TLR signaling-mediated differential histone modification at IL-10 and IL-12 promoter region leads to functional impairments in tumor-associated macrophages

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Tumor-associated macrophages (TAM) are severely compromised for the induction of proinflammatory mediators following toll-like receptor (TLR) activation. Here, we reported that the defective TLR response in TAM was due to the malfunctioning of the myeloid differentiation primary response gene 88 (MyD88)-dependent signaling cascade in concert with downregulation of tumor necrosis factor receptor-associated factor (TRAF) 6 and interleukin-1 receptor-associated kinase (IRAK) 1. However, the expression of toll-interleukin-1 receptor domain-containing adapter-inducing interferon beta (TRIF) and TRAF 3, which act via the TRIF-dependent pathway of TLR signaling, were found to be unaffected in TAM. Although, TRIF-mediated signal inducers, lipopolysaccharide or poly (I:C), induced high level of extracellular signal-regulated kinase (ERK)-1/2 mitogen-activated protein kinase (MAPK) phosphorylation, but they were failed to induce significant p38MAPK phosphorylation in TAM. Consequently, ERK-1/2-dependent histone phosphorylation at the IL-10 promoter elicited enhanced interleukin (IL)-10 production by TAM. Whereas, the lack of transcription favorable histone phosphorylation at the IL-12 promoter was accompanied with a very low amount of IL-12 expression in TAM. Moreover, ERK-1/2 MAPK activation resulted in enhanced IRAK M induction in TAM, a specific inhibitor of MyD88 pathway. Therefore, for the first time, we decipher an unexplored TLR signaling in TAM where ERK-1/2 activation in a MyD88-independent pathway results in transcription favorable histone modification at the IL-10 promoter region to enhance IL-10-mediated immunosuppression. Additionally, by enhancing IRAK M induction, it also polarizes TAM toward a more immunosuppressive form.

Introduction

Solid tumors consist of neoplastic cells, non-malignant stromal cells and migratory hematopoietic cells and complex interactions among these cell populations regulate inflammation, metastasis, angiogenesis and tumor progression (1,2). With tumor progression, tumor-instructed-microenvironment tilt the associated cell populations even host supportive cells toward its own favor (3–5). One of the most important cell types of this tumor microenvironment is the macrophage. Tumor-derived chemokines attract blood-circulating monocytes (6,7), which appear to be initiators in the activation of nuclear factor-kappaB (NF-jB) and MAPK in macrophages (20). Currently, it is reported that the defective NF-jB activation is responsible for the suppressive phenotype of TAM (13,21). However, there is no report on the TLR-mediated p38 and extracellular signal-regulated kinase (ERK)-1/2 MAPK signaling in TAM. In many diseases, the differential regulation of the p38 and ERK-1/2 MAPK signaling in macrophages determines the induction of either proinflammatory or anti-inflammatory response (22). ERK-1/2 MAPK activation is associated with enhanced IL-10 induction (23) and decreased IL-12 induction (24) in macrophages. Moreover, ERK-1/2 is also involved in core histone modification at the il-10 promoter region of macrophages (25). Therefore, it will be interesting to study its role in the context of chromatin modification at the il-10 and il-12 promoter region of TAM following TLR stimulation.

Moreover, ERK-1/2 MAPK also induce the expression of IRAK M (26), a negative regulator in MyD88-dependent TLR downstream signaling which disrupts the interaction between IRAK 4 and IRAK 1 and inhibits the MyD88-dependent TLR activation (27). Since the MyD88-dependent pathway is selectively disrupted in TAM (21,28), therefore, it will be interesting to investigate the role of ERK-1/2 MAPK for the induction of IRAK M in TAM.

In the present study, we have addressed the issue of higher IL-10 induction by TLR ligands from TAM in the face of tumor-associated pathogenesis by investigating underlying molecular mechanism. Here, we have hypothesized that established tumors selectively disrupt the MyD88-dependent pathway of TLR activation in TAM and utilize the MyD88-independent or TRIF-dependent pathway for their own favor. To explore the pathway involved in enhanced IL-10 induction, we stimulated TAM with poly (I:C) and CpG ODN which activate the TRIF-dependent and MyD88-dependent pathways of TLR signaling, respectively. Stimulation of TAM with poly (I:C) produced higher amount of IL-10. Interestingly, TAM were unresponsive to CpG ODN. Our findings also suggest that ERK-1/2 MAPK activation trigger a biphasic alteration in TAM by enhancing the expression of anti-inflammatory IL-10 and by upregulating the expression of (8,9). Classical macrophages produce higher amount of inflammatory mediators like interleukin (IL)-12, tumor necrosis factor (TNF) receptor-associated factor, reactive oxygen species and nitrogen intermediates when activated via lipopolysaccharide (LPS) or interferon (IFN)-γ (10,11). Interestingly, these tumor-associated macrophages (TAM) exhibited an altered phenotype and produced higher amount of immunosuppressive cytokine IL-10; however, the IL-12 expression was not significant enough in response to macrophage activators like LPS (12,13). Moreover, growing evidences from preclinical and clinical studies also support abundance of TAM with poor prognosis (14–17).

