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Wnt5a–Rac1–NF-κB Homeostatic Circuitry Sustains Innate Immune Functions in Macrophages

Debdut Naskar,* George Maiti,* Arijit Chakraborty,* Arunava Roy,†
Dhrubajyoti Chattopadhyay,‡ and Malini Sen*

Macrophages play a critical role in innate immunity. Differentiation Ags present on macrophages such as CD14 orchestrate the first line of defense against infection. The basal/homeostatic signaling scheme that keeps macrophages unengaged for innate immune functions remains unresolved. Wnt5a–Fz5 signaling being a primordial event during cell differentiation, we examined the involvement of Wnt5a–Fz5 signaling in the maintenance of innate immune functions. In this study, we demonstrate that innate immune functions of macrophages ensue at least partly through a homeostatic Wnt5a–Fz5–NF-κB (p65) circuit, which is Rac1 dependent. The autocrine/paracrine Wnt5a–Fz5–Rac1–p65 signaling cascade not only maintains basal levels of the immune defense modulating IFNs and CD14; it also supports macrophage survival. Wnt5a–Fz5–Rac1 signaling mediated p65 homeostasis in turn sustains Wnt5a expression in a feed-forward mode. The natural immune response of macrophages to Escherichia coli/LPS and virus is accordingly sustained. The depiction of sustenance of innate immune functions as an outcome of a homeostatic Wnt5a–p65 axis unfolds previously unidentified details of immune regulation and provides new insight into homeostatic cell signaling. The Journal of Immunology, 2014, 192: 000–000.

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M.S. designed research, analyzed data, and wrote the paper; D.N. performed experiments, analyzed data, and assisted in writing the paper; G.M. performed some experiments; A.C. helped in animal experiments; and A.R. and D.C. provided viral stock and helped in virus-related experiments.

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a similar regulatory scheme (9). Blockade of Wnt5a signaling in activated synovial fibroblasts from patients with rheumatoid arthritis results in inhibition of cytokine secretion, suggesting the occurrence of context-dependent Wnt5a-mediated immune stimulation (28, 29). Wnt5a signaling in activated cells involves the differential action of several signaling intermediates, such as phosphatidyl inositol, protein kinase C, and Ca2+ calmodulin-dependent protein kinase II, which can vary between cell types; transcriptional activators of several immune response and prosurvival genes, such as NF-κB acting as further downstream mediators (16–18, 30, 31). Interestingly, NF-κB has also been reported to promote Wnt5a expression in stimulated macrophages (11). In such a scenario, one might fittingly question whether Wnt5a signaling in macrophages is important for maintaining immune homeostasis and whether such a scheme involves NF-κB.

Dimeric NF-κB transcription factors are formed combinatorially from p50, p52, p65 (also known as RelA), cRel, and RelIB. These dimers are activated through different pathways during cell signaling. In the classical pathway, preexisting NF-κB homodimers and heterodimers composed of p65, p50, and cRel are released from their IκB-bound states in the cytoplasm, in response to different stimuli and translocated to the nucleus, where transcription occurs. The key feature of the classical pathway is the activation of a kinase known as IκB kinase (IKK). Activated IKK phosphorylates IκB, which then becomes a target for ubiquitination and 26S proteasome-mediated degradation. Thus, unbound NF-κB dimers are free to translocate to the nucleus, where they can promote cell survival and induce immune response genes under different cellular contexts (6, 7). Low levels of nuclear NF-κB (p65) have been observed in several cell lines even in the absence of activating stimuli (32, 33). Whether a similar NF-κB (p65) pool also exists in macrophages, how the pool of nuclear p65 is maintained and what functions it serves, however, remain unclear.

The current study demonstrates that a basal Wnt5a–Fz5 signaling keeps macrophages prepared for innate immune functions while also supporting their survival, at least partly through transcriptional activation of NF-κB (p65). The constitutively nuclear p65 in turn sustains Wnt5a expression. Accordingly, the basal IκB/CD14 level of macrophages along with their natural immune response to Escherichia coli–LPS and virus is retained. The homeostatic Wnt5a–NF-κB circuit in macrophages appears to be RAC1 dependent.

Materials and Methods

Mice

C57BL/6 mice were purchased from the National Institute of Nutrition (Hyderabad, India) and maintained in the institutional animal house facility. All the experimental procedures were approved by the Institutional Animal Ethics Committee.

Generation of bone marrow–derived macrophages

Bone marrow–derived macrophages (BMDM) were generated by following published protocols (34, 35) with minor modifications. Both femur and tibia were collected from the hind legs of C57BL/6 mice at 10–12 wk of age. Muscles attached to bones were removed using clean and sterile scissors and forceps.Bone marrow was isolated by flushing the marrow cavity with a syringe filled with RPMI 1640 media into a sterile 50 ml tube. Upon centrifugation at 2000 rpm for 5 min, cells were resuspended in RPMI 1640 media supplemented with 20% FBS, 30% L cell–conditioned medium (L-CM) as a source of M-CSF, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM l-glutamine. Subsequently, the cells were plated on 90-mm nontissue culture–treated plates (Axxygen) at a density of 4 × 10⁶ cells/ml and incubated at 37°C in a 5% CO₂ atmosphere. Three days after seeding the cells, an extra 10 ml of fresh RPMI 1640 (supplemented with 20% FBS, 30% L-CM, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM l-glutamine) was added to each plate, and incubation continued for additional 3 d. On the sixth day, supernatants were discarded and the attached cells were resuspended in RPMI 1640 supplemented with 10% FBS, 5% L-CM, 2 mM l-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin after being dislodged by Trypsin-EDTA. Finally, the cells were counted and seeded on tissue culture plates for 24 h before the experimental procedure, during which the culture medium was replaced by RPMI 1640 growth medium with 5% FBS.

