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Wnt5a Induces a Tolerogenic Phenotype of Macrophages in Sepsis and Breast Cancer Patients

Caroline Bergenfelz,* Catharina Medrek,* Elin Ekström,*,† Karin Jirström,‡ Helena Janols,§ Marlene Wullt,§ Anders Bredberg,§ and Karin Leandersson*

A well-orchestrated inflammatory reaction involves the induction of effector functions and, at a later stage, an active downregulation of this potentially harmful process. In this study we show that under proinflammatory conditions the noncanonical Wnt protein, Wnt5a, induces immunosuppressive macrophages. The suppressive phenotype induced by Wnt5a is associated with induction of IL-10 and inhibition of the classical TLR4-NF-κB signaling. Interestingly, this phenotype closely resembles that observed in reprogrammed monocytes in sepsis patients. The Wnt5a-induced feedback inhibition is active both during in vitro LPS stimulation of macrophages and in patients with sepsis caused by LPS-containing, Gram-negative bacteria. Furthermore, using breast cancer patient tissue microarrays, we find a strong correlation between the expression of Wnt5a in malignant epithelial cells and the frequency of CD163+ anti-inflammatory tumor-associated macrophages. In conclusion, our data point out Wnt5a as a potential target for an efficient therapeutic modality in severe human diseases as diverse as sepsis and malignancy. The Journal of Immunology, 2012, 188: 5448–5458.

Inflammation is a process where innate immune cells detect and respond to “danger-associated” molecules (e.g., exogenous pathogens, endotoxins [pathogen-associated molecular patterns (PAMPs)], or endogenous alarmins in tissue damage [also referred to as danger-associated molecular patterns (DAMPs)]) through pattern recognition receptors expressed on their surface (1, 2). The response leads to production of proinflammatory cytokines and chemokines. The environmental signals to which the monocyes-macrophages (Mo-M) are exposed define their phenotype and functions with the two extremes being the classic, M1, macrophages or alternative, M2, macrophages (3, 4). This results in the Mo-M lineage being very plastic in nature. M1 macrophages are generally considered as potent effector cells that produce large amounts of proinflammatory cytokines and effectively kill both pathogens and tumor cells. However, excessive inflammation also causes tissue damage and toxicity that is harmful to the host. Therefore, strong inducers of inflammation also activate homeostatic mechanisms that can limit the inflammation (5). In contrast, M2 macrophages are able to suppress the inflammatory response, skew the immune response toward an adaptive Th2 immunity, promote tissue remodeling, and repair mechanisms as well as induce angiogenesis. The mechanism by which M2 macrophages tune the inflammatory responses is through production of anti-inflammatory cytokines such as IL-10, TGF-β, and PGE2. Some of these factors can be induced by LPS via TLR4 activation (5). Although it is known that differentiation into alternative M2 macrophages requires specific cytokines (e.g., IL-4, IL-13, and IL-10) (3), the molecular mechanisms behind the homeostatic switch of Mo-M during inflammation remains elusive (5). Some proposed mechanisms include defective NF-κB activation (p50 homodimer formation), increased STAT3, CREB, AP-1, and IFN regulatory factor 3 activation, and a decreased STAT1, MAPK, and IFN regulatory factor 5 activity (5). Tumor-associated macrophages (TAMs) have been shown to resemble the immunosuppressive M2 macrophages in many aspects. Other cell populations inducing a strong immunosuppression are myeloid-derived suppressor cells, and the novel human CD14+ Mo suppressor sub-populations present in cancer patients. These cells stem from the myeloid lineage and are immature cells with a low HLA-DR expression pattern (CD14+CD33+CD11b+HLA-DRlow and CD14+CD33+CD11b+HLA-DR−low) (6–9).

