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Interleukin-4-induced β -catenin regulates the conversion of macrophages to multinucleated giant cells

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Abstract

The cytokine interleukin-4 (IL-4) exerts pleiotropic effects on macrophages as it plays a key role in the immune response to infectious agents, allergens, and vaccines. Macrophages exposed to IL-4 drastically change their gene expression and metabolic state to adjust to new functional requirements. IL-4 also induces macrophages to fuse together and form multinucleated giant cells (MGCs). MGC formation is associated with chronic inflammation resulting from persistence of pathogenic microorganisms or foreign materials in tissues. Very little is known, however, about the mechanisms regulating IL-4-induced macrophage-to-MGC conversion. We observed a dramatic increase in β -catenin protein but not mRNA amount in mouse macrophages following exposure to IL-4. To investigate the role of β -catenin in macrophages, we generated mice with a myeloid cell-specific deletion of the β -catenin gene. Ablation of β -catenin expression did not affect the viability of macrophages or impair expression of known IL-4-inducible genes. Intriguingly, β -catenin-deficient macrophages incubated with IL-4 formed MGCs with markedly greater efficiency than wild-type macrophages. Similar increases in multinucleated cell formation were detected in the peritoneal cavity of myeloid cell-specific β -catenin knockout mice injected with chitin, which is known to induce endogenous IL-4 production. Our findings reveal β -catenin as a novel regulator of macrophage responses to IL-4, and suggest that therapeutic modulation of its expression or function may help enhance the effectiveness or ameliorate the pathology of IL-4-driven immune responses.

Keywords

Macrophage; Cytokine; Beta-catenin

1. Introduction

Macrophages are a heterogeneous group of myeloid cells that play a key role in immune defense and tissue homeostasis. Although present in some abundance in normal tissues, macrophages are recruited in greater numbers to inflamed body sites. Macrophages serve a wide variety of functions, ranging from phagocytic removal of invading pathogens to

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production of signaling molecules orchestrating inflammatory responses and tissue repair (Murray and Wynn, 2011). Further, specific subpopulations of macrophages are known to regulate metabolic processes as diverse as bone mineral resorption, iron recycling, and fatty acid catabolism (Novack and Teitelbaum, 2008; Ganz, 2009; Chawla et al., 2011). The versatility of macrophages is attributable to their phenotypic plasticity: macrophages undergo shifts in gene expression and adopt distinct functional characteristics when exposed to different cytokines. Stimulation by the cytokines interferon (IFN)- γ and interleukin (IL)-4, for instance, results in the expression of distinct phenotypes, referred to as M1 and M2, respectively (Lawrence and Natoli, 2011; Sica and Mantovani, 2012). These two macrophage fates are geared to contrasting immune defense mechanisms effective against different types of pathogenic microorganisms.

During an active immune response, IL-4 is produced by T cells, mast cells, eosinophils, and basophils (Seder et al., 1991; Voehringer et al., 2004). IL-4 functions to promote Th2 effector cell development and antibody-mediated immunity by exerting pleiotropic effects on multiple cell types (Paul and Zhu, 2010). Other cellular sources of IL-4 have recently been identified that come into play in various physiological contexts. Most notably, adipocytes and tumor cells have been suggested to produce IL-4 and thereby induce fat- and tumor-associated macrophages to differentiate into M2 phenotype (Kang et al., 2008; Gocheva et al., 2010). The precise role of M2-polarized macrophages has not been unequivocally determined, but evidence suggests a link to tissue repair, metabolic control, and tumor growth (Gordon and Martinez, 2010). IL-4 signals to reprogram gene expression in macrophages and other target cells. IL-4 binding to its receptor leads to activation of the transcription factor Stat6, which is required for the expression of many IL-4-inducible genes (Goenka and Kaplan, 2011).

