Objective—Sepsis is a major cause of death for intensive care patients. High concentrations of inflammatory cytokines are characteristic of severe systemic inflammation and activated monocytes are their predominant cellular source. To identify targets for antiinflammatory intervention, we investigated the response of human macrophages to inflammatory and antiinflammatory mediators.

Methods and Results—We profiled gene expression in human macrophages exposed to lipopolysaccharide (LPS) and interferon (IFN)-γ in the presence or absence of recombinant activated protein C (APC) or IL-10 and identified Wnt5A as one of the transcripts most highly induced by LPS/IFN-γ and suppressed by APC and IL-10. We confirmed regulation of Wnt5A protein in macrophages and detected it in sera and bone marrow macrophages of patients with severe sepsis. We established that a functional Wnt5A/frizzled-5/CaMKII signaling pathway was essential for macrophage inflammatory activation. To prove the essential contribution of Wnt5A we measured inflammatory cytokines after stimulation with Wnt5A, silenced Wnt5A by siRNA, and blocked receptor binding with soluble Frizzled–related peptide-1 (sFRP1).

Conclusion—Wnt5A is critically involved in inflammatory macrophage signaling in sepsis and is a target for antiinflammatory mediators like APC or antagonists like sFRP1. (Arterioscler Thromb Vasc Biol. 2008;28:504-510)

Key Words: geneexpression • macrophages • activated protein C

Sepsis is a suspected or proven infection with a systemic inflammatory response. In severe sepsis, organ dysfunction also occurs and it is associated with a high mortality and morbidity. Severe sepsis still causes about 9.3% of all deaths in the USA.1,2

See accompanying article on page 400

During sepsis, the extent of plasma protein C depletion correlates with the severity of the outcome.3 In animal studies4 and clinical trials APC prevented death from severe sepsis or septic shock.5 Although this beneficial effect of APC is mostly ascribed to its anticoagulant properties, antiinflammatory effects of APC have also been proposed.6 The direct modulation of inflammation by APC has recently been described in gene expression profiling studies with human endothelial cells.7,8 Recently, recombinant human APC has been introduced as a therapeutic agent for treatment of patients with severe sepsis because of its unique anticoagulant and antiinflammatory properties; however, the exact mechanism of antiinflammatory action is still unknown.9

Macrophages play a central role in inflammation by responding to and releasing of numerous inflammatory cytokines and chemokines, leading to severe systemic inflammation and septic shock. However, the knowledge of antiinflammatory interactions on the level of monocytes/macrophages is scant. Therefore, we decided to expand our investigations on antiinflammatory effects of APC on this cellular system. In the present study, we were using a whole genome expression analysis approach, to define novel targets of APC in an in vitro model of inflammatory macrophage activation. Using probes obtained from human macrophages stimulated by INF-γ (IFN-γ) and endotoxin (LPS), we consistently found Wnt5A to be one of the genes induced by inflammatory stimuli and LPS which was blocked by APC at the transcriptional level.

Wnt5A is a member of the Wnt family of secreted signaling molecules, homologs of the Wingless proteins in Drosophila species.10 Wnt proteins are involved in embryonic development, in differentiation of white blood cells during mammalian hematopoiesis, and in tumorigenesis.11 The canonical Wnt signaling pathway controls target gene transcription via the central component β-catenin.12 Recently, regulation of β-catenin dependent gene transcription has been
elicitated, and in mammalian cell systems it predominantly involves Wnt3. However, in our proinflammatory activated human macrophage cellular system, Wnt3 expression was extremely low (see supplemental Table II), available online at http://atvb.ahajournals.org, and the canonical signaling pathway was not affected by either proinflammatory activation or by APC and IL-10. Recently, a member of the Drosophila Wnt protein family, WntD, has been linked to Toll/NF-κB signaling and shown to be involved in antibacterial defense against Listeria monocytogenes in a septic fly model. Furthermore, in a recent study Wnt5A and its receptor Frizzled-5 (FZD5) are involved in regulation of the response to microbial stimulation in human mononuclear phagocytes.

Here we show that Wnt5A acts through Ca2+/calmodulin-dependent protein kinase (CaMKII) and that this pathway contributes to the inflammatory response of human macrophages. APC and IL-10 modulate Wnt5A/CaMKII signaling in an antiinflammatory manner. The presence of high levels of Wnt5A in sera of patients with severe sepsis or septic shock and in activated macrophages from the bone marrow of septic shock patients suggests a critical role for Wnt5A in systemic inflammation and sepsis. Taken together, our study shows for the first time that, first, Wnt5A signaling is essential to the general inflammatory response of human macrophages and, second, APC acts antiinflammatory in activated human macrophages by interfering with Wnt5A signaling.

**Methods**

**Cell Culture**

Human PBMC derived macrophages were cultured as described in the supplement (available online at http://atvb.ahajournals.org).

**Gene Array Experiments**

Gene expression profiling was performed by competitive dual-color hybridization of complementary RNA probes on human 44K 60-mer oligonucleotide microarray chips (Agilent Technologies) as described in the supplement.

**Quantitative Real Time RT-PCR**

Real-Time PCR is described in the supplement.

**Antibodies**

The following antibodies were used for Western blotting and immunofluorescence: goat-anti-Wnt5A (1:1000, R&D Systems), rabbit-anti-FZD5 (1:1000, Abcam), rabbit-anti-CaMKII (1:1000, Abcam), rabbit-anti-active CaMKII (pT286; 1:500, Promega).

**Western Blotting**

Detection of FZD5 and CaMKII protein expression was performed by Western blotting and is described in the supplement.

**Immunohistochemistry and Immunofluorescence**

Details about immunohistochemistry and immunofluorescence experiments are given in the supplement. Fluorescent signal intensity was quantified using SigmaScan-Pro software (Systat-Software Inc).

**Quantitation of Secreted Cytokines**

Measurement of secreted IL-6, IL-8, IL-1β, and MIP-1β is described in the supplement.

