Wnt6 Is Expressed in Granulomatous Lesions of Mycobacterium tuberculosis–Infected Mice and Is Involved in Macrophage Differentiation and Proliferation

Kolja Schaale, Julius Brandenburg, Andreas Kispert, Michael Leitges, Stefan Ehlers and Norbert Reiling

J Immunol 2013; 191:5182-5195; Prepublished online 11 October 2013;
doi: 10.4049/jimmunol.1201819
http://www.jimmunol.org/content/191/10/5182

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/10/11/jimmunol.1201819.DC1

Why The JI?
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References
This article cites 60 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/191/10/5182.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2013 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Wnt6 Is Expressed in Granulomatous Lesions of Mycobacterium tuberculosis–Infected Mice and Is Involved in Macrophage Differentiation and Proliferation

Kolja Schaale,*1,2 Julius Brandenburg,*1 Andreas Kispert,† Michael Leitges,‡ Stefan Ehlers,§ and Norbert Reiling*

The Wnt signaling network, an ancient signaling system governing ontogeny and homeostatic processes, has recently been identified to exert immunoregulatory functions in a variety of inflammatory and infectious disease settings including tuberculosis. In this study, we show that Wnt6 is expressed in granulomatous lesions in the lung of Mycobacterium tuberculosis–infected mice. We identified foamy macrophage-like cells as the primary source of Wnt6 in the infected lung and uncovered a TLR–MyD88–NF-kB–dependent mode of induction in bone marrow–derived macrophages. Analysis of Wnt6–induced signal transduction revealed a pertussis toxin–sensitive, ERK–mediated, but β-catenin–independent induction of c–Myc, a master regulator of cell proliferation. Increased Ki-67 mRNA expression levels and enhanced thymidine incorporation in Wnt6–treated macrophage cultures demonstrate a proliferation–promoting effect on murine macrophages. Further functional studies in M. tuberculosis–infected macrophages using Wnt6 conditioned medium and Wnt6–deficient macrophages uncovered a Wnt6–dependent induction of macrophage Arginase-1 and downregulation of TNF-α. This identifies Wnt6 as a novel factor driving macrophage polarization toward an M2-like phenotype. Taken together, these findings point to an unexpected role for Wnt6 in macrophage differentiation in the M. tuberculosis–infected lung. The Journal of Immunology, 2013, 191: 5182–5195.

Macrophages are the primary host cells for Mycobacterium tuberculosis. The pathogenesis of tuberculosis is characterized by the formation of granulomatous lesions in the lung, which temporally and spatially coincides with immune–mediated containment of the infection. The granuloma can proceed either to localized sterilization of the infection and mineralization of the lesion or to localized caseation and necrosis that culminates in the release of infectious bacteria into the airways (recently reviewed in Ref. 1). Aerosol infection models in mice have been used successfully to identify key mechanisms governing granuloma formation and integrity, as well as bacterial replication (reviewed in Ref. 2). The immune response to mycobacterial infections has been studied in great detail; however, still little is known about how pathogenic mycobacteria manipulate their host cell to achieve persistence.

One successful approach to identify novel factors that influence antimicrobial effector mechanisms has been the use of systematic gene expression profiling of macrophages infected with mycobacteria or treated with conserved bacterial structures (3). We and others have recently identified a regulatory role for components of the Wnt signaling network to be operative at the interface between innate and adaptive immunity. In essence, Wnts can exert both proinflammatory and anti–inflammatory functions on macrophages and other cells of the immune system (reviewed in Refs. 4, 5). Wnt signaling is well–known as an ancient and highly conserved principle among metazoan species, which executes essential functions in embryogenesis and tissue homeostasis (reviewed in Refs. 6, 7). In the adult, dysregulation of Wnt signaling is often associated with cancer and degenerative diseases. In mice and men, 19 ligands, secreted palmitoylated glycoproteins of the Wnt family, 10 seven–transmembrane receptors of the Frizzled (Fzd) family, as well as several coreceptors or alternative receptors are known. Wnt proteins can fuel at least three distinct signal transduction pathways (Wnt/β–catenin, Wnt/planar cell polarity and Wnt/Ca2+ signaling; reviewed in Ref. 6).

Wnt5a was the first member of the Wnt family that was described to be induced in multiple inflammatory disease settings such as rheumatoid arthritis (8), tuberculosis (9), sepsis (10), psoriasis (11), atherosclerosis (12), and very recently also in obesity (13) and asthma (14). Our group described Wnt5a and its receptor Fzd5 to be induced in human macrophages challenged with M. tuberculosis or conserved bacterial structures (9). Furthermore, we could assign a proinflammatory function to Wnt5a, a finding later also reported by other groups (10, 15); Wnt5a exerts proinflammatory functions on macrophages (4, 9, 10), endothelial cells (15) and adipocytes (13).
Unlike Wnt3a, Wnt3b has been referred to as a "classical" or "canonical" member of the Wnt family characterized by the induction of the Wnt/β-catenin signaling pathway. In an inflammatory context, activation of the Wnt/β-catenin pathway has been associated with a reduced migration of monocytes (16) and lately with the induction of tolerance in dendritic cells (17). A key player in Wnt/β-catenin signaling is the glycosyl synthase kinase β3 (GSK3β), which is also a well-known regulator of NF-κB-dependent gene transcription. GSK3β inhibition, as it happens during activation of Wnt/β-catenin signaling, was shown to suppress proinflammatory cytokine expression in different experimental settings (18, 19). This holds true also in mycobacterial infections, as we recently demonstrated the suppression of TNF-α inhibition in mycobacteria-infected macrophages by Wnt3a or inhibition of GSK3β, respectively (4, 20).

We have now systematically screened the expression of the 19 Wnt homologs in mice experimentally infected with M. tuberculosis via the aerosol route. In this study, we identified Wnt6 to be induced in the lung of M. tuberculosis-infected mice and localized Wnt6 expression exclusively to macrophage clusters within granulomatous lesions. In vitro experiments using bone marrow–derived macrophages (BMDMs) uncovered a TLR-, MyD88-, and NF-κB-dependent, but TNF-α-independent, induction of Wnt6 gene expression. Studies on Wnt6 function identified a new role for this Wnt homolog in murine macrophage differentiation and proliferation, suggesting a distinct immunomodulatory potential.

Materials and Methods

Mice and macrophages

C57BL/6, TLR2−/−, MyD88−/−, NLR−/−, NMRI Wnt6−/−, Wnt6+/+ and Wnt6−/− (21) mice were raised and maintained under specific pathogen-free conditions. Murine BMDMs were generated as previously described (22). Cells were allowed to attach overnight before proceeding further.

