The Wingless homolog WNT5A and its receptor Frizzled-5 regulate inflammatory responses of human mononuclear cells induced by microbial stimulation

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Microarray-assisted gene-expression screens of human macrophages revealed WNT5A, a homolog of Wingless, a key regulator of *Drosophila melanogaster* embryonic segmentation and patterning, to be consistently up-regulated following stimulation with different mycobacterial species and conserved bacterial structures. The expression of WNT5A required Toll-like receptor signaling and NF-κB activation, which identifies a novel induction pathway for a Wingless homolog. We show that human peripheral-blood mononuclear cells express the WNT5A receptor Frizzled-5 (FZD5). Both WNT5A and FZD5 also were detected in granulomatous lesions in the lungs of *Mycobacterium tuberculosis*–infected patients. Functional studies showed that WNT5A and FZD5 regulate the microbially induced interleukin-12 response of antigen-presenting cells and interferon-γ production by mycobacterial antigen-stimulated T cells. Our findings implicate the evolutionarily conserved WNT/Frizzled signaling system in bridging innate and adaptive immunity to infections. (Blood. 2006;108:965-973)

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Introduction

Immunity to infections depends on the successful integration of innate and adaptive defense strategies.[1] Cells of the innate immune system, such as macrophages and dendritic cells, recognize pathogen-associated molecular patterns shared by many microbes but not found in higher eukaryotes, via members of the Toll-like receptor (TLR) family.[2,3] TLR-dependent signaling pathways can directly induce macrophage antimicrobial programs but also initiate inflammatory cell recruitment and help prime cells of the adaptive immune system to amplify bactericidal effector mechanisms. Experimental infections with microorganisms have been used successfully to uncover the intricacies governing the interplay between innate and adaptive immunity.[4,5] For example, cell-wall components of *Mycobacterium tuberculosis*, the causative organism of tuberculosis, critically depend on TLR2 and TLR4 to induce secretion of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin (IL-12, necessary for differentiating T-helper 1 cells).[6] Subsequently, mycobacteria-primed T cells secrete interferon gamma (IFN-γ) as a critical macrophage-activating agent. Eradication of mycobacteria is achieved only when both arms of the immune system are fine-tuned for full antimicrobial potency.

Functionally, the Toll-mediated induction of antimicrobial effector systems is highly conserved between *Drosophila melanogaster*

(*D melanogaster*) and *Homo sapiens*.[7,8] Another example for human evolutionarily ancient effector mechanisms is granulysin (a granule-stored bactericidal molecule), which is homologous to the amoebapores of *Entamoeba histolytica*.[9,10]

Thus, there is ample precedence for evolutionarily conserved signatures governing individual facets of the immune response. To uncover novel regulatory pathways in innate responses to infection, we performed a microarray-based gene-expression screen with human macrophages infected with mycobacteria or conserved bacterial structures. We found mRNA for *WNT5A*, a homolog of Wingless in *Drosophila* species, to be consistently up-regulated in response to all stimuli.

Wingless was originally characterized as a segment polarity gene in *D melanogaster,* which is essential in embryonic segmentation and patterning (reviewed in Klingensmith and Nusse[11]). Various homologs of the Wingless protein, termed WNT, are involved in embryonic development of nonvertebrates and vertebrates,[12,13] where WNT signaling determines cell motility, differentiation, and apoptosis.[14] In mammalian hematopoiesis, WNT signaling is essential for stem-cell homeostasis[15] and lymphocyte differentiation.[16,17] Most recently, one member of the WNT family of proteins, WntD, was shown to be involved in regulating antibacterial defenses in Drosophila.[18] To date, however, WNT

proteins have not been directly implicated in the human immune defense against infections.

Our functional analyses demonstrate that both WNT5A and Frizzled-5 (FZD5) regulate mycobacteria-induced IL-12 production and IFN-γ release during mycobacterial antigen-driven T-cell stimulation. This implies an unexpected and novel role for WNT/FZD signaling in orchestrating adaptive immunity in response to microbial stimulation of innate immune cells.

Materials and methods

Bacteria, antibodies, and reagents

M avium (strain SE01), *M tuberculosis* (strain H37Rv), synthetic lipopeptide (Pam$_3$CSK$_4$; kindly provided by Dr K. H. Wiesmüller, EMC Microcollections, Tübingen, Germany), lipopolysaccharide (LPS; *Salmonella enterica*, serovar friedenau H909; kindly provided by Prof H. Brade, Research Center Borstel, Germany), phorbol-12-myristate13-acetate (PMA), calcium ionophore A23187 (both Sigma Aldrich, Taufkirchen, Germany), and phytohemagglutinin (PHA) were used for stimulation. To rule out the presence of lipopolysaccharides (LPSs) in the assays, mycobacterial strains were tested in a limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). The effective LPS concentration in the experiments when ratios of 10:1 were used was below 2 pg/mL.

The following antibodies, proteins, and inhibitors were used: anti–human TLR2 (kindly provided by Genentech, San Francisco, CA); anti–human TNF-α (Infliximab; Centocor, Horsham, PA); anti-CD3, anti-CD28 (clone X35 and clone CD28.2; Beckmann Coulter, Krefeld, Germany); anti–murine WNT5A (R&D Systems, Minneapolis, MN), shown to neutralize WNT5A-induced repopulation of immune-deficient NOD/SCID mice by primitive subsets of human hematopoietic cells[19]; mouse IgG1 (BD Biosciences, San Jose, CA); goat IgG (Jackson Immunoresearch, West Grove, PA); anti–human FZD5[20] (Upstate, Charlottesville, VA); recombinant human Fzd5/Fc chimera (R&D Systems). BAY11-7082 was purchased from Calbiochem (EMD Biosciences, San Diego, CA). Compound 406 was a kind gift from Dr K. Brandenburg (Research Center Borstel).

Isolation, differentiation, and cultivation of human leukocyte subsets

Mononuclear cells were isolated from peripheral blood (PBMC) of healthy volunteers by density gradient centrifugation. Lymphocytes and monocytes were separated (purity consistently > 97%) by counterflow elutriation. Macrophages were generated from highly purified monocytes as described.[21] Stimulation was done in RPMI 1640 with 10% fetal calf serum (FCS) and 4 mM L-glutamine (all from Biochrom, Berlin, Germany). All experiments performed were approved by the Research Ethics Board of the University of Lübeck, Germany.

