Notch Signaling Determines the M1 versus M2 Polarization of Macrophages in Antitumor Immune Responses

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Abstract

Macrophages are important tumor-infiltrating cells and play pivotal roles in tumor growth and metastasis. Macrophages participate in immune responses to tumors in a polarized manner: classic M1 macrophages produce interleukin (IL) 12 to promote tumoricidal responses, whereas M2 macrophages produce IL10 and help tumor progression. The mechanisms governing macrophage polarization are unclear. Here, we show that the M2-like tumor-associated macrophages (TAM) have a lower level of Notch pathway activation in mouse tumor models. Forced activation of Notch signaling increased M1 macrophages which produce IL12, no matter whether M1 or M2 inducers were applied. When Notch signaling was blocked, the M1 inducers induced M2 response in the expense of M1. Macrophages deficient in canonical Notch signaling showed TAM phenotypes. Forced activation of Notch signaling in macrophages enhanced their antitumor capacity. We further show that *RBP-J*–mediated Notch signaling regulates the M1 versus M2 polarization through SOCS3. Therefore, Notch signaling plays critical roles in the determination of M1 versus M2 polarization of macrophages, and compromised Notch pathway activation will lead to the M2-like TAMs. These results provide new insights into the molecular mechanisms of macrophage polarization and shed light on new therapies for cancers through the modulation of macrophage polarization through the Notch signaling. *Cancer Res; 70(12); 4840–9. ©2010 AACR.*

Introduction

Macrophages are one of the major populations of tumor-infiltrating immune cells (1). In most solid tumors, however, the existence of macrophages is advantageous for tumor growth and metastasis. These macrophages, or tumor-associated macrophages (TAM), produce interleukin (IL) 10 and transforming growth factor (TGF) β to suppress general antitumor immune responses (2). Meanwhile, TAMs promote tumor neoangiogenesis by the secretion of proangiogenic factors (3) and define the invasive microenvironment to facilitate tumor metastasis and dissemination (4). Based on these findings, it is suggested that the modulation of macrophage activation might have therapeutic potentials for cancers (5).

Macrophages respond to environmental cues in a polarized manner (6). The M1 activation is triggered by interferon-γ (IFN-γ), bacterial lipopolysaccharide (LPS), or tumor necrosis factor α (TNFα), and is mediated by several signal transduction pathways involving signal transducer and activator of transcription (STAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and mitogen-activated protein kinases (MAPK; ref. 7). These events enhance the production of microbicidal agents such as the reactive oxygen species and nitric oxide (NO) (8, 9), and promote the subsequent inflammatory immune responses by increasing antigen presentation capacity and inducing the Th1 immunity through the production of cytokines such as IL12 (10–12). In contrast, M2 macrophage activation is ambiguously used to describe macrophages activated in ways other than the M1 activation (13, 14), including IL4/IL13-stimulated macrophages (15–19), IL10-induced macrophages (20–22), and immune complex–triggered macrophages (23, 24). Among many molecular differences between M1 versus M2 activation (25), the ratio of IL12 and IL10 production is critical to distinguish M1 and M2 macrophages (6). Noticeably, TAMs share many properties of M2 macrophages.

The Notch signaling pathway is highly conserved and specifies cell fates during development (26). The DNA-binding protein RBP-J/CBF1 mediates the major transactivation signals of Notch receptors (27). A relationship between Notch signaling and macrophage activation has been suggested by several studies. The upregulation of Notch ligands, Notch receptors, and *RBP-J* has been reported in activated macrophages (28, 29). Notch activation has been proved to modulate macrophage

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gene expression and activation (30–32). In this study, we unveil the role of Notch signaling in the determination of M1 versus M2 polarization in macrophage activation in tumors.

Materials and Methods

Animals

The *RBP-J*-floxed, *Mx-Cre*, and green fluorescent protein (GFP) transgenic mice were raised, bred, genotyped, and induced with poly(I)-poly(C) exactly as described in ref. (33). Animal experiments were approved by the Animal Experiment Administration Committee of the university.

For tumor-bearing mouse models, B16 melanoma or Lewis lung carcinoma (LLC) cells were mixed with macrophages and were injected s.c. on one side of the rear back of normal mice. Tumor growth was monitored by measuring tumor length (L) and short (S) with a sliding caliper (tumor size = $L \times S^2 \times 0.51$). At the end of the experiments, the mice were sacrificed, and the tumors were excised and were used further for fluorescence-activated cell sorting (FACS) or reverse transcription-PCR (RT-PCR).

Macrophage culture

Bone marrow was flushed from femurs and tibias. Nucleated cells (2×10^7) were incubated with 20 μL of biotinylated anti-Gr1 (Ly-6G) on ice for 15 minutes, and positively labeled cells were collected with the antibiotin magnetic beads (Miltenyi Biotec GmbH). The cells (2×10^6) were cultured in 24-well plates in 1 mL of RPMI1640 medium containing 20% FCS, 2 mmol/L L-glutamine, and 40 ng/mL murine granulocyte macrophage colony stimulating factor (Pepro Tech, Inc.) for the indicated periods of time. LPS (1 μg/mL, Sigma), IL4 (5 ng/mL, Pepro Tech), or TNFα (1 μg/mL, Pepro Tech) was added to the culture 12 hours before the end of the culture. In some experiments, γ-secretase inhibitor IX (GSI; Calbiochem) was added at the concentration of 75 μmol/L with DMSO as the control.

For the coculture of macrophages and OP9 cells, OP9-Dll1 or OP9-GFP cells (2×10^5; ref. 34) were seeded in 24-well plates. After cell adherence, Gr1$^+$ monocytes (2×10^6) were seeded and cultured with granulocyte macrophage colony-stimulating factor (GM-CSF) as above. In some experiments, the F4/80$^+$ macrophages were further isolated from the coculture system by using the F4/80-biotin (BM8, eBioscience) and the antibiotin magnetic beads.

The transfusion of Gr1$^+$ cells was performed by the injection of 2×10^6 magnetically sorted Gr1$^+$ cells, which were preincubated with 10 μL of DIO (Invitrogen) dye at 37°C for 20 minutes in dark and washed thrice with PBS, through the tail vein of normal congenic mice. Ten days later, the mice were used in further experiments.