Stimuli and inhibitors

M. tuberculosis (strain H37Rv, ATCC 27294; American Type Culture Collection, Manassas, VA), GFP-expressing M. tuberculosis H37Rv, and M. avium (strain SEO1) were grown as previously described (25, 26). GFP-expressing M. tuberculosis was generated using the plasmid 32362; pM9N37 (Addgene, Cambridge, MA), kindly provided by Prof. M. Niederweis (University of Birmingham, Birmingham, AL) (27). In vitro experiments, bacterial aliquots were centrifuged for 10 min at 835 × g and resuspended in the appropriate cell culture medium using a syringe and a 26-gauge syringe needle. LPS (Salmonella enterica serotype Friedenau F090) was kindly provided by Prof. H. Brade (Research Center Borstel, Borstel, Germany). The synthetic lipopeptide Pam3CSK4 was kindly provided with a combined permeabilization step with 0.1% saponin hematosylin and analyzed with a BX41 microscope (Olympus, Hamburg, Germany) and the NIH-Elements software (Nikon, Badhoevedorp, The Netherlands). Frozen tissue sections (5 μm) were deparaffinized, blocked with PBS containing 5% (v/v) FCS, and stained for Wnt6. Biotin-SNP-conjugated Fab(ab)2, Fragment Rabbit Anti-Sheep and Peroxidase-conjugated Streptavidin (Jackson Immunoresearch) were used for detection. To visualize M. tuberculosis and Wnt6 in the same lung section, we stained deparaffinized tissue sections for acid-fast bacteria using a Ziehl-Neelsen (ZN) carbol-fuchsin solution (Merck, Darmstadt, Germany) and stained for Wnt6 as described earlier. Slides were counterstained with Giils hematosylin and analyzed with a BX41 microscope (Olympus, Hamburg, Germany) and the NIS-Elements software (Nikon, Badhoevedorp, The Netherlands). Frozen tissue sections (5 μm) were air-dried, fixed (acetone/chloroform or 10% [v/v] ice-cold formalin), and blocked with 5% FCS or 10% normal donkey serum (Pan-Biotek, Aidenbach, Germany) with a combined permeabilization step with 0.1% Triton X-100. After staining for Wnt6, slides were stained with 4,4-diamidino-6,1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503; Molecular Probes, Eugene, OR) and stained for M. tuberculosis via the aerosol route was performed under BSL3 conditions as described previously (22). Mice were sacrificed at days 1, 21, and 42 postinfection (p.i.). Analyses were performed as previously described (20).

Real-time quantitative PCR

Total RNA was isolated from lung tissue (~20 mg homogenized in lysis buffer) or macrophages (BMDM: 0.4 × 106 to 1.5 × 106 cells; pMφ: 1 × 106 cells) using the High Pure RNA Isolation Kit, the High Pure RNA Tissue Kit (Roche Applied Science, Mannheim, Germany), or the Directzol RNA MiniPrep (Zymo Research, Irvine, CA). For reverse transcription, the Maxima First Strand cDNA Synthesis Kit for real-time quantitative PCR (RT-qPCR) (Fermentas, St-Leon-Rot, Germany) was used. Gene-specific primer pairs and TaqMan probes (Universal Probe Library, Roche Applied Science) were designed with the UPL assay design center (ProbeFinder Version 2.45 and earlier versions; sequences and probes are given in Supplemental Table 1). RT-qPCR was performed using the LightCycler 480 Probe Master Kit and the LightCycler 480 II system (Roche Applied Science) (20). Ct values of target and reference gene (hypoxanthine-guanine phosphoribosyltransferase) were determined by the second derivative maximum method. Relative gene expression was calculated using the 2−DDCT method.

SDS-PAGE and Western blot analysis

BMDMs (0.5 × 106 cells) were lysed in 125 μl 2× SDS sample buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 100 mM DTT) and Western blot were performed as previously described (25). Primary Abs were phospho-NF-κB p65 (Ser536), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204), 20G11, phospho-p38 MAPK (Thr180/Tyr182), phospho-protein kinase C (PKC) (pan; beta II Ser660), all Cell Signaling, Danvers, MA), purified anti–β-catenin (BD Bioscience, Heidelberg, Germany), GAPDH Ab (HyTest, Turku, Finland), or human Wnt-6 Affinity Purified Polyclonal Ab Sheep IgG (R&D Systems, Wiesbaden, Germany). Secondary Abs were AlexaFluor 680 Donkey Anti-Mouse IgG (Invitrogen, Darmstadt, Germany), IRDye800 Conjugated Affinity Purified Anti-Rabbit IgG (Rockland, Gilbertsville, PA), or peroxidase-conjugated Ab (F(ab)2) Fragment Donkey Anti-Sheep (Jackson Immunoresearch, Suffolk, U.K.). Western blots were analyzed using the Odyssey infrared imaging system (Ver. 2.1; Li-Cor Biotechnology, Lincoln, NE) or the Amer sham ECL Western blotting Detection Reagent, Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, U.K.) and the X-Omat M35 film processor (Kodak, Stuttgart, Germany).

Immunohistochemistry and immunofluorescence analysis

two μm filter and stored at –80°C. The synthetic lipopeptide Pam3CSK4 was kindly pro-vided with a combined permeabilization step with 0.1% saponin hematosylin and analyzed with a BX41 microscope (Olympus, Hamburg, Germany) and the NIS-Elements software (Nikon, Badhoevedorp, The Netherlands). Frozen tissue sections (5 μm) were air-dried, fixed (acetone/chloroform or 10% [v/v] ice-cold formalin), and blocked with 5% FCS or 10% normal donkey serum (Pan-Biotek, Aidenbach, Germany) with a combined permeabilization step with 0.1% Triton X-100. After staining for Wnt6, slides were stained with 4,4-diamidino-6,1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503; Life Technologies) to visualize lipid droplets (29) or with an anti-CD68 Ab (BA-11; Abcam, Cambridge, U.K.). Macrophages grown on glass coverslips were fixed (4% PFA), permeabilized using 90% methanol, and stained for Wnt6 (see earlier) or anti–Ki-67 (Clone M1B-5; DAKO, Glostrup, Denmark). The following secondary Abs were used: DyLight 488-conjugated goat anti-rat and Cy5-conjugated donkey anti-sheep (Diana nova, Hamburg, Germany) or Cy5-conjugated AffiniPure F(ab)2 Fragment Goat Anti-Mouse (Jackson Immunoresearch). Nuclei were stained with DAPI (Roche Applied Science). Slides were analyzed with an Axio Observer microscope with ApoTome and the AxioVision Software (Carl Zeiss AG, Oberkochen, Germany).