Microarray analyses

Microarray analyses were performed using Affymetrix GeneChips Human Genome U95Av2 consisting of 12 558 human genes and expressed sequence tags including all human WNT homologs. After 4 hours of stimulation total RNA from human macrophages was isolated by adding TriFastFL (PEQLAB, Erlangen, Germany), reverse transcribed, labeled, and hybridized according to the manufacturer's standard protocol. Primary image analysis of arrays and data analysis were performed using GeneChip Microarray Analysis Suite Version 5.0, MicroDB and Data Mining Tool (Affymetrix, Santa Clara, CA) as described previously.[22]

RT-PCR

Total RNA was isolated from cell cultures (0.4×10^6 macrophages; 1×10^6 lymphocytes) (SV Total RNA Isolation System; Promega, Mannheim, Germany) and reverse transcribed using oligodT nucleotides. For reverse transcriptase–polymerase chain reaction (RT-PCR) the following gene-specific primer pairs were used: beta-2-microglobulin (B2M) forward 5′-GCTGTGCTCGCGCTACTCTC-3′, reverse 5′-GCGGCATCT-TCAAACCTCCAT-3′; WNT5A forward 5′-ACACCTCTTTCCAAACAG-GCC-3′, reverse 5′-GGATTGTTAAACTCAACTCTC-3′[23]; WNT5A (quantitative PCR) forward 5′-GTCTTGAGCTTGGGGC-3′, reverse 5′-ACGTCCATGTCTATAACGA-3′, IL-2 forward 5′-TTACAAGAATC-CCAAACTCACC-3′; reverse 5′-TAGCAAACCATACATTCAACAA-3′; Fzd5 forward 5′-TGTCTGCTCTTTCTCGGC-3′, reverse 5′-CCGTCCAAA-GATAAACTGCT-3′. For quantitative RT-PCR, LightCycler technology (Roche Diagnostics, Mannheim, Germany) was used. Gene expression is displayed as relative expression normalized to B2M. Specificity of amplified products was confirmed by sequence analyses.

In situ hybridization and immunohistology

Cytospins of human macrophages were prepared after 4 hours of cultivation and fixed overnight in HOPE (HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid]-glutamic acid buffer-mediated organic solvent protection effect).[24] HOPE-fixed, paraffin-embedded sections of human lung tissue were deparaffinated. PCR product amplified with the WNT5A specific primer pair or nonspecific DNA (1356 bp of the bacterial vector pcDNA 3 cleaved by *Pst*I) was labeled with the DIG-High-Prime system (Roche) according to the manufacturer's instructions. Hybridization (2 ng/μL freshly denatured probe, 0.1% sodium dodecyl sulfate (SDS), 50% formamide, and 250 μg/mL yeast tRNA [Roche]) with both probes was carried out overnight in moist chambers at 46°C, and detection was performed using an antidigoxigenin antibody conjugated with alkaline phosphatase (anti–DIG-AP, Roche) as previously described in detail.[25] For immunohistochemistry, anti-FZD5 antibody (Upstate) was used. Detection was performed by a horseradish peroxidase–labeled streptavidine-biotin technique (LSAB2, Dakocytomation, Glostrup, Denmark) using aminoethylcarbazole as chromogenic substrate. Stained cytospins and tissue sections were analyzed with a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) equipped with the following C Plan objective lenses: ×20/0.4 NA; ×40/0.65 NA; and ×10/0.22 NA. Images were acquired as JPEG files using the Nikon Digital Sight DS-5M imaging system and software (Nikon, Düsseldorf, Germany). Minor adjustments of brightness, contrast, and color adjustment solely for the purpose of legibility were made with Adobe Photoshop 8.0 for Windows (Adobe Systems, San Jose, CA).

Generation of the anti–Fzd5 antiserum

Rabbits were immunized with a 21–amino acid peptide (CPILKESH-PLYNKVRTGQVPN) derived from the cysteine-rich extracellular domain of human FZD5 that has previously been used for the successful generation of a functionally active anti-FZD5 antiserum.[26] Immunogenicity of the peptide was enhanced by conjugation to keyhole limpet hemocyanine. Prior to immunization preimmune serum was obtained as a control. Peptide specificity of the obtained antiserum was monitored by competitive solid phase enzyme-linked immunosorbent assay (ELISA). An irrelevant peptide of the same size (amino acids 27-48 of human IL-8 receptor II; E. Brandt, Research Center Borstel) was used as an internal control in ELISA.

Cytokine detection

Supernatants were assayed by ELISA according to the manufacturer: IL-12p40, IL-2 (Duoset, R&D Systems), IFN-γ, TNF-α (Dr H. Gallati, Intex; Muttenz, Switzerland), and IL-8 (Cytoset, Biosource, Camarillo, CA).