The TLR family of pattern recognition receptors plays a key role in mediating inflammatory responses (10). TLR signaling leads to activation of the transcription factor family NF-κB/Rel, a necessary event in promoting transcription of proinflammatory genes. The NF-κB family consists of five members: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel, which can form different homo- and heterodimers leading to regulation of specific target genes (11). Formation of p50 or p52 homodimers leads to repression of the NF-κB response because of the lack of transcription activation domains in these proteins (11). Both M2 macrophages and TAMs are defective in NF-κB activation in response to M1 polarizing signals, probably a result of p50 homodimer formation (12–15). The molecular mechanism behind is not fully understood. The sterile tumor microenvironment contains endogenous TLR ligands (such as high-mobility group protein B1...
Mammalian noncanonical Wnt protein Wnt5a is capable of in- 
antagonist of TLR signaling represented by noncanonical Wnt signaling in Mo-M differentiation have not been investigated in de- 
sertive groups have also been shown to act mainly as a tumor suppressor, although 
reduction in inflammatory TAMs (CD163+) in breast cancer patients; these 
lethal damage and septic shock. The 
function of Mo-M to produce proinflammatory cyto-
noncanonical Wnt protein that induces specific signals (Ca2+, 
contrast to canonical Wnt proteins, Wnt5a is a nontransforming, 
functions are crucial to prevent lethal damage and septic shock. The 
and importantly also show upregulation of TLR4 
other studies were primarily based on mRNA 
levels of HLA-DR and coreceptor 
showing growth of 
Endotoxin tolerance is a documented clinical state of Mo-M 
reached adjuvant chemotherapy and 96 (67%) endocrine therapy. Approval 
patients (25), but for a major suppressor, although 
will be driven by the noncanonical Wnt β-catenin pathway. It is involved in embryonic 
and polarization. The mRNA levels of Wnt5a do not correlate to the protein levels 
Wnt5a is a secreted glycoprotein of the Wnt protein family. In 
noncanonical Wnt proteins, Wnt5a is a nontransforming, 
noncanonical Wnt protein that induces specific signals (Ca2+, 
protein kinase C, Rho-GTPases, and JNK) not induced by the 
endotoxins in sepsis patients (5, 17). It is 
well described that prior exposure of Mo-M to low doses of 
endotoxins (e.g., LPS) results in a cellular reprogramming of Mo-M 
reduced TNF-α serum levels, increased p50 homodimer forma-
tion, increased pinocytosis, but decreased cytoxic- and Ag pre-
sepsis patients show a reduced expression level of HLA-DR and coreceptor 
downregulation and importantly also show upregulation of TLR4 
Wnt5a staining was scored as the density of CD163+ cells with a Mo-M mor-
the mRNA levels of Wnt5a do not correlate to the protein levels 
(21, 22). Because Wnt5a deficiency is lethal, information about 
the role of Wnt5a in hematopoiesis is limited, although it has been 
implicated in self-renewal of HSCs and B cell-acute lympho-
levels of Wnt5a and its receptors. In addition, the 
upregulation of Wnt5a was interpreted as a functional marker for 
inflammation in already activated Mo-M derived from sepsis 
patients (25). However, the functional consequences of Wnt sign-
ing in Mo-M differentiation have not been investigated in 

day. Importantly, the noncanonical Drosophila WntD protein was 
previously shown to act as a negative feedback inhibitor of the 
NF-κB homolog Dorsal upon TLR signaling (30). Also, the 
WntD mutants had immune defects and exhibited increased levels of Wnt5a 
early published paper (32). Mo or CD44 naïve 
T cells were isolated using MACS, according to the manufacturer’s 
instructions (Monocyte isolation kit II, Naive CD4+ T cell isolation kit II; 
Milenyi Biotec., Bergisch Gladbach, Germany). 

Cell culture 

Mo were differentiated into macrophages in OptiMEM (OptiMEM was 
chosen after several comparisons using different serum-free media and was 
found to be the media that affected the M1/M2 status the least on its own) 
supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) 
using recombinant human (rh)GM-CSF for 5 d and subsequently polarized 
into M1 or M2 macrophages for 2–3 d using LPS and rhIFN-γ or rhIL-4, 
respectively, with or without Wnt5a or Wnt3a. Wnt5a and Wnt3a were 
added on days 1 and 5 of culture. The murine macrophage cell line 
RAW264.7 and the human breast cancer cell line MDA-MB-231 were 
restraining M1 and 2 polarization. Abs used for flow cytometry were the following:  
CD80, CD86, CD1a, CD206, and CCR7 (all from BD Biosciences), and analysis were performed using 7-
epithelial cells 0 (no staining) to 3 (high intensity).

Compounds 

All recombinant human cytokines were obtained from R&D Systems 
(Minneapolis, MN), and the following concentrations were used in all experiments: 10 ng/ml GM-CSF, 20 ng/ml IL-4, 20 ng/ml IFN-γ, 10 ng/ml 
IL-10, 50 ng/ml IL-6, 0.125–0.25 μg/ml Wnt3a, and 0.5 μg/ml Wnt5a.

Endotoxin levels were below detection levels (<0.1 pg/μl) in a 
Linus 

Leukocytes were collected from freshly (2–3 h) prepared leukocyte de-
pletion filters (Serpacell RZ 2000) of blood from healthy blood donors 
according to a previously published method (32). Mo or CD44 naïve T cells were isolated using MACS, according to the manufacturer’s instructions (Monocyte isolation kit II, Naive CD4+ T cell isolation kit II; Miltenyi Biotec., Bergisch Gladbach, Germany).