Aerosol infection

High- (1000 CFU) and low-dose (100 CFU) infection of C57BL/6 mice with M. tuberculosis H37Rv via the aerosol route was performed under BSL3 conditions as described previously (22). Mice were sacrificed at days 1, 21, and 42 postinfection (p.i.). Analyses were performed as previously described (20).

Downloaded from http://www.jimmunol.org/ by guest on November 13, 2017
Proliferation assays

Real-time proliferation assays (1.25 × 10⁴ BMDMs) were performed with the xCELLigence System (Roche Applied Science). Impedance measurement was carried out using plates with incorporated sensor array (E-Plate) and the Real-Time Cell Analyzer SP instrument. Data obtained were analyzed using the Real-Time Cell Analyzer Software 1.2 (Roche Applied Science). After 3 d, an additional 100 µl fresh medium (including stimuli) was added. For [³H]thymidine incorporation assays, 1.25 × 10⁴ BMDMs were pulsed on day 4 of the experiment for 12 h with tritiated thymidine ([³H]TdR; 2 Ci/mmol, 0.2 µCi/culture). Cells were detached and harvested on glass-filter mats. Differences in amounts of incorporated radioactive material were expressed as cpm/per culture.

Determination of TNF-α and nitrite release

Cell-culture supernatants were harvested at the times indicated and frozen at −20˚C until analysis. The concentrations of TNF-α were determined using quantitative ELISA (Mouse TNF-α DuoSet; R&D Systems) as recommended by the manufacturer. The formation of nitrite was performed using the Griess reaction as previously described (30).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). For real-time PCR data from in vivo experiments, four to five mice per time point were analyzed with a two-tailed Mann–Whitney U test not assuming parametric distribution (*p < 0.5, **p < 0.01). Real-time PCR data of three to five independent in vitro experiments were log-transformed according to Willems et al. (31), and statistical comparison was carried out using either Student two-tailed t test for two groups or one-way ANOVA for multiple-group comparison (*p < 0.5, **p < 0.01, ***p < 0.001). All data are shown as mean ± SEM.

Results

Expression of Wnt genes in the lung of M. tuberculosis–infected mice

C57BL/6 mice were infected with 1000 CFU M. tuberculosis H37Rv in an aerosol infection chamber. As previously described (22), a rapid rise of the bacterial burden was observed between days 1 and 21 p.i. followed by a plateau of M. tuberculosis replication until day 42 (data not shown). A systematic screen for the expression of all 19 Wnt genes was carried out using RT-qPCR. All 19 Wnt genes were detected at all time points analyzed. Statistically significant changes in expression levels are shown in Fig. 1 (uninfected mice [day 0] versus day 21 or 42, respectively). mRNA expression of Wnt2, Wnt3a, Wnt4, Wnt5a, Wnt7a, and Wnt10b was significantly reduced at days 21 and 42 p.i. (Fig. 1A). mRNA levels of Wnt8a and Wnt2b were reduced during the course of infection, but statistically significant differences were only obtained at day 21 or 42, respectively (Fig. 1A). For Wnt5b, Wnt9a, Wnt9b, Wnt11, and Wnt16, a tendency to lower expression during the course of infection was observed, whereas Wnt3 and Wnt7b showed no clear tendency to higher or lower expression (data not shown). Wnt1 and Wnt10a were expressed to a signifi-

FIGURE 1. mRNA expression of Wnt ligands and Wnt/β-catenin signaling target genes in the lung of M. tuberculosis–infected mice. RNA was isolated from lung tissue of C57BL/6 mice infected with 1000 CFU M. tuberculosis H37Rv and analyzed by RT-qPCR for Wnt ligands, Axin2, c-Myc, and NOS2. Samples from five mice per time point (day 0 [uninfected mice] and days 21 and 42 p.i.) were analyzed. Relative expression levels of Wnt ligands that were found to be significantly downregulated or upregulated at day 21 and/or 42 p.i. compared with day 0 are shown in (A) and (B), respectively. Fold induction of the Wnt/β-catenin target genes Axin2 and c-Myc, as well as of inducible NOS2, a common marker for M. tuberculosis/Th1–induced macrophage activation (C). All data are presented as mean ± SEM. Statistical analysis was performed using the Mann–Whitney U test (day 0 versus day 21 or 42, respectively; *p < 0.05, **p < 0.01). n.s., Not significant.
cantly higher extent at day 42 p.i. (Fig. 1B). However, expression levels of Wnt1 were extremely low compared with most other Wnts. Only mRNA expression of Wnt6 was increased significantly at days 21 and 42 p.i. (Fig. 1B).

In parallel, the expression of the inducible NO synthase (NOS2) and known Wnt target genes were analyzed to correlate the induction of inflammatory pathways to the activation of the Wnt/β-catenin pathway (shown in Fig. 1C). NOS2 expression was strongly increased 21 d and also 42 d p.i. The expression of the well-described Wnt/β-catenin pathway target gene Axin2 (32) was reduced to ~50% at days 21 and 42 p.i. The Wnt/β-catenin target gene c-Myc (33) showed no change in expression levels.

**Wnt6 expression in the lung of M. tuberculosis–infected mice is localized exclusively in macrophage clusters within granulomatous lesions**

To determine the cellular source of Wnt6 expression in the M. tuberculosis–infected lung, we analyzed formalin-fixed lung sections by immunohistochemistry using an anti-Wnt6 Ab (Fig. 2A–D). No Wnt6+ cells were detected in uninfected control lungs (Fig. 2A). When mice were infected with 1000 CFU, lungs showed large areas of cellular infiltrates primarily around the bronchi at 21 and 42 d p.i. (Fig. 2B–D). Demarcated clusters of Wnt6+ cells were located within these granulomatous structures. Wnt6+ cells showed a foamy, macrophage-like morphology characterized by a large cytoplasm and prominent nuclei (Fig. 2C, insert), suggesting macrophages as main cellular source. In mice aerogenically infected with 100 CFU M. tuberculosis, the size of lung granulomatous lesions was considerably smaller; however, Wnt6-expressing cells were easily identified at day 42 p.i. (Supplemental Fig. 1), whereas stainings of mouse lungs at day 21 were still negative (data not shown). This indicates that the intensity of Wnt6 expression is related to the number of M. tuberculosis CFU and the extent of M. tuberculosis–induced inflammation present in the infected mouse lung. In an independent study of murine M. avium infection, immunohistochemical analysis of infected mouse lungs also identified macrophage-like cells to be positive for Wnt6 (data not shown). To further characterize the identity of Wnt6+ cells, we double stained frozen sections of M. tuberculosis–infected lung tissue for Wnt6 and the macrophage marker CD68, and analyzed by fluorescence microscopy (Fig. 2E). CD68+ cells were found in large numbers in regions with high cellular density (as determined by DAPI staining). Wnt6+ cells were again found exclusively in dense cellular clusters within these infiltrates. The staining for Wnt6 was spotlike, reminiscent of a vesicular distribution. All Wnt6+ cells were also positive for CD68, indicating a macrophage-restricted