Pull-down of IKK enzyme complex and in vitro kinase assay

The in vitro kinase assay was performed following the manufacturer’s (Biobharati Life Science, Kolkata, India) protocol using RAW 264.7 and BMDM. Cells were washed twice with ice cold PBS, resuspended in PBS, centrifuged at 2000 rpm for 5 min at 4°C and subsequently lysed using cytoplasmic extraction buffer (10 mM HEPES-KOH, pH 7.9, 250 mM NaCl, 0.5% NP-40, 1 mM EDTA, 2 mM DTT, freshly added Protease Inhibitor Cocktail, 1 mM PMSF and 0.1 mM Na3VO4). Cytoplasmic extract (CE) was collected by centrifugation at 600g for 10 min at 4°C. Collected CE was used to immunoprecipitate the IKK enzyme complex. Accordingly, CE was pre-cleared with protein-G plus agarose beads by rotating for 30 min at 4°C and the precleared extract was incubated with anti IKKγ Ab for 1 h at 4°C in rotating condition following which 10 μl of protein-G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each tube and again rotated at 4°C overnight. Immunoprecipitated IKK complex-beads were washed twice with CE buffer and incubated with kinase assay buffer (20 mM HEPES, pH 7.7, 20 mM β-glycerophosphate, 10 mM MgCl₂, 100 mM NaCl, 100 μM NaF, 100 μM NaVO₃, 10 mM NaF, 1 mM PMSE) to which 20 μl of reaction mixture (0.5 μg GST-IκBα [1–54 aa], 5 μCi [γ³²P]-ATP [Perkin Elmer], 20 μM cold ATP) was added for 30 min at 30°C. Reactions were stopped by adding 10 μl of SDS loading dye, proteins separated by SDS-PAGE, and radioactive bands estimated using phosphorimager. Efficiency of pull-down of IKK complex was estimated by immunoblot with rabbit antisemur against IKK-2/β.

Peritoneal macrophage isolation

Peritoneal macrophages were isolated from C57BL/6 mice (10–12 wk old) following standard protocol. Mice were sacrificed, and the outer skin was surgically removed. Keeping the peritoneal cavity intact, the peritoneum was washed with 6 ml ice cold PBS. Peritoneal lavage obtained was centrifuged to pellet down the cells. RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin was used to resuspend the cells. Subsequently, cells were allowed to adhere to tissue culture plates overnight in normal RPMI 1640 growth medium for appropriate assays.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (CHIP) assay was performed following published protocol (36) with minor modifications. Cells were fixed with 1% formaldehyde for 10 min at 37°C. Excessive cross-linking was blocked by adding glycerine to a final concentration of 125 mM and rocking for 5 min. The adherent cell monolayer was then rinsed twice with ice-cold PBS, after which cells were scraped from the surface and centrifuged at 2000 rpm for 5 min. Cell pellets were subsequently lysed in SDS lysis buffer (50 mM Tris-Cl, pH 8.1, 1% SDS, 10 mM EDTA and protease inhibitor mixture). Extracts were sonicated to make genomic DNA fragments of 100–500 bp. After centrifugation at 15,000 rpm for 10 min at 4°C to pellet insoluble materials, extracts were pre-cleared using 10 μg of salmon sperm DNA (Life Technologies-Invitrogen) and protein G plus agarose (rotation for 1 h at 4°C) subsequently, 5 μg of rabbit anti-p65 (Santa Cruz Biotechnology) or isotype control, protein G-agarose beads (Santa Cruz Biotechnology) and salmon sperm DNA were added to the precleared extract. A similar DNA–protein complex was pulled down by centrifugation after overnight rotation. The bead-Ab-chromatin complex was washed successively with low-salt immune complex buffer (20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton-X100, 150 mM NaCl), high salt immune complex buffer (20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton-X100, 500 mM NaCl) and LiCl immune complex buffer (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 0.25 M LiCl, 1% deoxycholic acid, 1% Triton X-100). Finally, the immune complex was washed with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Protein-chromatin complex was reverse cross-linked by adding 100 μl of chex100 and then heated at 100°C for 10 min. Subsequently, the protein was digested by protease K at 55°C for 30 min, after which the chromatin was precipitated by phenol-chloroform extraction and ethanol precipitation. Precipitated DNA was quantified by semi-quantitative PCR analysis using primers specific for kβ site on murine (CD43) promoter增强 region. (forward: CACCTGTGATAGGAATTCGC; and reverse: ATGTAAGGGCCCATGATTTCG; 227 bp). As a negative control primer pairs (forward: AGGCAGAGATAAAGCTCAAAG; and reverse: AGGCAGAGATAAAGCTCAAAG; and reverse:
from Biobharati Life Science. Anti-mouse CD14 Abs were purchased from

Luciferase assay
RAW264.7 cells were cotransfected with the NF-kB-LUC and Renilla Luciferase expression vectors (a gift from Prof. G. Ghosh, University of California–San Diego) at 50:1 ratio using Lipofectamine LTX reagent (Life Technologies). After 48 h of transfection, medium was replaced with fresh RPMI 1640 medium and cells were treated with either IWP-2 (0.05 μM) or DMSO for an additional 48 h. Cell lysate was prepared, and luciferase activity was measured using a dual Luciferase assay kit (Promega) in a luminometer (Promega). Relative luciferase activities were calculated by dividing the values of firefly luciferase activity by the corresponding values of Renilla luciferase activity.