**Immunoprecipitation of Wnt5A in Sera**

Detection of Wnt5A in the sera of septic patients and healthy individuals was performed by immunoprecipitation as described in the supplement.

**Generation of Small Interfering RNA (siRNA) and Transfection of Macrophages**

Wnt5A siRNA silencing experiments were performed as described in the supplement.

**Statistical Analysis**

Data were analyzed with the use of Graphpad-Prism version 4.0 statistical software. We used an unpaired 2-tailed Student t test or, for comparison of data among groups, 1-way ANOVA followed by the Newman–Keuls test. Probability values <0.05 were considered statistically significant.

**Results**

**Identification of Targets for the Antiinflammatory Action of IL-10 and APC by Gene Expression Profiling**

To define novel targets for antiinflammatory intervention, preferably at an early stage of the inflammatory response, we applied microarray based comparative transcriptome analysis in our model of monocyte derived macrophages.

The mRNA expression profiles of macrophages incubated with LPS/INFγ with or without IL-10 or APC for 8 hours were compared with the expression profiles of untreated macrophages (common reference, cultured in parallel) by competitive 2-color hybridization on human whole genome oligonucleotide array chips. The cytokine IL-10, which has well described antiinflammatory properties, was used to compare its effect with the suspected but not elucidated antiinflammatory action of APC in our setting of inflammatory activated macrophages. Analysis of experiments with macrophages from 3 different donors revealed a set of genes induced by LPS/INFγ (see supplemental Table I). Complete data are available in the ArrayExpress database with the following accession number, E-MEXP-927. In addition to several known inflammatory genes such as IL1β (87-fold induction compared with untreated cells), IL6 (15-fold), IL8 (3-fold), CCL2 (17-fold), and CCL4 (14-fold), Wnt5A was strongly induced by LPS/INFγ (79-fold, see supplemental Table I). Antiinflammatory stimuli reduced Wnt5A induction 2.7-fold by APC and 4.2-fold by IL-10 (supplemental Figure I). Furthermore, the FZD5 and CaMKII genes, which encode proteins involved in Wnt signaling pathways, were also differentially expressed. Both the genes for the receptor FDZ5 and for the signaling enzyme CaMKII are not upregulated by LPS/INFγ in presence of APC or IL10.

**IL-10 and APC Regulate the Wnt5A/Ca2+ Pathway at the mRNA Expression and Protein Level**

Quantitative RT-PCR verified that the expression and regulation of Wnt5A mRNA was induced by LPS/INFγ in human macrophages and that the level of induction of Wnt5A mRNA was reduced by the action of IL-10 and APC (Figure 1a). Transcriptional expression of Wnt5A was maximal at 8 hours in activated macrophage and declined rapidly within 24 to 48 hours to normal levels compared with unstimulated...
cells (data not shown). To test whether Wnt5A is also induced by signals targeting Toll-like receptors (TLR), we investigated a representative array of TLR agonists targeting TLR 1-9, and found that only PamCSK4 (TLR 1/2 agonist) and Imiquimod (TLR 7 agonist) did not induce Wnt5A mRNA transcription. All other TLR agonists induced Wnt5A expression more than 10-fold (Figure 1b). Immunocytochemistry was used to assess the levels of accumulated Wnt5A protein in activated macrophages (Figure 1c). After 24-hour incubation with LPS/INF-γ/H9253, Wnt5A protein expression had increased in macrophages compared with untreated control cells. In contrast, after treatment with LPS/INF-γ/H9253/IL-10 or LPS/INF-γ/H9253/APC Wnt5A protein expression was unchanged in macrophages compared with untreated control cells (see Figure 1d). This observation leads us to the assumption that Wnt5A expression is induced by LPS/INF-γ and that this effect is suppressed by IL-10 and APC.

Figure 1. Wnt5A expression is induced by LPS/INF-γ and TLR agonists and is blocked by IL-10 and APC. a and b, Wnt5A mRNA of unstimulated (control) or macrophages treated with the indicated stimuli. c, Immunofluorescence of Wnt5A protein in macrophages treated for 24 hours with the indicated stimuli. d, Quantitative fluorescence signal intensity of Wnt5A in control and activated macrophages. Detailed information to each figure is given in the supplement (available online at http://atvb.ahajournals.org).

FZD5 and CaMKII Are Present and Contribute to Wnt5A Signaling in Macrophages that Can Be Blocked by a sFRP1

Western blot analysis showed FZD5 and CaMKII expression in macrophages, providing further evidence that these important components of the Wnt5A signaling pathway are present (Figure 2a). FZD5 protein was present as a single band with a molecular weight of 250 kDa. There were no detectable differences in the amount of protein in response to different treatments. CaMKII was present as a single band with a molecular weight of 60 kDa. Although it was not possible to establish whether the amount of CaMKII protein increased in macrophages stimulated with LPS/INF-γ, CaMKII protein expression clearly decreased because of the action of APC. To address CaMKII activation by Wnt5A, we used a specific polyclonal antibody that recognizes only the phosphorylated form of CaMKII. Activation of CaMKII by Wnt5A and by LPS/INF-γ was confirmed by immunofluorescence in cultured macrophages (Figure 2b and 2c). To corroborate our evidence for the contribution of Wnt5A to the inflammatory response of human macrophages, we blocked Wnt5A signaling at the ligand/receptor stage. sFRP1 is a member of the sFRP family that contains a cysteine-rich domain homologous to the putative Wnt binding site of Frizzled proteins, and it can act as a soluble modulator of Wnt signaling by specifically binding to Wnt5A21. Incubating macrophages with sFRP1 in addition to LPS/INF-γ or recombinant Wnt5A reduced CaMKII phosphorylation, indicating that the Wnt5A signaling pathway was activated by inflammatory stimuli and that sFRP1 blocked this activation by preventing binding of Wnt5A to its receptor, FZD5 (Figure 2b and 2c).