M2 gene expression and phenotype apart, IL-4 induces radical changes in macrophage morphology and behavior: IL-4-exposed macrophages aggregate and fuse together, forming syncytia called multinucleated giant cells (MGCs). MGCs are most frequently observed in tissues afflicted with chronic inflammation. Persistent microbial infection and foreign body implantation can create such tissue environments, and are indeed associated with MGC formation (Helming and Gordon, 2009a). IL-4-induced macrophage fusion and MGC formation likely reflect an attempt to increase the capacity of macrophages to contain and destroy invading non-self entities. Studies employing gene knockout and knockdown approaches and the use of function-blocking antibodies (Kyriakides et al., 2004; Yagi et al., 2005; Helming and Gordon, 2007; Jay et al., 2007; Moreno et al., 2007; Pajcini et al., 2008; Helming et al., 2008; Helming et al., 2009b; MacLauchlan et al., 2009; Van den Bossche et al., 2009; Yu et al., 2011) revealed that, in addition to the IL-4 receptor and Stat6, MGC formation depends on several other proteins in diverse functional categories: CD36, DC-STAMP, E-cadherin, and TREM-2 (cell surface interaction); CCL2 (chemotaxis); Rac1, Dock180, DAP12, and Syk (intracellular signaling); MMP9 (proteolysis); and NF- κ B p105/p50 (gene transcription). Analysis of Dicer-deficient macrophages has shown that a microRNA-based mechanism is at work to hold MGC formation in check (Sissons et al., 2012). However, few proteins have been documented to serve as negative regulators of IL-4-induced MGC formation.

Here we discover that β -catenin functions to inhibit the conversion of IL-4-exposed macrophages to MGCs. By generating and investigating mice with a deletion of the β -catenin gene, *Ctnnb1*, in myeloid cells, we find that ablation of β -catenin expression in macrophages leads to marked increases in the efficiency of the formation of MGCs and multinucleated cells of smaller size in vitro and in vivo. Our study provides new insight into how IL-4 signaling and macrophage fusion are regulated, and identify a new role for β -catenin in macrophage biology.

2. Materials and Methods

2.1. Animals

C57BL/6J mice were used to isolate bone marrow and prepare bone marrow-derived macrophages. Mice with floxed (fl) *Ctnnb1* alleles (Brault et al., 2001) and *LysMCre* knockin mice (Clausen et al., 1999), both on a C57BL/6J background, were obtained from the Jackson Laboratory. These mice were crossed to generate myeloid cell-specific *Ctnnb1* knockout mice (*Ctnnb1^{fl/fl}-LysMCre*). All animal studies were conducted under IACUC-approved protocols.

2.2. Macrophage preparation and culture

Bone marrow isolated from the tibia and femur of mice were cultured in a differentiation medium containing high-glucose Dulbeccos' modified Eagles medium (DMEM), 10% fetal bovine serum, and 10 ng/ml macrophage-colony stimulating factor (M-CSF; PeproTech) for seven days. Plastic Petri dishes were used to facilitate recovery of trypsin- and EDTA-treated macrophages. Macrophages in tissue culture plates were treated with lipopolysaccharide (LPS; Sigma-Aldrich) and the following cytokines: granulocyte-macrophage colony stimulating factor (GM-CSF), IFN- γ , IL-4 and IL-6 (all from PeproTech); and RANK ligand (RANKL) and IL-10 (all from R&D Systems).

2.3. Protein and RNA analysis

Whole cell lysates were prepared as described (Park et al. 2004) and analyzed by immunoblot using antibodies against the following proteins: arginase-1 (sc-18354), iNOS (sc-651), IRF4 (sc-6059), IRF8/ICSBP (sc-6058; all from Santa Cruz Biotechnology); β -catenin (610153; BD Biosciences); IRF5 (10547-1-AP; Proteintech); E-cadherin (3195; Cell Signaling Technology); Ym-1 (01404; Stemcell); and actin (A4700; Sigma-Aldrich). Total RNA was extracted from cultured macrophages using Trizol (Life Technologies). cDNA was synthesized from total RNA using a SuperScript II cDNA synthesis kit (Life Technologies). Real-time quantitative PCR (qPCR) was performed using Fast SYBR Green master mix (Life Technologies). The oligonucleotide primers used in real-time qPCR are listed in Table 1.

2.4. MGC formation in vitro

Macrophages were incubated in Permanox chamber slides (Lab-Tek) at 2×10^6 /ml for 16 h, treated with IL-4 (25 ng/ml), and further incubated for 7 d. Cells on the slide were stained with 0.09% crystal violet and analyzed by bright-field microscopy.