RAW264.7 was cultured with RPMI1640 supplemented with 10% FCS and 2 mmol/L L-glutamine, and was transfected with Lipofectamine 2000 (Invitrogen) according to the recommended protocol.

Flow cytometry

FACS analysis was performed with routine protocols using the FACSCalibur flow cytometer (BD Immunocytometry Systems), with antibodies listed in Supplementary Table S1. For cytoplasmic staining, cells were treated with Brefeldin A, and were stained and analyzed by using a Fixation & Permeabilization kit (eBioscience) following the recommended protocols.

ELISA and immunohistochemistry

Culture supernatants were collected and were assayed using different ELISA kits (Jinmei Biotec). Immunohistochemistry analysis for CD31, vascular endothelial growth factor receptor 2, and Hif1α in tissues was performed as previously described (35). Microvessels labeled by CD31 and vascular endothelial growth factor receptor 2 (VEGFR2) were counted. The images were imported into the Image Pro Plus 5.1 software, and the pixels for each color were analyzed to quantitatively represent positively stained signals.

Mixed lymphocyte reaction

Macrophages (2×10^5) were seeded in 24-well plates. Naïve T cells were negatively isolated from the lymph nodes of congeneic mice with magnetic beads. The T cells (2×10^6) labeled with carboxyfluorescein diacetate succinimidylester were mixed with the irradiated allogenic bone marrow macrophages (stimulator) and were added to each well. Five days later, the nonadherent T cells were collected and were labeled by anti-CD3 for FACS. In some experiments, naïve CD4$^+$ T cells were negatively isolated by using magnetic beads and were used in the coculture system. The nonadherent T cells were collected and seeded at 1×10^6 cells per well in the 96-well plates for a 3-day culture before further analyses.

Cytotoxic assay

The crude B16 tumor antigen was prepared by repeated freeze and thaw of 5×10^6 cells in 1 mL PBS and were loaded to macrophages for 24 hours. The macrophages (2×10^5) were irradiated and were cocultured with T cells (1×10^6) in 96-well plate for 3 days. The B16 cells as targets were then added into the culture with the ratio to T cells of 1:10, 1:20, 1:40, and 1:80. The cytotoxicity against the B16 cells was assessed by using the lactate dehydrogenase (LDH) assay kit (Cayman Chemical Company). In some cases, the macrophages (2×10^5) were injected s.c. into normal mice. On day 5 after the injection, CD3$^+$ T cells (1×10^6) were isolated from the draining lymph nodes, and the ability of killing was detected as above.

NO production

Macrophages (1×10^5) were seeded in 96-well plates and were cultured in the presence of LPS for 3 days. The culture supernatants (100 μL) were added to 100 μL Griess reagent, and the absorbance at 540 nm was measured with a microplate reader.

Reverse transcription-PCR

Total RNA was prepared from the cultured or sorted macrophages, reverse transcribed, and cDNA was used to amplify mannose receptor, YM1, iNOS, and migration

inhibitory factor, with β-actin as an internal control. Real-time PCR was performed using a kit (SYBR Premix EX Taq, TaKaRa) and the ABI PRISM 7500 Real-time PCR system, with β-actin as a reference control. Primers used in PCR were shown in Supplementary Table S2.

The coding region of the mouse suppressor of cytokine signaling (SOCS3) cDNA was amplified by PCR from a mouse embryonic cDNA library, was cloned, and was confirmed by sequencing. The *SOCS3* cDNA fragment was inserted into pIRES-EGFP.

Statistics

The statistical analysis was performed with the SPSS 12.0 program. Results were expressed as the means ± SD. The comparisons between groups were undertaken using the unpaired Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Differential activation of Notch signaling in M1 and M2 macrophages in tumors

We generated macrophages *in vitro* from bone marrow–derived monocytes cultured in the presence of GM-CSF (36) and inoculated normal mice with B16 or LLC cells mixed with LPS-activated macrophages. Tumor growth was monitored by tumor size and weight. As shown in Fig. 1A and B and Supplementary Fig. S1, LPS-stimulated macrophages significantly repressed the growth of the B16 and LLC tumors.

We next analyzed the polarization of macrophages in the tumors. Macrophages were obtained from *GFP* transgenic

mice, stimulated with LPS, and were used to inoculate with tumor cells. On day 5, 10, and 15 after inoculation, the tumors were dissected and macrophages were analyzed by FACS for the cytoplasmic expression of IL12 and IL10, hallmark cytokines of M1 and M2 polarization, respectively (Supplementary Fig. S2). As shown in Fig. 1C and Supplementary Fig. S3, GFP+ macrophages were M1 polarized (more IL12+ cells), whereas the endogenous GFP− macrophages were M2 polarized (more IL10+ cells).

We sorted GFP+ (M1) and GFP− (M2) macrophages from the B16 tumors and examined the expression of the Notch-related molecules. The results showed that GFP+ macrophages in tumors expressed higher levels of *Notch* ligands and receptors, and the downstream molecule *Hes1*, suggesting a higher level of Notch activation in the M1-polarized macrophages in tumors (Fig. 1D).