Wnt5A Stimulates the Release of Proinflammatory Cytokines in Macrophages, and IL-10 or APC Block the Inflammatory Action of Wnt5A

The biologic response of macrophages to an inflammatory stimulus is the synthesis and secretion of an array of inflammatory cytokines and chemokines. In a next series of experiments we wanted to know whether Wnt5A itself could account for a comparable effect on inflammatory cytokine production as LPS/IFN-γ. To investigate the biologic response to Wnt5A we quantified the levels of proinflammatory cytokines IL-6, IL-8, IL-1β, and MIP-1β in supernatants of macrophage cultures treated for 24 hours with recombinant mouse Wnt5A. As shown in Figure 3, recombinant Wnt5A, expressed in a mammalian cell system and without detectable endotoxin concentrations (see method section), did indeed stimulate the release of proinflammatory cytokines in macrophages (black bars) in a comparable manner to cells that were stimulated with LPS/INF-γ (data not shown). Furthermore, IL-10 (gray bars) or APC (open bars) prevented the inflammatory cytokine release on stimulation with Wnt5A (Figure 3). There were again common and distinct effects of IL-10 and APC. The antiinflammatory effects of IL-10 and APC were comparably strong reducing IL-1 and MIP-1 production, whereas the effect of APC on IL-6 or IL-8 production was not as impressive as IL-10.
Blocking Wnt5A Signaling Influences the Expression of Inflammatory Cytokines

To examine the Wnt5A contribution to the inflammatory response in macrophages further, we generated small interfering RNA (siRNA) against Wnt5A by transcription and dicing of a human Wnt5A specific template and investigated whether inhibition by siRNA of Wnt5A transcription influences the transcription and secretion of inflammatory cytokines. Transfection of unstimulated macrophages with anti-Wnt5A siRNA decreased Wnt5A activity by 80%, but did not significantly affect macrophage viability (data not shown). However, in LPS/INF-γ stimulated macrophages, knockdown of Wnt5A significantly decreased transcription of Wnt5A (Figure 4a). To ensure specificity of the observed effect of siRNA directed against Wnt5A, we measured expression of the housekeeping gene HPRT and found no significant change (Figure 4a). Moreover, knockdown of Wnt5A significantly decreased transcription and secretion of the inflammatory cytokines IL-1β, IL-6, and IL-8 (Figure 4b). Transfection with siRNA directed against luciferase GL3 duplex had no effect on the response to LPS/INFγ compared with mock-transfected cells (data not shown). These results further support a mechanism by which Wnt5A influences the transcription and release of inflammatory cytokines. Because of our previous observation that sFRP1 blocked CaMKII phosphorylation induced by LPS/INFγ stimulation we incubated stimulated human macrophages with sFRP1. We verified that sFRP1 inhibited the release of IL-1β, IL-6, IL-8, and MIP-1β in macrophages cultured under inflammatory conditions, which confirms that Wnt5A signaling caused secretion of proinflammatory cytokines (Figure 4c).

Wnt5A Protein Is Present in the Serum and Bone Marrow of Patients With Severe Sepsis or Septic Shock

Because of the high levels of Wnt5A induced by inflammatory stimuli in vitro, we determined whether Wnt5A is present in vivo. Because Wnt5A is secreted, we suggested its presence in sera of septic patients. Thus we performed immunoprecipitation in archive sera samples from patients with severe sepsis or septic shock defined according to the criteria set forth by the 2003 International Sepsis Definitions Conference16 using a Wnt5A specific polyclonal antibody. Supernatants of transfected cells expressing and secreting large amounts of Wnt5A served as controls (Figure 5a). Densitometric analysis showed a distinct difference between the amounts of Wnt5A protein in patient samples compared with sera from healthy individuals (Figure 5b). Wnt5A concentration in sera of patients could be affected by kidney failure or disturbances of liver function, which occur often in patients with severe sepsis or septic shock. Serum creatinine concentrations were normal in all sera tested. In addition, neopterin, a marker of activated human macrophages, was significantly elevated only in the sera of sepsis patients (Figure 5b). The finding of elevated Wnt5A in sera of patients suggests an active role for secreted Wnt5A in the pathophysiology of sepsis. To confirm this observation, we demonstrated Wnt5A expression in macrophages of bone marrow samples of patients with fatal sepsis.17 Immunohistochemical analysis showed that there was intense staining for Wnt5A in hemophagocytic macrophages in these patients compared with bone marrow from healthy individuals. Again, sections of Wnt5A expressing cells and L-cells served as positive and negative controls, respectively, as they were fixed and stained in the same manner. The intensity of Wnt5A staining in the untransfected L-cells (background staining) was comparable to that found in bone marrow sections of healthy individuals (Figure 5c).
Discussion

In this study we found that expression of Wnt5A is pivotal in a pathway involved in sustained inflammatory macrophage activation during sepsis. Our initial studies aimed to identify novel targets of the antiinflammatory activity of APC in pharmacological doses used in sepsis patients, in macrophages.

Beyond modulation of coagulation activation, the pleiotropic antiinflammatory activities of APC that are directed to different cellular targets are proposed to contribute to the uniquely beneficial activity of this agent in patients with severe sepsis. In a genome wide transcriptional screen of macrophages stimulated with LPS and IFN-γ, we have identified Wnt5A as one of the most highly induced genes. Suppression of inflammation-driven expression by APC and IL-10 implicated Wnt5A in an active role in the inflammatory response. Both the inflammatory driven transcriptional expression of Wnt5A and its suppression by APC and IL-10 were confirmed by independent methods at the mRNA and protein levels. Furthermore, high levels of immunoprecipitatable Wnt5A in sera from patients with severe sepsis provided direct evidence of an active role for secreted Wnt5A in the pathophysiology of the systemic inflammatory response during sepsis. Although our studies did not intend to identify the cellular source of Wnt5A secretion during sepsis, demonstration of Wnt5A positive macrophages within the bone marrow of patients with fatal sepsis suggests that activated macrophages play an important role in this disease setting.