Toll-like receptors (TLR) are very important components of innate immunity (18). They are expressed constitutively on the surface as well as on the endosomal membrane of macrophages, dendritic cells and activate these cells by recognizing molecular pattern of either microbial- or host-derived origin (18,19). Depending on the nature of the pathogen, TLR transduce the activation signal to the cells either by the myeloid differentiation primary response gene 88 (MyD88) dependent or the toll-interleukin1 receptor domain-containing adapter-inducing interferon-beta (TRIF) dependent signaling pathway (18,20). Both of these pathways of TLR signaling ultimately result in the activation of nuclear factor-kappaB (NF-kB) and mitogen-activated protein kinase (MAPK) to mount a strong proinflammatory response against the invaders (18,20). The MyD88-dependent pathway activates NF-kB and MAPK in a TNF receptor-associated factor (TRAF) 6-dependent mechanism utilizing MyD88, interleukin-1 receptor-associated kinase (IRAK) 4 and IRAK 1 signaling molecules (20). TRIF-dependent pathway utilizes both of the TRAF 6-dependent and TRAF 6-independent mechanism for the activation of NF-kB and MAPK in macrophages (20). Currently, it is reported that the defective NF-kB activation is responsible for the suppressive phenotype of TAM (13,21). However, there is no report on the TLR-mediated p38 and extracellular signal-regulated kinase (ERK)-1/2 MAPK signaling in TAM. In many diseases, the differential regulation of the p38 and ERK-1/2 MAPK signaling in macrophages determines the induction of either proinflammatory or anti-inflammatory response (22). ERK-1/2 MAPK activation is associated with enhanced IL-10 induction (23) and decreased IL-12 induction (24) in macrophages. Moreover, ERK-1/2 is also involved in core histone modification at the il-10 promoter region of macrophages (25). Therefore, it will be interesting to study its role in the context of chromatin modification at the il-10 and il-12 promoter region of TAM following TLR stimulation.

Abbreviations: ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; IFN, interferon; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; LPS, lipopolysaccharide; mRNA, messenger RNA; NF-kB, nuclear factor-kappaB; PCR, polymerase chain reaction; siRNA, small-interfering RNA; TAM, tumor-associated macrophage; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor.
IRAK M, thereby polarizing the TAM toward a suppressive phenotype. Together, our present study revealed a previously unknown mechanism of defective TLR activation response in TAM, which can be further exploited as a target to develop novel therapeutic strategies.

Materials and methods

Reagents and chemicals

RPMI-1640 medium, penicillin and streptomycin, PD098059 (ERK inhibitor), SB203580, LPS, collagenase and TRI Reagent were from Sigma (St Louis, MO). Poly (IC) and CpG ODN were obtained from Invivogen (San Diego, CA). Fetal calf serum was purchased from Gibco BRL (Grand Island, NY) and enzyme-linked immunosorbent assay (ELISA) Assay Kit (Quantikine M) for mouse IL-12, IL-10, TNF-α, IFN-γ and transforming growth factor (TGF)-β were from R&D Systems (Minneapolis, MN). Deoxynucleoside triphosphates, RevertAidTM M-MuLV Reverse Transcriptase, oligo dT, RNase inhibitor and other chemicals required for complementary DNA synthesis were from Fermentas (Ontario, Canada). Anti-phospho-H3 and TRIF Abs were obtained from Abcam and chromatin immunoprecipitation (ChiP) assay kits were purchased from Millipore (Bedford, MA). MyD88, IRAK 1, IRAK M, TRAF 6, TRAF 3, TLR 4, TLR 3, TLR 9, β-actin, phosphorylated and dephosphorylated forms of p38 and ERK-1/2 antibodies and MyD88 small-interfering RNA (siRNA) and control siRNA were obtained from Santa Cruz Biotechnology (San Jose, CA).