Isolation of Mo and CD44+ T cells 

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Pinocytosis assay

The pinocytic activity of macrophages was analyzed using FITC–dextran uptake. The cells were incubated with 1 mg/ml FITC–dextran (Sigma-Aldrich) at 37°C for 20 min and subsequently analyzed using flow cytometry. Pinocytosis was controlled using cells that were incubated at 4°C (data not shown).

Allogeneic MLR

The cultured primary macrophages were harvested on day 8 of culture, reseeded in 96-well plates, and incubated with freshly isolated naive CD4+ T cells at stimulator–responder ratios ranging from 1:10 to 1:100. On day 4 of coculture, 1 μCi [methyl-3H]thymidine was added for 18 h, and incorporation was determined in a Microbeta Counter (PerkinElmer).

Luciferase reporter assay

A total of 0.5 μg pNFκB-luciferase (BD Biosciences) or pIL-10 promoter luciferase (a gift from Prof. L. Ziegler-Heitbrock) plasmid and 0.05 μg TK-renilla-luciferase or CMV-renilla-luciferase (Promega, Madison, WI) plasmids were cotransfected into RAW264.7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) Wnt5a (24 + 24 h) or LPS (24 h).

ELISA

Supernatants from differentiated macrophages were collected, and the amount of IL-10, IL-12, and TGF-β was determined using ELISA (R&D Systems), according to the manufacturer’s instructions. Ratios of IL-10/IL-12 concentrations were calculated for each well.

Cytotoxicity assay

The cytotoxic effect of macrophages was determined by measuring lactate dehydrogenase (LDH) activity using a Cytotoxicity detection kit according to the manufacturer’s instructions (Roche Applied Science, Roche Diagnostics, Mannheim, Germany). Briefly, macrophages and MDA-MB-231 were cocultured (10:1 ratio) in OptiMEM supplemented with penicillin-streptomycin for 24 h, after which the LDH levels were measured (33). The cytotoxic capacity of untreated M1 cells were set to 100%.

Native PAGE, EMSA ELISA, and EMSA

Nuclear extracts were prepared as described previously (34). For native PAGE electrophoresis, a 5% native PAGE with 5% glycerol was run with nuclear extracts and subsequently transferred to polyvinylidene difluoride membranes using normal SDS-PAGE transfer buffer. Immunoblotting was...
performed using p50 and p65 Abs. Lamin B was used as a loading control in SDS-PAGE gels. The EMSA ELISA for p50 and p65 was purchased from TransAM Active Motif (Carlsbad, CA) and performed according to the manufacturer’s procedure. For EMSA, DNA probes were labeled with γ[32P]ATP by incubation with T4 polynucleotide kinase (New England Biolabs), annealed, and purified using Micro Bio-Spin P-30 Tris Chromatography Columns (Bio-Rad). For supershifts, the mixture of nuclear extracts was incubated with anti-p50, -p52, or -p65 Abs for 10 min prior to addition of the probe. The samples were separated on 5% polyacrylamide TBE gels that were subsequently analyzed by autoradiography at ~70°C. Oligonucleotides used were 5′-GAGTTGGGAGCTTCCAGGCTC-3′ and 5′-GAGCCCTGAAAGTCCCCACTC-3′.