2.5. Immunofluorescence microscopy

Cells fixed with 4% formaldehyde and permeabilized with cold 0.5% Triton X-100 were incubated with phalloidin-tetramethylrhodamin B isothiocyanate (Sigma-Aldrich) and DAPI (Invitrogen). After treatment with the anti-fading agent VectaShield (Vector Laboratories), the signal was visualized using an A1R confocal microscope system with an x60 oil-immersion lens (Nikon).

2.6. Chitin-induced peritonitis

Chitin (Sigma-Aldrich) was suspended in phosphate-buffered saline (PBS), filtered through a 70- μ m strainer, centrifuged, and resuspended in ten times the packed pellet volume of PBS. Mice were injected intraperitoneally with 0.2 ml of 10% chitin suspension. Peritoneal lavage was collected 6 d after chitin injection, and the cells were mounted on slides by Cytospin centrifugation, stained with Diff-Quik solutions (Siemens Healthcare Diagnostics), and analyzed by bright-field microscopy.

3. Results

3.1. IL-4 induces a drastic increase in β -catenin protein but not mRNA amount in macrophages

Growing evidence suggests a role for β -catenin in macrophage biology (Otero et al., 2009; Yang et al., 2010). We sought to determine the role of β -catenin in cytokine-induced changes in macrophage phenotype, and first examined β -catenin expression in bone marrow-derived macrophages exposed to various cytokines. The cytokines used were chosen so that the functional states of macrophages induced by them represented diversity, ranging from M1 to M2, pro- to anti-inflammatory, and myeloproliferative to osteoclastogenic. In our experimental condition, macrophages treated with IFN- γ and IL-4 displayed the predicted patterns of M1 and M2 marker gene expression: the expression of iNOS induced by IFN- γ , and that of Ym-1, arginase-1, and E-cadherin by IL-4 (Fig. 1A). Of note, GM-CSF also induced some but not all M2 marker genes. This analysis was extended to the expression of β -catenin as well as some transcription factors known to play a role in macrophage polarization. We observed a strong induction of β -catenin in response to IL-4 (Fig. 1B). Interferon regulatory factor (IRF) 4, which drives M2 macrophage marker gene expression (Sato et al., 2010), was also induced by IL-4. On the other hand, IRF5 and IRF8 were induced by IFN- γ (Fig. 1B), consistent with their implication in M1 macrophage function (Krausgruber et al., 2011; Xu et al., 2012). The response of macrophages to GM-CSF was mixed, showing an induction of β -catenin, IRF4, and IRF5.

We examined β -catenin protein and mRNA abundance in macrophages during the response to IL-4. A gradual increase in β -catenin protein amounts was evident in macrophages exposed to IL-4 (Fig. 2A). This induction was, however, not accompanied by an increase in β -catenin mRNA amounts (Fig. 2B). Rather, IL-4-treated macrophages exhibited a decline in β -catenin mRNA abundance (to approximately half the original amount over 24 h). These observations suggested that the induction of β -catenin expression was mainly at the level of protein synthesis or stability. The contrary effect of IL-4 on β -catenin mRNA was relatively small in magnitude, and presumably eclipsed by its far greater effect on β -catenin protein.

3.2. The expression of known IL-4-inducible genes remains intact in macrophages lacking β -catenin

To determine the physiological function of macrophage-specific β -catenin signaling, we generated mice in which the deletion of *Ctnnb1*, the gene encoding β -catenin, was targeted to myeloid cells. These mutant mice, designated β -catenin Δ M, were born and grew to adulthood without manifesting any apparent abnormalities (data not shown). Macrophages formed normally when bone marrow cells from β -catenin Δ M mice were cultured in the presence of M-CSF (data not shown). In these macrophages, β -catenin expression was almost completely abolished (Fig. 3A). Therefore, macrophages derived from β -catenin Δ M mice were referred to as β -catenin-knockout (KO) macrophages.