Statistical analysis

For statistical analysis, raw data were $\log_{10}$ transformed prior to analysis. Data obtained from independent experiments were compared using a *t* test for paired comparisons. mRNA levels or cytokine concentrations under different culture conditions were correlated to control cultures (normalized to 100%) and are represented as mean ± standard deviation (SD).
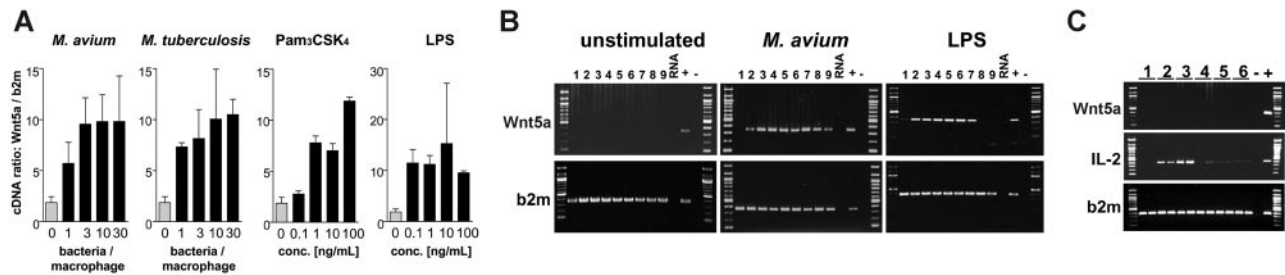
Figure 1. Induction of WNT5A expression in human monocyte-derived macrophages, but not lymphocytes. Human cells were stimulated as indicated. Total RNA was isolated, reverse transcribed, and used for quantitative (A) or conventional RT-PCR (B,C). Representative results of 1 at least 3 independent experiments are shown. (A) Dose response of Wnt5a expression in human macrophages after 4 hours of stimulation with *M avium*, *M tuberculosis*, synthetic lipopeptide (Pam$_3$CSK$_4$), or LPS, monitored by quantitative RT-PCR normalized to B2M mRNA levels; mean ± SD of duplicate determinations of 1 of 3 experiments are shown. (B) Kinetics of WNT5A and B2M mRNA expression in human macrophages induced by *M avium* (multiplicity of infection [MOI] 3) or LPS (10 ng/mL) analyzed by RT-PCR. Lane 1, 0 hours; lane 2, 2 hours; lane 3, 4 hours; lane 4, 6 hours; lane 5, 8 hours; lane 6, 10 hours; lane 7, 24 hours; lane 8, 48 hours; lane 9, 72 hours; RNA, RNA without reverse transcription; +, cDNA from human macrophages stimulated with *M tuberculosis* for 4 hours; and -, no cDNA. (C) Isolated human lymphocytes were left unstimulated (lane 1) or were stimulated with anti-CD3 (lane 2), anti-CD3/anti-CD28 (lane 3), 1 μg/mL (lane 4), or 5 μg/mL (lane 5) PHA or 10 ng/mL PMA/A23187 (lane 6). Cells were stimulated in duplicates as indicated for 4 hours; total RNA was isolated and reverse transcribed. Expression of WNT5A, IL-2, and beta-2-microglobulin (b2m) were analyzed by RT-PCR. One of 3 experiments is shown. +, cDNA from human macrophages stimulated with *M tuberculosis* for 4 hours (WNT5A); cDNA from PBMC stimulated with anti-CD3 for 4 hours (IL-2); -, no cDNA.

Results

Differential expression of genes of the WNT/FZD signaling pathway in human macrophages

A microarray-based gene-expression screen was performed on human primary macrophages stimulated with mycobacteria (*M tuberculosis* strain H37Rv, *M avium* strain SE01) or conserved bacterial structures (LPS, synthetic lipopeptide [Pam$_3$CSK$_4$]). In addition to a number of cytokines (eg, TNF-α, IL-1β), chemokines (eg, RANTES, MIP-1α), and signal-transducing molecules (eg, TRAF1, NF-kB1 [p105]), the gene encoding for one member of the WNT family, WNT5A, was found to be up-regulated by 3- to 5-fold in response to all stimuli (data not shown). Quantitative RT-PCR confirmed that on top of a low basal transcription, WNT5A mRNA expression in response to intact mycobacteria as well as synthetic lipopeptide and LPS was induced by 5- to 10-fold in human macrophages in a dose-dependent manner (Figure 1A). Two additional genes that encode for proteins involved in WNT signaling pathways also were found to be differentially expressed: dishevelled-3 (2- to 3-fold up-regulation) and *FRAT2* (3- to 15-fold down-regulation, depending on the microbial stimulus) (data not shown).

WNT5A mRNA expression is restricted to professional antigen-presenting cells

A similar induction of WNT5A mRNA expression in response to microbial stimuli also was observed in primary human monocytes and dendritic cells (data not shown). Under all conditions analyzed, up-regulation of WNT5A mRNA expression was apparent 2 hours after stimulation and persisted for 24 to 72 hours (Figure 1B). WNT5A mRNA expression by human macrophages was independently confirmed by in situ hybridization[25] with a WNT5A-specific probe in human monocyte-derived macrophages and alveolar macrophages in uninvolved (tumor-free) human lung tissue derived from tumor patients (Figure 2). Next we investigated WNT5A expression in human lymphocytes. To this end, the lymphocyte fraction of peripheral-blood mononuclear cells (PBMCs), which comprise T- and B-lymphocytes as well as NK cells, was analyzed by RT-PCR. Both in the absence or presence of polyclonal stimuli (eg, crosslinking CD3 and CD28, addition of phytohemagglutinin or

phorbol ester/calcium ionophore), there was no detectable WNT5A mRNA expression in the lymphocyte fraction, whereas IL-2 mRNA expression was readily induced (Figure 1C). These data indicate that activation-induced WNT5A expression in human blood–derived leukocytes is restricted to cells of the myeloid lineage.

WNT5A expression in macrophages is mediated by Toll-like receptors and the NF-κB signaling pathway

Receptors of the innate immune system that recognize conserved pathogen-associated molecular patterns (PAMPs) are called pattern recognition receptors (PRR). One family of PRR, the family of TLRs, mediates cell activation induced by defined conserved microbial ligands.[1] Intact mycobacteria and lipoproteins require TLR2 signaling to activate cells; activation by LPS is mediated by the TLR4/MD2 complex (reviewed in Medzhitov[1]). Our analyses of TLR signaling in human macrophages showed that inhibitory anti-TLR2 antibodies selectively blocked mycobacteria-induced induction of WNT5A mRNA expression (Figure 3A). Analogously, the addition of the synthetic tetra-acyl LPS precursor Ia (compound
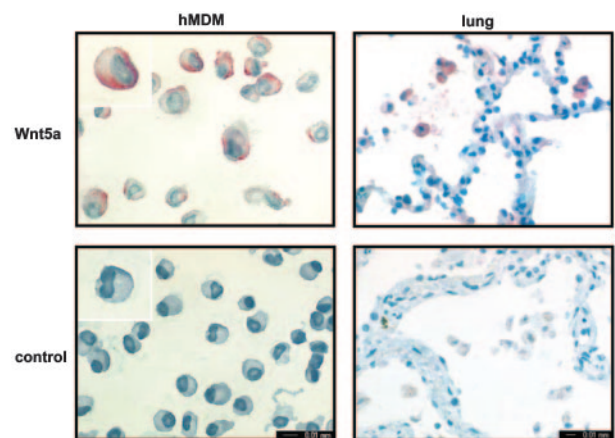
Figure 2. WNT5A expression in human monocyte-derived and alveolar macrophages. Qualitative detection of WNT5A mRNA expression by in situ hybridization with a WNT5A-specific probe (top row). Human monocyte-derived macrophages (hMDM) were analyzed after cultivation for 4 hours. Human uninvolved lung tissues obtained during tumor surgery (5 individuals; one representative shown) were analyzed. (Bottom row) In situ hybridization with a nonspecific scrambled DNA probe.