**FIGURE 2.** Expression of Wnt6 in the lung of M. tuberculosis–infected mice. (A–D) Formalin-fixed lung sections (2 μm) of uninfected (A) and M. tuberculosis H37Rv–infected C57BL/6 mice [1000 CFU; (B): day 21 p.i.; (C): day 42 p.i.] were stained with an Ab against Wnt6 and an appropriate peroxidase-conjugated secondary Ab, and visualized using 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin. (D) As control, a serial section of day 21 p.i. incubated with the secondary Ab alone is shown.

(E and F) Frozen sections of infected lungs (day 42 p.i., 5 μm) were analyzed by fluorescence microscopy for the expression of Wnt6 and the macrophage marker CD68 (E), and lipid droplet staining agent BODIPY 493/503 (F). Sections were incubated sequentially with the primary and appropriate fluorescence-labeled secondary Abs and counterstained with DAPI.
production of Wnt6; but not all CD68+ cells contained Ags reactive with the Wnt6-targeted reagent, indicating that only a sub-set of CD68+ cells were producers of Wnt6.

To address whether Wnt6 expression may be indeed increased in macrophages with a foamy macrophage-like morphology in vivo, we stained frozen sections of lungs of M. tuberculosis–infected mice for Wnt6 and BODIPY 493/503, a dye that identifies the presence of neutral lipids (29). As shown in Fig. 2F, the majority of Wnt6-expressing cells was also positive for the BODIPY staining, which demonstrates that a staining with Wnt6 mainly identifies cells carrying lipid droplets, a characteristic feature of foamy macrophages. However, not all lipid droplet-carrying cells were positive for Wnt6, illustrating the heterogeneity of the foamy macrophage population in vivo.

To visualize mycobacteria and Wnt6 in the same section, we prestained lung sections of infected mice (day 42 p.i.) by ZN staining. In the secondary Ab control (processed without addition of the anti-Wnt6 Ab), large mycobacteria-containing areas within the granulomatous lesions were detected (Fig. 3A). Using a higher magnification, it became apparent that primarily clusters of macrophage-like cells contained ZN-stained, often aggregated mycobacteria (Fig. 3B, 3C). Analysis of ZN and Wnt6 double-stained sections revealed that ZN+ areas were generally also positive for Wnt6 (Fig. 3D). It appeared that most cells harboring mycobacteria were clearly positive for Wnt6, whereas ZN− neighboring cells showed only a faint or no staining for Wnt6 (Fig. 3E, 3F). In particular, huge cells harboring larger aggregates of mycobacteria frequently showed a very intense staining for Wnt6.

To characterize Wnt6 mRNA expression upon microbial stimulation, we infected murine BMDMs of C57BL/6 origin with M. tuberculosis or M. avium for 4 and 24 h. In parallel, cells were stimulated with the TLR agonists LPS (TLR4) or the synthetic lipopeptide Pam3CSK4 (TLR1/TLR2). The expression levels of all 19 Wnt homologs were analyzed by RT-qPCR. Expression of Wnt3a, Wnt7a, Wnt7b, and Wnt8b was not detectable at all under any of the conditions. In addition, Wnt2, Wnt3, Wnt9b, and Wnt16 were detected only at very low levels and not in all of the samples, precluding qualitative or even quantitative conclusions (data not shown). The expression of the remaining Wnt genes is shown in Fig. 4A (only time point 4 h): the heat map shows that the family members Wnt1, Wnt10a, Wnt5b, Wnt6, Wnt11, Wnt2b, Wnt9a, Wnt5a, and Wnt10b were detected in BMDMs. With regard to an upregulation of Wnt genes in BMDMs by mycobacteria and conserved bacterial structures, Wnt1 expression was enhanced after 4 h by all four stimuli, but expression levels were extremely low. Only Wnt6 showed a comparably high and enhanced expression in response to all four stimuli, including M. tuberculosis, analyzed at 4 and 24 h p.i. Several other Wnt genes showed slightly enhanced expression in response to LPS (Wnt2b), LPS and Pam3CSK4 (Wnt10b), or LPS, Pam3CSK4, and M. avium (Wnt10a) after 4 h. After 24 h, a slightly elevated expression was detected for Wnt10a in response to LPS and for Wnt5b and Wnt11 in response to M. tuberculosis.

Gene expression levels of Wnt6 were analyzed in more detail by means of dose–response experiments (see Fig. 4B). After 4 h,
Wnt6 was induced in BMDMs ~2- to 4-fold in response to different doses of LPS, M. avium, and M. tuberculosis, and 6- to 8-fold in response to Pam3CSK4. However, no clear dose dependency was observed for any of the stimuli at this time point. After 24 h, all stimuli induced an even stronger expression of Wnt6 (LPS: 10- to 16-fold, Pam3CSK4: 4- to 10-fold, M. avium: 8- to 25-fold, M. tuberculosis: 2- to 14-fold) in a strictly dose-dependent manner. We also addressed the expression of Wnt6 in M. tuberculosis–infected human primary cells and infected human monocyte-derived macrophages of four healthy blood donors with M. tuberculosis (MOI 1) by fluorescence microscopy. Twenty-four hours p.i., cells were fixed and stained for Wnt6. Nuclei were stained with DAPI. No positive signals for Wnt6 could be shown using BMDMs derived from M. tuberculosis–deficient mice (Fig. 5A, 5B). In addition, Wnt6 expression by TLR ligands and mycobacteria as described earlier suggests a mechanism operative via MyD88-dependent signaling also in case of TLR4 and Pam3CSK4 when compared to LPS, M. avium, and M. tuberculosis–infected lungs (Fig. 2E).