**Results**

Wnt5a blocks M1 macrophage differentiation

It was previously reported that Wnt5a mRNA transcripts were expressed at high levels in human macrophages exposed to LPS and IFN-γ [proinflammatory M1 (25, 26)]. When we analyzed the protein expression levels of Wnt5a in primary human Mo, Mo-M with the proinflammatory phenotype (M1), and Mo-M with the alternatively activated phenotype (M2), we found that Wnt5a protein was not detected in Mo but expressed at low levels in M1 cells and also in M2 cells (Supplemental Fig. 1A). We therefore set out to analyze whether Wnt5a might affect the differentiation of primary human Mo-M in cultures, primed with a proinflammatory stimulus (LPS and IFN-γ; “M1”), or alternative stimuli (IL-4; “M2”), together with recombinant Wnt5a (rWnt5a) or rWnt3a as a control. First, we could see that addition of Wnt5a to the Mo-M cultures led to a slight but nonsignificant decrease in numbers of CD14+CD209− Mo-M (Supplemental Fig. 1B, 1C). When we analyzed the effect of the Wnt proteins on induction of M1 versus M2 macrophages, we found that M1 differentiation, but not M2 differentiation, was significantly blocked by Wnt5a but not by Wnt3a (Fig. 1A, Supplemental Fig. 1D, 1E). Interestingly, the Wnt5a-treated cells were not of M2 phenotype (Fig. 1A, Supplemental Fig. 1E) but rather had a phenotype resembling the novel suppressor Mo populations (CD14+HLA-DR−/low and coreceptor−/low) described in different human cancers (Fig. 1B–D) (7–9). Much like the suppressive Mo previously described, the HLA-DR−/low cells expressed CD14, CD33, CD16, and CD11b (Fig. 1C, Supplemental Fig. 1F) but lacked CD1a, CD209, and CCR7 or expressed CD80 and CD86 at lower levels (Fig. 1C, 1D). Similar phenotypic cell populations have also been described in sepsis patients as a result of endotoxin tolerance (5), although the relationship and molecular similarity between these populations is to our knowledge not known. We therefore also evaluated whether using a typical endogenous TLR4-ligand (HMGB1) together with Wnt5a, thus representing a noninfectious situation such as cancer, would induce the same HLA-DR−/lowCoreceptor−/low phenotype. As shown in Supplemental Fig. 2A, the combination of HMGB1 and Wnt5a indeed promoted the accumulation of CD14+HLA-DR−/lowCoreceptor−/low cells. To ensure that the effect was Wnt5a specific and not caused by endotoxin contamination, we analyzed whether addition of Wnt5a prior to M1 stimulation with LPS/IFN-γ gave rise to the same populations. We found that the block of M1 differentiation was only achieved when we treated the Mo–differentiation cultures with Wnt5a throughout the whole culture.
period (at GM-CSF addition day 1 and LPS/IFN-γ addition day 5) but not if we added Wnt5a on day 1 or 5 only. This ruled out a possible endotoxin contamination, because this would have displayed the same effect whether Wnt5a were solely added on day 1 (Supplemental Fig. 2B). The rWnt3a and rWnt5a used in this study were also negative in endotoxin Limulus amebocyte lysate assays (data not shown). The morphology of the cells was similar to M1 cells (Fig. 2A, 2B) with the exception that there were a larger number of cells with secretory vesicles upon Wnt5a treatment (Fig. 2B, lower left). Finally, addition of rWnt5a only to freshly prepared Mo did not give rise to CD14+HLA-DR<sup>low</sup> cells while exclusion of IFN-γ from the cultures or exchanging GM-CSF for M-CSF did (data not shown).

The Wnt5a-induced HLA-DR<sup>low</sup> Mo-M have an alternative activation

To investigate whether the Wnt5a-induced HLA-DR<sup>low</sup> Mo-M were biologically functional, we analyzed different macrophage properties of the cultured cells. As seen in Fig. 2C, the Wnt5a-treated M1 cells (HLA-DR<sup>low</sup>) produced significantly more ROS than cultures of M1 cells or cells treated with rWnt3a (Fig. 2C). Upon analysis of the cytotoxic capacity of the Wnt-treated M1 cells, we found that only Wnt5a-treated M1 cells had a significantly reduced cytotoxic capacity (Fig. 2D). In line with this, the T cell stimulatory capacity of Wnt5a-treated M1 cells was reduced in allogeneic MLR (Fig. 3A) as measured by [3H] incorporation. Noteworthy, the Wnt5a-treated M1 cells displayed a similar IL-10/IL-12 ratio to Wnt3a-treated M1 cells, as measured by ELISA (Fig. 3B), although the relative levels of IL-10 and TGF-β were induced specifically in the rWnt5a-treated M1 cultures (Fig. 3C). Also, Wnt5a did not induce the IL-10/IL-12 ratio in M2 cultures significantly whereas rWnt3a did (Fig. 3B). Finally, rWnt5a-treated M1 cells had a higher pinocytosis capacity as measured by FITC-dextran uptake, similar to M2 cells (Fig. 3D).

Wnt5a inhibits NF-κB signaling

The major intracellular signal cascade in proinflammatory cell signaling is NF-κB. It is well known that LPS induces activation of