Cell culture

TAM and peritoneal macrophages were cultured in complete RPMI-1640 medium containing 10% fetal calf serum (vol/vol), 2 mMolar glutamine and 100 U/ml penicillin and streptomycin. TLR stimulation was performed in complete RPMI-1640 medium with the following ligand concentrations: poly (IC), 30 µg/ml; LPS, 100 ng/ml; CpG ODN, 1 µM. For different treatments, PD098059 (a specific pharmacological inhibitor of ERK-1/2 MAPK) (10 µM) or SB203580 (a specific pharmacological inhibitor of p38MAPK) (1 µM) was added to the cells prior to the TLR ligand stimulation.

Preparation of TAM and peritoneal macrophages

Male 8-week-old C57BL/6 mice were purchased from the National Centre for Laboratory Animal Sciences, India. For tumor development, mice were inoculated subcutaneously with 10⁶ B16F10 melanoma cells as described elsewhere (29). TAM were isolated 3 weeks after tumor implantation. TAM were isolated following a standard protocol, briefly the tumor tissue was kept in collagenase for 20 min with gentle agitation using a magnetic stirring rod. By 20 min, most of the tissue had been digested and the sample was passed through a 70 µm mesh to provide a single cell suspension. These cells were then spun at 1500 r.p.m., the collagenase containing supernatant was aspirated and the pellet was washed with calcium and magnesium free Hanks’ balanced salt solution (HBSS). Cells were washed twice before being finally resuspended in complete HBSS at a concentration of 10 million cells/ml. Cell viability was assessed using a trypan blue exclusion test and was >98%. The cells were plated on 10 cm tissue culture dishes at 5 ml per dish and allowed to adhere for 40 min at 37°C. After this time, the plates were washed with HBSS to remove all non-adherent cells. Such a short time of adherence was required to prevent contamination of the cell population by tumor cells, which have begun to adhere after 1 h. The adherent population contained >95% macrophages as identified by the morphologic and functional criteria (30). Additionally, they are also identified by the surface expression of macrophage markers like F4/80 and CD68.

Enzyme-linked immunosorbent assay

The cell-free supernatant from control peritoneal macrophages and TAM were assayed for mouse IL-12, IL-10, TNF-α, IFN-γ and TGF-β cytokines with the use of the sandwich ELISA kit (Quantikine M; R&D Systems). The assay was performed according to the manufacturer’s instructions.

Preparation of cell lysate and immunoblot analysis

Cell lysates were prepared as described elsewhere (32). Equal amounts of protein (50 µg) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were subsequently transferred to a nitrocellulose membrane. The membrane was blocked overnight with 5% bovine serum albumin in Tris–saline buffer (pH, 7.5), and immunoblotting was performed to detect TLR 3, TLR 4, TLR 9, MyD88, TRAF 6, IRAK 1, IRAK M, TRIF; TRAF 3, β-actin and phosphorylated or dephosphorylated forms of p38MAPK, ERK-1/2 as described elsewhere (33).

Coimmunoprecipitation

Coimmunoprecipitation experiments were carried out to detect the TLR 4–MyD88, TLR 9–MyD88, MyD88–IRAK 1 and IRAK 1–IRAK M interactions using a standard protocol that has been described elsewhere (33,34).

Nitrile generation

Nitrile level in culture was measured using the Nitric Oxide Colorimetric Assay kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) (35). Cell-free supernatants were collected from different experimental sets and nitrile levels were estimated in accordance with the manufacturer’s instructions. Data were expressed in micromoles of nitrile.

ChiP assay

ChIP assays were conducted using the ChiP Assay kit following the manufacturers Protocol (Millipore). Briefly, 1×10⁶ peritoneal macrophages and TAM were plated overnight in six-well plates. Cells were stimulated as described in figures, and then fixed for 10 min at 37°C in 1% paraformaldehyde. Cells were washed on ice with ice-cold HBSS containing 1 mM phenylmethylsulfonyl fluoride, harvested and then lysed in sodium dodecyl sulfate lysis buffer. DNA was sheared by ultrasonication using a High Intensity Ultrasonic Processor (hielscher) for 3 × 10⁻⁶ s pulses at 20% amplitude. Lysates were cleared by centrifugation and diluted in ChIP dilution buffer. Lysates were precleared using salmon sperm DNA/protein A-agarose and a sample of ‘input DNA’ was collected at this point. Protein–DNA complexes were immunoprecipitated with 5 µg of Ab overnight at 4°C. Ab-protein–DNA complexes were then captured using salmon sperm DNA/protein A-agarose and a sample of ‘input DNA’ was collected at this point. Washes were performed twice with low and high salt, LiCl, and Triton-X 100. The buffer was then removed, and the DNA was eluted with 1% sodium dodecyl sulfate, 0.1 M NaHCO₃ buffer and disrupted by heating at 65°C for 4 h. DNA was then extracted using phenol/chloroform extraction and ethanol precipitation. Polymerase chain reaction (PCR) was conducted using promoter-specific primers of il-10, il-12p40 and il-12p35. PCR amplified product was subsequently size fractioned on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. For relative quantification of promoter levels, real-time PCR was also performed.