Notch activation promotes M1 polarization of macrophages

We then examined the effects of Notch activation on macrophage polarization by using OP9 cells overexpressing Dll1 (OP9-Dll1), with OP9-GFP as a control (34). Normal bone marrow monocytes were cultured with different OP9 cells in the presence of GM-CSF, and the resulting macrophages were stimulated with LPS or IL4. Cytoplasmic staining of IL12 and IL10 expression in F4/80+ cells showed that with forced Notch activation, LPS induced a remarkably stronger M1 response, as manifested by the significantly increased IL12-expressing cells (Supplementary Fig. S4, top; Fig. 2A). Interestingly, in the presence of Notch activation, IL4 induced not only IL10-expressing cells (M2 activation), but also cells
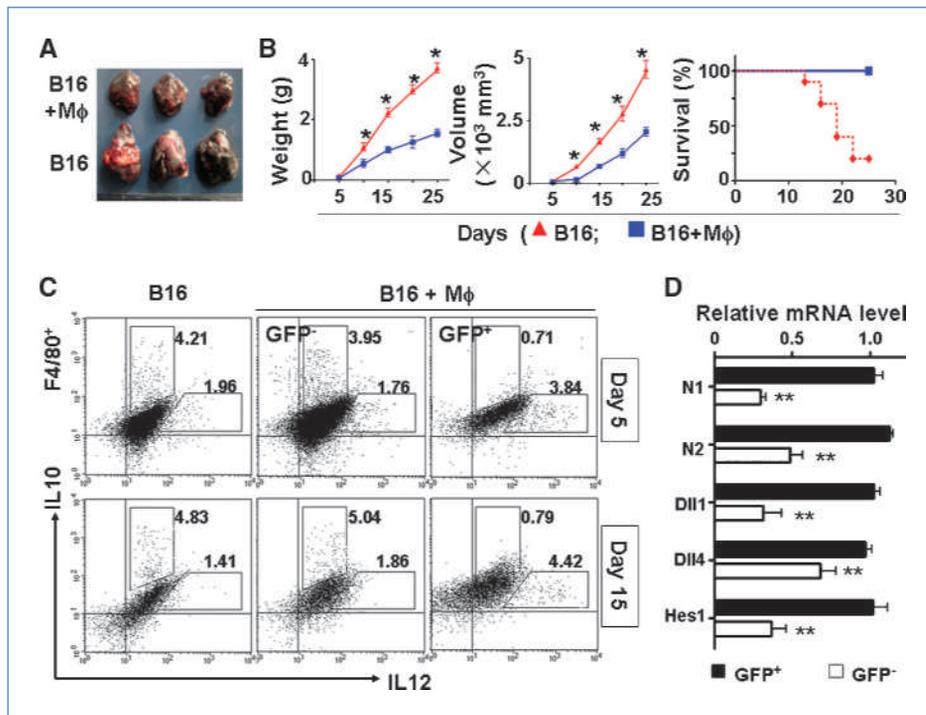
Figure 1. M1 macrophages inhibited tumor growth and expressed higher level of Notch-related molecules. A, B16 cells were mixed with or without GFP+ bone marrow–derived macrophages pretreated with LPS and were injected s.c. into wild-type mice. Tumors were dissected 15 d after inoculation and were photographed. B, tumor weight, tumor volume, and the survival of mice were compared on different days after tumor inoculation. C, tumors were minced, and cell suspensions were analyzed by FACS for cytoplasmic IL10 and IL12. The results from tumors on day 5 and 15 were shown. D, GFP+ and GFP− macrophages (1 × 10^6) in tumors were sorted and analyzed by real-time RT-PCR. Columns, mean; bars, SD. *, $P < 0.05$; **, $P < 0.01$; $n = 3$.
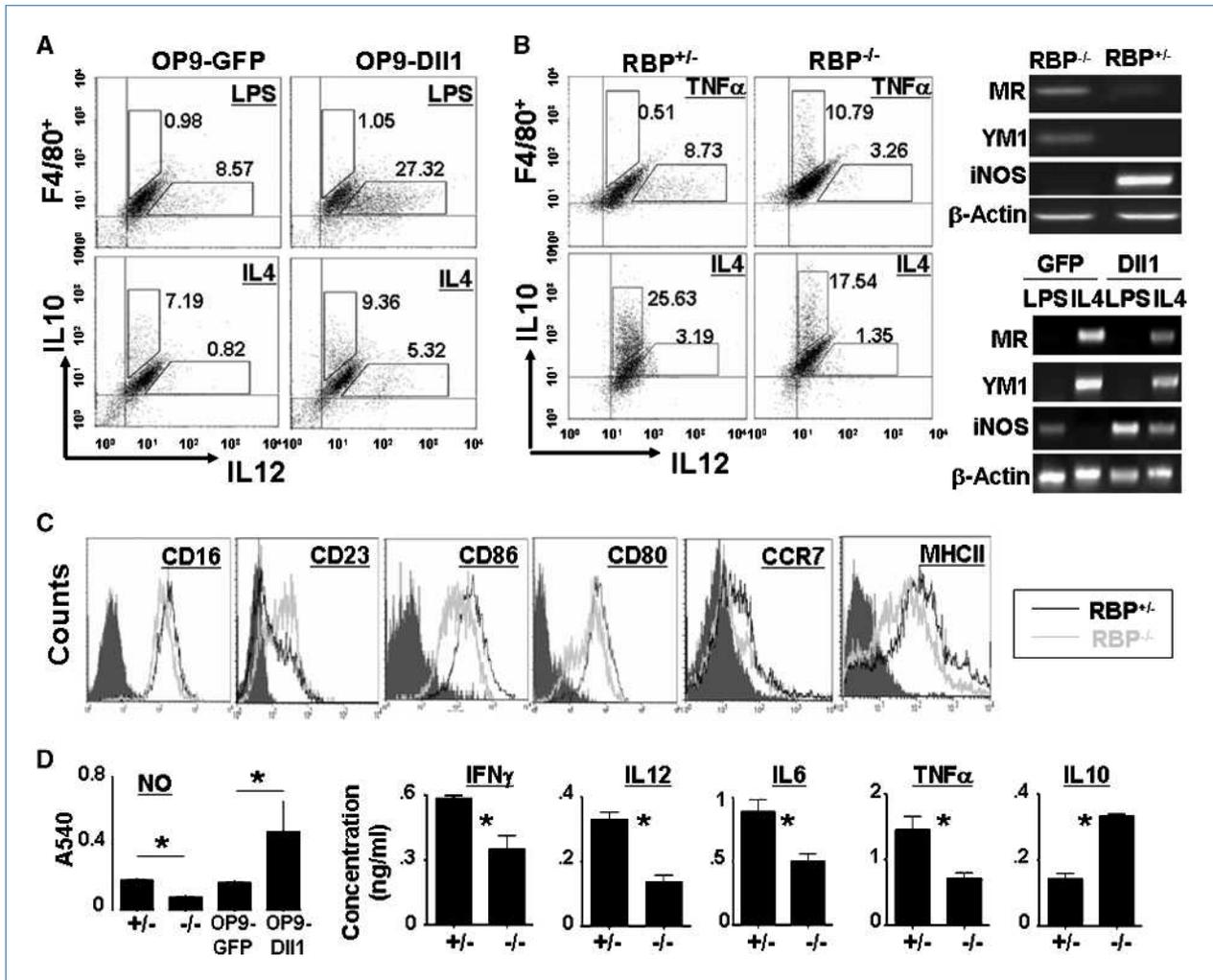
Figure 2. Modulation of Notch signaling subverted macrophage polarization. A, normal Gr1^{+} monocytes were cocultured with OP9-GFP or OP9-Dll1 cells in the presence of GM-CSF. The macrophages were then stimulated with LPS or IL4, and the cytoplasmic IL10 and IL12 were analyzed by FACS. B, macrophages were cultured from the *RBP-J* knockout (*RBP*^{−/−}) and the control (*RBP*^{+/−}) mice, stimulated with TNFα or IL4, and were analyzed as above. C, LPS-stimulated *RBP*^{+/−} and *RBP*^{−/−} macrophages were analyzed by FACS (left) and RT-PCR (right). For RT-PCR, *RBP*^{+/−} and *RBP*^{−/−} macrophages were stimulated by LPS (top right), or normal macrophages were stimulated with LPS or IL4, in the presence of OP9-GFP or OP9-Dll1 (bottom right). cDNA fragments for MR, YM1, iNOS, and β-actin were amplified. The results represented three independent experiments. D, NO and cytokine production. Macrophages (1 × 10^{5}) with different genotypes or prestimulations were cultured in 96-well plates for 3 d. The supernatants (100 μL) were harvested and measured. For the cytokine production by the macrophages, the supernatants of the LPS-stimulated macrophages cultured as in B were collected and measured by ELISA. Columns, means (*n* = 3); bars, SD. *, *P* < 0.05.