Recently a distinct role for Wnt family members in inflammation and immunity has been recognized. The Drosophila Wnt protein family member WntD regulates the innate immune response to infection in the fly. In these studies, WntD expression is shown to be controlled by Toll signaling, a highly conserved pathway known to induce antimicrobial and inflammatory responses also in the human macrophage. Furthermore, WntD signaling in the fly was independent of the common β-catenin pathway, as it is the case in the inflammatory human macrophage system described herein. Moreover, WntD was identified as the first secreted regulator of Toll signaling in the fly. This is in line with an earlier study that described Wnt5A being secreted by activated antigen presenting cells and by inflammatory synoviocytes from rheumatoid arthritis joints. This was the first report of Wnt5A expression associated with an inflammation in humans. However, it became unclear in this study how Wnt5A expression was linked with inflammatory cytokine production, because the signaling of Wnt5A through FFZD5 was not delineated. More recently, Wnt5A expression is shown to be induced by mycobacterial cell wall components and endotoxin in human antigen presenting cells and is dependent on activation of the central inflammatory regulator NF-κB. In this report it is clearly shown that Wnt5A expression required Toll-like receptor (TLR)2 and TLR4-dependent signaling. This finding is completely in line with the finding of TLR-dependent WntD expression in the fly, indicating that these pathways are
intracellular level of β-catenin. This canonical pathway is the most intensively studied pathway of Wnt action in cell development and differentiation. We were unable to detect any evidence for canonical signaling in our inflammatory activated macrophages. The noncanonical Wnt signaling pathway elucidated in the present study induced a rise in intracellular calcium levels and activated CaMKII, which, among other effects, leads to activation and nuclear translocation of the transcription factor NF-AT.20 Cell specific characteristics and the coreceptor environment in particular determine which of these pathways are activated on ligation of a frizzled receptor with its Wnt ligand. Ca2+ release and the associated phosphorylation of CamKII are extensively studied signaling events that are critically involved in macrophage activation.21,22 Our finding that recombinant Wnt5A increased phosphorylation of CamKII provides experimental evidence that Wnt5A is capable of activating the proinflammatory Ca2+/CamKII pathways in macrophages. This effect is completely abrogated by sFRP1, a soluble Wnt binding protein that specifically binds to and impairs Wnt5A interaction with its cognate cell surface receptor.18 This implies that a specific Wnt5A/FDZ5 interaction mediates the Wnt5A activity observed in our experiments and excludes nonspecific effects imparted by small molecular contaminants such as endotoxin. Again, this is fully in line with the findings of others that FDZ proteins are receptors for Wnt and that Wnt signaling is modulated by the specific cysteine-rich domain of soluble frizzled related peptides.18 Our finding, that sFRP1 also inhibited the LPS/IFN-γ-induced CamKII phosphorylation, implies a causal link between Wnt5A secretion triggered by inflammation and LPS/IFN-γ-induced macrophage activation. Again, this Wnt5A/FDZ5 mediated activation of the noncanonical signaling pathway may thus act as a positive regulatory mechanism that sustains and enhances inflammatory macrophage activation induced by exogenous proinflammatory agents. Our finding that Wnt5A activates the noncanonical signaling pathway in macrophages is compatible with its capacity to exert inflammatory effects in rheumatoid synoviocytes and in antigen presenting cells. Furthermore, treatment of macrophages with exogenous Wnt5A initiated secretion of inflammatory cytokines in our study. However, the subsequent observation that Wnt5A knockdown by siRNA significantly decreased the transcription and secretion of inflammatory cytokines and that inhibition of autocrine Wnt5A signaling by sFRP1 almost completely reversed LPS/INFγ-induced cytokine secretion suggests that Wnt5A is not just another inflammatory macrophage activator but also a pivotal regulator of macrophage activation during inflammation. In light of the findings in the septic fly model, where Wnt is critically involved in the control of infection, the findings of induced Wnt5A on mycobacterial infection of human monocytes, and the identification of Wnt5A in inflamed synoviocytes of rheumatoid arthritis, our present findings suggest that Wnt5A is a highly specific autocrinous and paracринous macrophage-derived effector molecule triggering inflammation through a well defined pathway. Therefore, our results suggest that the Wnt5A pathway is an attractive candidate target for therapeutic intervention in inflammatory diseases such as sepsis or rheumatoid arthritis. First, significant amounts of Wnt5A are secreted in septic patients. One could well assume from this finding and from the observations of Wnt5A being released by

Figure 5. Wnt5A protein is present in the serum and bone marrow of patients with severe sepsis or septic shock. a, Immunoprecipitation of Wnt5A in serum from patients with sepsis (lanes 5–8) and from healthy individuals (lanes 1–4). Lane 9 shows Wnt5A conditioned medium (Wnt5A-CM) that was immunoprecipitated following the same protocol. b, Left graph, densitometric analysis of bands detected by immunoprecipitation. c, Wnt5A in sections from bone marrow biopsies of patients with fatal sepsis and from healthy individuals was detected by immunohistochemistry as described in Methods.

linked together and are highly conserved throughout species. However, it was not elucidated whether activation of other TLRs induce Wnt5A or other Wnt homologues.15 Our study extends the idea that Wnt5A secretion constitutes a highly conserved response to inflammatory macrophage activation by demonstrating that Wnt5A mRNA is not only induced by LPS and the classical macrophage stimulator INFγ, but also by a representative array of TLR agonists targeting TLR 1-9.

After secretion, Wnt5A is proposed to act by ligation to its receptor FDZ5, which we and others have shown to be expressed in human macrophages.13 It was, however, not clear which pathway of Wnt5A signaling was involved in macrophages in the setting of inflammatory activation. Different signaling pathways may be triggered subsequently by Wnt5A/FDZ5. The canonical signaling pathway acts by enhancing the
synoviocytes\(^1^9\) that Wnt5A is elevated also in the sera of rheumatoid arthritis patients. Second, interference with the Wnt5A signaling pathway acts by downregulation of Wnt5A expression and controlling inflammation at one of its critical checkpoints, partial suppression of Wnt antagonists might be a valuable strategy for modulating Wnt signaling. Although our results suggest that the development of Wnt antagonists might be a valuable strategy for achieving in vivo suppression of inflammatory Wnt signaling. Our results suggest that the development of Wnt antagonists might be a valuable strategy for controlling inflammation at one of its critical checkpoints, partial Wnt control during sepsis may yet be achieved by treatment with APC, which acts by downregulation of Wnt5A expression and thus by modulating inflammatory Wnt activity.