RNA isolation and RT-PCR
RNA was isolated from human monocytes, mouse BMDM, or RAW 264.7 cells using Trizol. cDNA was generated using M-MLV RT following instructions provided by the manufacturer (Biobharati Life Science). For primer pairs that were used for RT-PCR on the cDNA generated, see Table I.

Nuclear and cytoplasmic extract preparation
Macrophages washed with cold PBS were centrifuged at 2000 rpm for 5 min at 4˚C. The pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 1 mM PMSF, 1 mM Na3VO4) and incubated for 10 min on ice. The cells were lysed by the addition of 0.5% Nonidet P-40, followed by vigorous vortexing for 20 s. The nuclei were pelleted at 600 g × 20 s. The nuclei were pelleted at 600 g, and the supernatant was treated as cytoplasmic extract. Pelleted nuclei were resuspended in hypertonic nuclear extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, and 1 mM Na3VO4), and kept on ice for 15 min. The nuclear extracts were then collected after centrifuging at 12,500 rpm for 15 min at 4˚C. USF-2 and α-tubulin have been used as the nuclear and cytoplasmic marker, respectively, to cancel out cross-contamination of nuclear extract with cytoplasmic extract or vice versa. Anti-USF-2 (N-18; catalog no. SC-861) and anti-α-tubulin (catalog no. SC-5286) were purchased from Santa Cruz Biotechnology.

SDS-PAGE and Western blot
Cell lysate was made using ice-cold lysis buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1% Triton X-100, 1 mM EDTA, 20 mM PMSF). Concentration of total protein was measured by Bradford reagent (BioRad). Equal amounts of protein from total cell lysates or nuclear/cytoplasmic extracts before transfection in six-well tissue culture plates (approximately 106 cells/well) of a 96-well tissue culture plate. Wnt5a siRNA or control siRNA–transfected cells were grown for 65 h before the MTT assay. Twenty microliters of MTT solution in PBS (5 mg/ml) was added to each culture well, and plates were incubated at 37˚C for 3.5 h. After removal of the medium, the formazan precipitate was dissolved in 150 μl DMSO and agitated for 15 min, and absorbance was measured at 550 nm. For PI exclusion assay, 10 μl PI solution (1 mg/ml) was added to transfected cells for 10 min just before analysis by flow cytometry.

Table I. Primer pairs used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’–3’)</th>
<th>Reverse (5’–3’)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Wnt5a</td>
<td>CAGGTGACAGCCGCGTCTCAC</td>
<td>CTACCTGGCACAAAATGTTCCAC</td>
<td>287</td>
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<tr>
<td>Human Fzd5</td>
<td>GGCAGATGCCTCGGCTGAC</td>
<td>ATCATTGGCACAGGACTCCG</td>
<td>450</td>
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<tr>
<td>Mouse Wnt5a</td>
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<td>ACAATCCCTGGCTACTTCTCC</td>
<td>265</td>
</tr>
<tr>
<td>Mouse Fzd5</td>
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<td>CGGAGAGACCTCTGCTG</td>
<td>250</td>
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<tr>
<td>Mouse CD14</td>
<td>TGCCAGCGTCTCGGCTGAC</td>
<td>CCTGGAGACGACTCTTGG</td>
<td>281</td>
</tr>
<tr>
<td>Mouse MCL1</td>
<td>AGGCAGCTGCTCGGAAATC</td>
<td>GCAGAGACGCATCTTCTGAT</td>
<td>315</td>
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<tr>
<td>Mouse Bcl-xl</td>
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<td>AGGATGCGCTCGGACGACCC</td>
<td>385</td>
</tr>
<tr>
<td>Mouse BCL-2</td>
<td>CCACCTGGCTCTTCAGCTC</td>
<td>TCAACAGACGATCCACTCACC</td>
<td>337</td>
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<tr>
<td>Mouse Bax</td>
<td>TCCGGCAATGGATGAGTGAC</td>
<td>TCCAGAAGTGGAGAGGAGGC</td>
<td>378</td>
</tr>
<tr>
<td>Mouse IFN-β</td>
<td>CGAGAGGACAGGACAGGAC</td>
<td>TGAGAGGTTCGGAGGAC</td>
<td>319</td>
</tr>
<tr>
<td>Human/Mouse GAPDH</td>
<td>ACCACAAGCTCCGAGCTCAC</td>
<td>TCCACCACTGGCTCTGTA</td>
<td>450</td>
</tr>
<tr>
<td>CHPV Replication</td>
<td>TGAGAAAGTGGAGGAGATTCGA</td>
<td>TCCAGAAGTGGAGAGGAGGC</td>
<td>150</td>
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</tbody>
</table>
Confocal microscopy
PMØ from C57BL/6 mice were grown on coverslips at 37°C in 5% CO2. Cells were washed several times with PBS and fixed with methanol for 10 min at room temperature. Adhered macrophages were treated first with Ab against p65 overnight and subsequently with anti-rabbit IgG–Alexa Fluor 561 for 2 h. DAPI solution (1 μl) was added for nuclear staining, and photographs were taken using an Andor Revolution XD Spinning Disk Microscope with an Andor iXon 897 EMCCD camera at 600× magnification.