expressing IL12, a marker of M1 response (Supplementary Fig. S4, bottom; Fig. 2A). These results suggested that the activated Notch signaling could enhance the M1 response, even in the presence of an M2 inducer (IL4). Notch signaling alone did not activate macrophages (data not shown).

Blocking of Notch signaling results in M2 polarization even in the presence of M1 inducers

We then investigated the role of Notch signaling in macrophage polarization by using GSI that abrogates Notch signaling. The results showed that when Notch signaling was blocked, both LPS and TNFα induced the increase of IL10-expressing cells and the decrease of IL12-expressing cells

compared with the controls (Supplementary Figs. S5), suggesting M2 rather than M1 response. This treatment did not alter the expression of Toll-like receptor (TLR) 4 and its downstream molecule Myd88 as detected by using RT-PCR and FACS (data not shown).

Next, Gr1^{+} monocytes were recovered from the poly(I)-poly(C)–induced *MxCre-RBP*[f/f] (*RBP-J*^{−/−}) mice (33) and cultured in the presence of GM-CSF to generate *RBP-J*^{−/−} macrophages, with *RBP-J*^{+/−} macrophages as a control, which have no detectable phenotypic difference from wild-type macrophages (data not shown). *RBP-J*^{−/−} macrophages were generated at a higher efficiency than *RBP-J*^{+/−} in this culture system (data not shown). As shown in Fig. 2B and Supplementary

Figs. S6 and S7, TNFα and LPS induced an M1 response in *RBP-J*^{+/−} macrophages, with a remarkable increase of IL12-expressing cells, whereas few IL10-expressing cells were observed. In the TNFα-stimulated *RBP-J*^{−/−} macrophages, however, the IL10^{+} cells increased significantly, whereas the IL12^{+} cells decreased significantly. In the LPS-stimulated cells, although M1 activation still existed, many cells expressed the M2 marker IL10. So the disruption of Notch signaling by *RBP-J* deletion switched macrophages to M2 polarization despite of the presence of M1 inducers. IL4 induced comparable M2 response in *RBP-J*^{+/−} and *RBP-J*^{−/−} macrophages.

Macrophages undergoing M1 or M2 activation have characteristic markers and cytokine profiles (reviewed in refs. 1, 6, 7, 37). FACS analysis showed that (Fig. 2C, left of cytographs), compared with the control, LPS-stimulated *RBP-J*^{−/−} macrophages expressed mildly lower level of CD16/CD32, CD80, CD86, CCR7, but higher level of CD23, compatible with an M2 phenotype. LPS-stimulated *RBP-J*^{−/−} macrophages had lower level of MHC II expression, probably due to the autocrine effect of IL10. The expression of