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Disclosures

None.

References

Wnt5A/CaMKII Signaling Contributes to the Inflammatory Response of Macrophages and Is a Target for the Antinflammatory Action of Activated Protein C and Interleukin-10

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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Cell culture
Human macrophages were prepared from buffy coats of healthy blood donors (Swiss Red Cross, Zurich, Switzerland), by a strictly standardized protocol. Briefly, after separation by Ficoll gradient (Ficoll-Paque™ PLUS, Amersham Biosciences) and three washes with Mg²⁺/Ca²⁺-free phosphate-buffered saline (PBS; Gibco Europe), cells were suspended in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen Europe) supplemented with 10% heat-inactivated pooled human serum (Human Serum, off the clot; PAA, Austria) and seeded at a density of 10⁷ cells/mL in 6-well tissue culture plates (Falcon Oxnard, USA). Macrophages were obtained at a purity of >98% (determined by Giemsa staining) after 2 h incubation under standard cell culture conditions (37°C, 5% CO₂) in a SteriCult tissue culture incubator (Forma Scientific, USA). The cells were washed four times in warmed Gey’s balanced salt solution and incubated for 24 h as before. Medium was then replaced by IMDM supplemented with 2% human pooled serum and cells were cultured for 48 h. For the last 8 h or 24 h cells received the following treatments prior to mRNA or protein expression analysis respectively: 100 U/mL human recombinant IFNγ, 5 ng/mL IL-10 (both from PeproTech, Rocky, Hill, USA); 10 ng/mL LPS (from Escherichia coli 055:B5; Difco Laboratories, Detroit, USA); 5 μg/mL human recombinant APC (Drotregocin alpha activated; Xigris™, Eli Lilly, Switzerland); 250 ng/mL recombinant mouse Wnt5A, 10 μg/mL recombinant human soluble frizzled-related peptide-1 (sFRP1: both expressed in chinese hamster ovary cells, no endotoxin detectable, from R&D Systems Europe Ltd., Abington, UK); and human TLR 1–9 agonist, 5 μM oligodeoxynucleotides (ODN), 0.5 μg/mL ssRNA40/LyoVec, 0.5 μg/mL Imiquimod, 500 ng/mL FSL1, 5 μg/mL Flagellin, 25 μg/mL Poly I:C, 10⁸ cells/mL HKLM and 0.5 μg/mL PamCSK4, 10 ng/mL E.coli K12 LPS (tlrl-kit, InvivoGen, USA).

DNA microarray hybridization and analysis
Differential gene expression profiling of human macrophages was performed by competitive dual-color hybridization of cRNA probes with untreated and treated cells cultured for 48 h on human 44k 60-mer oligonucleotide microarray chips (Agilent Technologies Schweiz AG, Basel, Switzerland). Total RNA was purified using the RNeasy Mini Kit protocol (Qiagen AG, Hombrechtikon, Switzerland) with on-column
DNase digestion according to the manufacturer’s instructions. For quality control purposes and to quantify samples, each RNA sample (1 μL) was analyzed on RNA 6000 Nanochips using a Bioanalyzer 2100 instrument and 2100 Expert software (Agilent Technologies,). High quality total RNA typically had an 18/28S ribosomal RNA ratio >1.5 and was stored frozen in aliquots at −70°C. Labeled cRNA probes were synthesized from 1 μg total RNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) and cyanine-3-CTP or cyanine-5-CTP (Amersham Biosciences Europe GmbH, Freiburg, Germany). Labeled cRNA probes were purified on RNeasy Mini Spin Columns (Qiagen) and quantified in a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Anaheim, USA). Equal quantities of Cy3- and Cy5-labeled probes (1 μg of each) were mixed, spiked with control targets and incubated in fragmentation buffer for 30 min at 60°C in the dark. After fragmentation, the gasket slides were completely filled with the respective probe mixtures. The hybridization chambers were assembled with Human 1A Oligo Microarray slides (Agilent Technologies) and hybridized for 17 h at 60°C in the dark with mixing at 4 rpm. After washing twice in SSPE/N-Lauroylsarcosine for 1 min, the slides were carefully washed in acetonitrile for exactly 30 sec and dried. Finally, slides were directly scanned using a dual-laser microarray scanner and analyzed with Feature Extraction software (Agilent Technologies). Data mining was achieved using the Rosetta Resolver Gene Expression Data Analysis System (Rosetta Biosoftware, Seattle, USA, www.rosettabio.com).

RNA isolation and quantitative real-time reverse transcription PCR (RT-PCR)