Isolation of RNA and real-time PCR

Total RNA extracted from macrophages (TRI reagent; Sigma) according to the standard protocol (36,37) was reverse transcribed using Revert Aid M-MuLV reverse transcriptase (Fermentas). Real-time PCR was performed using SYBR Green mix and the ABI 7500 real-time PCR system (Applied Biosystems, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. Sequences of the PCR primers are listed in Table 1. The reaction conditions consisted of an initial activation step (5 min at 95°C) and cycling step (denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 1 min at 72°C for 40 cycles), after which melt curve analysis was performed. Detection of the dequenched probe, calculation of threshold cycles and further analysis of these data were done using Sequence Detector software (version 1.4; Applied Biosystems). Relative changes in iNOS2, TLR 3, TLR 4, TLR 9 and cytokine messenger RNA (mRNA) expression were compared with unstimulated control, normalized to GAPDH and quantified using the 2⁻ΔΔCT method.

Statistical analysis

The experiments were performed in triplicate. The data, represented as mean values ± standard deviations, are from one experiment that was performed at least three times. Student’s t-test was employed to assess the significance of the differences between the mean values of control and experimental groups. A value of *P < 0.05 was considered to be significant and *P < 0.001 was considered to be highly significant.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental animal protocols received prior approval from the Institutional Animal Ethical Committee (Bose Institute, Registration Number: 95/99/CPCS/EAA).

Results

TAM exhibit defective induction of inflammatory mediators in response to different TLR ligands

To explore the different TLR activities in TAM, we stimulated them with TLR 3, TLR 9 and TLR 4 ligands, which in turn activate the
Interestingly, CpG ODN was also unable to induce significant IL-10 expression in TAM (Figure 1A and B). Although, CpG ODN induced high level expression in TAM compared with the control peritoneal macrophages (Figure 1A and B). However, both of the poly (I:C) and LPS treatment failed to induce any significant NO generation or iNOS2, IL-12, TNF-α expression in TAM compared with the control peritoneal macrophages (Figure 1A and B).

Table I. List of primer sequences

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<tr>
<th>Target</th>
<th>Primer sequences</th>
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<tr>
<td>TLR 3</td>
<td>Forward: 5'-TAGCAGTCATTCCAAAGAATCAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AACCTTCAGAGTTGATTGATG-3'</td>
</tr>
<tr>
<td>TLR 9</td>
<td>Forward: 5'-CTGCCTGAACGCTTGTCAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGGGGAAGCAGCATCAG-3'</td>
</tr>
<tr>
<td>IL-12</td>
<td>Forward: 5'-CGGAAAGACAAATATCAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TACTCCCGTGTCAGGCTTA-3'</td>
</tr>
<tr>
<td>iNOS2</td>
<td>Forward: 5'-TACCTTCAGTGCCTGGCTGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCAACACTGAAAACAGTGT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-TGATCCCGTGTCAGGCTGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCAACACTGAAAACAGTGT-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: 5'-GACAGTGTCCTGACGGCTTA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GACAGTGTCCTGACGGCTTA-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward: 5'-ACACTGCATCTTGGCTTTGCAGCT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TCACCTTCAGGTCCTGGCTGA-3'</td>
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<tr>
<td>TGF-β</td>
<td>Forward: 5'-AAGGGAAACGATAAGGGAGGCT-3'</td>
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<tr>
<td></td>
<td>Reverse: 5'-TGGCTAGTGCTTCAGGCTTA-3'</td>
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<tr>
<td>IL-12p35</td>
<td>Forward: 5'-GACAGTGTCCTGACGGCTTA-3'</td>
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<td>IL-12p40</td>
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<td>IL-23p19</td>
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<td>Reverse: 5'-GACAGTGTCCTGACGGCTTA-3'</td>
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<tr>
<td>TLR-4</td>
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<td></td>
<td>Reverse: 5'-GACAGTGTCCTGACGGCTTA-3'</td>
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MyD88-independent, MyD88-dependent pathway and both of the pathways, respectively (18,20). Poly (I:C) and LPS treatment induced very high level of IL-10 and TGF-β expression in TAM compared with that of the control peritoneal macrophages (Figure 1A and B). However, both of the poly (I:C) and LPS treatment failed to induce any significant NO generation or iNOS2, IL-12, TNF-α and IFN-γ expression in TAM compared with the control peritoneal macrophages (Figure 1A and B). Although, CpG ODN induced high level of IL-12, TNF-α, IFN-γ and iNOS2 expression in control peritoneal macrophages but it failed to do so in case of TAM (Figure 1A and B). Interestingly, CpG ODN was also unable to induce significant IL-10 and TGF-β expression in TAM compared with its untreated state (Figure 1A and B).

