration-purified, γ-irradiated LPS from Salmonella typhosa was used in this study with >99% purity. FITC-conjugated rat anti-mouse CD106 (mVMCAM-1, catalog no. 553332), CD54 (mICAM-1, catalog no. 553252), CD16 (CD32) (FcyRIII), catalog no. 553144), Fc block (2.4G2, catalog no. 553142), rat anti-mouse FDC-M1 (catalog no. 551320), biotinylated anti-rat ICAM chain (catalog no. 553871), rat IgG1b (catalog no. 553988), rat IgG2a (catalog no. 554688), hamster IgG1 (catalog no. 553971) isotype controls, streptavidin-HRP (catalog no. 550946), and the diaminobenzidine substrate kit (catalog no. 550880) were from BD Pharmingen. FITC-conjugated rat anti-mouse TLR4 was from Imgenex (catalog no. IMG-428C). Anti-biotin conjugated rat anti-mouse TLR4 was from Miltenyi Biotec. Rabbit anti-mouse TLR4 was from Imgenex (catalog no. IMG-428C). Anti-β-actin (a gift from Virginia Commonwealth University Institutional Animal Care and Use Committee).

Immunohistochemical labeling of TLR4, FDC-M1, and GL-7 in situ

Popliteal lymph nodes (LNs) were collected from normal BALB/c mice and frozen in CryoForm embedding medium (I:EC). Frozen sections of 10 μm thickness were cut on a Leica (Jung Frigocut 2800E) cryostat and air dried. Following absolute acetone fixation, the sections were dehydrated and the endogenous peroxidase activity was quenched with the Universal Block. The sections were washed and saturated with 10% BSA. Serial sections were incubated with unlabeled rabbit anti-mouse TLR4 or rat anti-mouse FDC-M1. Sections were washed and then incubated with biotin-conjugated goat anti-rabbit or anti-rat IgG, followed by streptavidin-HRP. The sections were developed using a diaminobenzidine substrate kit. The sections were developed using a diaminobenzidine substrate kit. For GC B cell labeling, 10-μm cryostat sections of axillary LNs from OVA-immunized BALB/c mice were labeled with IgM rat anti-mouse T and B cell activation Ag (GL-7, Ly-77) (eBioscience 14-5902-85), followed by biotinylated goat anti-rat IgM (Southern Biotechnology Associates 3020-08) and phosphatase-labeled streptavidin (Kirkegaard & Perry Laboratories 15-30-00). The sections were developed with SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/NBT alkaline phosphatase substrate (Sigma-Aldrich B5655). Rabbit polyclonal IgG (Abcam ab227478), rat IgG2c (BD Biosciences 553982), and rat IgM (eBioscience 14-3431) isotype controls were similarly treated. Images were captured with Optronics digital camera and analyzed with Bioquant Nova software.

DC, FDC, and B cell isolation

DCs were isolated from splenic leukocytes using the CD11c micro bead kit from Miltenyi Biotec. FDCs were isolated by positive selection from LNs (axillary, lateral axillary, inguinal, popliteal, and mesenteric) of irradiated animals as described (29). One day before isolation, mice were irradiated with 1000 rad to eliminate most lymphocytes, and then mice were killed, and LNs were collected, opened, and treated with 1.5 ml of collagenase D (22 mg/ml, C-1088882; Roche), 0.5 ml of DNase I (5000 U/ml, D-4527; Sigma-Aldrich), and 2 ml of DMEM with 20 mM HEPES. After 45 min at 37°C in a CO2 incubator, released cells were washed in 5 ml of DMEM with 10% FCS. Cells were then sequentially incubated with FDC-specific Ab (FDC-M1) for 45 min, 1 μg of biotinylated anti-rat λ chain for 45 min, and 20 μl of anti-biotin microbeads (Miltenyi Biotec) for 15–20 min on ice. The cells were layered on a MACS LS column and washed with 10 ml of ice-cold MACS buffer. The column was removed from the VarioMACS, and the bound FDCs were released with 5 ml of MACS buffer, B220+ B cells were isolated from BALB/c LNs using CD45R (B220) MicroBeads from Miltenyi Biotec.

FDCs and B220+ B cells isolated from BALB/c mice were incubated with mouse Fc-Block (BD Pharmingen) for 15 min on ice, followed by FITC-conjugated rat anti-mouse TLR4 or isotype control for 60 min in the dark at 4°C. After washing, the cells were analyzed using an FC500 flow cytometer and Cytomics RXP analysis software. Histograms were gated for FDCs based on their forward and side scatter properties established with FDC phenotypic markers, including FDC-M1, CD21/35, and CD32 (29). The figures were plotted using WinMDI software (Scripps Research Institute).