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**FIGURE 3.** Wnt5a inhibits NF-κB signaling. (A) Wnt5a treatment leads to a significantly reduced APC function of primary human Mo-M. Allogeneic MLR of primary human Mo-M and primary human CD4<sup>+</sup> T cells as measured by [3H] incorporation. The relative level of T cell stimulatory capacity in cultures of 1:10 Mo-M:T cells as compared with 1:100 Mo-M:T cells reveals a decreased APC function with increased Mo-M:T cell ratio in Wnt-treated cells. n = 6. Error bars represent SEM. Student t test, ***p < 0.001. (B) Wnt treatment leads to an increased ratio of IL-10:IL-12 production in primary human M1 cultures. The levels of IL-10 and IL-12 were measured using ELISA, and the relative values were calculated to be able to compare supernatants from the same well. Only rWnt 3a treatment of M2 cultures led to a significantly increased IL-10:IL-12 (right panel). n = 10. Error bars represent SEM. Student t test, *p < 0.05. (C) IL-10 and TGF-β ELISA on supernatant from cultures of M1, rWnt3a-treated M1, or rWnt5a-treated M1 cultures. The relative values with M1 set to 1 are shown. n = 3. Error bars represent SEM. Student t test, *p < 0.05. (D) Pinocytosis as measured by FITC-dextran uptake of primary human Mo-M cultures. Proinflammatory (M1) macrophages have a lower pinocytic capacity as compared with alternatively activated (M2) macrophages (Ref. 44 and right histogram). The relative level of cells within the M1 phenotype gate in M1 cultures (white bars; left panel) and M2 phenotype gate in M1 cultures (gray bars; right panel) are displayed as compared with untreated control (M1) cells. Wnt5a treatment leads to significantly reduced levels of cells displaying an M1 pinocytosis capacity as compared with both control cells and Wnt3a-treated cells (left panel). The amount of cells displaying an M2 pinocytosis capacity were increased, although not significantly (right). n = 5. Error bars represent SEM. Student t test, *p < 0.05.
NF-κB upon binding to its receptor TLR4 (10). To investigate whether this proinflammatory pathway was active in macrophages upon Wnt5a treatment, we performed NF-κB luciferase assays upon RAW264.7 mouse macrophages. As shown in Fig. 4A and in contrast to what was previously proposed, Wnt5a did not induce a proinflammatory NF-κB signal by itself but actually inhibited LPS induced NF-κB activation, similar to the noncanonical Drosophila WntD protein (30).

Wnt5a induces a nonclassical NF-κB p50 activation in combination with LPS

We next analyzed the NF-κB signaling pathway in Wnt5a M1 cells at the molecular level. We detected an increased cleavage of p105 (Fig. 4B, mouse macrophages left and primary human macrophages center) and an increased phosphorylation of p105 (Fig. 4B, right panel). We also detected an increased DNA-binding complex consisting of p50 homodimers as judged by EMSA (Fig. 4C, left panel; OD measurements, center panel), an increased DNA-binding fraction of p50 as compared with p65 (measured by EMSA ELISA on primary human macrophages; Fig 4C, right), and an increased p50 nuclear localization as compared with p65 (Fig. 4D) in Wnt5a-treated M1 cells, just as has previously been shown for M2 cells and endotoxin tolerance reprogrammed Mo (13, 15). Lamin B was used as a loading control for nuclear lysates (Fig. 4D, lower panel). This should be compared with the simultaneous decrease in NF-κB activity found in the luciferase assays upon Wnt5a addition (Fig. 4A). We cannot exclude that the nonclassical p52 NF-κB pathway is activated; however, this would not influence our conclusions. Reprogramming of M1 cells has previously been shown to involve a downregulation of TLR4 expression (35). Indeed, the Wnt5a-treated M1 cells had significantly lower levels of TLR4 expression (Fig. 4E) compared with the other cultures. Differentiation into alternatively activated M2 macrophages both demands and induces activation of certain signaling cascades such as STAT3 (36). As shown in Supplemental Fig. 2C, a short (3 h) stimulation of human primary Mo with Wnt5a also induced STAT3 activity whereas Wnt3a did not.

Blockade of IL-10 signaling partly restores the M1 phenotype

To explain how Wnt5a can increase the IL-10/IL-12 ratio in M1 cultures, we considered previous results suggesting that LPS increases IL-10 transcription in M2 macrophages because of
a change in transcription factor activation profiles (36). We thus assayed whether Wnt5a could affect the outcome in an IL-10 promoter luciferase assay (Fig. 5A). Indeed, when we added Wnt5a to the RAW264.7 cell cultures, we found that Wnt5a alone activated the IL-10 promoter, an effect that was stable also in combination with LPS. Also, treating primary human Mo with rWnt5a for 3 h induced IL-10 mRNA and protein production significantly as measured by quantitative PCR and ELISA (Supplemental Fig. 2D, 2E).

Because IL-10 is suggested to be an important LPS-induced feedback inhibitor, we investigated whether blockade of IL-10 signaling would restore the M1 phenotype in Wnt5a-treated M1 cells by using flow cytometry. By blocking IL-10R signaling in the NF-κB reporter assay, we could show that the Wnt5a-induced inhibition of NF-κB activity probably was mediated by IL-10 (Fig. 5B). Indeed, when we blocked IL-10 signaling in Wnt5a M1 cells, the HLA-DR<sup>hi</sup> phenotype was partially restored compared with untreated Wnt5a M1 cells (Fig. 5C, 5D). Blocking IL-6 signaling did not restore this population (Fig. 5C).