To further characterize this unusual response of TLR activation in TAM, the TLR 3, TLR 4 and TLR 9 expression in TAM were analyzed. We observed no significant difference in TLR 3, TLR 4 or TLR 9 expression at the mRNA or protein level in TAM compared with the control macrophages in untreated state. Interestingly, Poly (I:C) and LPS treatment induced a sharp increase in the TLR 3 and TLR 4 expression both at the mRNA and protein level compared with its resting state (Figure 1C and D). In contrast, the same stimulus induced high level of TLR 9 expression in control macrophages both at the mRNA and protein level (Figure 1C and D). These findings suggested that there was a defect in the MyD88-dependent pathway in TAM. Whereas, the enhanced IL-10 and TGF-β induction in TAM was not dependent on the MyD88-dependent pathway of TLR signaling.

**ERK activation is crucial for IL-10 production in TLR-stimulated TAM**

Since, ERK-1/2 and p38MAPK are associated with TLR-mediated cytokine production in macrophages (18,20), we investigated their activation in TLR-stimulated TAM. LPS and poly (I:C) induced higher level of ERK-1/2 phosphorylation in TAM compared with the control macrophages (Figure 2A). Although, CpG ODN treatment would show the representative of replicate experiments. (Solid bars represent TAM and the clear bars represent peritoneal macrophages).
failed to induce ERK-1/2 phosphorylation in TAM, it induced moderate level of ERK phosphorylation in control macrophages (Figure 2A). Interestingly, LPS, poly (I:C) or CpG ODN failed to induce any significant p38MAPK phosphorylation in TAM compared with the control macrophages (Figure 2A). Since, in many diseases, ERK and p38MAPK reciprocally regulate each other (22), we investigated whether the inhibition of either of these two MAPK could lead to enhanced activation of the other in TAM. Surprisingly, inhibition of

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**Fig. 2.** Differential MAPK signaling in TAM from B16F10 melanoma bearing C57BL/6 mice. Peritoneal macrophages (2 × 10⁶ cells) and TAM (2 × 10⁶ cells) were stimulated with LPS (100 ng/ml), poly (I:C) (30 μg/ml) or CpG ODN (1 μM) for 30 min. The cells were then lysed and subjected to western blot analysis with anti-pERK-1/2, pp38MAPK, p38MAPK and ERK-1/2 antibodies as described in Materials and Methods (A). In a separate experiment, macrophages or TAM were pretreated with PD98059 (10 μM), a specific inhibitor of ERK-1/2, for 30 min followed by stimulation with LPS (100 ng/ml), poly (I:C) (30 μg/ml) or CpG ODN (1 μM) for 30 min. After which the cells were lysed and subjected to western blot analysis with pp38MAPK and p38MAPK antibodies as described in Materials and Methods (B). In a separate set of experiments, peritoneal macrophages (2 × 10⁶ cells) and TAM (2 × 10⁶ cells) were pretreated with PD98059 (10 μM) for 2 h followed by treatment with poly (I:C) (30 μg/ml), CpG ODN (1 μM) or LPS (100 ng/ml) for 24 or 48 h. The cell-free supernatants obtained from differently treated cells after 24 h were subjected to ELISA for the detection of IL-12, IL-10, TNF-α, IFN-γ and TGF-β release (D). Whereas, the cell-free supernatants obtained from differently treated cells after 48 h were subjected to Griess method assay for the detection of nitrite generation (D). In another set of experiments, peritoneal macrophages (2 × 10⁶ cells) and TAM (2 × 10⁶ cells) were pretreated with SB203580 (1 μM) for 2 h followed by treatment with poly (I:C) (30 μg/ml), CpG ODN (1 μM) or LPS (100 ng/ml) for 24 h. After which the cells were lysed and subjected to western blot analysis with anti-pERK-1/2 and ERK-1/2 antibodies (C). The blots shown here are representative of triplicate experiments.
ERK by PD98059 or abrogation of p38MAPK by SB203580 failed to induce significant p38MAPK or ERK phosphorylation in TAM in response to LPS, poly (I:C) or CpG ODN compared with the control macrophages (Figure 2B and C).