Detection of TLR4 mRNA in purified FDCs by RT-PCR

Total cellular RNA was extracted from 2 × 106 FDCs purified from LNs or spleens as well as control splenic DCs of BALB/c mice using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions, and 100 ng was reverse transcribed into cDNA using GeneAmp Gold RNA PCR Core Kit. One-tenth of cDNA was primed with TLR4-specific primers 5′-GCCTTCACCTCCTGCCTAC-3′ and 3′-CGAGGCTTTTCACTCTACA-5′ and amplified by PCR. PCR products were electrophoresed in 1.5% agarose, stained with ethidium bromide, and visualized by UV transilluminator.

Analysis of FcyRIIB, VCAM-1, and ICAM-1 on LPS-treated FDCs in vivo and in vitro

FDCs were positively selected with FDC-M1 from the draining LNs of irradiated BALB/c mice 3 days after injecting 25 μg of LPS in the four limbs. Control FDCs were isolated from saline-injected mice. In addition, 1 × 106 purified FDCs were treated in vitro with 10 μg of LPS in 1 ml of medium for 3 days, and the surface expression of FcyRIIB, VCAM-1, and ICAM-1 was compared with 1 × 106 control FDCs cultured in medium without LPS. Analysis was done by incubating FDCs with mouse Fc-Block (BD Pharmingen) for 15 min on ice, followed by FITC-conjugated rat Abs against FcyRIIB, ICAM-1, and VCAM-1 or isotype control for 60 min in the dark at 4°C. After washing, the cells were analyzed by flow cytometry.

Adoptive transfer

C3H/HeJ and C3H/HeN recipients were exposed to 600 rad of irradiation, and 1 day later they were reconstituted with 2 × 108 LPS-responsive C3H/HeN total splenic leukocytes injected behind the neck. The mice were challenged with LPS for 3 days, and the FDCs were isolated after 3 days.

Analysis of FcyRIIB, VCAM-1, and ICAM-1 on LPS-treated C3H/HeN and C3H/HeJ FDCs

FDCs were positively selected from the LNs of C3H/HeN or C3H/HeJ mice 3 days after injecting 25 μg of LPS in each limb. Control FDCs were isolated from saline-injected mice. FDCs were first incubated with mouse Fc-Block for 15 min on ice, followed by FITC-conjugated rat Abs against FcyRIIB, ICAM-1, and VCAM-1 or isotype control for 60 min in the dark at 4°C. After washing, the cells were analyzed by flow cytometry.

Intracellular labeling of phospho-IΚB-α in purified FDCs treated with LPS in vitro

Purified FDCs were serum starved for 24 h, and then treated with 10 μg/ml LPS overnight in DMEM with 10% FCS. Control groups of unstimulated FDCs as well as macrophage cell line J774 were similarly treated. Cells were washed and then fixed and permeabilized using Fix & Perm cell permeabilization kit (Caltag Laboratories). Intracellular phospho-IκB-α was labeled with rabbit anti-phospho-IκB-α (Cell Signaling Technology), followed by PE-conjugated goat anti-rabbit IgG, and cells were analyzed with flow cytometry.
Cell culture and ELISA assessment of OVA-specific IgG

LN cells from OVA-immunized BALB/c mice were cultured, in the presence of 100 ng/ml OVA-anti-OVA ICs, with FDCs isolated from LPS-treated mice, and compared with control FDCs at ratios of 64, 128, and 256 lymphocytes/FDC. The medium was changed after 1 wk, and OVA-specific IgG was assessed by ELISA 7 days later, as previously described (30).

Results

Immunohistochemical labeling of TLR4 on FDCs, but not on GC B cells in situ

As shown in Fig. 1, adjacent sections of LNs were labeled for TLR4 and the FDC-specific marker FDC-M1. TLR4 colocalized with the FDC-M1, as indicated by the characteristic labeling of FDC network or reticulum made up by interactive FDC dendrites can be seen. B. Lack of TLR4 labeling on GC B cells. Adjacent sections from axillary LNs of OVA-immune animals were labeled with GL-7, anti-mouse T and B cell activation Ag (Ba), and anti-TLR4 (Bc). As shown between arrowheads, GL-7 + B cells did not label with anti-TLR4. Isotype controls (Ab, Ad, Bh, and Bd) are shown to the right of the appropriate adjacent section. C. Flow cytometry was used to assess TLR4 labeling of purified FDCs in vitro.