Figure 3. Induction of WNT5A expression is TLR and NF-κB dependent. Cells were stimulated for 4 hours. Total RNA was isolated, reverse transcribed, and analyzed by quantitative RT-PCR. (A, top) Human macrophages from 3 individual donors were stimulated with *M avium (M av)*, *M tuberculosis (M tb*; MOI 3), or LPS (10 ng/mL) in the presence of an anti-TLR2 antibody or an isotype control 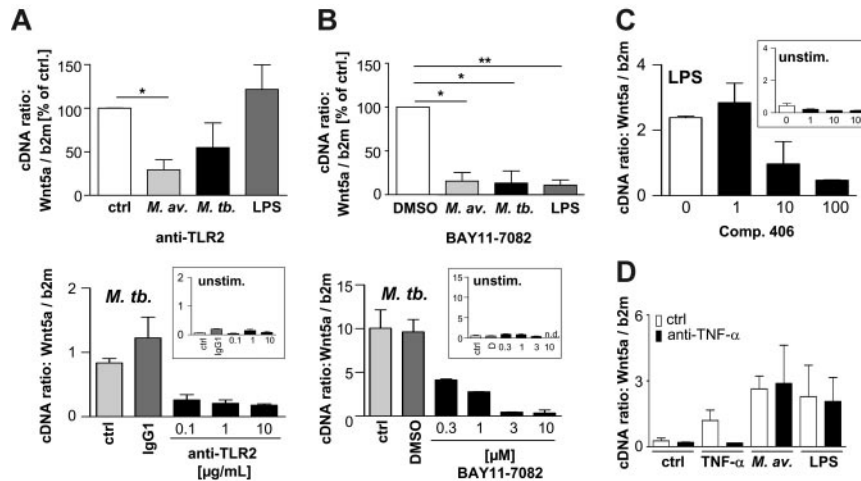antibody (10 μg/mL or as indicated). Statistical analysis was performed on data obtained from 3 independent experiments by normalizing WNT5A expression in the absence of antibody to 100% (ctrl) (*$P < .05$). (A, bottom) For the dose-response curve, means ± SD of duplicate determinations of 1 representative experiment of 3 is shown. (B, top) Human macrophages from 3 individual donors were stimulated with *M avium (M av)*, *M tuberculosis (M tb*; MOI 3), or LPS (10 ng/mL) in the presence of BAY11-7082 (inhibitor of IκB-α-phosphorylation), dimethyl sulfoxide (DMSO) as solvent control, or left untreated (ctrl), and WNT5A mRNA expression was analyzed. For statistical analyses, results from 3 individual donors in the presence of an optimal inhibitor concentration of 3 μM were compared to DMSO-treated cultures normalized to 100% (*$P < .05$; **$P < .005$). (C) Detection of LPS-induced (10 ng/mL) WNT5A expression in human macrophages in the presence of Compound 406 (1-100 ng/mL). Mean ± SD of duplicate determinations of 1 representative experiment of 3 is shown. (D) Human macrophages were stimulated for 4 hours with recombinant human TNF-α, LPS (each 10 ng/mL), or *M avium* (MOI 10) in the presence of anti–TNF-α antibodies (10 μg/mL). Total RNA was isolated, reverse transcribed, and analyzed for WNT5A expression. Mean ± SD of duplicate determination of 1 representative experiment of 3 are shown.

406), an antagonist of LPS-mediated activation in human monocytes,[27] dose-dependently inhibited LPS-induced WNT5A expression (Figure 3C). We infer that WNT5A expression induced in macrophages by intact mycobacteria or purified microbial structures critically depends on TLR-mediated signaling. This TLR-mediated induction of WNT5A in human macrophages represents a hitherto undescribed pathway of WNT induction.

We further analyzed whether cellular activation by TLR ligation might be sufficient to induce WNT5A expression. To this end, human embryonic kidney cells (HEK293) transfected with the human TLR2 (HEK293-TLR2) were stimulated with mycobacteria or synthetic lipopeptide and functionally compared to control cells. HEK293 cells lacking TLR2 expression responded to TNF-α, but not to bacterial stimulation, by secreting IL-8. TLR2-positive HEK293 cells additionally released IL-8 in response to mycobacteria and synthetic lipopeptide but not to LPS (Figure S1, available on the *Blood* website; see the Supplemental Figures link at the top of the online article). However, WNT5A expression in TLR2-transfected HEK293 cells was not further enhanced by stimulation with TNF-α or TLR2 agonists (Figure S1). This suggests that the presence of TLR2 alone may not be sufficient to regulate WNT5A expression and that the cellular background or differentiation status also plays an important role. This is further supported by our findings that WNT5A expression cannot be induced by bacterial stimuli in human myeloid premonocytic cell lines such as HL-60, U937, and MonoMac6 (data not shown).

TLR-mediated activation of antigen-presenting cells involves various signaling cascades, the NF-κB pathway being crucial for a broad spectrum of cellular responses that orchestrate the interplay between innate and adaptive immune responses.[1] To demonstrate that WNT5A mRNA expression in response to bacterial stimulation depends on the activation of NF-κB, human macrophages were incubated in the presence of a highly specific pharmacologic inhibitor of IkBα phosphorylation (BAY11-7082).[28] The inhibitor dose-dependently reduced WNT5A mRNA induction in response

to either TLR2-targeted (mycobacteria) or TLR4-targeted (LPS) stimuli (Figure 3B, and data not shown).

Early WNT5A expression in macrophages is not due to secondary effects induced by TNF-α

Macrophages react to bacterial infection by rapidly releasing proinflammatory cytokines, such as TNF-α. Recently, it has been shown that WNT5A and WNT10A expression in a gastric cancer cell line was promoted by TNF-α.[29] To investigate whether the observed WNT5A expression was primarily due to a secondary effect mediated by an early autocrine action of TNF-α, we cultivated human macrophages with TNF-α or mycobacteria in the presence or absence of inhibitory anti–TNF-α antibodies (Figure 3D). Anti–TNF-α antibodies completely abrogated TNF-α–induced WNT5A mRNA expression. However, the presence of inhibitory anti–TNF-α antibodies had no effect on WNT5A mRNA expression in human macrophages stimulated with either mycobacteria or LPS (Figure 3D). These results demonstrate that early induction of WNT5A mRNA by mycobacteria or LPS can occur independently of TNF-α.