We examined how deficiency in β -catenin function affected IL-4-induced M2 marker gene expression. Ym-1 and arginase-1 protein induction by IL-4 was intact in β -catenin-KO macrophages (Fig. 3B). To assess the effect of β -catenin ablation on the expression of a larger number of IL-4-inducible genes, we performed real-time qPCR analysis (Fig. 3C). Wild-type and β -catenin-KO macrophages exhibited comparable mRNA induction of the genes encoding syndecan-4 (*Sdc4*), E-cadherin (*Cdh1*), Ym-1 (*Chi3l3*), arginase-1 (*Arg1*), C-type mannose receptor 1 (*Mrc1*), matrix metalloproteinase-13 (*Mmp13*), thymus and activation regulated chemokine (*Ccl17*), and monocyte chemotactic protein-1 (*Ccl2*). Therefore, the ability to link IL-4 receptor signaling to the expression of known IL-4-inducible genes seemed to be spared in β -catenin-KO macrophages.

3.3. Loss of β -catenin enhances IL-4-induced macrophage-to-MGC conversion

Given the involvement of β -catenin in several cell differentiation processes, we conceived of β -catenin playing a role in the conversion of macrophages into cells with different morphological and functional properties. We explored whether IL-4-induced macrophage fusion and MGC formation were dependent on or regulated by β -catenin. Neither wild-type nor β -catenin-KO macrophages formed MGCs in the absence of IL-4 in the culture medium. In its presence, macrophage fusion was detectable as early as three days after treatment with the cytokine. Remarkably, β -catenin-KO macrophages produced a greater number of MGCs than wild-type counterparts (Fig. 4, A and B). Besides, individual MGCs from β -catenin-KO macrophages appeared to be larger in size. To obtain single-cell images that present further details of MGC structure, the cells were stained for filamentous actin (F-actin) and DNA, and analyzed by confocal microscopy. F-actin in MGCs was mainly distributed to the cell circumference (Fig. 4C). MGCs derived from β -catenin-KO macrophages included cells with atypical shapes such as those displaying a hollow core. The number of nuclei enclosed with the F-actin boundary indicated the extent to which macrophage fusion had occurred to form the MGC. The average number of nuclei per MGC was higher for β -catenin-KO than wild-type cells (Fig. 4D). The subpopulation of MGCs with more than fifteen nuclei was particularly expanded in the β -catenin-KO cell group.

3.4. MGC formation during chitin-induced peritonitis is regulated by myeloid cell-specific β -catenin function

We next examined whether β -catenin in macrophages contributes to limiting MGC formation *in vivo*, particularly in a setting that involves the recruitment of macrophages and their exposure to endogenously produced IL-4. Chitin-induced peritonitis seemed to meet these criteria, as it has been shown that when administered into the peritoneal cavity, chitin triggers innate immune signaling that leads to rapid infiltration of IL-4-producing leukocytes, such as eosinophils and basophils, and subsequently IL-4-induced macrophage polarization (Reese et al., 2007; Satoh et al., 2010). These responses were recapitulated when we injected chitin into wild-type and β -catenin Δ M mice and examined peritoneal lavage fluid 6 days later: In both mice, eosinophil infiltration was detected (Fig. 5A) and the peritoneal macrophages expressed M2 marker genes (data not shown). Importantly, multinucleated macrophages were abundant in the lavage fluid from β -catenin Δ M mice whereas few such cells were detected in wild-type mice (Fig. 5, A and B). The distribution of numbers of nuclei per multinucleated cell in the chitin-induced peritonitis experiment shifted to a lower range compared to that of MGCs generated *in vitro* (Fig. 4D & Fig. 5C). This may reflect a difference between the *in vitro* and *in vivo* settings in the efficiency of macrophage fusion or the strength of IL-4 signaling. Nevertheless, our findings from the chitin-induced peritonitis model were consistent with the results of our *in vitro* experiments and indicated a role for β -catenin in regulating multinucleated macrophage formation *in vivo*.