FIGURE 2. RT-PCR analysis of TLR4 mRNA in purified FDCs. Total RNA extracted from purified FDCs of mouse LNs (A) and spleen (B) were primed with TLR4-specific primers and compared with purified DCs isolated from mouse spleen (C). A single 361-bp band shared between DCs and FDCs is seen, providing evidence for TLR4 expression in FDCs.

Cell culture and ELISA assessment of OVA-specific IgG

LN cells from OVA-immunized BALB/c mice were cultured, in the presence of 100 ng/ml OVA-anti-OVA ICs, with FDCs isolated from LPS-treated mice, and compared with control FDCs at ratios of 64, 128, and 256 lymphocytes/FDC. The medium was changed after 1 wk, and OVA-specific IgG was assessed by ELISA 7 days later, as previously described (30).

Results

Immunohistochemical labeling of TLR4 on FDCs, but not on GC B cells in situ

As shown in Fig. 1A, adjacent sections of LNs were labeled with FDC-specific marker FDC-M1 (Fig. 1Aa) and anti-TLR4 (Fig. 1Ac). TLR4 colocalized with the FDC-M1, as indicated by the
FIGURE 3. Up-regulation of FcγRIIB, ICAM-1, and VCAM-1 on LPS-treated FDCs in vivo (A) and in vitro (B). FDCs were purified from the LN of LPS-treated BALB/c mice and compared with control FDCs isolated from saline-injected mice (A). Likewise, purified FDC preparations treated in vitro with LPS for 3 days were compared with control FDCs cultured in medium without LPS (B). FDCs were labeled with FITC-conjugated rat Abs against FcγRIIB (a), ICAM-1 (b), and VCAM-1 (c) or isotype control. The MFI of FITC-labeled FDCs were almost double the corresponding isotype controls in vivo and in vitro, although the intensity of VCAM-1 up-regulation on FDCs stimulated in vitro was comparatively less. These data are representative of three separate experiments of this type.

TLR4 labeling in the area between the arrows. The characteristic labeled FDC network or reticulum made up by interactive dendrites of FDCs can be seen. GC B cells intermingle with FDCs, and we sought to determine whether they label with anti-TLR4 and contribute to the labeling in Fig. 1. GC B cells are activated and labeled with anti-mouse T and B cell activation Ag GL-7 (Fig. 1A). GC B cells were activated and labeled with anti-TLR4 and anti-mouse T and B cell activation Ag GL-7 (Fig. 1A). In contrast with FDCs, they did not label with TLR4 in adjacent LN sections (Fig. 1B). Thus, TLR4 labeling in GCs appeared to be attributable to FDCs and not B cells. Isotype controls (Fig. 1D) were included and shown to the right of the corresponding labeled sections.

TLR4 labeling of purified FDCs

To confirm expression of TLR4, FDCs were purified by positive selection using the mAb FDC-M1, and labeled with anti-TLR4 FITC. As illustrated in Fig. 1C, flow cytometric analysis revealed that virtually the entire FDC population shifted to the right when labeled with anti-TLR4 with more than a 2-fold increase in mean fluorescent intensity (MFI) over the background control. Analysis was restricted to viable cells, indicating that TLR4 is expressed on the surface of FDC membranes. B cells positively selected with B220, an Ag expressed on B lineage cells throughout their development, but not on plasma cells, also appeared to shift to the right as a consequence of anti-TLR4 labeling (Fig. 1D). However, in contrast with FDCs, in which the MFI more than doubled upon labeling with anti-TLR4, the increased MFI for the B cells was only 0.2 above the isotype control. In short, murine B cells may express some TLR4, but it is unlikely that they are making a significant contribution to the strong TLR4 labeling in GC areas where the FDC-reticulum identified by FDC-M1 is present.

TLR4 mRNA in purified FDCs

RNA extracted from purified FDCs from murine LNs (Fig. 2A) and spleen (Fig. 2B) was reverse transcribed into cDNA. TLR4-specific primers were used to amplify the relevant DNA, and the results were compared with similarly treated purified splenic DCs as a positive control (Fig. 2C). Gel electrophoresis of amplified PCR products revealed a single 361-bp band shared between DCs and FDCs, indicating TLR4 expression by FDCs.