Expression of the WNT5A receptor FZD5 by human monocytes/macrophages and lymphocytes

WNT proteins bind to 7-pass transmembrane receptors of the FZD family and initiate at least 3 different intracellular signaling pathways, resulting in the regulation of gene expression and/or changes in cell behavior.[30] FZD5 has been shown to be a functional receptor for WNT5A.[31] We therefore analyzed the presence of FZD5 in human macrophages and lymphocytes (Figure 4A,B). Quantitative RT-PCR showed a basal expression of FZD5 mRNA in human macrophages, which is up-regulated in response to mycobacteria (5-fold) and LPS (50-fold) 4 hours after stimulation. A substantial up-regulation (10-fold) also was observed in lymphocytes following stimulation by anti-CD3 or anti-CD3/anti-CD28
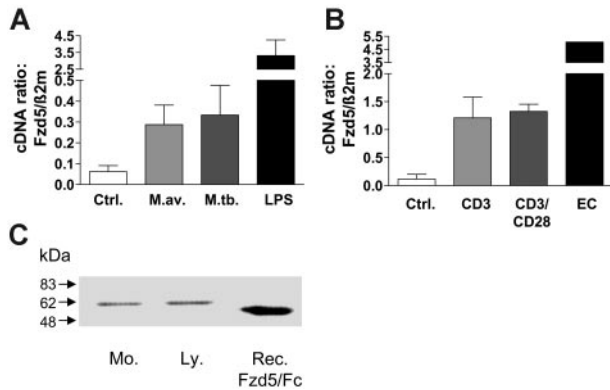
Figure 4. Expression of FZD5 in human monocyte-derived macrophages and lymphocytes. (A) Human macrophages were incubated in the absence or presence of *M avium* or *M tuberculosis* (MOI 3) or LPS (10 ng/mL) for 4 hours. Total RNA was isolated, reverse transcribed, and quantitative RT-PCR for detection of FZD5 mRNA expression was performed in duplicates. One representative experiment of 3 is shown. Ctrl indicates unstimulated macrophages. (B) Human lymphocytes were incubated for 4 hours in the absence or presence of anti-CD3 or anti-CD3/CD28 antibodies. Total RNA was isolated and used for quantitative RT-PCR as described. Ctrl indicates unstimulated lymphocytes; EC, cDNA from unstimulated human umbilical high venule endothelial cells (endothelial cells have been shown to express FZD5[55,67]; serving as positive control). (C) Protein detection of FZD5 in lysates of human immune cells. 1×10^6 monocytes (Mo.) resp. lymphocytes (Ly) were lysed, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the proteins transferred to a nitrocellulose membrane by Western blot.[66] Recombinant human FZD5/Fc chimeric protein (50 ng) served as control (rec. FZD5). After incubation with an anti-FZD5 antibody[20] blots were incubated with a peroxidase-conjugated goat-antirabbit secondary antibody, and visualization was performed by enhanced chemiluminescence.

antibodies. These data are the first to demonstrate that stimulation of human myeloid cells by Toll-like receptor agonists or T-cell stimulation induce transcription of the FZD5 gene.

To detect FZD5 at the protein level, a specific anti-FZD5 antibody was used.[20] A single band at 62 kDa was detected by Western blot in lysates of unstimulated human monocytes and lymphocytes (Figure 4C). A recombinant FZD5/Fc fusion protein was added as a positive control and was detected at around 55 kDa as described by the manufacturer. In macrophages stimulated with mycobacteria as well as with LPS, no detectable changes in FZD5 protein level were observed (data not shown). These data demonstrate that FZD5 is expressed by human monocytes/macrophages as well as lymphocytes. The presence of FZD5 protein in human mononuclear cells and its transcriptional regulation in response to stimulation suggest that Wnt proteins may exert direct functional activities on human immune cells.

WNT5A and FZD5 are present in granulomatous lesions from *M tuberculosis*–infected patients

We wished to determine whether WNT5A and FZD5 would be present at the site of macrophage–T-cell interaction in an in vivo infection scenario. The granuloma is the structural correlate of the host's strategy to successfully fight mycobacterial infection and is a site of ongoing activation and interaction between macrophages and lymphocytes.[32,33] We therefore analyzed the presence of WNT5A mRNA and FZD5 protein expression in granulomatous lesions of patients freshly diagnosed with pulmonary tuberculosis. Figure 5 depicts that FZD5 protein is detectable in various cells with mononuclear morphology using the antibody described in Figure 4. The arrows indicate that FZD5-positive cells are found predominantly in areas rich in mononuclear-cell infiltration associated with the granuloma. The parallel analysis of WNT5A mRNA expression by in situ hybridization showed mostly cells with a

larger cytoplasm, indicative of macrophage morphology, to be positive for WNT5A. These data demonstrate that WNT5A- and FZD5-expressing cells are present in mononuclear infiltrates at the site of infection in *M tuberculosis*–infected patients. The immunohistochemical analyses show that not all macrophages and lymphocytes are positive for FZD5 protein WNT5A mRNA. The specific characteristics of the FZD5- as well as the WNT5A-expressing subsets are currently unknown.

Selective inhibition of IL-12 and IFN-γ release by anti-WNT5A and anti-FZD5 antibodies

IFN-γ plays a central role in the cell-mediated immune response to intracellular infections by activating microbicidal defense mechanisms in macrophages.[34] To investigate a potential role for WNT5A at the interface between antigen-presenting cells and lymphocytes, we stimulated PBMCs of "purified protein derivative of *M tuberculosis*" (PPD)–reactive healthy donors with PPD[35] and analyzed the antigen-induced, T-cell–dependent IFN-γ production in the presence of a neutralizing anti-WNT5A antibody.[19] The PPD-induced release of IFN-γ was reduced in a dose-dependent manner by anti-WNT5A antibodies but not by isotype control antibodies (Figure 6A). The PPD-induced IL-2 and TNF-α release, as well as the proliferative response of human PBMCs, was not affected (Figure 6A, and data not shown). Analyses of the combined results from 3 independent donors showed that anti-WNT5A–mediated inhibition of the antigen-triggered IFN-γ response was reproducible and statistically significant (Figure 6B).