4. Discussion

Macrophages have the potential to acquire diverse functional properties and opt for a specific phenotype under the guidance of extrinsic factors such as cytokines. Intracellular signaling pathways and gene regulatory mechanisms serve to reprogram macrophage functions at the instruction of cytokines. In this study we have identified β -catenin as a negative regulator of IL-4-induced macrophage fusion and MGC formation. Given that β -catenin has a dual mechanism of action, acting as both a component of adherens junctions (AJ) and a nuclear transcription factor (Valenta et al., 2012), we can draw multiple hypotheses as to how it exerts regulatory functions to limit macrophage-to-MGC formation in response to IL-4. Notably, E-cadherin, another AJ component, was also induced in IL-4-

treated macrophages. An AJ-like structure could therefore be produced in IL-4-stimulated macrophages and engage in regulating MGC formation. Although E-cadherin has been shown to promote MGC formation (Moreno et al., 2007), it is still conceivable that β -catenin in the complex with E-cadherin exerts an opposite effect. Alternatively, β -catenin may function in the nucleus to participate in gene transcription events that underlie the IL-4-induced phenotype. Although β -catenin deficiency had no significant effect on the expression of IL-4-inducible genes tested in this study, it remains a possibility that β -catenin-KO macrophages are defective in the expression of as-yet-unidentified genes.

Macrophage polarization into M1 and M2 phenotypes is a useful model linking cytokine signaling to alternative functional states. Apart from the major findings discussed above, our study presents evidence that the two macrophage phenotypes are not always expressed in a mutually exclusive manner: GM-CSF treatment brought about a mixed gene expression pattern with regard to macrophage polarization. Specifically, GM-CSF induced the expression of several IL-4-responsive proteins (arginase-1, E-cadherin, β -catenin, and IRF4). Simultaneously, GM-CSF-treated macrophages produced greater amounts of the M1-associated transcription factor IRF5. Interestingly, conflicting evidence exists in the literature that supports a role for GM-CSF in skewing macrophage polarization in either direction (Chen et al., 2007; Kuroda et al., 2009; Fleetwood et al., 2009; Krausgruer et al., 2011). It remains to be seen whether the GM-CSF-induced phenotype corresponds to immunological ambidexterity or represents an intermediate in the continuum of functional state between M1 and M2 polarization.

Bone marrow-derived macrophages expressed high amounts of β -catenin after IL-4 and GM-CSF stimulation. In addition to its relevance to MGC formation, this induction likely indicates an increased demand for β -catenin activity in various other processes that follow cytokine exposure. The findings of this study and the availability of β -catenin Δ M mice will help understand the implications of macrophage fusion, MGC formation, and myeloid cell-specific β -catenin activity in immune defense and cytokine-mediated pathological conditions.

5. Conclusions

IL-4 and GM-CSF induce β -catenin expression in macrophages. Macrophages exposed to IL-4 produce higher amounts of β -catenin protein but not mRNA. Macrophage-to-MGC conversion is regulated by β -catenin; loss of β -catenin expression in macrophages results in MGC formation with greater efficiency. Our study presents β -catenin as a new regulator of cytokine-induced changes in macrophage functional state.

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Abbreviations

AJ	adherens junctions
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
IL	interleukin
IRF	Interferon regulatory factor

KO	knockout
MGC	multinucleated giant cell
PBS	phosphate-buffered saline
qPCR	quantitative PCR
RANKL	RANK ligand

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Highlights

IL-4 induces β -catenin protein but not mRNA in mouse macrophages.

Macrophages lacking β -catenin form MGCs with greater efficiency after IL-4 exposure.

MGC formation in vivo is regulated by myeloid cell-specific β -catenin function.

Fig. 1. Induction of β -catenin and other macrophage proteins by cytokines. (A) Mouse bone marrow-derived macrophages were left unstimulated or stimulated with LPS (100 ng/ml), IFN- γ (50 ng/ml), IL-4 (25 ng/ml), IL-6 (25 ng/ml), IL-10 (25 ng/ml), GM-CSF (10 ng/ml), or RANKL (25 ng/ml). Whole cell lysates were prepared after 24 h and subjected to immunoblot analysis with antibodies against the macrophage polarization marker proteins indicated on the left. (B) Mouse bone marrow-derived macrophages were treated and whole cell lysates were prepared as in (A). Immunoblot analysis was performed with antibodies specific for the transcription factor proteins indicated on the left. Arrow and asterisk indicate specific and non-specific signals, respectively.

Fig. 2.