Wnt5a ex vivo restimulation of Mo from sepsis patients potentiates the phenotype of alternative Mo activation

Because it had previously been suggested that Wnt5a would be a factor produced and involved in activation of proinflammatory Mo in sepsis patients, we next analyzed the phenotype of Mo in patients with urosepsis with regard to pro- or anti-inflammatory properties. As expected, the CD14<sup>+</sup> Mo from sepsis patients had a similar phenotype as compared with our Wnt5a M1 cells with regard to HLA-DR expression (Fig. 6A, 6B) and coreceptor expression (Fig. 6C), however, differed substantially from those taken from normal healthy blood donors (Fig. 6A–C). Similar to what has previously been described, but in contrast to our in vitro-

![FIGURE 5. Blockade of IL-10 signaling partly restores the M1 phenotype. (A) Dual luciferase IL-10 promoter assay using murine Mo-M RAW246.7 cells. The relative dual luciferase units (RLU) were measured using the pRL-CMV vector as a control. Wnt5a alone activates the IL-10 promoter significantly. LPS treatment or LPS plus Wnt5a treatment also induces the IL-10 promoter. n = 11. Error bars SEM. Students t test, *p < 0.05. (B) Blocking IL-10R signaling in a dual luciferase NF-κB promoter assay using murine Mo-M RAW246.7 cells. The relative dual luciferase units were measured using the pRL-TK vector as a control. RLU of LPS-stimulated RAW246.7 cells was set to 1. The Wnt5a-induced inhibition of NF-κB signaling is mediated by IL-10. n = 5. Error bars SEM. Student t test, **p < 0.01. (C) Addition of IL-6R or IL-10R blocking Abs in M1 primary human Mo-M cultures treated or not with Wnts. The relative effect of HLA-DR<sub>high</sub> cells using IL-6R (n = 3) or IL-10R (n = 9) blocking Abs in M1 primary human Mo-M cultures is shown as compared with the respective control cultures without blocking Abs (ratios). The IL-10R blocking Ab partly restores the effects of Wnt5a on M1 phenotype in M1 cultures. Error bars SEM. Student t test, *p < 0.05. (D) The lower panel shows representative histogram plots of HLA-DR expression.](http://www.jimmunol.org/)

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cultured Wnt5a M1 cells, CD14+ Mo from sepsis patients had an increased CD163 expression (Fig. 6C, Supplemental Fig. 1E) (37, 38). The reason for this discrepancy is not clear, but importantly, we could further show that Wnt5a/LPS treatment of ex vivo sepsis Mo lead to a prominent increase in CD163 expression (Fig. 6D) as compared with healthy controls (Supplemental Fig. 3A). Also, as shown in Fig. 6E, treatment with Wnt5a alone downregulated coreceptor levels on CD14+ Mo from sepsis patients even further but importantly did not affect coreceptor expression levels in healthy controls.

**Wnt5a expression in breast tumors correlates with the presence of CD163+ TAMs**

In a tumor or sterile inflammation, the TLR ligands present are endogenous DAMPs. Because we had already seen that the endogenous TLR4-ligand HMGB1 could induce reprogramming of Mo-M in the presence of Wnt5a, we next analyzed whether intratumoral Wnt5a levels would correlate with the presence of anti-inflammatory TAMs in breast cancer. The breast cancer cohort analyzed consisted of 144 patient samples arranged in a TMA. Consecutive sections of 117 samples were scored because of loss of material or low tumor cell content in the remaining samples. We could see that a high tumoral Wnt5a expression correlated significantly \( (p \leq 0.001) \) with a high density of CD163+ TAMs (Fig. 7). CD163+ cells were present in 95 (81%) cases, and Wnt5a was expressed in 87 (74%) cases. The CD163 staining was scored as the density of CD163+ cells, ranging from 0 (absent) to 3 (high density) in both the stromal and malignant epithelial cell areas. The pan-macrophage marker CD68 was analyzed in consecutive sections and was also expressed in the same areas and correlated significantly with CD163 expression (data not shown). The Wnt5a staining was scored as the intensity of expression in malignant epithelial cells 0 (no staining) to 3 (high intensity). The expression of tumoral Wnt5a did not correlate with the presence of macrophages expressing the pan-macrophage marker CD68 (data not shown). We could also see that CD163+ TAMs expressed Wnt5a (Fig. 7B, Supplemental Fig. 3B).