The induction of IFN-γ critically depends on the presence of IL-12 at the site of infection.[36] Figure 6B shows that anti-WNT5A antibodies significantly inhibit the mycobacteria-induced IL-12 release of human macrophages. A similar effect was observed when cells were stimulated with conserved bacterial structures such as LPS (data not shown). The formation of TNF-α and IL-8 in these experiments was not affected (data not shown), corroborating the specificity of the observed effect.

The same anti-WNT5A antibody was used to specifically detect WNT5A in lysates and supernatants of WNT5A overexpressing NIH-3T3 fibroblasts[37] (Figure S2). Attempts to demonstrate the presence of WNT5A at the protein level by Western blot in cultured primary human mononuclear cells were not successful, possibly
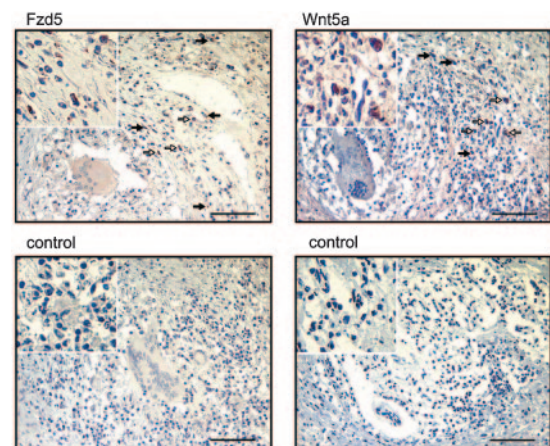
Figure 5. WNT5A and FZD5 expression in lung tissue of *M tuberculosis*–infected patients. Sections from lung biopsies of *M tuberculosis*–infected patients were stained for the presence of FZD5 protein (left column) and WNT5A mRNA (right column) as described in "Materials and methods." Arrows indicate a selection of FZD5 resp. WNT5A-positive cells. Inserts represent a 3-fold magnification of the areas with positive cells defined by white arrows. Bar = 100 μm.

Figure 6. WNT5A- and FZD5-dependent regulation of Th1 cytokine responses. (A) PBMCs of PPD-reactive donors were stimulated with PPD (10 μg/mL) i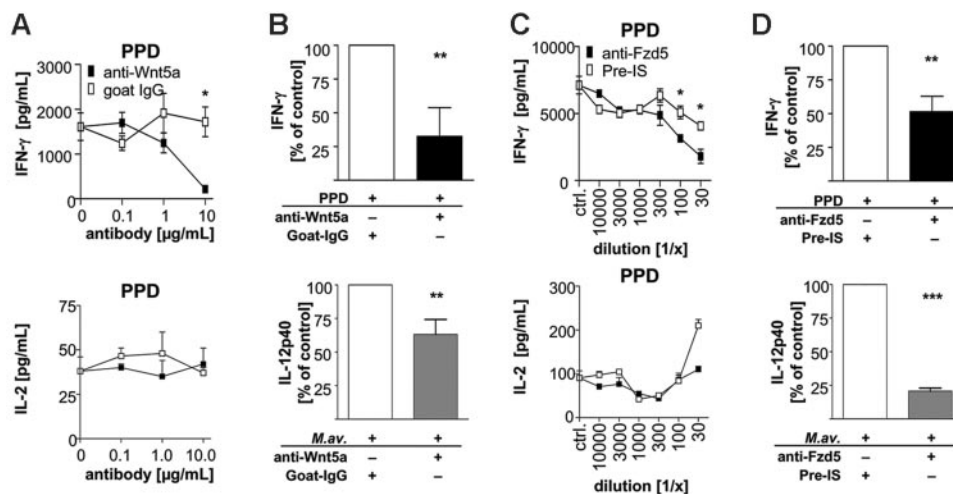n the presence of anti-Wnt5a or isotype-matched control antibodies. IFN-γ (96 hours) and IL-2 (24 hours) concentrations in supernatants were analyzed by ELISA. Stimulation was performed in triplicates: mean ± SD of 1 of 3 independent experiments are depicted. (B) PPD- or *M avium*–induced (10 μg/mL and MOI 3, respectively) IFN-γ and IL-12p40 release by PBMCs in the presence of optimal inhibitory anti-WNT5A antibody concentration (10 μg/mL) was determined. Three independent experiments using cells of individual donors were compared by normalizing control conditions (cultures treated with goat-IgG) to 100%. (C) PBMCs of PPD-reactive donors were stimulated with PPD (10 μg/mL) in the presence of an anti-FZD5 antiserum and preimmune serum. IFN-γ (96 hours) and IL-2 (24 hours) concentration in supernatants were analyzed by ELISA. Experiments were performed in triplicate; mean ± SD of 1 of 3 independent experiments are depicted. (D) PPD- or *M avium*–induced (10 μg/mL and MOI 3, respectively) IFN-γ and IL-12p40 release by PBMCs in the presence of optimal inhibitory dilution of the anti-FZD5 antiserum (1/100) were determined. Three independent experiments using cells of individual donors were compared by normalizing control conditions (cultures treated with preimmune serum) to 100% ($*P < .05$; $**P < .01$; $***P < .001$).

due to the limited sensitivity of the assay or a low level of overall WNT5A protein expression. It bears mentioning that the literature contains numerous studies confirming WNT protein expression in overexpressing systems; however, detection of endogenous WNT proteins in primary cells appears to be very difficult.[12,13]

The FZD5-specific antibody[20] used to demonstrate the protein expression of FZD5 (Figures 4C, 5) is directed against an intracellular proportion of FZD5 and can therefore not be used as an inhibitory agent to interfere with ligand receptor interactions. To investigate a potential functional role of FZD5 in the regulation of cytokine release, we generated an antiserum against the extracellular N-terminal cysteine-rich domain of FZD5, which is essential for WNT-ligand binding.[38] The specificity of the antiserum was measured in a competition ELISA (Figure S3). The PPD-induced IFN-γ release of PBMCs was dose-dependently and significantly decreased with increasing concentrations of this anti-FZD5 antiserum, when compared with the effect of the corresponding preimmune serum (Figure 6C,D). In contrast, the PPD-induced IL-2 and TNF-α release, as well as the proliferative response of PBMCs, was not influenced by the anti-FZD5 antiserum (Figure 6C, and data not shown). Further experiments addressing the effect of the anti-FZD5 antiserum on mycobacteria-induced IL-12 release by macrophages showed a significant inhibition of IL-12 production compared with cultures treated with the preimmune serum (Figure 6D). From our data we conclude that WNT5A and its receptor, FZD5, play an important role in the regulation of key cytokines of the antimicrobial defense.