Total cellular RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Basel, Switzerland), which included a DNase digest. Total RNA was quantified spectrophotometrically and equal amounts (5 μg) were transcribed into cDNA with oligo(d)T primers and StrataScript RT Reverse Transcriptase using the StrataScript First-Strand Synthesis System (Stratagene, Rotkreuz, Switzerland). Duplicates of cDNA were amplified by RT-PCR with gene-specific primers using the 7500 Fast Real-Time PCR system (Applied Biosystems, Inc., Rotkreuz, Switzerland) and the Power SYBR Green Master Mix (Applied Biosystems). Sequence-specific primers were selected using Primer Express v2.0 software (Applied Biosystems). The following primers were employed for Wnt5A and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Wnt5A forward, 5’-AGT TGC CTA CCC TAG C-3’; Wnt5A
reverse, 5'-GTG CCT TCG TGC CTA T-3'; GAPDH forward, 5'-AAC AGC GAC ACC CAC TCC TC-3'; GAPDH reverse, 5'-GGA GGG GAG ATT CAG TGT GGT-3'. Primers for cytokines were as follows: IL1β-forward, 5'-CAG AAA ACA TGC CCG T-3', IL1β reverse, 5'GCA CTA CCC TAA GGC AG-3', IL6 forward, 5'-CCT GAC CCA ACC ACA AA-3', IL6 reverse, 5'-AGT GTC CTA ACG CTC ATA C-3', IL8 forward, 5'-AGA CAG CAG AGC ACA CAA GC-3, IL8 reverse, 5'-ATG GTT CCT TCC GGT GGT-3', HPRT forward, 5'-GAC TGT AGA TTT TAT CAG ACT GA-3', HPRT reverse, 5'-TGG ATT ATA CTG CCT GAC CAA-3'. PCR was carried out with an initial denaturation step (10 min, 95°C) followed by 40 cycles of denaturation (15 sec, 95°C), annealing (30 sec, 55°C), and extension (30 sec, 72°C). Fluorescence was measured at the end of each extension. Relative mRNA levels were quantified by RQ Study SDS Software v1.3.1 (Applied Biosystems) using the comparative Ct method. The expression level of each gene was normalized to GAPDH levels in each experimental sample. Final data were expressed as mRNA expression in treated cells relative to expression in untreated cells. A melting curve analysis was performed for each amplicon to verify the specificity of each amplification step.

**Western blotting**

For assaying FZD5 and CaMKII protein expression, cells were lysed with Mammalian Cell Lysis/Extraction reagent (Sigma-Aldrich Chemical Co., Buchs, Switzerland) supplemented with complete mini protease inhibitor cocktail tablets (Roche Diagnostics Schweiz AG, Rotkreuz, Switzerland). After clearing the lysates by high-speed centrifugation, protein concentrations of each sample were determined using a Protein Bradford assay (Bio-Rad Laboratories AG, Reinach, Switzerland). For immunoblotting, 20 μg total protein of each sample was resolved on SDS 4–15% gradient polyacrylamide gels and transferred to PVDF membrane (Millipore AG, Zug, Switzerland). After transfer, the membranes were incubated for 1 h in blocking solution (5% non-fat milk in PBS containing 0.1% Tween-20) and then 1 h with the respective antibody in blocking solution. Antibody binding was detected with a horseradish peroxidase-coupled donkey-anti rabbit secondary antibody diluted 1:10’000 followed by enhanced chemiluminescence (ECL) detection (ECL Plus, Amersham Pharmacia Biotech, Inc., Uppsala, Sweden).
Immunofluorescence staining

For immunofluorescent microscopic analysis of Wnt5A protein expression, macrophages were grown on sterile, endotoxin-free 12 mm glass coverslips pretreated with poly-D-Lysine (Invitrogen AG, Basel, Switzerland). In brief, 100 µL of a 2 x 10^8 cells/mL mononuclear cell suspension were seeded on coverslips in 24-well cluster plates, incubated in IMDM/10% pooled human serum for 2 h under standard cell culture conditions, washed and cultured for 48 h with and without stimuli as described above. After incubation, coverslips were fixed with 3% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100. The coverslips were then exposed to primary antibodies for 60 min, followed by Alexa 594 labeled rabbit anti-goat or Alexa 488 labeled goat anti-rabbit (1:1000: Molecular Probes, Invitrogen Europe), for 60 min. Coverslips were washed three times with PBS, pH 7.4, after each antibody incubation. Nuclei were counterstained with 10 μg/mL diamidino-phenylindole (DAPI; Sigma-Aldrich). Images were taken with an Axioskop 2 microscopy system equipped with an AxioCam MRc digital camera using AxioVision v4.4 software (Carl Zeiss, Feldbach, Switzerland). For quantification of immunofluorescent signals, 5 images were registered for each treatment and were analysed using SigmaScan Pro software (San Jose, CA, USA).

Immunohistochemical staining

Bone marrow samples were retrieved from the archives of the Department of Pathology of the University Hospital Zurich. They consisted of autopsy samples from patients that had died from severe sepsis or septic shock according to the criteria set forth by the 2003 International Sepsis Definitions Conference. Autopsy samples from patients that had died from gun-shot suicides (age 31–88 yr) served as controls. Microtome sections (4 μm) were cut from formalin-fixed, decalcified and paraffin-embedded marrow samples. Immunohistochemistry was performed on an automated immunohistochemistry module (Bond (TM), Vision BioSystems., Newcastle Upon Tyne, UK) using affinity purified WNT5A polyclonal goat antisera (R&D Systems, Minneapolis; USA), diluted 1:120 for 60 min followed by the Bond polymer system using diaminobenzidine for visualization. Pretreatment for heat induced epitope retrieval was carried out for 30 min with the EDTA based pH8.8 Bond Epitope Retrieval Solution 2 (Vision BioSystems). Nuclei were counterstained with hematoxylin for bright-field microscopy. Cross-species experiments were performed.
to rule out the possibility of non-specific binding of the secondary antibodies. As additional positive and negative controls for Wnt5A immunohistochemistry, we used sections prepared from a 1:10 mixture of regular L-cells (no Wnt5A overexpression) and Wnt5A transfected L-cells (ATCC, CRL-2648/CRL-2814). Cell pellets were formalin-fixed, paraffin-embedded and stained in parallel to the human bone marrow samples.

Quantitation of secreted cytokines
The inflammatory cytokines IL-1β, IL-6, IL-8, and MIP-1β were quantified in undiluted cell culture supernatants collected 24 h after treatment using the Bio-Plex Human Cytokine Multiplex Assay on the Bio-Plex 2200 platform according to the manufacturer’s instructions (Bio-Rad, Hercules, USA). After preparation and culture in IMDM/10% human serum for 24 h, medium was changed to IMDM/2% human serum and cells were treated for an additional 24 h with the stimuli outlined above (see Cell culture section). Complete medium containing 2% human serum was used as a blank. Data were analyzed on the Bio-Plex Reader using Bio-Plex v3.0 software (Bio-Rad, Hercules, USA).