**Discussion**

The Mo-M are cells of the innate immune system that are highly plastic in nature. The plasticity is generally affected by extracellular signals, such as the cytokine environment in tumors that skews macrophages toward alternatively activated TAMs (3, 4). Although the functional differences between the populations are quite well described, there are still questions to be answered regarding the molecular mechanisms behind their programming (7–9). During sepsis, a systemic infection causes an acute innate proinflammatory response that immediately is counteracted by a self-regulated anti-inflammatory reaction. The clinical manifestations and prognoses of these patients are dependent on the balance between these pro- and anti-inflammatory mediators. This can be attributed to the proposal that proinflammatory mediators can give rise to systemic...
inflammatory response syndrome and shock and the anti-inflammatory mediators to an immune paralysis known as compensatory anti-inflammatory response syndrome (5, 17). Similarly, in a local inflammation, a proinflammatory immune response is necessary to eradicate the pathogen or "danger-associated" molecules but must then be stopped to avoid serious tissue damage. Whether the danger signal eliciting the inflammation is "nonself" (PAMP; represented by LPS in this study) or "self" (DAMP; represented by HMGB1 in this study) will probably not matter (1, 2). The immune system has developed different regulatory mechanisms for this purpose (17). It is tempting to speculate that also in a cancer context, the altered tumor-associated Ags and DAMPs (e.g., HMGB1 and S100 family proteins) (16) might indeed elicit an inflammatory process, but similar to a local inflammation or in sepsis patients, it is turned off through self-regulatory reprogramming of the Mo-M.

Wnt5a has previously been implicated in various forms of cancer, mainly as a tumor suppressor but also as a tumor promoter (20). There are no definite explanations for how Wnt5a can have a contradicting effect on prognosis in the different cancer types. Because it was reported that Wnt5a was expressed in macrophages in breast cancer (29) and concurrently was suggested to be an important inflammatory effector molecule in sepsis (25, 26)—to our point of view, two similar immune mechanisms—we decided to investigate the functional consequences of Wnt5a on Mo-M in detail. We show that expression of tumoral Wnt5a correlates significantly with the presence of anti-inflammatory CD163+ TAMs but not with CD68, a general macrophage marker in a breast cancer TMA. We also show that Wnt5a together with LPS can induce reprogramming of Mo-M in vitro and in sepsis patients. It is interesting to note that previous studies concerning Wnt5a expression in macrophages all represent diseases that have a large M2 population (25, 27–29). In our experimental setup, we mainly used LPS as a proinflammatory mediator, although we did have similar effects using the endogenous TLR4-ligand HMGB1 instead of LPS, showing that a similar mechanism can be accounted for in sterile inflammations and tumors.

The noncanonical Drosophila WntD protein has previously been shown to act as a negative feedback inhibitor of the NF-κB homolog Dorsal upon TLR signaling, describing a secreted feedback antagonist of TLR signaling represented by noncanonical Wnt proteins in Drosophila (30). In this study, we show that the mammalian noncanonical Wnt protein Wnt5a is capable of inducing a similar negative feedback loop of NF-κB upon TLR signaling in Mo. We also show that the inhibition lies in the formation of NF-κB p50 homodimers. The principal mechanism accounting for alternative macrophage activation and endotoxin tolerance is believed to be IL-10 feedback inhibition (5, 36). The mechanism describing how the IL-10 gene is activated in Mo-M with a proinflammatory phenotype has been under intense investigation. One explanation is that a nonclassical NF-κB activity (p50- or p52-homodimer formation) induces IL-10 transcription together with certain transcription factors (e.g., cAMP-induced CREB activity) (39). Wnt5a induces cAMP and CREB activa-
tion (40). We suggest that the mechanism behind Wnt5a-induced macrophase reprogramming is that Wnt5a acts as a feedback antagonist of TLR signaling with subsequent induction of anti-inflammatory cytokines such as IL-10. Indeed, we could partially restore the M1 phenotype by blocking IL-10 signaling. A similar mechanism has previously been described for PGE2 (41, 42). Interestingly, although addition of rWnt5a alone to freshly prepared Mo led to a robust increase in IL-10, rWnt5a alone did not give rise to CD14HLA-DRcells, indicating that additional signals apart from IL-10 is required for reprogramming. Also noncanonical Wnt signaling can affect NF-kB activity as has previously been suggested (43), although the mechanism behind this phenomenon is very different, suggesting that Wnt5a has unique effects on macrophage cell signaling as compared with canonical β-catenin Wnt signaling.