LPS-mediated up-regulation of FDC-FcγRIIB, FDC-ICAM-1, and FDC-VCAM-1

Injecting LPS increased the levels of FcγRIIB, ICAM-1, and VCAM-1 on FDCs from the draining LNs (Fig. 3A), where virtually the entire FDC population shifted to the right when labeled with Abs reactive with these markers and the MFI approximately doubled in each case. Similarly, treating purified FDCs in vitro (Fig. 3B) with LPS for 3 days increased the levels of FcγRIIB, ICAM-1, and VCAM-1 expression on FDCs, although the intensity of VCAM-1 up-regulation was comparatively less.

FDCs purified from TLR4-mutated C3H/HeJ mice did not respond to LPS even when reconstituted with leukocytes from TLR4 wild-type mice

FDCs from wild-type LPS-responsive C3H/HeN mice up-regulated FDC-FcγRIIB, FDC-ICAM-1, and FDC-VCAM-1 when injected with LPS (Fig. 4A) much like the BALB/c mice shown in Fig. 3. Moreover, this response persisted after irradiation and reconstitution with LPS-responsive C3H/HeN total splenic leukocytes (Fig. 4B). In marked contrast, FDCs from TLR4-mutated C3H/HeJ mice failed to up-regulate FDC-FcγRIIB, FDC-ICAM-1, and FDC-VCAM-1 after LPS injection (Fig. 4C) even after reconstitution with wild-type TLR4 LPS-responsive C3H/HeN leukocytes (Fig. 4D). This suggests that LPS engagement of TLR4 on the FDC is important, and that FDC activation is not simply a response to cytokines being produced by other TLR4-responsive leukocytes.

LPS-mediated up-regulation of intracellular phospho-1κB-α in FDCs indicative of NF-κB activation

Signaling through TLR4 typically involves the NF-κB pathway, prompting the hypothesis that stimulation of FDCs with LPS...
would up-regulate intracellular phospho-IκB-α in FDCs. Purified FDCs were incubated with 10 μg/ml LPS overnight. Control groups included unstimulated FDCs and murine macrophage cell line J774 that were similarly treated. Flow cytometric analysis revealed that the MFI of intracellular phospho-IκB-α in the control macrophage cell line about doubled after exposure to LPS (Fig. 5A). The FDC response to LPS was very similar, and the increase in MFI was even higher (Fig. 5B). Similar results were obtained with 0.1 and 1 μg of LPS, but the results were most apparent with the 10 μg dose illustrated in Fig. 5.

FIGURE 4. Lack of up-regulation of FcγRIIB, ICAM-1, and VCAM-1 on FDCs purified from TLR4-mutated C3H/HeJ mice even when reconstituted with leukocytes from TLR4 wild-type mice. The levels of expression of FcγRIIB, ICAM-1, and VCAM-1 in TLR4-intact LPS-responsive C3H/HeN were assessed using flow cytometry 3 days after LPS injection (A) or after irradiation, reconstitution with C3H/HeN leukocytes, and LPS injection (B). Similarly, FcγRIIB, ICAM-1, and VCAM-1 in TLR4-mutated C3H/HeJ mice were analyzed 3 days after LPS injection (C) or after irradiation, reconstitution with C3H/HeN leukocytes, and LPS injection (D). FDCs from TLR4-intact LPS-responsive C3H/HeN mice demonstrated up-regulation of FcγRIIB, ICAM-1, and VCAM-1 when injected with LPS, and that persisted after irradiation and reconstitution. On the contrary, FDCs purified from TLR4-mutated C3H/HeJ mice failed to up-regulate their FcγRIIB, ICAM-1, and VCAM-1 upon encountering LPS, and that persisted even after reconstitution with TLR4-intact LPS-responsive C3H/HeN leukocytes. These data are representative of three separate experiments of this type.

FIGURE 5. Up-regulation of intracellular phospho-IκB-α in purified FDCs treated with LPS in vitro. Purified FDC preparations were treated with 10 μg/ml LPS overnight, and control groups of unstimulated FDCs as well as macrophage cell line J774 were included. FDC intracellular phospho-IκB-α rose after LPS treatment to levels comparable to those seen in the control macrophage cell line. These data are representative of three separate experiments of this type.