Estimation of E. coli uptake
The laboratory strain E. coli DH5α (multiplicity of infection [MOI] ~ 2) was added to RAW or BMDM ~60 h after transfection. Two hours after infection, extracellular bacteria were extensively washed with ice cold PBS, and infected cells were subsequently lysed by adding distilled water. The diluted aliquots were then spread on LB agar plates, and CFUs were counted after incubating the plates overnight at 37°C.

IWP-2 liposome formulation
Preparation of liposome with or without incorporation of IWP-2 (Santa Cruz Biotechnology) has been described in detail previously (9). To prepare liposome-IWP2, a mixture of L-α-phosphatidylcholine, octadecylamine, and IWP-2 in a 20:2:0.1 ratio (100 μM IWP-2) was dissolved in 1 ml chloroform, and the solvent was evaporated under low pressure in a rotary evaporator. After overnight desiccation, the thin, dry film was dispersed in 1 ml cold PBS and sonicated twice for 30 s in an ultrasonic disruptor. The suspension was incubated in ice for 2 h and ultracentrifuged (100,000 × g, 30 min, and 4°C) twice to remove excess free drug from liposome entrapped IWP2. Free liposome was prepared similarly without adding IWP-2.

Inhibitors
IKK inhibitor (TPCA-1; catalog no. 401481) and Rac1 inhibitor (NSC23766; catalog no. 553502) were purchased from Calbiochem.

Rac1 activity assay
Rac1 activity in RAW cells was measured following the manufacturer’s protocol (Pierce - Thermo Scientific). Sixty hours after transfection, RAW cells were washed with ice cold PBS several times before lysis with lysis buffer; 500 μg of the lysate was mixed with 0.1 mM GTP-γS and incubated at room temperature for 30 min prior to the addition to glutathione beads prebound to 20 μg of the PK-βBD GST fusion protein. After incubation for 1 h at 4°C in rotation condition, the sample mix was washed four times. Finally, the pelleted beads were resuspended in 50 μl Laemmli sample buffer and subjected to SDS-PAGE. GTP-γS bound (activated) Rac1 was detected by immunoblotting using anti-Rac1antibody provided in the kit.

Chandipura virus infection and plaque assay
Chandipura virus (CHPV), a pathogenic virus known to infect murine macrophages was propagated in Vero E6 cells following a published protocol (37). A confluent monolayer of RAW cells or BMDM (~105 cells) was infected with CHPV at MOI of 0.05 or 0.5. After adsorption for 1 h at 37°C, the cells were washed extensively with PBS and incubated with RPMI 1640 culture medium for 6 or 14 h at 37°C and 5% CO2. RNA was isolated from the infected cells for subsequent analysis. Plaque assay was performed using culture supernatant from RAW cells infected for 14 h following published protocol (38) with slight modifications. A confluent monolayer of Vero E6 cells was infected with culture supernatant (1:10) from RAW for 1 h. Next, Vero cell monolayers were washed extensively with PBS approximately three times, after which 2 ml DMEM–low-melting agarose (Sigma-Aldrich) mixture (1 ml 2× DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine plus 1 ml 2.5% low-melting agarose) was layered carefully on the cell monolayers. After solidification, incubation was completed at 37°C and 5% CO2 in a tissue culture incubator for 24–48 h until plaques became visible. Crystal violet solution (0.4% crystal violet, 40% methanol) was used to stain the plate. The number of visible plaques was counted.

LPS stimulation assay
Sixty hours after transfection, RAW cells were treated with E.coli O55:B5 LPS (1 ng/ml) from Sigma-Aldrich for 2 h and culture supernatants were collected for estimation of TNF-α and IL-6 by ELISA and total RNA was prepared to estimate IFN-β mRNA level.

Statistical analysis
Results were analyzed with unpaired Student t test using GraphPad Prism 6 software. Bar graphs are expressed as mean ± SEM; p ≤ 0.05 was considered statistically significant. Significance was annotated as follows: *p ≤ 0.05, **p < 0.005, ***p ≤ 0.0005. Densitometry analysis was performed by Image J software (National Institutes of Health).