We also examined the TLR-mediated IL-12, TNF-α, IFN-γ, IL-10, TGF-β production and NO generation in TAM in ERK-abrogated condition. Interestingly, inhibition of ERK activation resulted in a slight increase in the IL-12 production by TAM. However, there was no significant change in the TNF-α, IFN-γ production and NO generation in TAM compared with the control macrophages in response to LPS, poly (I:C) or CpG ODN (Figure 2D). Although, abrogation of ERK activation resulted in significant reduction of IL-10 production, but it failed to downregulate TGF-β expression significantly in both of the TAM and control peritoneal macrophages (Figure 2D). These observations indicated that TLR-mediated ERK-1/2 MAPK activation was a crucial event for the regulation of IL-10 and IL-12 induction in TAM.

Changes in histone modifications at the IL-10 and IL-12 promoter of TAM result in differential production of IL-10 and IL-12

To investigate the mechanism behind the regulation of IL-10 and IL-12 induction in TLR-activated TAM, we examined the core histone modification at the il-10 and il-12 promoter by ChIP assays. LPS and poly (I:C) treatment were accompanied with high amount of phosphorylated histones at the il-10 promoter region of TAM compared with the control macrophages (Figure 3A). However, TAM were unresponsive to CpG ODN for the induction of histone phosphorylation at the il-10 promoter (Figure 3A). In contrast, control macrophages showed moderate amount of histone phosphorylation at the il-10 promoter in response to the three different TLR ligands (Figure 3A).

To confirm the involvement of ERK activation in chromatin modification at the il-10 promoter, we abrogated ERK activation, which showed significantly reduced H3 phosphorylation at the il-10 promoter in both of the TAM and control macrophages (Figure 3D). Interestingly, LPS, poly (I:C) and CpG ODN failed to induce any detectable histone phosphorylation at the il-10 promoter (Figure 3B) compared with the control macrophages receiving the same stimuli (Figure 3B). Interestingly, ERK abrogation resulted in very high level of histone phosphorylation at the il-12p40 promoter in control peritoneal macrophages, but there was a slight increase in the phosphorylation of histones at the il-12p40 in case of TAM (Figure 3E).

We also investigated the histone phosphorylation at the il-12p35 promoter in both of the TAM and control macrophages under normal as well as in ERK-inhibited condition. Interestingly, LPS, poly (I:C) or CpG ODN failed to induce any detectable histone phosphorylation in TAM in either of the normal or ERK-inhibited condition (Figure 3C).
and F). Whereas, LPS, poly (I:C) and CpG ODN induced very high level of histone phosphorylation at the \( \text{il-12p35} \) promoter in control peritoneal macrophages (Figure 3C). Interestingly, abrogation of ERK activation failed to induce further increase in histone phosphorylation at the \( \text{il-12p35} \) promoter of control peritoneal macrophages (Figure 3F). These findings suggested that ERK activation led to histone phosphorylation specifically at the \( \text{il-10} \) promoter in TAM and the lack of histone phosphorylation at the \( \text{il-12p40} \) as well as at the \( \text{il-12p35} \) promoter might be the reason for the reduced IL-12 induction in TAM.

Defective p38MAPK activation is due to the downregulation of MyD88-dependent signaling molecules in TAM

As TRAF 6 is an essential molecule for the activation of MAPK and NF-\( \kappa \)B following TLR stimulation (18,20), we analyzed the TRAF 6 expression in TAM. TRAF 6 expression was very much lower in TAM compared with the control macrophages (Figure 4A). We also observed that IRAK 1 expression level was significantly downregulated in TAM compared with the control macrophages (Figure 4A). However, the MyD88, TRIF and TRAF 3 expression level in TAM were almost similar to that of the control macrophages (Figure 4A).

Since TLR–MyD88 and MyD88–IRAK 1 interactions are essential prerequisites for the activation of the MyD88-dependent pathway of TLR signaling (18,20), we observed these interactions in TAM following stimulation with LPS and CpG ODN. There was no detectable MyD88–TLR 4 and MyD88–TLR 9 interactions in TAM (Figure 4B and C) and the MyD88 and IRAK 1 interaction was also significantly downregulated in comparison with the control macrophages (Figure 4D). These findings indicated that the defective activation of the MyD88 dependent in TAM was due to the malfunctioning of the downstream signaling molecules of this pathway.