Immunoprecipitation of Wnt5A in sera
Archived serum samples from patients with severe sepsis or septic shock (as above according to the criteria set forth by the 2003 International Sepsis Definitions Conference¹, n = 5; average age 34 years, range 18–62) and from healthy individuals (n = 5; average age 30 years, range 25–45) were stored frozen at –70°C until analysis. None of the patients with sepsis or septic shock had received APC (Drotregocin alpha activated; Xigris™, Eli Lilly, Switzerland).
Aliquots of sera (300 µL) from patients and healthy individuals were incubated with 2 µg of anti-Wnt5A (R&D Systems Europe Ltd., Abington, UK) overnight at 4°C and then adsorbed to protein G beads (Sigma-Aldrich) overnight at 4°C. Samples were washed twice with 1 x IP buffer (Sigma-Aldrich) containing 0.5 M NaCl, four times with 1 x IP buffer and once with 0.1 x IP buffer. Finally, samples were eluted with Laemmli sample buffer (Bio-Rad). The eluate was analyzed by 10% SDS–polyacrylamide gel electrophoresis and Western blot onto PVDF membranes (Millipore) using the same anti-wnt5A antibody and chemiluminometric detection as described above.
To investigate the inflammatory status of the sera and to exclude impaired clearance of Wnt5A, due to renal insufficiency in patients with sepsis we analyzed neopterin, a common inflammatory metabolite released by activated macrophages and serum creatinine. Neopterin was measured by HPLC for pteridins after oxidation as described previously. Creatinine was measured by ISO certified analysis using kinetic Jaffe reaction and a Beckman Unicel DXC 600 analyser. Protein was measured by Protein Bradford assay (BioRad).

**Generation of small interfering RNA (siRNA) and transfection of macrophages.** Template DNA probe specific for human Wnt5A (RZPDP3000B068D) was obtained from RZPD (Berlin, Germany). From this probe, double-stranded RNA was synthesized with T7 enzyme, and 22 bp small interfering RNA probes (siRNA) directed against Wnt5A were prepared by dicing using the X-tremeGENE siRNA Dicer kit (Roche Diagnostics, Germany) according to the manufacturer’s instructions. The siRNA was column-purified and RNA concentrations were measured with a NanoDrop spectrophotometer. Human macrophages were transfected for 5 hr at 37°C with 2.0 μg siRNA with the X-tremeGENE siRNA transfection reagent (Roche Diagnostics) in IMDM with 10% human pooled serum. Cells were then washed and incubated in IMDM/10% human pooled serum with 100 U/mL INFγ and 1 ng/mL LPS for 8 h (optimal time for mRNA expression) or 24 h (optimal time point for protein secretion). siRNA directed against luciferase GL3 duplex (Dharmacon, Lafayette, USA) served as both transfection and negative control. To ensure specificity of the observed effect of siRNA directed against Wnt5A, we measured expression of the housekeeping gene HPRT using the primers and PCR conditions as described above.

**References**

FIGURE I

Figure I. Gene expression profile analysis of human macrophages.
Human macrophages were cultured as described in Methods. They were treated with LPS (10 ng/mL) and INFγ (100 U/mL) for the last 8 h in culture and the differential mRNA transcription pattern was characterized by oligonucleotide microarray with Agilent 44k gene chips and analysis using Rosetta Biosoftware as described in supplemental material. The ratio plot displays combined results from three
independent experiments (mean ± SD of selected genes). The x-axis (Log intensity) shows baseline gene expression and the y-axis (Log ratio) shows the ratio of the signal intensities in the two hybridized samples. Individual genes are represented as blue (significantly upregulated, \( p \leq 0.001 \)), green (significantly downregulated, \( p \leq 0.001 \)). Selected genes (Wnt5A, CaMKII and FZD5) are shown in red. (b) Re-ratio plot of human macrophages treated with LPS/INF\( \gamma \) and IL-10 (5 ng/mL) versus human macrophages treated with LPS/INF\( \gamma \). Plots represent combined results from three independent experiments, each. The re-ratio analysis tool of Rosetta Biosoftware was used for direct comparison between two samples that were hybridized against a common reference. (c) Re-ratio plot of human macrophages treated with LPS/INF\( \gamma \) and APC (5 \( \mu \)g/mL) versus human macrophages treated with LPS/INF\( \gamma \) for 8h.
Table I
Selected genes related to inflammation that are upregulated in human macrophages treated with LPS/INFγ. Influence of IL10 or APC on the expression of these genes.

<table>
<thead>
<tr>
<th>Sequence Name(s)</th>
<th>Accession #</th>
<th>Sequence Description</th>
<th>LPS/INFγ vs Untreated control</th>
<th>LPS/INFγ/IL10 vs LPS/INFγ#</th>
<th>LPS/INFγ/APC vs LPS/INFγ#</th>
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<td>IL1B</td>
<td>NM_000576</td>
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Fold changes (up or down) of gene expression were calculated using the ratio or re-ratio# function of Rosetta Biosoftware that allows direct comparison between two samples that were both hybridized against a common reference. Data are from three independent experiments, p < 0.001.
Table II

Wnt family members and Wnt pathway related gene expression in human macrophages treated with LPS/INFγ versus untreated cells (p<0.001)

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<td>Homo sapiens wingless-type MMTV integration site family, member 5A</td>
<td>+79.2</td>
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Figure 1. Wnt5A expression is induced by LPS/INFγ and TLR agonists and is blocked by IL-10 and APC.