Changes in β -catenin protein and mRNA amounts in IL-4-treated macrophages. (A) Mouse bone marrow-derived macrophages were stimulated with IL-4 (25 ng/ml) for the indicated durations. Whole cell lysates were prepared and subjected to immunoblot analysis with antibodies against β -catenin and actin. (B) Mouse bone marrow-derived macrophages were treated with IL-4 as in (A). Total RNA was isolated and subjected to real-time qPCR analysis using primers specific to *Ctnnb1* (encoding β -catenin) and *Ppia* (encoding cyclophilin; standard gene for normalization). Values represent percent mRNA amounts relative to that in unstimulated cells (0 h).

Fig. 3.

IL-4-induced gene expression in macrophages lacking β -catenin. (A, B) WT and β -catenin-KO mouse macrophages were left unstimulated or stimulated with IL-4 (25 ng/ml) for 24 h. Whole cell lysates were prepared and subjected to immunoblot analysis with antibodies against the proteins indicated on the left. Arrow denotes the truncated form of β -catenin protein resulting from the deletion within *Ctnnb1*. (C) WT and β -catenin-KO mouse macrophages were treated with IL-4 as in (A) for 4 h. Total RNA was isolated and subjected to real-time qPCR analysis using primers specific to the genes indicated on the left. *Ppia* was the standard gene for normalization as in Fig. 2B. Values represent mRNA amounts relative to that in unstimulated WT cells (left panel) and percent mRNA amounts in KO cells relative to those in WT cells 4 h after IL-4 treatment (right panel).

Fig. 4. MGC formation in β -catenin-deficient macrophages treated with IL-4. (A, B) WT and β -catenin-KO mouse macrophages in chamber slides were left unstimulated (None) or stimulated with IL-4 (25 ng/ml) for 7 d. Cells were visualized by crystal violet staining and bright-field microscopy (A). The inset images depict cells in the same chamber slides in higher magnification. Scale bar, 500 μ m. Relative MGC abundance indicates MGC numbers in image fields of the same area and represents mean $\pm$ standard deviation from three independent areas (B). ** $P < 0.01$. (C, D) WT and β -catenin-KO mouse macrophages were treated as in (A), stained for F-actin (red) and DNA (blue), and visualized by confocal microscopy (C). Scale bar, 50 μ m. The distribution of numbers of nuclei per MGC in each cell population is shown using the color code indicated on the right (D), which matches the colors with the ranges of numbers of nuclei per MGC (e.g. cyan for two to five nuclei per MGC).

Fig. 5. Multinucleated cell formation in β -catenin Δ M mice following chitin injection. (AC) WT and β -catenin Δ M mice (β -catenin-KO) were subjected to chitin-induced peritonitis, and peritoneal lavages were collected and analyzed by Diff-Quik staining and bright-field microscopy. (A). Red and blue arrowhead indicate multinucleated cells and eosinophils, respectively. Scale bar, 50 μ m. The four small panels on the right are images of individual multinucleated cells from β -catenin Δ M mice in higher magnification. Relative multinucleated cell abundance (B) and the distribution of numbers of nuclei per multinucleated cell (C) are presented as in Fig. 4.

Table 1

Oligonucleotide primers used in real-time qPCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>Arg1</i>	caagacagggctccttcag	ttccaagagtgggttcac
<i>Ccl17</i>	caggaagttggtgagctggt	catccctggaactccact
<i>Ccl2</i>	gccagctctctctctcca	cccagaagcatgacaggac
<i>Cdh1</i>	gagaacgggtggtcaaagagc	tgccccgggtatcatcatct
<i>Chi3l3</i>	ccagcatatgggcatacctt	gggcaccaattccagtctta
<i>Ctnnb1</i>	cagatgcagcgactaagcag	gctgcactagagtccaagg
<i>Mmp13</i>	tttattgttgctgccatga	ggctcctggagtgatccaga
<i>Mrc1</i>	agtgatggaacccagtgac	gttctcatggcttgctctc
<i>Ppia</i>	atggtcaacccaccgtgt	ttcttgctgtctttggaacttgtc
<i>Sdc4</i>	atctggatgacacggaggag	gcattctcagggatgtggtt