**Induction of Wnt6 depends on TLR–MyD88–NF-κB signaling but is independent of TNF-α production**

The induction of Wnt6 expression by TLR ligands and mycobacteria as described earlier suggests a mechanism operative via TLR, MyD88, and NF-κB. Dependency on TLR-MyD88 signaling could be shown using BMDMs derived from TLR2- and MyD88-deficient mice (Fig. 5A, 5B). In TLR2−/− and MyD88−/− macrophages, Wnt6 mRNA expression levels were significantly reduced in response to M. tuberculosis and Pam3CSK4 when compared with wild type macrophages. Similar data were obtained when cells were infected with M. avium (data not shown). In response to LPS, Wnt6 mRNA expression was unchanged in case of TLR2 deficiency, but clearly reduced in MyD88−/− macrophages, pointing to a role for MyD88-dependent signaling also in case of TLR4 stimulation. NF-κB dependency was analyzed using a small molecule...
inhibitor of IκB-α phosphorylation (Bay 11-7082). Preincubation with this inhibitor of NF-κB signaling for 30 min dose-dependently reduced the expression of Wnt6 in response to M. avium and Pam3CSK4 (Fig. 5C). The influence of TNF-α was analyzed using macrophages from TNF-α-deficient mice (Fig. 5D). There was no apparent difference observed in Wnt6 expression in TNF−/− versus TNF+/+ macrophages, when stimulated with LPS, Pam3CSK4, or M. avium, nor was there an induction of Wnt6 expression observed after stimulation with rTNF-α in either TNF−/− or TNF+/+ macrophages, demonstrating TLR-induced Wnt6 induction to be TNF-α independent.

Exogenous Wnt6 induces expression of c-Myc in a β-catenin signaling–independent manner

To investigate Wnt6-induced signaling in macrophages, we used Wnt6 CM produced by Wnt6-overexpressing NIH3T3 cells (28). RT-qPCR analysis of Wnt6-treated murine macrophages revealed a differential induction of the Wnt/β-catenin target genes Axin2 and c-Myc. Whereas Wnt6 CM induced c-Myc expression ~9-fold, no difference was observed in Axin2 expression compared with cells treated with CCM (Fig. 6A). Wnt3a as well as the GSK-3β inhibitor SB216763 induced Axin2 but not c-Myc expression in macrophages (data not shown). Western blot analysis corroborated a β-catenin-independent induction of c-Myc expression because there was no accumulation of β-catenin observed in macrophage lysates after stimulation of the cells with Wnt6 CM (Fig. 6B). The GSK3 inhibitor SB216763 (34) was used as a positive control to mimic the activation of Wnt/β-catenin signaling and leading to accumulation of β-catenin.

Wnt6 induces phosphorylation of PKC, p38, and ERK

Aside from Wnt/β-catenin signaling, several other Wnt-induced signaling pathways have been described in macrophages. Recently, we reported a Wnt-mediated activation of NF-κB and MAPKs (4). In addition, Pereira et al. (10) suggested an activation of Wnt/Ca2+ signaling by Wnt5a. Thus, we analyzed a potential Wnt6-mediated activation of the NF-κB and MAPK pathways using phospho-specific Abs against p65–NF-κB and the MAPKs JNK1/2, p38, and ERK1/2, respectively. Activation of Wnt/Ca2+ signaling was monitored using a pan-phospho-PKC Ab because PKC was described as a downstream target of Wnt/Ca2+ signaling (reviewed in Ref. 35). Western blot analysis revealed a fast but transient increase in ERK and p38 phosphorylation induced by Wnt6 CM (Fig. 6B). An activation of the MAPK JNK by Wnt6 CM was not observed. A strong signal for phospho-PKC isoforms was detectable already in control (CCM-treated) macrophages (Fig. 6B). The GSK3 inhibitor SB216763 (34) was used as a positive control to mimic the activation of Wnt/β-catenin signaling and leading to accumulation of β-catenin.

Wnt6 induces phosphorylation of PKC, p38, and ERK

Aside from Wnt/β-catenin signaling, several other Wnt-induced signaling pathways have been described in macrophages. Recently, we reported a Wnt-mediated activation of NF-κB and MAPKs (4). In addition, Pereira et al. (10) suggested an activation of Wnt/Ca2+ signaling by Wnt5a. Thus, we analyzed a potential Wnt6-mediated activation of the NF-κB and MAPK pathways using phospho-specific Abs against p65–NF-κB and the MAPKs JNK1/2, p38, and ERK1/2, respectively. Activation of Wnt/Ca2+ signaling was monitored using a pan-phospho-PKC Ab because PKC was described as a downstream target of Wnt/Ca2+ signaling (reviewed in Ref. 35). Western blot analysis revealed a fast but transient increase in ERK and p38 phosphorylation induced by Wnt6 CM (Fig. 6B). An activation of the MAPK JNK by Wnt6 CM was not observed. A strong signal for phospho-PKC isoforms was detected already in control (CCM-treated) macrophages (Fig. 6B). The GSK3 inhibitor SB216763 (34) was used as a positive control to mimic the activation of Wnt/β-catenin signaling and leading to accumulation of β-catenin.
Wnt6-induced c-Myc expression is sensitive to PTX and ERK inhibition

Several Wnt-mediated effects depend on heterotrimeric G proteins (reviewed in Ref. 36). To investigate the participation of heterotrimeric G proteins, we analyzed the sensitivity of Wnt6 CM–induced signaling events to PTX, an inhibitor of the \( \alpha \) subunit of \( Gi, Go \) and \( Gt \). As shown in Fig. 7A, Wnt6 CM–induced expression of c-Myc in murine BMDMs is reduced in a dose-dependent manner by preincubation with PTX. With regard to Wnt6-induced signaling in BMDMs, we observed PTX-sensitive and -insensitive effects. Whereas the Wnt6 CM–induced phosphorylation of PKC remained unchanged in the presence of PTX, ERK phosphorylation was reduced \( \sim 30\% \) as determined by quantification of Western blots (Fig. 7C). To further elucidate a possible role for MAPKs in the Wnt6-driven c-Myc expression, we carried out experiments with small molecule MAPK inhibitors (37). As shown in Fig. 7B, Wnt6 CM–induced c-Myc expression was sensitive only to inhibition by U0126, which has been described as an inhibitor of the ERK pathway. Incubation with SB203580, an inhibitor of the p38 MAPK pathway, had no effect.