Elevated IRAK M expression in TAM is associated with TLR-mediated ERK activation

As TAM exhibited defective MyD88–IRAK 1 interaction upon stimulation with TLR ligands, we investigated the IRAK M expression in resting and TLR-activated TAM. In consistent with other studies (38), unstimulated TAM showed high level of IRAK M expression (Figure 5A); however, when stimulated with LPS and poly (I:C), the IRAK M expression level was significantly enhanced compared with the control macrophages (Figure 5A). However, CpG ODN treatment could not induce any significant induction of IRAK M expression in TAM compared with its resting state (Figure 5A).

To investigate the inhibitory role of IRAK M in TAM, we analyzed the IRAK 1 and IRAK M interaction in TLR-activated TAM. We observed significantly higher IRAK 1 and IRAK M interaction in LPS or poly (I:C)-treated TAM in comparison with the control macrophages (Figure 5A). However, CpG ODN treatment failed to induce IRAK 1–IRAK M interaction in TAM (Figure 5A). Recent findings suggest that ERK activation is associated with enhanced IRAK M induction in macrophages (26). To investigate the role of ERK in IRAK M induction, we inhibited ERK activation in TAM by PD98059 treatment. Results showed a significant reduction in IRAK M expression and IRAK 1–IRAK M interaction in TAM following stimulation with LPS, poly (I:C) and CpG ODN (Figure 5B). These results indicated that TLR-mediated ERK activation was crucial for the enhanced IRAK M production in TAM.
To investigate whether MyD88 was involved in enhanced IRAK M induction in TAM, we treated TAM with a MyD88-specific siRNA or the control siRNA before stimulation with LPS, poly (I:C) or CpG ODN. Interestingly, we observed no significant reduction in the IRAK M expression or IRAK 1-IRAK M interaction in MyD88 siRNA-treated TAM compared with that of the control siRNA-treated TAM (Figure 5C and D). Therefore, the enhancement of IRAK M expression and IRAK 1-IRAK M interaction in TAM following TLR ligand stimulation was not dependent upon the MyD88-dependent pathway of TLR signaling. Collectively, these findings suggested that the enhanced IRAK M induction in a MyD88-independent pathway abrogated the MyD88-dependent pathway of TLR signaling in TAM.

Discussion

In the present study, we have investigated the molecular basis of defective TLR signaling in TAM. In consistent with previously reported defective MyD88-dependent pathway in TAM (21,28), CpG ODN failed to induce any significant increase in the IL-10, IL-12, TNF-α, IFN-γ, TGF-β and NO production compared with the control macrophages (Figure 1A and B). Interestingly, poly (I:C) treatment led to increased IL-10 and TGF-β production (Figure 1A and B), but there was no detectable IL-12, TNF-α, IFN-γ expression or NO generation in TAM compared with the control macrophages (Figure 1A and B). These findings indicated that the MyD88-independent pathway might be partially functional in TAM and seems to be responsible for enhanced IL-10 production.

Since p38 and ERK-1/2 MAPK are important downstream effectors of TLR signaling (18,20), we observed their activation in TLR-stimulated TAM. Interestingly, LPS and poly (I:C) that act via the MyD88-independent pathway induced significantly higher level of ERK-1/2 MAPK phosphorylation in TAM compared with the control macrophages (Figure 2A). Whereas, there was no significant p38MAPK phosphorylation in TAM in response to poly (I:C) and LPS compared with the control macrophages (Figure 2A). These findings clearly indicated that the TRIF-dependent pathway was partially functional in TAM, since it could not induce p38MAPK activation. However, CpG ODN did not induce any significant MAPK activity in TAM compared with the control macrophages (Figure 2A), which supported further the presence of a defective MyD88-dependent pathway in TAM.

Although in disease condition, ERK-1/2 and p38MAPK are known to be reciprocally regulated in macrophages (22,39); however, this did not hold true in case of TAM. Abrogation of either of the ERK or p38MAPK activation by their pharmacological inhibitors could not induce any significant enhancement of p38MAPK or ERK activation in TAM upon stimulation with LPS, CpG ODN or poly (I:C)
compared with the control macrophages (Figure 2B and C). Although abrogation of ERK activation significantly reduced IL-10 production in both the TAM and control macrophages in response to LPS and poly (I:C) (Figure 2D), however, it could not lead to any significant changes in the expression of TNF-α, IFN-γ, TGF-β or NO in both of the TAM and control macrophages. Interestingly, inhibition of ERK activation resulted in significant enhancement of IL-12 expression in control macrophages, although there was very little increase in IL-12 induction in TAM in ERK-inhibited condition. Therefore, ERK activation was associated with enhanced IL-10 induction in TAM and it was also inhibiting the IL-12 induction in TAM in concert with other factors.