Results
Basal/homeostatic Wnt5a–Fz5 signaling sustains a threshold level of NF-κB activity and IFN production in macrophages
Because NF-κB is at the center of immune regulation and is constitutively present in the nuclei of resting monocytic cells (6, 7, 32), investigating the existence of a basally active pool of NF-κB in macrophages was deemed essential for a basic understanding of the mechanism of immune homeostasis at the cellular level. Accordingly, we confirmed the presence of a nuclear pool of NF-κB (p65) in BMDM and mouse PMØ in the absence of activating stimuli (Fig. 1A). All BMDM used for experiments expressed the macrophage marker F4/80 (Supplemental Fig. 1A), thus validating macrophage differentiation from bone marrow progenitor cells. Furthermore, the generation of BMDM was accompanied by increase in Wnt5a expression, suggesting that augmented expression of Wnt5a is a characteristic feature of macrophage differentiation (Supplemental Fig. 1B). Expression of Wnt5a as well as Fz5 (putative receptor for Wnt5a) was also present in mouse peritoneal macrophages, human macrophages, and the RAW264.7 macrophage cell line (Supplemental Fig. 1C, 1D). Given that Wnt5a plays a role in NF-κB responsive cytokine gene regulation (29), we then examined the role of basal Wnt5a signaling in nuclear NF-κB homeostasis. Thus, Wnt5a or Fz5 expression was separately knocked down in RAW 264.7, mouse BMDM, and peritoneal macrophages with the use of the appropriate siRNAs, and the effect of suppression of Wnt5a/Fz5 expression on the nuclear translocation of NF-κB subunits was evaluated. As documented in immunoblots of nuclear and cytoplasmic extracts, nuclear translocation of p65 was significantly inhibited in Wnt5a siRNA (W si) transfected RAW 264.7 and BMDM, when compared with the corresponding controls (C si) (Fig. 1B, 1C). Transfection of similar cultures of macrophages with Fz5 siRNA (F si) yielded analogous results (Supplemental Fig. 2A). Suppression of Wnt5a expression in mouse peritoneal macrophages also resulted in diminution of nuclear p65 (Fig. 1D). Interestingly, there was no significant change in the nuclear level of c-Rel or p50 in Wnt5a knocked down cells. Inhibited nuclear translocation of p65 in Wnt5a siRNA transfected cells correlated with more than 2-fold enhancement in the levels of IkB-α and IkB-β and significantly diminished activity of IkB kinase β (IKK2) compared with the corresponding controls (Fig. 1E, 1F), validating that a basal Wnt5a-mediated signal transduction contributes to maintenance of a steady NF-κB activity through activation of IKK β.

Interestingly, reduced NF-κB (p65) nuclear translocation in Wnt5a knocked down BMDM correlated with more than 50% reduced expression and secretion of IFN-β (Fig. 1G, 1H), a known modulator of innate immune responses, implying that basal p65 activity regulates the synthesis of IFN-β as a way of keeping macrophages prepared for combating invading pathogens. This concept is in agreement with the documented induction of IFN-β by p65 as a transcriptional coregulator with IRF-3 (39, 40). A similar correlation was found even with respect to the secretion of IFN-γ, another important player in innate immune signaling (Fig. 1I), corroborating the occurrence of an autocrine–paracrine mode of homeostatic Wnt5a signaling. Furthermore, administration of an IKK2 inhibitor, which blocks the nuclear localization of NF-κB (p65)
suppressed the basal expression of Wnt5a in macrophages (Supplemental Fig. 2B), suggesting that Wnt5a–Fz5 mediated basal activation of NF-κB sustains Wnt5a expression in macrophages in a feed-forward mode. This suggestion is supported by the presence of NF-κB binding sites in the Wnt5a promoter sequence (43). Thus, a self-sustaining Wnt5a–p65 immune homeostasis is maintained.

Basal NF-κB activity along with IFN production is inhibited by the inhibitor of Wnt production, IWP-2

Previous studies have documented the inhibitory effect of IWP-2 on Wnt secretion (9, 44). It has furthermore been demonstrated that IWP-2 has a differential effect on Wnts, Wnt5a secretion being influenced much more than that of Wnt3a at low doses of ∼0.05 μM (9, 45). Similar results were obtained using mouse macrophages (Supplemental Fig. 3). Accordingly, the effect of IWP-2 administration on a basal level of Wnt5a-mediated NF-κB activity was evaluated. As demonstrated in Fig. 2A, treatment of mouse BMDM with IWP-2 (0.05 μM) resulted in ∼50% reduction in Wnt5a expression, corroborating published results (9). Reduced secretion of Wnt5a correlated with more than 50% inhibition in the nuclear translocation of p65 (Fig. 2B), as observed previously (Fig. 1). Concurrently, as revealed by kinase activity assay (Fig. 2C), IKK2/β activity of the IWP-2 treated cells was also significantly compromised. Similar reduction in nuclear localization of p65 upon IWP-2 administration was also observed in RAW264.7 cells (Fig. 2D). Moreover, IWP-2–mediated reduction in the constitutive nuclear p65 level was validated by ∼40% reduced luciferase activity in IWP-2–treated RAW cells transfected with a p65-luciferase reporter plasmid (Fig. 2E). In addition, i.p. administration of liposome-encapsulated IWP-2 into C57BL/6 mice suppressed the basal expression of Wnt5a in macrophages (Supplemental Fig. 2B), suggesting that Wnt5a–Fz5 mediated basal activation of NF-κB sustains Wnt5a expression in macrophages in a feed-forward mode. This suggestion is supported by the presence of NF-κB binding sites in the Wnt5a promoter sequence (43). Thus, a self-sustaining Wnt5a–p65 immune homeostasis is maintained.
Expression of the innate immune response mediator CD14 is sustained by a homeostatic Wnt5a–Fz5–NF-κB (p65) axis