Wnt6 modulates the inflammatory response of mycobacteria-infected macrophages

To address a potential regulatory role of Wnt6 in mycobacterial infections, we used murine BMDMs and analyzed the inflammatory response to mycobacteria in the presence or absence of Wnt6: we infected macrophages with \( M. \) tuberculosis (Fig. 8) and \( M. \) avium (Supplemental Fig. 2). Wnt6 CM–treated \( M. \) tuberculosis–infected cells showed a 30% decrease in TNF-\( \alpha \) expression after 4 h of incubation (Fig. 8A). The mRNA expression level of NOS2, which encodes a key enzyme of the antimycobacterial response of murine macrophages, was slightly reduced in Wnt6 CM–treated \( M. \) tuberculosis–infected cells, although this did not reach statistical significance (Fig. 8B). At the same time, we observed that expression of Arginase-1 (\( \text{Arg-1} \)), a marker for murine alternatively activated macrophages (38, 39), was strongly upregulated (Fig. 8C). Although Wnt6 CM alone did not alter Arg-1 expression significantly, it showed a synergistic effect with \( M. \) tuberculosis infection, resulting in a \( \sim 4\)-fold increase compared with infected cells treated with CCM. Similarly, Wnt6 CM induced enhanced mRNA levels of mannose receptor 1 (\( \text{MRC-1} \)), another well-known marker of alternative macrophage activation (39) (Fig. 8C). Also, BCL-2, a protein with antiapoptotic activities (reviewed in Ref. 40), was induced by Wnt6 CM in uninfected macrophages (Fig. 8D).
These data suggested that exogenously added Wnt6 actively modulates the inflammatory response of mycobacteria-infected macrophages.

To further substantiate these findings, we used macrophages from Wnt6-heterozygous and Wnt6-deficient mice and infected them with M. tuberculosis: Fig. 9A demonstrates that Wnt6-heterozygous mice show ~50% of the expression of WT mice, whereas Wnt6 mRNA was completely absent in Wnt6-deficient mice at all time points analyzed. The analysis of TNF mRNA expression and release showed that Wnt6−/− BMDMs revealed a significantly enhanced formation of this antimycobacterial mediator (Fig. 9B). Similar data were obtained when cultures of resident peritoneal cells, mainly consisting of in vivo differentiated macrophages (pMΦ), were used (Fig. 9F). These data sets demonstrate a proinflammatory phenotype of Wnt6−/− macrophages, when infected with M. tuberculosis. We also observed that M. tuberculosis–induced NOS2 mRNA levels were significantly reduced in Wnt6−/− cells (Fig. 9C). However, the magnitude of nitrite formation of Wnt6+/− and Wnt6−/− macrophages, reflecting the total NO formation of the two cell types, was comparable, although the overall level of nitrite formation was very low. Notably, NO formation of LPS-stimulated Wnt6−/− cells was significantly decreased compared with Wnt6+/− macrophages (data not shown), suggesting a reduced activity of NOS-2 in response to this TLR4 agonist. At the same time, M. tuberculosis–induced Arg-1 mRNA expression in Wnt6−/− mice was reduced by >75–90%, when compared with Wnt6+/− cells (Fig. 9D). Similarly, MRC-1 mRNA expression was significantly decreased in Wnt6-deficient, uninfected macrophages (Fig. 9D). Notably, the analysis of MRC-1 gene expression in M. tuberculosis–infected cells did not lead to differences between the two cell types (data not shown). BCL-2 mRNA levels were found to be dose dependently induced by M. tuberculosis infection of Wnt6+/− but not Wnt6−/− BMDMs (Fig. 9E).

Wnt6 induces proliferation in macrophages

Having observed that exogenous Wnt6 drives c-Myc expression in macrophages, we used Wnt6-deficient macrophages to address whether also endogenously formed Wnt6 would influence macrophage c-Myc expression: already basal levels of c-Myc expression were significantly lower in Wnt6−/− BMDMs (data not shown) and peritoneal macrophages (Fig. 9F), when compared with Wnt6+/− macrophages. In addition, we observed a significant reduction in M. tuberculosis–induced c-Myc mRNA expression in Wnt6-deficient macrophages when compared with control cells. This data set complements our findings obtained with exogenously added Wnt6 and independently points to a link between Wnt6 and the function of c-Myc in macrophages. An enhanced expression of c-Myc has previously been associated with promotion of the cell cycle also in macrophages (41). Thus, we monitored a potential effect of Wnt6 on macrophage proliferation, using both c-Myc mRNA expression and analysis of the proliferation marker Ki-67 as readout systems. RT-qPCR analysis revealed an enhanced Ki-67 mRNA expression in Wnt6 CM–stimulated BMDMs after 24 h (Figs. 10A, 11). Analysis of Ki-67 protein expression by fluorescence microscopy identified an enhanced number of Ki-67+ nuclei in Wnt6 CM–treated macrophage cultures (Fig. 10B). A similar effect was observed when macrophages were treated with CSF-1, used as a positive control. These data indicate that Wnt6 may promote proliferation of murine macrophages. To further support these data, we monitored macrophage cell proliferation using the xCELLigence system (Roche Applied Science). This real-time system allows measurements of the electrical impedance across the bottom of the cell-culture plate (Cell Index) and has been shown to be equivalent to assays measuring metabolic activity (42). Fig. 10C documents that Wnt6 CM–treated cells showed a steeper ascent and a higher overall level of the Cell Index compared with CCM-treated cells, suggesting an enhanced proliferation and higher cell numbers after 3 d. Restimulation by addition of fresh CM after 3 d resulted in a further rapid increase of the Cell Index in Wnt6 CM–treated cells. An increase of the Cell Index was also observed in CSF-1–treated macrophage cultures, although different kinetics were observed: the Cell Index rose slowly but continuously to a level comparable with that obtained with Wnt6 CM–treated cells after 6 d. To independently confirm our real-time proliferation data, we performed [3H]thymidine in-
and MRC-1 cated groups were compared by Student two-tailed experiments. For statistical analysis data were log-transformed and indi-

Methods

H37Rv as indicated. mRNA expression was determined by RT-qPCR: ([3H]TdR incorporation when compared with CCM-treated mac-