two M2 macrophage-specific molecules, mannose receptor (MR) and YM1, were higher in the LPS-stimulated *RBP-J*^{−/−} macrophages than in the *RBP-J*^{+/−} macrophages (Fig. 2C, right of electrophoretic graphs). The expression of inducible NO synthesase (iNOS) and macrophage migration inhibitory factor (data not shown), two M1 markers, was barely detectable in the LPS-stimulated *RBP-J*^{−/−} macrophages (Fig. 2C, right of electrophoretic graphs), in contrast to the significant bands in the controls. The low expression of iNOS in the LPS-stimulated *RBP-J*^{−/−} macrophages was in accordance with their low NO production (Fig. 2D). LPS-stimulated macrophages cocultured with OP9-Dll1 showed higher iNOS expression and NO production (Fig. 2D), whereas IL4-stimulated macrophages cocultured with OP9-Dll1 expressed not only MR and YM1, but also iNOS (Fig. 2C, right of electrophoretic graphs), consistent with the FACS data (Fig. 2A). Analysis of the cytokine production profile showed that the LPS-stimulated *RBP-J*–deficient macrophages produced a lower level of M1 cytokines (IFNγ, IL12, IL6, and TNFα), but higher level of the M2 cytokine IL10 (Fig. 2D, right).
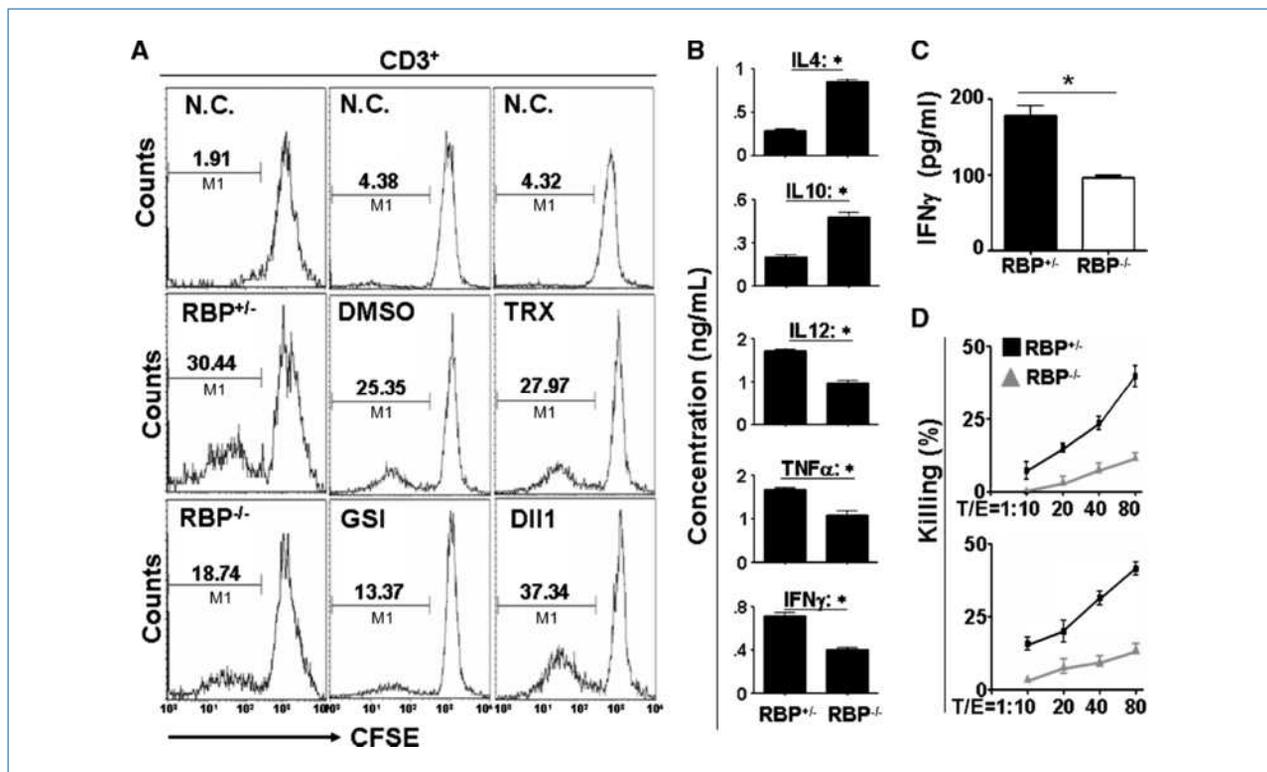
Figure 3. The interference of Notch signaling in macrophages modulated their antigen presentation activity. A, mixed lymphocyte reaction. LPS-stimulated macrophages with the indicated genotypes or treatments were cocultured for 5 d with carboxyfluorescein diacetate succinimidylester (CFSE)–loaded syngeneic T cells prestimulated with the irradiated allogenic cells. The proliferation of T cells was examined by FACS. N.C., negative control without macrophages. B, the *RBP*^{+/−} and *RBP*^{−/−} macrophages were stimulated with LPS and were cocultured with negatively sorted CD4^{+} T cells for 24 h. The T cells were collected and were cultured for 3 more days, before the supernatants were analyzed by ELISA. C, *RBP*^{+/−} or *RBP*^{−/−} macrophages were injected into the hind footpads of normal mice. CD3^{+} T cells in the draining lymph nodes were isolated 5 d later and were cultured for 3 d. The supernatants were analyzed by ELISA. D, cytotoxicity. Tumor antigen–loaded macrophages were irradiated and were cocultured with T cells for 3 d. B16 cells as targets were added into the culture. Cytotoxicity was assessed using the LDH assay kit. Bottom, the macrophages were injected into normal mice. CD3^{+} T cells were isolated 5 d later from the draining lymph nodes, and the ability of killing was detected as above. Points, means (n = 3); bars, SD. *, P < 0.05.
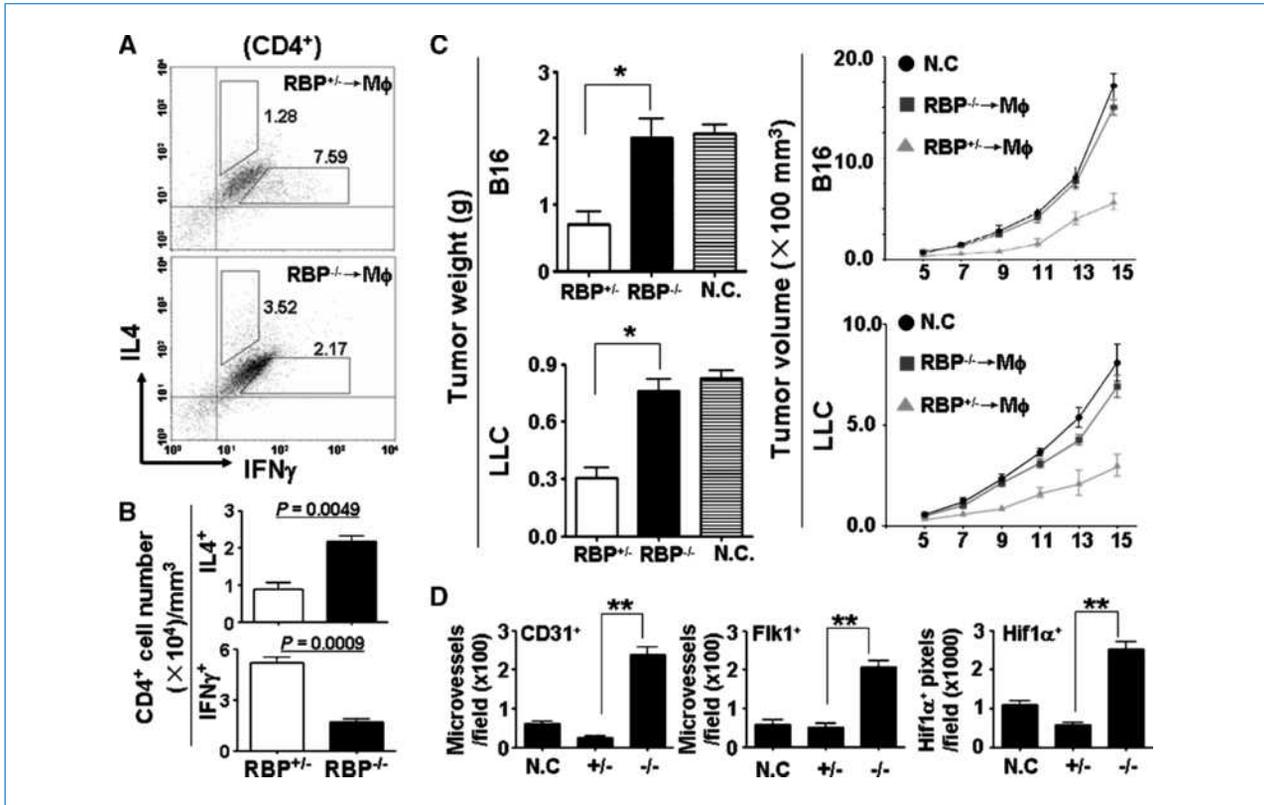
Figure 4. RBP-J–deficient macrophages displayed TAM-like phenotypes. A and B, B16 cells were inoculated s.c. with LPS-activated $RBP^{+/-}$ or $RBP^{-/-}$ macrophages in normal mice. CD4$^+$ T cells in the tumors were analyzed 15 d later by FACS. The absolute number of different populations of cells per 200 mm^3 of tumors in A was calculated and compared in B. C, B16 and LLC cells were inoculated as in A; the weight (left) and volume (right) of tumors were measured. D, B16 tumors in C were stained by immunohistochemistry. Microvessels labeled by CD31 (left) and VEGFR2 (middle) were counted, and the microvessel densities (microvessels per field) were compared. The Hif1α–positive signals were quantified and were compared (right). Columns, means (**, $P < 0.01$; $n = 3$); bars, SD.