CD14, a macrophage differentiation Ag, is an important innate immune response mediator required for the recognition of microbial/viral Ags and TLR-mediated signaling as a means of restraining infection (46–51). Bioinformatic analysis of possible kB sites within 10 kb upstream and 1 kb downstream of the transcriptional start site of the CD14 gene resulted in the identification of five putative NF-κB binding sites (Fig. 3A). Accordingly, using anti-p65 Ab, we confirmed p65 binding to a specific site (#5: Fig. 3A) of the CD14 enhancer/promoter by CHIP-PCR assay in unstimulated RAW264.7 macrophages. To ascertain that the identified region is indeed transcriptionally active, similar CHIP analysis was also performed with Ab against RNA polymerase II using primer pairs spanning the TSS on the CD14 promoter (Fig. 3B). We further established that in Wnt5a knocked down cells, there is at least a 50% reduced level of p65 recruitment to the designated kB site (Fig. 3C). Consequently, we evaluated the influence of basal Wnt5a–Fz5–NF-κB (p65) signaling on CD14 expression in macrophages. Knocking down expression of endogenous Wnt5a and Fz5 in RAW264.7 and BMDM by siRNA transfection (Wnt5a, Wsi; Fz5, Fsi) resulted in greater than 50% inhibition of CD14 expression at mRNA and protein levels (Fig. 3D–3F). Similar results were obtained when mouse peritoneal macrophages were transfected with Wnt5a siRNA (Fig. 3G). Moreover, pretreatment of RAW 264.7 cells, BMDM, and peritoneal macrophages with IWP-2, which is documented to reduce Wnt5a secretion (Fig. 2A), yielded analogous results (Fig. 3H, 3I). It is to be noted that Wnt5a siRNA transfection did not significantly affect the expression of other macrophage markers, such as F4/80 and CD11b, that are not NF-κB responsive (Supplemental Fig. 4). The necessity of NF-κB activity for a basal level of CD14 expression was furthermore validated by a stepwise decline in CD14 protein level in RAW 264.7 macrophages exposed to different concentrations of an inhibitor shown to be specific for IKKβ/IKK2 activity; thus, NF-κB mediated transcriptional activation (52, 53) (Fig. 3J). Taken together, these findings suggest that a Wnt5a–Fz5–NF-κB homeostatic circuit programs macrophages significantly for sustained CD14 expression. Further validation of this concept comes from the fact that blockade of NF-κB (p65) expression by p65 siRNA in BMDM blocks CD14 expression (Fig 3K).

Wnt5a–NF-κB–CD14 axis in macrophages is Rac1 dependent

Because Wnt5a stimulation correlates with Rac1 activity (9, 23, 24, 27), we investigated whether the homeostatic Wnt5a arbitrated basal NF-κB activity in macrophages with concurrent CD14 expression is Rac1 dependent. Initially, we confirmed that Wnt5a-depleted macrophages harbor reduced levels of basally activated Rac1 by demonstrating an ∼50% reduced level of GTP-γS bound Rac1 in the Wnt5a siRNA-transfected RAW264.7 macrophages compared with the corresponding controls (Fig. 4A). Subsequently, as depicted in Fig. 4B, we found analogous reduction in CD14 expression upon exposing RAW cells to a Rac1 inhibitor at a dose of ∼10 μM (54, 55). Rac1 inhibition also correlated with a similar reduction in IKK2 activity and nuclear localization of p65 (Fig. 4C, 4D), as observed previously in Wnt5a siRNA-transfected macrophages.

Wnt5a–Rac1–NF-κB–mediated CD14 expression correlates with sustained innate immune response of macrophages to E. coli/LPS and virus

While being operational as a coreceptor for LPS, CD14 is also involved in bacterial and viral recognition and internalization, thereby triggering TLR-dependent cytokine and type I IFN responses.

caused at least 40% reduction in basal IFN-γ compared with the corresponding vehicle control (Fig. 2F), suggesting that reduction in basal NF-κB (p65) activity in vivo correlates with reduced secretion of NF-κB (p65) responsive innate immune defense mediators. Blockade in IFN-γ secretion by peritoneal macrophages upon administration of IWP-2 was also confirmed in vitro (Fig. 2G).

FIGURE 2. IWP-2 inhibits basal NF-κB activity in macrophages. (A) IWP-2–mediated inhibition of Wnt5a secretion (0.5 μM IWP-2 treatment for 48 h) from BMDM as quantified by ELISA. (B) Immunoblot analysis demonstrating a significantly lower level of nuclear p65 in IWP-2–treated BMDM than its control (DMSO-treated BMDM). (C) IWP-2–treated BMDM exhibit compromised IKK-β activity as estimated by in vitro kinase assay. The bar graph represents the relative average intensity of pGST-IκBα in three independent experiments. Equal pull-down of IKK-α/IKK-β complex is depicted by immunoblot with antiserum against IKK-2/IKK-β. (D) Bar graph depicts IWP-2–mediated reduction in luciferase activity in RAW 264.7 cells cotransfected with NF-κB luciferase plasmid and Renilla luciferase plasmid. Relative luciferase activity from three independent experiments is calculated by dividing NF-κB luciferase activity units by Renilla luciferase activity units. (E) ELISA demonstrating that in vivo (i.p. injection) administration of liposome encapsulated IWP-2 (LI) into C57BL/6 mice for 2 consecutive days causes ~40% reduction in the secretion of IFN-γ (F) as compared with free liposome (L) administration. n = 4. (G) ELISA from three independent experiments demonstrating significantly lower secretion of IFN-γ from IWP-2–treated PMØ (in vitro) than its control. *p ≤ 0.05, **p ≤ 0.005.
Moreover, CD14 plays a role in the containment of bacterial and viral infection in macrophages (46–51, 56, 57). Thus, we evaluated how Wnt5a–CD14 homeostasis influences immune responses to E. coli/LPS and virus in macrophages. Accordingly, we demonstrated ∼3-fold reduced internalization of E. coli (MOI ∼ 2) in Wnt5a siRNA transfected RAW264.7 cells and BMDM that expressed significantly lower level of CD14 when compared with the appropriate controls (Fig. 5A–5C). Reduced internalization of E. coli (DH5α) correlated with reduction in IL-6 and TNF-α secretion (Fig. 5D, 5E). IL-6 and TNF-α correlated with reduction in IL-6 and IFN-α secretion levels in RAW264.7 cells, which were also considerably compromised in Wnt5a knocked down RAW264.7 cells, compared with the corresponding controls (Fig. 5F–5H). In addition, upon infection with a pathogenic negative-stranded RNA virus of the rhabdovirus (vesicular stomatitis virus) family known to infect murine macrophages, namely CHPV (37, 58, 59) at MOI of ∼0.05 (Fig. 6A, 6B) or 0.5 (Fig. 6C), Wnt5a-depleted BMDM and RAW 264.7 macrophages displayed at least 50% less IFN-β response at both mRNA and protein level compared with the appropriate control, as judged by RT-PCR analysis and ELISA (Fig. 6D–6E). A significant increase in viral replication correlated with IFN-β reduction in Wnt5a-deficient cells, as demonstrated by the level of CHPV RNA (Fig. 6A–6C, lower panels). Wnt5a-deficient RAW cells also released significantly higher numbers of viral particles, of ∼1 log difference compared with controls at MOI of 0.5, as demonstrated by plaque assays (Fig. 6F), suggesting that Wnt5a/CD14-deficient macrophages are less potent than normal macrophages in restraining CHPV infection. Because basal Wnt5a signaling could have a significant effect on molecules other than CD14, it perhaps strengthens innate immune responses to bacteria or viruses also independently of CD14.