FIGURE 9. Wnt6 deficiency affects the effector functions of in vitro and in vivo differentiated macrophages in response to M. tuberculosis. BMDMs (A–E) or peritoneal macrophages (pMΦ) (F) from NMR1 Wnt6+/+ (wt), Wnt6−/− and Wnt6+/− mice were infected with M. tuberculosis H37Rv as indicated. mRNA expression was determined by RT-qPCR: (A) Wnt6, 4 h and 24 h; (B) TNF-α, 24 h; (C) NOS2, 24 h; (D) Arg-1, 24 h and MRC-1, 4 h; (E) BCL-2, 24 h and (F) c-Myc, 24 h. The release of TNF-α (B, F) and Nitrite (C) were measured as described in Materials and Methods. Depicted is the mean ± SEM of three to five independent experiments. For statistical analysis data were log-transformed and indicated groups were compared by Student two-tailed t test. *p < 0.05, **p < 0.01. n.s., Not significant.

corporation assays. After 4 d of treatment with CM, BMDMs were pulsed with [3H]TdR for a total time of 12 h. BMDM lysates of cells stimulated with Wnt6 CM showed a 25-fold increased [3H]TdR incorporation when compared with CCM-treated mac-

Discussion

Wnt signaling has been primarily described as a master regulatory pathway during ontogeny and homeostatic processes. There is now growing evidence demonstrating a regulatory role for Wnt signaling also in inflammatory and infectious diseases. In this study, we monitored the expression of all 19 Wnt homologs in mice experimentally infected with M. tuberculosis via the aerosol route. We identified Wnt6 to be induced and localized its expression exclusively to macrophage clusters within granulomatous lesions. In vitro experiments using BMDMs uncovered a TLR-, MyD88-, and NF-κB-dependent but TNF-α–independent induction of Wnt6 gene expression. Analysis of Wnt6-induced signaling using Wnt6 CM suggests a PTX-sensitive and ERK-dependent, but β-catenin–

subset of macrophages facilitating bacterial persistence rather than eradicating the pathogen. This line of argument is further corroborated by the notion that Wnt signaling is broadened during the course of infection in the lungs: we observed that mRNA expression levels of the majority of Wnt homologs (Wnt2, Wnt2b, Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt8a, and Wnt10b) were also significantly reduced during M. tuberculosis infection. The potential physiological relevance of this counter-regulation of inflammatory versus Wnt/β-catenin–driven anti-inflammatory and homeostatic processes has been discussed only recently (4, 20). A growing body of experimental data from independent laboratories underscores the anti-inflammatory potential of Wnt/β-catenin signaling by: 1) reduction of proinflam-

matory cytokine formation in macrophages (4, 20) and also septic shock models (18, 19, 43), 2) inhibition of transendothelial mono-

cyte migration (16), and 3) induction of tolerance by reprogram-

ming dendritic cells (17).

Therefore, our discovery that some Wnts (Wnt1, Wnt10a, and Wnt6) are induced in an inflammatory environment such as the lung of M. tuberculosis–infected mice is of particular interest. We identified CD68+ cells with a foamy macrophage-like morphology within granulomatous lesions as the main cellular source of Wnt6. Our observation that Wnt6+ cells contain lipid vesicles as shown by BODIPY 493/503 staining supports the notion that Wnt6 indeed stains a subset of foamy macrophages in vivo. These clusters of foamy macrophages within large cellular infiltrates of primarily lymphoid origin have been proposed to be the habitat of M. tu-

berculosis in experimental mouse models and also in human disease (44, 45). Therefore, Wnt6+ cells may represent a specific subset of macrophages facilitating bacterial persistence rather than eradicating the pathogen. This line of argument is further supported by our data obtained from in vitro experiments using murine BMDMs: a systematic expression screen of macrophages treated with pathogenic mycobacteria and conserved bacterial structures revealed Wnt6 and Wnt1 as only Wnt ligands that are induced 2-fold by all stimuli used, including M. tuberculosis. Analysis of M. tuberculosis–infected cell cultures by fluorescence microscopy suggests that only infected macrophages, but not by-

stander cells, contain detectable amounts of Wnt6. This may indi-
cate a mechanism requiring direct contact with microbial structures rather than a paracrine induction by secreted mediators such as inflammatory cytokines.
FIGURE 10. Wnt6 has proliferation-inducing effects on murine macrophages. BMDM derived from C57BL/6 mice were incubated with Wnt6 CM, CCM, or rCSF-1 (50 ng/ml) for 1–6 d with a restimulation at day 3. (A) Ki-67 expression after 24 h was determined by RT-qPCR. Data were log-transformed and groups compared using a paired Student two-tailed t test. Depicted is the mean ± SEM of four independent experiments. *p < 0.05. (B) Cells were stained for Ki-67 after 24 h and analyzed by fluorescence microscopy. Nuclei were stained with DAPI. Shown are representative pictures from one out of two independent experiments. Original magnification ×250. (C) Over a period of 6 d the cellular growth (expressed as cell index) of 12,500 cells/well was determined by real-time impedance measurement using the xCELLigence system (Roche). (D) Cells were seeded as described for (C). At day 4, cells were pulsed with [3H]-thymidine for a period of 12 h. Depicted is mean ± SEM of the relative [3H]-thymidine incorporation (counts per minute/culture normalized to CCM treated control) of two independent experiments.

The induction of Wnts has been only poorly described in inflammatory settings: to date, an upregulation of Wnt6 has been almost exclusively observed in microarray-based studies, for example, in M. tuberculosis–infected murine macrophages (46), in the bladder of mice after stimulation with LPS, in a rat renal allograft model, in coloscopic biopsies of ulcerative colitis patients, and most recently also in the lung of asthma patients (14, 47–49). Of note, Liu et al. (50) describe an induction of Wnt6 mRNA by Salmonella typhimurium infection of murine intestinal epithelia cells ex vivo. However, none of these studies defined the mode of induction or assigned a function of Wnt6. We now identified Wnt6 mRNA expression induced in BMDMs by microbial stimuli to be dependent on TLR-, Myd88-, and NF-κB signaling, but independent of TNF-α. Regarding the well-known property of TNF-α to activate NF-κB and to boost TLR-driven, NF-κB–mediated gene transcription, it can only be assumed that activation of NF-κB may be necessary but not sufficient to induce Wnt6 expression. These data also support the notion that Wnt6 expression is induced locally in macrophages that have direct contact to or are infected by mycobacteria. The ZN/Wnt6 double staining, which reveals that in vivo most Wnt6+ cells are infected, further strengthens this interpretation of our data. If this were to apply also to M. tuberculosis–infected macrophages within the granulomatous lesion, Wnt6 may become a useful biomarker.