We transplanted DIO-labeled $RBP\text{-}J^{+/-}$ and $RBP\text{-}J^{-/-}$ monocytes into normal congenic mice. F4/80$^+$ macrophages were isolated from bone marrow and the spleen of the recipients 10 days later. Whereas LPS or TNFα induced M1 activation of $RBP\text{-}J^{+/-}$ macrophages (DIO$^+$F4/80$^+$), the same treatment induced M2 in the expense of M1 activation of $RBP\text{-}J^{-/-}$ macrophages (Supplementary Fig. S8, top and middle; Supplementary Fig. S9). When LPS was injected into the peritoneal cavity of the recipient mice, DIO$^-$F4/80$^+$ endogenous macrophages and DIO$^+$F4/80$^+$ $RBP\text{-}J^{+/-}$ macrophages showed M1 differentiation. However, LPS evoked the M2 polarization in DIO$^+$F4/80$^+$ macrophages of mice accepting $RBP\text{-}J^{-/-}$ Gr1$^+$ cells *in vivo* (Supplementary Fig. S8, bottom; Supplementary Fig. S10). In summary, these data indicated that macrophages deficient in Notch signaling were polarized into M2 phenotypes in the expense of M1.

RBP-J–deficient macrophages show attenuated capacity to activate T cells

Macrophages activate T cells in immune responses (38, 39). Macrophages were activated with LPS, with their Notch signaling being blocked by *RBP-J* deletion or by the pretreatment with GSI. Syngenic T cells activated by irradiated allogenic cells were cocultured with different macrophages, and T-cell proliferation was assessed. The blockade of Notch signaling in macrophages attenuated their capacity of stimulating T-cell proliferation (Fig. 3A). On the other hand, if macrophages were pretreated with recombinant human Dll1 (40) and were activated by LPS, their ability to stimulate T-cell proliferation was enhanced (Fig. 3A, right).

$RBP\text{-}J^{-/-}$ and control macrophages were stimulated with LPS and were cocultured with CD4$^+$ naive T cells from normal congenic mice. T cells were then collected and cultured for 3 more days, and their cytokine production was assayed by ELISA. Compared with T cells cocultured with the $RBP\text{-}J^{+/-}$ macrophages, T cells cocultured with the $RBP\text{-}J^{-/-}$ macrophages showed the Th2 cytokine profile, with higher level of IL4 and IL10 but lower level of IL12, IFNγ and TNFα (Fig. 3B). Furthermore, the LPS-stimulated $RBP\text{-}J^{+/-}$ or $RBP\text{-}J^{-/-}$ macrophages were injected s.c. into normal mice. CD3$^+$ T cells in the draining lymph nodes were isolated 5 days later and cultured *in vitro*. ELISA of the supernatant showed that the T cells from mice accepting $RBP\text{-}J^{-/-}$ macrophages had lower capacity of producing IFNγ, compared with the controls (Fig. 3C).

We next examined the ability of *RBP-J*–deficient macrophages to activate the cytotoxicity of T cells. *RBP-J*^{+/-} and *RBP-J*^{-/-} macrophages were loaded with B16 tumor antigens, irradiated, and were cocultured with CD3^{+} T cells. B16 cells as targets were added into the culture with different ratios to T cells. Cytotoxicity against B16 cells was assessed. In another set of experiments, tumor antigen–loaded macrophages were injected s.c. into normal mice. On day 5 after the injection, CD3^{+} T cells were isolated from the draining lymph nodes, and the ability to kill B16 tumor cells was detected. The results showed that both *in vitro* (Fig. 3D, top) and *in vivo* (Fig. 3D, bottom), the *RBP-J*^{-/-} macrophage–activated T cells showed attenuated cytotoxic activity to tumor cells. These results suggested that the Notch signaling–blocked macrophages had lowered activity in activating T cells.
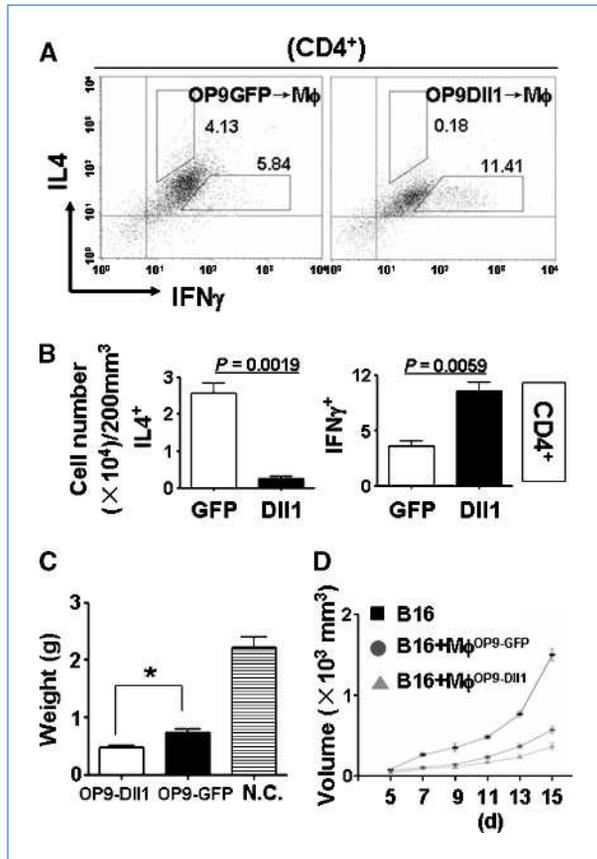
Figure 5. Macrophages with activated Notch signaling showed stronger tumor-repressing activity. A and B, normal macrophages prestimulated using OP9-GFP or OP9-Dll1 were isolated with anti-F4/80 magnetic beads. B16 cells were mixed with the macrophages and were inoculated s.c. in normal mice. The tumors were dissected 15 d after the inoculation, and CD4^{+} T cells were analyzed by FACS (A). The absolute number of different populations of cells per 200 mm³ of tumors was compared in B (*n* = 3). C and D, the tumor weight (C) and the tumor volume (D) were examined.