To investigate the mechanism of regulation of IL-10 and IL-12 expression, we extended our study to the chromatin modifications at the promoter region of il-10 and il-12 in TAM. Although, inhibition of ERK activation in TAM resulted in decreased histone phosphorylation at the il-10 promoter but there was no significant enhancement of histone phosphorylation at the il-12p40 or il-12p35 promoter (Figure 3D–F). Thus, the lack of IL-12 induction in TAM was due to the decreased histone phosphorylation at the il-12p40 and il-12p35 promoter and enhanced IL-10 production in TAM was dependent upon the ERK-1/2 MAPK-mediated histone modification at the IL-10 promoter.

To investigate the defect of MyD88-dependent pathway in TAM, the expression of the downstream signaling molecules of this pathway were analyzed. Although, the MyD88 expression was unaffected but other downstream signaling molecules like IRAK 1 and TRAF 6 were significantly downregulated in TAM compared with the control macrophages (Figure 4A). Furthermore, there was no significant TLR-MyD88 and MyD88–IRAK 1 interactions in TAM compared with the control macrophages (Figure 4B and C). Moreover, unstimulated TAM showed significantly higher level of IRAK M expression compared with the control macrophages (Figure 5A), which was consistent with other studies (38). LPS and poly (I:C) treatment induced further enhancement of the IRAK M expression and IRAK 1–IRAK M interaction in TAM (Figure 5A). The enhancement of IRAK M induction in TAM was found to be ERK mediated, since inhibition of ERK-1/2 activation by PD98059 resulted in significant reduction of IRAK M expression and IRAK 1–IRAK M interaction in TAM (Figure 5B). However, we were unable to identify the underlying molecular mechanism of reduced IRAK 1 and TRAF 6 expression in unstimulated TAM.

TRAF 6 is an important mediator for the activation of MAPK and NF-kB in macrophages following TLR stimulation (18,20) and it is utilized in both the MyD88- and MyD88-independent pathways (18,20). Interestingly, TRAF 6 knockdown macrophages upon stimulation with LPS exhibited significant induction of the ERK-1/2 MAPK phosphorylation by activation of the ikappaB kinase-defective CpG ODN-mediated signaling in TAM. To investigate whether a functional MyD88-IRAK 1–TRAF 6 insensitive pathway operates in TAM, we treated TAM with MyD88-specific siRNA or control siRNA before stimulation with the TLR ligands and analyzed the IRAK M expression and IRAK 1–IRAK M interaction. There was no significant reduction in either of the IRAK M expression or IRAK 1–IRAK M interaction in MyD88 siRNA-treated TAM compared with that of the control siRNA-treated TAM. However, further studies are required to elucidate the TRIF–TRAF 3-mediated activation of the NF-kB1 p105-TPL2-ERK-1/2 pathway in TAM.

Collectively, these findings illustrated that the MyD88-dependent and MyD88-independent pathways of TLR activation were differentially regulated in TAM. Tumors paralyzed the MyD88-dependent pathway but selectively modulate the TRIF-dependent pathway for the activation ERK-1/2 MAPK in TAM. The activation of ERK-1/2 MAPK led to transcription favorable chromatin modification (histone phosphorylation) at the il-10 promoter region of TAM resulting in enhanced IL-10 production following TLR stimulation. ERK-1/2 activation also induced enhanced IRAK M expression in TAM, which further disrupted the activation of MyD88-dependent pathway. Thus, ERK-1/2 MAPK activation primarily appeared as a prerequisite for the induction of suppressive phenotype in TAM. Although TLR signaling initiated by the accessibility of bacterial or viral products as TLR ligands. However, recent findings have confirmed the presence of endogenous TLR ligands within the host and also in the tumor site (19,41,42). Possibly, these endogenous TLR ligands at the tumor microenvironment constitutively activate TAM to maintain their protumor functionality. Therefore, this study precisely defined one of the important mechanisms of tumor-microenvironment-imposed alterations of macrophage activity in context of tumor pathogenesis. Further studies required for validating this altered signaling cascade as a target of cancer therapies that can restrain tumor angiogenesis, immunosuppression and metastasis.

Supplementary material

Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org.

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References


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