FIGURE 6. Activated FDCs have enhanced accessory cell activity in OVA-specific recall responses. OVA-specific lymphocytes were cultured, in the presence of OVA-anti-OVA ICs, with FDCs isolated from LPS-treated mice and compared with control FDCs at ratios of 64, 128, and 256 lymphocytes to 1 FDC. Two weeks later, OVA-specific IgG was assessed by ELISA. At all tested ratios, cultures containing activated FDCs promoted significantly higher anti-OVA IgG levels than cultures with control FDCs (p < 0.01; Student’s t test). Averages were calculated using data from three cultures, and results illustrate the means ± SEM.
LPS-activated FDCs exhibited enhanced accessory activity in promoting recall responses in vitro

FDCs from normal mice are known to enhance recall responses optimally at ratios between 1 FDC and 4–16 lymphocytes, although detectable accessory activity may be apparent with more lymphocytes (31). We reasoned that LPS-activated FDCs would have enhanced accessory activity, and that might be most apparent in IgG recall responses at low FDC to lymphocyte ratios. When cultured at a ratio of 1 FDC to 64 lymphocytes, the IgG anti-OVA produced in cultures containing activated FDCs was ~4 times higher than the production with FDCs from normal mice, which in turn produced anti-OVA IgG comparable to 1 activated FDC to 256 lymphocytes (Fig. 6). In marked contrast, FDCs from normal mice were without detectable activity at 1 FDC to 256 or even 128 lymphocytes (Fig. 6).

Discussion

Engagement of TLRs on DCs leads to physiological and phenotypic changes that dramatically alter their ability to present Ag to T cells (1, 32, 33). DC alterations include changes in the following: survival, chemokine receptor expression, chemokine secretion, migration, cell shape, and endocytic activity (34). LPS and other TLR agonists promote DC maturation and accessory activity in priming naive T cells, promoting clonal expansion, and stimulating differentiation of T cells into effectors (35). We reasoned that for the immune system to respond to microbial invasion as an integrated unit, microbial patterns encountered early in immune responses should activate not only the DC-T cell axis, but also the FDC-B cell axis. The data reported in this study indicated that FDCs express surface TLR4 and that engagement of this receptor in vivo and in vitro altered their physiology and phenotype, leading to FDC activation. Moreover, activated FDCs were far more effective in enhancing the production of specific IgG than FDCs from normal mice. IgC-bearing FDCs reside in GCs where B cells do the following: proliferate, isotype switch, somatically hypermutate, become memory cells, and transition from B cells into Ab-forming cells (reviewed in Refs. 13 and 36). Considerable data indicate that FDCs influence these B cell functions (reviewed in Refs. 13 and 14). An understanding of FDC activation could facilitate our ability to manipulate the immune system so that the appropriate adjuvants could be selected to activate FDCs and optimize humoral immune responses when vaccines are administered.

Analysis of mRNA by RT-PCR in the present study suggested that TLR4 expression in DCs and FDCs is comparable. The fact that FDCs are barely endocytic and retain Ag in ICs on their surfaces (37) allows the surface-expressed FDC-TLR4, as shown by flow cytometry, to engage LPS in an environment spatially associated with other FDC accessory functions, including IC trapping and receptor-mediated B cell-FDC clustering and interactions. The presence of TLR4 molecules inside the FDCs has not been excluded, but clearly, anti-TLR4 labels FDC membranes intensely. Cytokines help activate many cell types, and we reasoned that cytokines induced by LPS may play an important role in FDC activation. However, no support for this idea was found in the present study. When normal splenic leukocytes that are fully capable of secreting cytokines upon TLR4 engagement were injected into irradiated TLR4-mutated C3H/HeJ mice and challenged with LPS, the FDCs with mutated TLR4 still failed to activate (Fig. 4D). These results argue that functional TLR4 on FDCs is important. Moreover, when purified wild-type FDCs were treated with LPS in vitro, they responded well, suggesting that FDC-TLR4 is sufficient (Fig. 3B).
memory cell formation, transition from B cells to Ab-forming cells, selection, and affinity maturation. In addition to TLR4, preliminary studies using RT-PCR indicated that FDCs express TLR-2, TLR-3, and TLR-9. By use of flow cytometry with purified FDCs, we were able to confirm expression of TLR-2 and TLR-3. Stimulation of FDCs with the TLR-3 ligand poly(I:C) resulted in up-regulation of complement receptor 1/2, FcγRIIB, and VCAM-1. However, the data suggest that TLRs may differ in their impact on FDC phenotype and function, and further studies in this area are in progress. Nevertheless, an understanding of how FDCs can be optimally activated via TLR engagement could provide information needed to control and enhance the development of protective humoral immune responses.

Disclosures
The authors have no financial conflict of interest.

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