FIGURE 3. Wnt5a–NF-κB homeostatic circuit maintains CD14 expression in macrophages. (A) Schematic representation of mouse CD14 promoter/enhancer region (GenBank: AC027740.11) with putative NF-κB binding sites. Putative κB sites are in bold letters with their positions designated by #1–#5. The diagram depicts the putative κB sites represented by white boxes, with arrowheads indicating the position of encompassing primers. N.C (black box, negative control) represents a region on the promoter/enhancer that does not contain any putative κB site, with the appropriate primer pair encompassing the region. (B) CHIP-PCR assay demonstrating that p65 localizes to the CD14 promoter/enhancer (upper panel) and RNA polymerase II is present at the transcriptional start site (TSS) (lower panel), thus ensuring transcriptional activity at the identified region. (C) Wnt5a knocked down RAW cells have ∼50% reduced recruitment of p65 compared with control to a specific κB (# 5) site on the CD14 promoter/enhancer. The graph below represents the mean relative intensity of bands (W si versus C si) ± SEM (within the box), from two independent experiments. (D–G) Significant reduction in CD14 expression at mRNA and protein level in Wnt5a (W si) or Fz5 (F si) knocked down BMDM, RAW264.7, and PMØ, as compared with the corresponding controls. (H) IWP-2–mediated decrease (at least 50%) in CD14 expression in RAW264.7, BMDM, and PMØ by immunoblot assay. (I) ELISA depicting IWP-2–mediated reduction in Wnt5a secretion from PMØ from three independent experiments. (J) Immunoblot demonstrating reduction of CD14 expression in RAW 264.7 exposed to IKK inhibitor (5 and 10 μM) for 4 h from three independent experiments. (K) Representative immunoblot demonstrating that knockdown of p65 (p65 si) in BMDM reduces CD14 expression relative to its corresponding control (C si). Bar graphs demonstrate mean relative intensity ± SEM of p65 and CD14 bands from two independent experiments. Relative intensity was estimated by dividing band intensity value of p65 or CD14 with that of β-actin. ***p ≤ 0.0005.


**Wnt5a–NF-κB homeostasis supports macrophage survival**

Because macrophage survival is crucial for the sustenance of immune responses we assessed the effect of basal Wnt5a–NF-κB signaling on macrophage survival. Thus, the transcriptional profile of the prosurvival NF-κB (p65) responsive genes Bcl2, Bcl-xl, and Mcl1 was evaluated in BMDM upon curbing Wnt5a expression by Wnt5a–siRNA transfection. As demonstrated in Fig. 7A, Bcl2, Bcl-xl, and Mcl1 mRNA levels were significantly reduced in Wnt5a siRNA transfected cells (W si) as compared with the corresponding controls (C si). A simultaneous increase in the expression level of the proapoptotic Bax gene was also reflected. In corroboration with the mRNA results, the Wnt5a knocked down cells displayed diminished Bcl2 protein level as compared with the corresponding control and a simultaneous increase in the level of Bax protein (Fig. 7B). Decreased survival was also manifested in the heightened level of PI stain incorporated by the Wnt5a-deficient BMDM (Fig. 7C). Results supporting a role of Wnt5a in cell survival were separately obtained by MTT assay on both mouse BMDM and peritoneal macrophages after knocking down Wnt5a expression (Fig. 7D, 7E). A plausible mechanism of a homeostatic Wnt5a–NF-κB (p65)–mediated macrophage survival and immune response is depicted in Fig. 8.