Discussion

This study demonstrates that (1) WNT5A is expressed by human antigen-presenting cells in response to mycobacteria and conserved bacterial structures, (2) the expression of WNT5A is dependent on TLR signaling and the activation of the NF-κB pathway, (3) WNT5A and its receptor FZD5 are present on human mononuclear

cells in vitro and in vivo, and (4) WNT5A and FZD5 are functionally involved in the regulation of the human Th1 cytokine response, indicating their role in antimicrobial defenses.

Wingless, a morphogen in *Drosophila* species, and its phylogenetically highly conserved homologs (termed WNT) are critical regulators of cell polarity, motility, differentiation, apoptosis, and carcinogenesis in both nonvertebrates and vertebrates. In mammals, members of the WNT family (currently comprising 19 human and murine homologs) are involved in early T-cell differentiation and B-cell proliferation (reviewed by Staal and Clevers[39] and Reya et al[17]). Little is known about the expression and the potential function of WNT homologs under pathophysiologic conditions, such as in an inflammatory setting. In one report, transcripts for WNT1, 5A, 10B, 11, and 13 were detected in synovial fibroblasts from patients with rheumatoid arthritis.[23] WNT2 and WNT5A were detected in tumor-associated macrophages of the *Lamina propria* in colorectal cancer patients,[40] although the inducing stimulus was not determined. Recently, other investigators reported a differential expression of WNT5A in response to various microbial stimuli using microarrays for studying pathogen-induced gene-expression profiles of human macrophages and dendritic cells.[41,42] However, these findings were not confirmed by an independent method. To date, the role of WNT5A during the immune response, particularly as it relates to the defense against infections, has not been further characterized.

We identified WNT5A as a differentially expressed gene in response to mycobacteria (*M avium* and *M tuberculosis*). Further experiments demonstrated the induction of WNT5A expression after contact with various conserved microbial structures such as lipopolysaccharide and lipopeptide, which indicates that this molecule is commonly induced in myeloid cells in infectious processes. The identification of TLR2 and TLR4 in human macrophages as 2 important pattern recognition receptors initiating Wnt5a expression and the fact that the NF-κB pathway is critically involved in this process further corroborate the hypothesis that Wnt5a is part of a general signature in the immune response to

infections. Our analyses of various cell types indicate the activation-induced expression of WNT5A to be restricted to cells of the myeloid lineage such as monocytes, macrophages, and dendritic cells. Lymphocytes do not express WNT5A mRNA, even after stimulation by crosslinking CD3/CD28 or PMA/calcium ionophore. We therefore conclude that WNT5A expression is not an indicator of cellular activation in general, but a rather specific signal initiated by cells of the innate immune system in response to infectious agents. It will be interesting to elucidate whether specific triggering of other TLRs also leads to the induction of WNT homologs. However, it is noteworthy that TLR ligation itself is not sufficient to induce WNT5A expression, since stimulation of TLR-transfected HEK293 cells did not lead to an enhanced level of WNT5A mRNA expression. This suggests certain cell-type–specific requirements or environmental cues for the regulation of Wnt homolog expression.

In *Drosophila* species, wingless gene expression during embryonic development is induced by Hedgehog (Hh), a soluble factor, which binds to a 7-pass transmembrane receptor termed Patched (Ptc).[43] Homologs of Hh also have been identified in vertebrates. Cellular activation by Hh leads to the stabilization of the transcription factor Cubitus interruptus (Ci). Three homologs of the *ci*-gene were identified in vertebrates, all of which encode for Gli-proteins (from glioblastoma; reviewed in Ingham and McMahon[43]). The expression of WNT homologs in vertebrates can be induced by Hh-induced signaling and Gli-proteins.[44] Deletion of the murine Sonic hedgehog (Shh) led to a reduced Wnt5a expression during development of the genital organs.[45] A further direct relation between Shh and the regulation of Wnt5a expression was found in the morphogenesis of hair follicles.[46] Additionally, further Hh-independent mechanisms have been described to regulate Wnt5a expression. In a human epithelial cell line, a protein kinase C (PKC) and protein tyrosine kinase–dependent induction of WNT5A was observed.[47] The results of our present study clearly demonstrate a TLR-mediated, NF-κB–dependent mRNA expression of WNT5A in human macrophages. A direct relationship between TLR-induced signals and the expression of WNT homologs has not been described to date, neither in invertebrates nor in vertebrates. The current study therefore identifies a novel, thus far unknown, pathway leading to WNT5A expression.

TNF-α is an important mediator of the initiation and amplification of the immune response, for example, to intracellular pathogens. A recent report has demonstrated a role for TNF-α in the induction of WNT5A in fibroblasts in rheumatoid arthritis.[23] We extend this observation and show WNT5A induction by TNF-α in human macrophages. Using neutralizing anti–TNF-α antibodies, we also demonstrate that mycobacteria-induced, as well as LPS-induced, early induction of WNT5A is independent of TNF-α, excluding that the observed WNT5A expression in macrophages is due only to secondary stimulation by TNF-α. The fact that TNF-α alone is able to induce WNT5A may nevertheless be important, since it may contribute to augmenting WNT5A levels in inflamed tissue; for example, in granulomatous lesions. This hypothesis is confirmed by other studies that described a supportive effect of cytokines in the induction of WNT5A[29] and other WNT homologs (WNT10, WNT14).[48,49]

Studies on the presence of FZD receptors as potential interaction partners for WNT homologs on human immune cells have been focused primarily on their role in early T-cell development (reviewed in Staal and Clevers[39]). A recent report by Murphy and Hughes[50] described the expression of FZD5 mRNA by human T cells isolated from peripheral blood. The detection of FZD5 protein

in human mononuclear cells and its increased mRNA expression in macrophages and T cells in response to stimulation in vitro, as shown in our study, strongly suggest that FZD5 may have a function in immunologic responses. The presence of FZD5 on human immune cells further implies that WNT proteins may exert a direct functional activity on human immune cells in an autocrine or paracrine manner. Indeed, both WNT5A and FZD5 were found to be expressed under pathophysiologic conditions in vivo, in alveolar macrophages adjacent to tumor-bearing tissue (Figure 2), and in tuberculous granulomas (Figure 6), indicating a direct involvement in antimicrobial responses. The presence of the ligand and its receptor in granulomatous lesions of tuberculosis patients prompted us to investigate in which way WNT/FZD signaling might be important in the host response to mycobacterial infections.