Although this study mainly focuses on cells of the murine immune system, we also demonstrate a significant increase of Wnt6 mRNA expression in human monocyte-derived macrophages infected with M. tuberculosis H37Rv, indicating that this Wnt homolog may be subject to a similar regulation in human macrophages. Based on this study, which shows that immunohistochemical detection of Wnt6 protein is related to the number of CFU in a given mouse lung, and our recent observation that M. tuberculosis clinical isolates differ in their virulence patterns (26), detailed immunohistochemical studies are now needed to characterize the cells and the conditions leading to the expression of the Wnt6 protein during pulmonary tuberculosis in humans.

Secreted Wnt proteins are proposed to act predominantly, although not entirely, as short-range mediators (reviewed in Ref. 51). In fundamental work by Shimizu et al. (52), different Wnt homologs were characterized by their ability to morphologically transform C57MG epithelia cells and their potential to activate Wnt/β-catenin signaling. Wnts today classified as so-called canonical Wnts (e.g., Wnt1, Wnt3, and Wnt3a) induced strong morphological changes that could be correlated with their ability to induce β-catenin stabilization. Wnt6 was shown to induce only slight morphological changes and only in an autocrine but not in a paracrine manner. An activation of Wnt/β-catenin signaling was not observed. This was later corroborated by Gazit and colleagues (53), describing that again Wnt1, Wnt3, and Wnt3a, but not Wnt6, induced Wnt/β-catenin signaling in Fzd1-overexpressing HEK293T cells. We therefore investigated whether Wnt6 may influence macrophage-specific effector functions in primary cells and during infection. Wnt6 CM did not induce Wnt/β-catenin signaling in primary murine macrophages as determined by analysis of β-catenin protein levels and Axin2 mRNA expression. Remarkably, another dedicated Wnt/β-catenin target gene, c-Myc (33), turned out...
to be induced by Wnt6 CM in a β-catenin-independent manner. This finding is further complemented by the observation that c-Myc expression is significantly reduced in Wnt6−/− macrophages infected with M. tuberculosis. It is also well in line with our observation that Axin2, but not c-Myc expression, is reduced in the lung during the course of M. tuberculosis infection, suggesting a Wnt/β-catenin–independent expression of c-Myc also in vivo. Mechanistic studies in primary macrophages revealed that expression of c-Myc is dependent on activation of the MAPK ERK. The transcription factor c-Myc is an important regulator of cell proliferation in various cell types, including macrophages (reviewed in Ref. 36). But as a global regulator of chromatin, it exerts functions in virtually all cellular processes, for example, in cell metabolism, differentiation, apoptosis, and many more (reviewed in Ref. 54).

In-depth analysis of Wnt6-mediated signaling revealed that phosphorylation of ERK and the induction of c-Myc expression is sensitive to PTX, implying an involvement of heterotrimeric G proteins that has been discussed for several signaling events mediated by G protein–coupled receptors of the Frizzled family (reviewed in Ref. 41). It is currently not known whether a single receptor or multiple receptors mediate Wnt6-induced signaling. Notably, the Wnt6-induced change in PKC phosphorylation was not altered by pretreatment of BMDM with PTX, indicating that different receptors and downstream signaling systems may be involved. The m.w. of the additional PKC isoform that was detectable in Wnt6-treated macrophages could correspond to PKCe (55).

In recent years, the concept of macrophage polarization has been developed and further sophisticated: macrophages are now seen as a spectrum of phenotypic subtypes ranging from classically activated macrophages (M1) to the alternatively activated macrophages (M2) based on gene expression induced in response to pathogen- and cytokine-derived stimulation (38, 56). In this study, we show that Wnt6 induces the expression of Arg-1 and reduces the formation of TNF-α in M. tuberculosis–infected macrophages, which supports the notion that Wnt6 shifts macrophage polarization toward an M2-like phenotype. El Kasmi et al. (57) demonstrated that mycobacteria induce Arg-1 in a TLR- and Myd88-dependent but STAT6-independent manner. Our data are in line with these findings because we observe that Wnt6 expression is also TLR and Myd88 dependent. Thus, we may have identified Wnt6 as a so far unknown but important regulatory factor in this signaling chain driving macrophage M2 differentiation. The almost complete loss of Arg-1 mRNA levels in Wnt6−/− macrophages is accompanied by a significant, albeit incomplete reduction of c-Myc levels in these cells. Whether this correlation is indeed functionally related, for example, in the sense that the lack of c-Myc is causal for the reduced Arg1 expression, needs to be addressed in further analyses. This is not unlikely, because it was shown that c-Myc drives the expression of C/EBPβ (58), a transactivating factor essential for Arg-1 induction (57).

A recent study showed that alternative differentiation of human macrophages into a M2 phenotype requires c-Myc (59), a transcription factor that we find significantly induced also in murine macrophages by Wnt6. This prompted us to investigate a potential proliferation-inducing potential of Wnt6. Indeed, treatment of murine BMDMs with Wnt6 CM turned out to have a cell-cycle–promoting effect as shown by the analysis of Ki67 and thymidine incorporation assays in vitro. A more detailed analysis is needed to validate whether Wnt6 may support macrophage growth also in vivo. It is well-known that Wnt proteins promote cellular growth, even in inflammatory settings (60). With
regard to the M. tuberculosis-infected lung, there is currently no evidence supporting a potential Wnt6-induced proliferative effect of macrophages within the granulomatous lesion, a microenvironment regarded as a classical TH1 setting. However, the local mediator milieu may be decisive whether macrophages actively proliferate also in inflammatory conditions. Jenkins et al. (61) recently demonstrated the proliferation of tissue-resident macrophages during helminth-induced tissue inflammation, which is associated with IL-4 production, reflecting a prototype TH2-type immune response. In the context of tuberculosis, we propose that Wnt6 production in the granuloma drives macrophage differentiation rather than proliferation. Based on our observation that the majority of Wnt6-expressing cells contain lipid vesicles as shown by BODIPY 493/503 staining, it is intriguing to speculate that M. tuberculosis induces Wnt6 to promote the formation of foamy macrophages as a cellular habitat to persist and replicate within the host. Rı´ os-Barrera et al. (62) described foamy macrophages as a pathogen-induced anti-inflammatory and apoptosis-resistant macrophage phenotype promoting persistence or even proliferation of bacteria. The authors characterized this vacuolated or foamy macrophage phenotype by low expression of TNF-α and NOS2, both factors crucial for the control of mycobacterial growth (63). Our present study provides with Wnt6 a novel pathogen-induced factor produced by foamy macrophages and is a target for the antiinflammatory action of activated protein C and mediates metabolic distortion in obesity. Science 319: 454–457.