**Discussion**

The immune surveillance program requires macrophages to survive and remain prepared for preventing infections from invading pathogens (1). Although it is well documented that NF-κB is at the crux of the innate immune regulatory network, it is not clearly understood how sustenance of innate immune functions in mac-
rophages involves NF-κB (6, 7). In this study, we demonstrate that sustained expression of immune defense modulating IFNs and CD14 is supported at least partly by a homeostatic Wnt5a–Fz5–Rac1 signaling scheme via a basal pool of active NF-κB (p65) in the nucleus. Thus, recognition and internalization of bacteria, microbial Ags such as LPS, and viral nucleic acids by CD14 along with subsequent immune response through TLR signaling is at least partly an outcome of a homeostatic Wnt5a–Rac1–NF-κB circuitry. Moreover, transcriptional activation of NF-κB–responsive prosurvival genes such as Bcl2 in response to the homeostatic Wnt5a–NF-κB circuit promotes macrophage survival thus sustaining innate immune functions. The basal nuclear NF-κB (p65) pool in turn supports expression of Wnt5a, thus maintaining a self-sustaining Wnt5a–NF-κB homeostatic circuit. Overall, the depiction of a context-dependent IFN and cytokine response in macrophages as a consequence of a basal Wnt5a–Rac1–NF-κB axis unmasks unknown details of innate immune regulation and adds crucial parameters to our understanding of the immune system (Fig. 8). It is important to confirm whether conditional deletion of Wnt5a in macrophages in vivo has deleterious effects on innate immune responses.

The innate immune network is a tightly regulated scheme, which if tilted off balance can lead to pathologic outcomes. A key player in this balancing act is CD14. CD14 is crucial for establishing a robust immune response to pathogens, but uncontrolled stimulation of CD14 in an excessively pathogenic milieu can lead to severe complications (60). The beneficial effect of CD14 also depends largely on the types of microflora and Ags that it encounters. Whereas CD14 is required for immune defense against infections from Salmonella and Listeria monocytogenes, it also has been found to enhance mortality in an endotoxin-induced (i.e., LPS) mouse model of sepsis (49–51, 60, 61). The influence of Wnt5a in immune response could follow a similar pattern. The occurrence of a homeostatic Wnt5a–Rac1–NF-κB circuit for induction of innate immune responses that might curtail harmful viral and bacterial infections is of substantial importance. However, whereas a basal Wnt5a–NF-κB signaling in macrophages supports microbe and viral

**FIGURE 6.** Wnt5a–CD14–mediated response to CHPV. (A–C) RT-PCR analysis depicting significant reduction in IFN-β expression and elevated level of viral (CHPV-genome) replication in W si compared with C si–transfected BMDM (A) and RAW264.7 (B) upon infection with CHPV at an MOI of 0.05. Infection of RAW (C) with a relatively higher MOI of 0.5 showed the same result (n = 3). RNA from the infected cells was isolated after indicated time period, and cDNA was generated using either random hexamer (for replication studies) or oligo deoxythymidine (for IFN-β). Graphs at the bottom represent relative increase in viral replication in W si–transfected RAW or BMDM compared with their corresponding control. (D and E) ELISA using duplicates from three independent experiments depicting reduced secretion of IFN-β from RAW (D) or BMDM (E), first transfected with W si or C si and subsequently infected with CHPV (MOI = 0.5), 16 h after infection. (F) Representative picture and graph from duplicates of three independent experiments showing a greater number of plaques produced from culture supernatant of W si–transfected RAW (MOI = 0.5) than in the corresponding controls (C si) upon infection of Vero E6 cells. *p ≤ 0.05. P.I, postinfection.
recognition and IFN response and leads to other protective immune functions including cytokine secretion, as demonstrated in this study, Wnt5a signaling in excess can exacerbate several aspects of host-pathogen interactions (e.g., phagocytosis), thus prolonging inflammation (9). Furthermore, because Wnt5a signaling could influence gene regulation at different levels, its influence on immune response may be much beyond that of CD14. It is unclear at this stage whether there is any interplay between Wnt5a–NF-κB homeostasis and the nuclear liver X receptor pathway that also contributes to innate immunity (62).

Although an association between Wnt5a-stimulated signaling and NF-κB activity has been reported (11, 31), existence of a Wnt5a–NF-κB homeostatic circuitry and its significance in the establishment of innate immune response are not documented. Low levels of nuclear NF-κB have been observed in cell lines of the monocyte lineage (32). Maintenance of homeostatic conditions in embryonic fibroblasts by nuclear p65 has also been discussed (8, 33, 63, 64). However, it remains unclear how a pool of nuclear p65 is maintained in unstimulated or resting cells and what functions it serves in different cell types. To our knowledge, our studies suggest for the first time that the Wnt5a–Fz5–Rac1 signaling pathway maintains a self-sustaining circuitry for nuclear p65 homeostasis in macrophages to continue innate immune responses efficiently. Interestingly, whereas depletion of endogenous Wnt5a and Fz5 in RAW264.7, BMDM, and peritoneal macrophages reduces the basal nuclear translocation of p65 (NF-κB), nuclear levels of c-Rel or p50 do not change significantly (Fig. 1). Perhaps a basal Wnt5a–Fz5–Rac1 signaling maintains the phosphorylation and subsequent degradation of p65-bound IκBα and IκBβ to a certain level, thus preventing the sequestration of a pool of p65 homodimers in the cytoplasm of macrophages. Accordingly, a steady pool of nuclear p65 prevails and is responsible for sustaining CD14 expression and a context-dependent cytokine and IFN response.

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