The validation of a functional role for WNT5A and FZD5 in vivo, for example, in the well-established animal model of low-dose aerosol *M tuberculosis* infection in mice,[51,52] is hampered by the fact that mice deficient in WNT5A and FZD5 are severely compromised in development[53,54] and die in utero.[55] Therefore, we focused on a human in vitro model to investigate if the activation of antigen-presenting cells (IL-12-, TNF-α-, IL-8 release) and antigen-specific lymphocyte responses (IFN-γ and IL-2 release, and proliferation) were influenced by WNT5A and FZD5. The proliferation and cytokine response of lymphocytes in PBMCs of PPD-reactive individuals is based on the presence of memory T cells. These arise after initial contact with the antigen and react to restimulation with a substantial IFN-γ response,[56] whose initial priming is dependent on the presence of IL-12.[36]

Collectively, the data presented in our study demonstrate that both WNT5A and FZD5 are capable of regulating IL-12 and IFN-γ production in an antigen-driven setting. It is intriguing to see that either inhibition of the ligand or the receptor leads to the same phenotype regulating IL-12 and IFN-γ formation but does not affect IL-2, TNF-α, and IL-8 formation. Our results suggest that WNT5A mediates its effect via FZD5. However, it is possible that other FZD receptors also are involved in transmitting the WNT5A signal; for example, FZD4 or FZD2, both of which have been shown to act as receptors for WNT5A.[57,58] A potential regulatory function of WNT5A on cytokine expression is further supported by a recent observation that in patients with rheumatoid arthritis, WNT5A modulates IL-6 and IL-15 expression in synoviocytes.[26]

Based on the results of our present study, we would like to propose the following model of WNT5A and FZD5 as modulators of the interplay between antigen-presenting cells and T lymphocytes (Figure 7): in the context of an infectious process, WNT5A produced by antigen-presenting cells regulates the IL-12 response in an autocrine manner and thereby primes specifically activated T lymphocytes for IFN-γ release. In addition, lymphocytes also express FZD5, and therefore a paracrine effect of WNT5A on these cells directly regulating the IFN-γ response is possible. This scenario of a WNT protein modulating antimicrobial defenses has only recently been uncovered in the fruit fly, suggesting that this role has been conserved during evolution. Specifically, WntD was found to act as a negative feedback regulator of NF-κB–mediated induction of antimicrobial peptide secretion.[18] It therefore appears possible that different members of the WNT family of proteins might differentially act as positive or negative modulators of immune responses to infection.

The detailed molecular mechanism by which WNT5A and FZD5 regulate the cytokine release of primary human T cells and monocytes/macrophages are not yet clear: one scenario is the involvement of the so-called canonic signaling WNT signaling
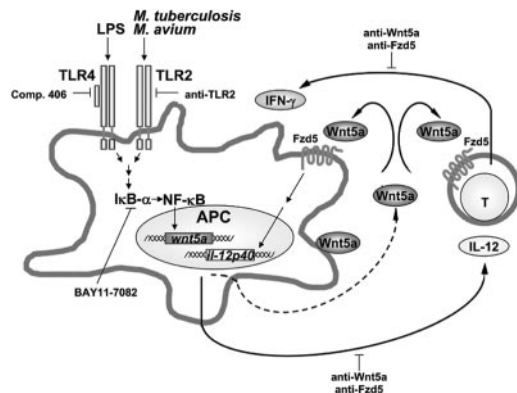
Figure 7. Model: WNT5A and FZD5 as modulators of the interplay between antigen-presenting cells and T lymphocytes. Toll-like receptor–induced, NF-κB–mediated activation of human antigen-presenting cells (APCs) leads to transcription of WNT5A, which, released or cell surface bound, is able to interact with FZD receptors (eg, FZD5). WNT5A and FZD5 regulate the microbial-induced IL-12 response in an autocrine manner and thereby prime specifically activated T lymphocytes for IFN-γ release. In addition, since lymphocytes also express FZD5, a paracrine effect of WNT5A on these cells directly regulating the IFN-γ response is possible. Reagents used to develop this model are indicated by ⊣ and ⊢ in the affected pathways.

pathway, which leads to the stabilization of β-catenin and subsequent activation of the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors (reviewed in Logan and Nusse[59]). A second pathway that affects planar-cell polarity (PCP), which involves the activation of JNK kinase, has been described (reviewed in Mlodzik[60]). The so-called noncanonic pathway leads to a rise in intracellular calcium concentrations and activation of Ca2+/calmodulin-dependent protein kinase II, which results in the activation and nuclear translocation of the transcription factor NF-AT (reviewed in Kühl et al[61]). WNT5A has been shown to induce axis formation via FZD5,[31] demonstrating its capacity to signal via the β-catenin pathway. In other systems WNT5A has been shown to act via the WNT/calcium pathway.[62,63] Cell-type–specific requirements, as well as the recently described differential recruitment of co-receptors,[64] may decide which of the above signaling pathways is activated by WNT5A. One may be tempted

to speculate that the WNT/calcium pathway plays a role in the regulation of cytokine responses, since several NF-AT binding sites have been described in the IFN-γ promoter.[65] Interestingly, a recent report shows that in human embryonic kidney cells WNT5A leads to activation of the mitogen-activated protein kinase (MAPK) pathway by activating TAK-1/NLK kinase, a kinase that also is a critical component of the TLR activation cascade involved in cytokine formation.[66] However, detailed future studies on the signaling pathways induced by WNT5A and FZD5 in human immune cells are necessary to elucidate the underlying mechanisms of WNT/FZD function in human differentiated immune cells.

A successful immune response to infection needs to be carefully choreographed. IL-12 and IFN-γ are critical mediators of cellular immunity to intracellular infections. We showed that WNT5A and FZD5, expressed by human mononuclear cells in response to microbial stimuli in a TLR- and NF-κB–dependent manner, are necessary for the regulation of IL-12 and IFN-γ in response to infectious agents. Both Toll and WNT signaling pathways are evolutionarily highly conserved and have only recently been found to intersect in *Drosophila*.[18] They may both have evolved to efficiently regulate the adaptive immune response in higher organisms. These findings, for the first time, implicate the phylogenetically conserved WNT/FZD system in bridging innate and adaptive immunity to infections.

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