(a) mRNA expression of Wnt5A measured by quantitative RT-PCR. Macrophages were unstimulated (control) or treated with LPS, INFγ, IL-10, and APC, as indicated. Changes in mRNA expression were normalized to changes in GAPDH expression and are expressed as mean ± SD from three independent experiments. (b) mRNA expression of Wnt5A measured by quantitative RT-PCR. Macrophages were unstimulated (control) or treated with TLR agonists as follows: 5 μM oligodeoxynucleotides (ODN, TLR9), 0.5 μg/mL ssRNA40/LyoVec (TLR8), 0.5 μg/mL Imiquimod (TLR7), 500 ng/mL FSL1 (TLR6/2), 5 μg/mL Flagellin (TLR5), 25 μg/mL Poly I:C (TLR3), 10^8 cells/mL HKLM (TLR2), and 0.5 μg/mL PamCSK4 (TLR1/2), 10 ng/mL E.coli K12 LPS (TLR4), (tlrl-kit, InvivoGen, USA). Data is expressed as described in (a). (c) Immunofluorescence staining of Wnt5A protein in macrophages. Cells grown on coverslips were treated for 24 h with LPS/INFγ, LPS/INFγ/IL-10 or LPS/INFγ/APC, in the same concentrations as used for microarray experiments. Fluorescence was visualized using a Zeiss Axioskop 2 fluorescence microscope and an AxioCam MRc digital camera using Axiovision v4.4 software. All photomicrographs within a series were taken using the same exposure time and frame. Magnification, 630X. (d) Quantification of the fluorescence signal intensity of Wnt5A. Bars represent total intensity per cell count (mean±S.E.M, n=15 pictures). p<0.05 (one-way ANOVA).

Figure 2. The Wnt5A signaling pathway is active in macrophages and is blocked by sFRP1.

(a) Detection of CaMKII and FZD5 protein in lysates of cultured macrophages treated with LPS, INFγ, IL-10 and APC, as indicated, for 24 h. (b) Immunofluorescent detection of phosphorylated CaMKII. Macrophages were cultured for 24 h prior to incubation for 30 min or 120 min with LPS, INFγ, sFRP1 or Wnt5A, as indicated. Control cells were untreated. Wnt5A and sFRP1 were mixed and pre-incubated for 30 min before addition to the cells. Phosphorylated CaMKII was detected using a specific anti-active CaMKII antibody (see Methods). Fluorescence was visualized using a Zeiss Axioskop 2 fluorescence microscope and an AxioCam MRc digital
camera using Axiovision v4.4 software. All photomicrographs within a series were taken using the same exposure time and frame. Magnification, 630X. (c) Quantification of the fluorescence signal intensity of phosphorylated CaMKII. Bars represent total intensity per cell count (mean±S.E.M, n=10 pictures). p<0.05 (one-way ANOVA).

**Figure 3. Induction of inflammatory cytokine secretion by Wnt5A signaling, its modulation by anti-inflammatory mediators IL-10 and APC.**

Concentrations of IL-6, IL-8, IL-1β and MIP-1β protein secreted by macrophages treated with Wnt5A in the absence and the presence of IL-10 or APC, respectively. Control cells were untreated. Cytokines were measured in cell culture supernatants collected 24 h after treatment using the Bio-Plex Human Cytokine Multiplex Assay on the Bio-Plex 2200 platform. Cytokine concentrations in treated cells were normalized to the concentrations in control cells and are presented as mean ± SEM from three independent experiments. *p<0.05.

**Figure 4. Effect of blocking Wnt5A signaling with siWnt5A on inflammatory cytokines secretion**

(a) Left graph, fold induction of Wnt5A mRNA expression in LPS/INFγ stimulated macrophages after transfection with siWnt5A. Right graph, HPRT mRNA expression in LPS/INFγ stimulated macrophages after transfection with siWnt5A The expression level was normalized to GAPDH mRNA in each experimental sample. Data are presented as mRNA expression in stimulated siWnt5A-(open bar) and mock-transfected (black bar) cells relative to the expression level in unstimulated transfected cells. Results are expressed as mean ± SEM of three independent experiments. *p<0.05 (b) IL-6, IL-8, IL-1β mRNA expression and secretion by LPS/INFγ stimulated macrophages after transfection with siWnt5A. Data are presented as mRNA expression in stimulated siWnt5A-(open bar) and mock-transfected (black bar) cells relative to the expression level in unstimulated transfected cells, and as the ratio of the concentration of cytokine secreted by stimulated cells to that secreted by unstimulated cells (in siWnt5A or mock transfected cells). Results are expressed as mean ± SEM of three independent experiments. *p<0.05 (c) Concentrations of IL-6, IL-8, IL-1β and MIP-1β secreted by macrophages. Cells were stimulated with LPS/INFγ in the absence or presence of
sFRP1. Control cells were untreated. Cytokine concentrations in treated cells were normalized to the concentrations in control cells and are presented as mean ± SEM from three independent experiments. *p<0.05.

**Figure 5. Wnt5A protein is present in the serum and bone marrow of patients with severe sepsis or septic shock.**

(a) Immunoprecipitation of Wnt5A in serum from patients with sepsis (lanes 5–8) and from healthy individuals (lanes 1–4). Lane 9 shows Wnt5A conditioned medium (Wnt5A-CM) that was immunoprecipitated following the same protocol. The molecular weight of the band visualized corresponds to ~40 kDa, as expected for Wnt5A. (b) Left graph, densitometric analysis of bands detected by immunoprecipitation using a ChemiDoc XRS system and Quantity One v4.5.0 software (Bio-Rad). The average density is normalized to the total protein concentration (mg/mL) of each individual sample. Right graph, serum neopterin concentrations (nmol/L) normalized to the respective creatinine concentration (μmol/L) in the samples used for Wnt5A immunoprecipitation. Data are from one representative of four independent experiments. p<0.05 (c) Wnt5A in sections from bone marrow biopsies of patients with fatal sepsis and from healthy individuals was detected by immunohistochemistry as described in Methods. As controls, sections of L-cells and Wnt5A cells mixed in a ratio of 10:1 were treated in the same manner. Photomicrographs were acquired with a Zeiss Axioskop 2 equipped with Axiocam MRc digital camera and Axiovision v4.4 software. Magnification, 400X (left panel) and 640X (right panel). Arrows point to Wnt5A positive macrophages.