RBP-J–deficient macrophages show TAM-like phenotypes

We then tested the effects of *RBP-J*^{-/-} macrophages on tumor growth. *RBP-J*^{+/-} and *RBP-J*^{-/-} macrophages were mixed with B16 cells and were inoculated s.c. in normal mice. Fifteen days later, the infiltrated CD4^{+} T cells in the tumors with *RBP-J*^{+/-} macrophages were Th1 dominated (IFNγ^{+}), but CD4^{+} T cells in the tumors with *RBP-J*^{-/-} macrophages were Th2 dominated (IL4^{+}; Fig. 4A and B). The T-cell number in the draining lymph nodes of mice bearing tumors containing *RBP-J*^{-/-} macrophages was lower than that of the control (1.95×10^6 versus 3.27×106, $P = 0.0068$).

Tumor growth was followed up for 15 days regarding the tumor size and the tumor weight. The results showed that in B16 and LLC tumors, although the control macrophages had significant tumor repression activity, RBP-J–deficient macrophages showed no such activity (Fig. 4C). Meanwhile, remarkably, more microvessels were detected in tumors containing *RBP-J*^{-/-} macrophages. These tumors also had higher Hif1α expression, suggesting less blood perfusion (Supplementary Fig. S11; Fig. 4D). These results suggested that RBP-J–deficient macrophages had TAM-like phenotypes.

Macrophages with activated Notch signaling show enhanced tumor-repressing activity

Normal macrophages prestimulated with OP9-GFP or OP9-Dll1 were mixed with B16 melanoma cells and inoculated s.c. T cells in the tumors were examined 15 days later. As shown in Fig. 5A and B, macrophages precultured with OP9-Dll1 promoted Th1 bias (with more IFNγ^{+} cells) of T cells in the tumors, compared with the control. Moreover, Notch-activated macrophages showed stronger tumor-repressive effects (Fig. 5C and D).

SOCS3 is critically involved in Notch signaling–mediated macrophage polarization

It has recently been shown that Notch signaling mediates Bacillus Calmette-Guerin–induced SOCS3 upregulation (41). We then examined *SOCS3* expression in macrophages with interrupted Notch signaling. LPS induced *SOCS3* expression in primary normal macrophages or macrophage cell line RAW264.7. Blocking of Notch signaling with GSI or *RBP-J* knockout abrogated LPS-induced *SOCS3* expression (Fig. 6A).

We next examined the role of SOCS3 in Notch-mediated M1/M2 polarization. Treatment of RAW264.7 with LPS and IL4 increased the cells expressing IL12 and IL10, respectively. GSI increased cells expressing IL10 and mildly decreased cells expressing IL12 concomitantly (Supplementary Fig. S12). RAW264.7 cells were then transfected with vectors expressing enhanced green fluorescent protein (EGFP) or EGFP+SOCS3 and were treated with LPS+DMSO or LPS+GSI. As shown in Fig. 6B and C, in cells expressing only EGFP, GSI subverted LPS-induced M1 polarization, consistent with previous data. However, in cells expressing both EGFP and SOCS3, the M1 polarization of LPS-stimulated macrophages was restored in the presence of GSI. These results, in combination with Narayana's data (41), suggested that SOCS3
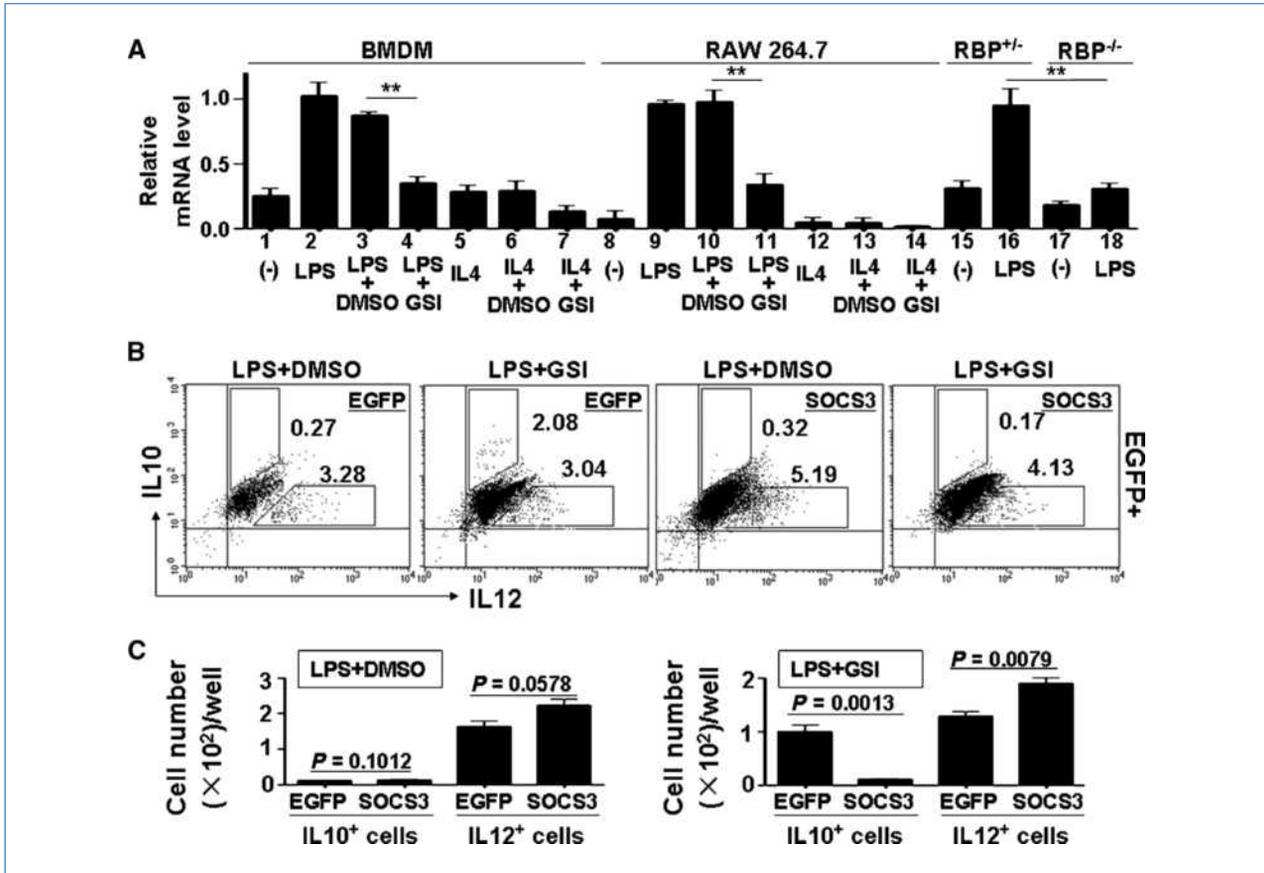
Figure 6. SOCS3 mediated the modulation of macrophage polarization by Notch signaling. A, bone marrow–derived macrophages (2×10^6) or RAW264.7 cell were stimulated with medium (–), LPS, or IL4, in the presence of DMSO or GSI for 12 h. Total RNA was prepared and analyzed by real-time RT-PCR. B and C, RAW264.7 cells were transfected with pIRES-EGFP or pIRES-EGFP-SOCS3. Three days after the transfection, the cells were stimulated with LPS+DMSO or LPS+GSI. The expression of IL10 and IL12 was analyzed by FACS (B). Absolute number of $IL12^+$ and $IL10^+$ cells was compared (C). Columns, means; bars, SD; **, $P < 0.01$ ($n = 5$).