To validate our in vitro findings, we were also able to show that the Wnt5a-treated M1 cells had a similar phenotype as Mo from sepsis patients. A functional consequence of ex vivo LPS resimulation of Mo from sepsis patients is downregulation of HLA-DR and coreceptors. We could repeat these findings and also showed that Mo from sepsis patients already had a decreased HLA-DR/coreceptor expression level. More importantly, we could also show that addition of Wnt5a/LPS induced an accumulation of CD163Mo and a further downregulation of coreceptors that was not seen in Mo from healthy blood donors. Taken together, our findings indicate that the noncanonical Wnt5a protein is a novel unique and important factor involved in reprogramming of Mo-M in diseases such as sepsis. In fact, also, the use of endogenous TLR4-ligand (HMGB1) together with Wnt5a resulted in the same reprogramming of primary human Mo-M in vitro. It will be interesting to investigate whether the same effects are seen using freshly prepared TAMS and endogenous TLR ligands and whether this can account for some of the contradictory effector mechanisms reported for Wnt5a in different tumors.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figures

Supplementary Fig. 1

(A) Western blot of Wnt5a levels in primary human monocytes (Mo) and cultured Monocyte-Macrophages (Mo-M). Monocytes lack Wnt5a expression while M1 and M2 Mo-M express Wnt5a. GAPDH is shown as a loading control.

(B) Primary human Mo-M treated with GM-CSF and Wnt3a or Wnt5a. The percentage of CD14^+CD209^- Mo-M decreased slightly in cultures treated with Wnt5a, as measured by flow cytometry. Relative values relate to cultures treated with GM-CSF only (set to 1). N=4. Error bars SD.

(C) Flow cytometric analysis of CD14^+CD209^- primary human Mo-M cultures.

(D) Differentiation of primary human Mo-M into alternatively activated (M2) macrophages in the presence of Wnt3a or Wnt5a (left). Wnt signaling does not affect the generation of M2 macrophages (HLA-DR^+CD163^+) significantly. M1; M1 phenotype gated on HLA-DR^+CD80^+CD86^++ or alternatively activated (M2; M2 phenotype gated on HLA-DR^+CD163^+) macrophages in the presence of Wnt3a or Wnt5a as compared to untreated M2 cultures (relative values). N=4. Error bars SD. Students t-test.

(E) CD163 levels remain unaffected by Wnt5a treatment of M1 and M2 cultures.

(F) Histogram plots showing CD11b expression on CD14^+HLA-DR^low gated cells.

Supplementary Fig. 2

(A) Differentiation of primary human Mo-M into M1 macrophages using the endogenous TLR4 ligand HMGB1 (1μg/ml upper row and 10μg/ml lower row) in the presence of Wnt3a or Wnt5a. HMGB1 induces an accumulation of CD14^+HLA-DR^lowCD86^low cells (% indicate HLA-DR^lowCD86^low cells in the CD14^+ gate) in combination with Wnt5a but not in combination with Wnt3a or used alone, just like LPS.

(B) Treatment with Wnt5a prior to the addition of LPS (1st inc) or at the time LPS was added only (2nd inc) does not lead to induction of the co-receptor^low (represented by CD86) population as compared to Wnt5a during the whole culture period (1st + 2nd inc).

(C) Western blot showing a Wnt5a, as compared to Wnt3a, induced (3h) activation of STAT3-PY in freshly prepared human primary monocytes.

(D) IL-10 Q-PCR of human primary monocytes treated with Wnt3a or Wnt5a for 3h.

(E) Induced secretion of IL-10 by human primary monocytes treated with Wnt3a or Wnt5a for 3h as measured by ELISA. The ratio represents the induced IL-10 levels compared to supernatant of untreated, cultured (3h) monocytes (set to 1). N=4. Error bars SEM. Students t-test. *** P<0.001

Supplementary Fig. 3
(A) *Ex vivo* LPS or Wnt5a stimulation of peripheral blood from healthy controls does not affect the anti-inflammatory CD163⁺ monocyte population.

(B) Immunofluorescent staining of nuclei (DAPI blue), CD163 (FITC green) and Wnt5a (TRITC red) in human tonsil. CD163⁺ macrophages should not be present in Germinal Centers (GC).
A

CD86

CD14+

Ctrl  Wnt3a  Wnt5a

10%  20%  40%

CD86

B

M1

Wnt5a

1st inc  2nd inc  1st + 2nd inc

CD33

CD86

C

Mo

STAT3-PY

STAT3

Wnt5a:  -  -  +  (3h)
Wnt3a:  -  +  -  (3h)

D

Mo IL-10 qPCR 3h

Wnt3a  Wnt5a

Ratio

E

Mo IL-10 ELISA 3h

Wnt3a  Wnt5a

Ratio

***
A 24h ex vivo PBL

Ctrl   Wnt5a

Ctrl   LPS

Ctrl   Wnt5a+ LPS

B Tonsil

CD163

Wnt5a

CD163/Wnt5a