was a downstream molecule to Notch signaling to modulate M1/M2 polarization of macrophages.

Discussion

Although macrophage activation is roughly classified into two poles, activated macrophages express different sets of molecules and show different functional properties strongly dependent on the stimuli in the environment (37). This is especially true for M2 activation. Evidence has shown that IL10- and immuno-complex–induced M2 activation is associated with IL10 production. But the relationship between IL4/IL13 and IL10 has been controversial, although some data have shown that IL4 and IL13 in combination with LPS could increase IL10 production (42). In our hand, IL4 treatment induced IL10 expression in macrophages. Taking the production of IL12 and IL10 as the criteria for M1 and M2 polarization, we concluded that Notch signaling represents a critical endogenous mechanism to determine M1 versus M2 polarization of macrophage activation. Interestingly, Notch signaling–deficient macrophages seemed to have

"complex" phenotypes of different subtypes of M2 polarization. They have a surface marker profile of alternatively activated macrophages and functionally bias naïve $CD4^+$ T cells into Th2. On the other hand, they express IL10 and show reduced T-cell activation ability and TAM-like activity when inoculated in solid tumors, which are all properties of regulatory macrophages. More studies are needed to clarify whether this is due to the heterogeneicity or the plasticity of macrophage activation.

SOCS family members are inducible inhibitors of cytokine signals and thus play a critical role in limiting inflammation responses (43). SOCS proteins could be induced by cytokines through the cytokine signaling pathway, and they in turn inhibit the cytokine signaling by several mechanisms. SOCS proteins could also be directly induced by Toll-like receptor signaling. In macrophages and dendritic cells, SOCS proteins not only regulate the sensitivity of cells toward cytokines but also modulate signaling through Toll-like receptor. Liu and colleagues (44) showed that unique expression of SOCS3 is essential for classic macrophage activation. Because SOCS3 is a downstream molecule of Notch signaling (41), it is most

likely that Notch signaling determines the M1 versus M2 polarization of macrophages through SOCS3.

Several reports and our unpublished data[3] have shown that the stimulation of macrophages with proinflammation factors results in not only the M1 type macrophage activation but also the upregulation of Notch pathway molecules, leading to the activation of canonical Notch signaling. Our unpublished results also showed that LPS-, parasitic antigens−, and TNFα-induced Notch ligand upregulation was dependent on RBP-J. Therefore, M1 activation of macrophages is accompanied by the activation of Notch signaling. Our results reported here have indicated that the Notch activation in macrophages could promote the M1 polarization while repressing the M2 polarization. Therefore, these stimulants might trigger an M1 polarization through a combination of transcription factors including STATs, NFκB, and MAPKs. This M1 polarization could be licensed and amplified through the activation of Notch signaling. On the other hand, literature has shown that the activation of canonical Notch signaling leads to the production of Hes family bHLH molecules, which could in turn repress the expression of Notch ligands to attenuate Notch signaling in neighboring cells (45). Therefore, as the M1 macrophage activation proceeds, this feedback mechanism could lead to the shutdown of the Notch signaling autonomously to direct macrophages to shift from an M1 polarization to an M2 polarization, re-

sulting in the fading of macrophage-triggered inflammation. This proposed scenario is reminiscent of the role of Notch signaling in endothelial cells, in which the proangiogenic signaling through VEGFR2 induces the expression of Notch-related molecules and the activation of Notch signaling, and the activated Notch signaling in turn represses angiogenesis through the expression of Hes family molecules. However, given the complexity of both macrophages population and Notch signaling pathway, more studies are needed to show the requirement of specific Notch pathway molecules in the polarization of specific subtype macrophages, as proposed by a recent report by Michelucci and colleagues (46).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[3] Our unpublished data.

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