REPRINTS
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 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 signaling cascade in concert with downstream regulation of tumor necrosis factor receptor-associated factor (TRAF) 6 and interleukin-1 receptor-associated kinase (IRAK) 1. However, the expression of 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 tilts the associated cell populations even host supportive cells over abundance of TAM with poor prognosis (14–17). Moreover, growing evidences from preclinical and clinical studies also support abundance of TAM with poor prognosis (14–17). Toll-like receptors are very important components of innate immunity (18). They are expressed constitutively on the surface as well as on the endosomal membrane of macrophages 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 signaling cascade in concert with downstream regulation of tumor necrosis factor receptor-associated factor (TRAF) 6 and interleukin-1 receptor-associated kinase (IRAK) 1. However, the expression of IRAK M induction, it also polarizes TAM toward a more immunosuppressive form.
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, PD98059 (ERK inhibitor), SB203580, LPS, collagenase and TRI Reagent were from Sigma (St Louis, MO). Poly (I:C) 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, RevertAMPTM M-MuLV Reverse Transcriptase, oligo (dT)20, RNase inhibitor, and other chemicals required for complementary DNA synthesis were from PerkinElmer (Ontario, Canada). Anti-phospho-ERK 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, TRL 4, TRL 3, TRL 9, b-actin, phosphorylated and dephosphorylated forms of p38 and ERK1/2 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 mmol/l glutamine and 100 U/ml penicillin and streptomycin. TAM stimulation was performed in complete RPMI-1640 medium with the following ligand concentrations: poly (I:C), 30 μg/ml; LPS, 100 ng/ml; CpG ODN, 1 μM. For different treatments, PD98059 (a specific pharmacological inhibitor of ERK-1/2 MAPK) (10 μM) was added to the cells prior to the TAM 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 resuspended in 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 been shown 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 (Supplementary Figure S1 is available at Carcinogenesis Online). Peritoneal macrophages were isolated on the same day under similar treatment conditions from control C57BL/6 mice as described elsewhere (31).

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 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 3% 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).

Coomiunoprecipitation
Coomiunoprecipitation 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).

Nitrite generation
Nitrite 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 nitrite levels were estimated in accordance with the manufacturer’s instructions. Data were expressed in micromoles of nitrite.

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 (Ibchers) for 3 × 10 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 for 1 h at 4°C. After washing beads with low and high salt, LiCl, and Tris-EDTA buffers, the protein–DNA complexes were eluted using 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 quantitation of promoter levels, real-time PCR was also performed.

Isolation of DNA and real-time PCR
Total DNA 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 I. 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 65°C and extension for 1 min at 72°C for 40 cycles), after which melt curve analysis was performed. Detection of the denatured 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 by 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: 9599/CPCSEA).

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
Table 1. 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’-TACGCACTCACAACAGAATCAT-3’&lt;br&gt;Reverse: 5’-AACCTTGTGATIGATTAGGTAATA-3’&lt;br&gt;Reverse: 5’-GGTTGAGCTGAGCTGAC-3’&lt;br&gt;Reverse: 5’-GGCTAGTGCTTCAGACAG-3’&lt;br&gt;Reverse: 5’-TACCCAGCTGAGCTCCAC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’</td>
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<td>TLR 9</td>
<td>Forward: 5’-GGTTGAGCTGAGCTGAC-3’&lt;br&gt;Reverse: 5’-GGCTAGTGCTTCAGACAG-3’&lt;br&gt;Reverse: 5’-TACCCAGCTGAGCTCCAC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’</td>
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<td>IL-10</td>
<td>Forward: 5’-GGTTGAGCTGAGCTGAC-3’&lt;br&gt;Reverse: 5’-GGCTAGTGCTTCAGACAG-3’&lt;br&gt;Reverse: 5’-TACCCAGCTGAGCTCCAC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’</td>
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<td>Forward: 5’-GGTTGAGCTGAGCTGAC-3’&lt;br&gt;Reverse: 5’-GGCTAGTGCTTCAGACAG-3’&lt;br&gt;Reverse: 5’-TACCCAGCTGAGCTCCAC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’</td>
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<td>iNOS2</td>
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<td>IL-10</td>
<td>Forward: 5’-GGTTGAGCTGAGCTGAC-3’&lt;br&gt;Reverse: 5’-GGCTAGTGCTTCAGACAG-3’&lt;br&gt;Reverse: 5’-TACCCAGCTGAGCTCCAC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’</td>
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<td>IL-12p40</td>
<td>Forward: 5’-GGTTGAGCTGAGCTGAC-3’&lt;br&gt;Reverse: 5’-GGCTAGTGCTTCAGACAG-3’&lt;br&gt;Reverse: 5’-TACCCAGCTGAGCTCCAC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’&lt;br&gt;Reverse: 5’-TGCGTAGCTGACAGCTCC-3’</td>
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<td>GAPDH</td>
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<tr>
<td>IFN-γ</td>
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<td>TGF-β</td>
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<tr>
<td>TLR-4</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, TGF-α, IFN-γ and iNOS2 expression in control peritoneal macrophages but it did not 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 mRNA as well as protein expression in TAM, which was almost comparable with the control macrophages receiving the same treatment (Figure 1C and D). However, when TAM were stimulated with CpG ODN, there was no significant increase in the TLR 9 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...
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
ERK by PD98059 or abrogation of p38MAPK by SB203580 failed to induce significant p38MAPK or ERK phosphorylation 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 promoters 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 show 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-12p35 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-κB 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). 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-mediated histone modification at the IL-10 promoter.

To investigate the defect of MyD88-dependent pathway in TAM, the expression of 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-MycD88 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 PD90859 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 skapab2 kinase-depend-ent NF-kB-p105-TPL2 pathway (40). TAM were defective in TRAF 6 expression; however, the expression of the TRIF adapter protein and TRAF 3 in TAM was similar to that of the control macrophages (Figure 4A). Since, both of the LPS and poly (I:C) act via the TRIF-dependent pathway, therefore, the enhanced ERK activation in TAM upon stimulation with LPS and poly(I:C) might be due to the presence of a MyD88–IRAK 1–TRAF 6 insensitive but TRIF–TRAF 3–Rip (receptor-interacting protein) 1-dependent pathway (20). However, being a TLR 9 ligand, CpG ODN acts only by the MyD88-dependent pathway and cannot activate this unique TRIF-dependent pathway of TLR signaling (40). It might be the possible reason for the 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-kB-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 of 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

Differential histone modification in TAM


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The combination of a novel immunomodulator with a regulatory T cell suppressing antibody (DTA-1) regress advanced stage B16F10 solid tumor by repolarizing tumor associated macrophages in situ

Sayantan Banerjee, Kuntal Halder, Sweta Ghosh, Anamika Bose, and Subrata Majumdar*

Division of Molecular Medicine, Bose Institute, Kolkata, India

Keywords: antigen presentation, DTA-1, IFN-γ receptor, melanoma, Mw, regulatory T cells, tumor associated macrophages

Abbreviations: APC, antigen presenting cell; IL, interleukin; LPS, lipopolysaccharide; MACS, magnetic-activated cell sorting; Mw, Mycobacterium indicus pranii; STAT, signal transducer and activator; TAM, tumor associated macrophages.

Macrophages are the most abundant immune cells found within most of the solid tumors and are attracted by the chemokines secreted by the growing tumor. Once within the tumor, the infiltrating macrophages, known as TAM, become alternatively polarized to a M2 phenotype and begin to secrete high level of anti-inflammatory cytokines, growth factors, angiogenic factors necessary for the growth and persistence of the tumor within the host. Moreover, TAM play a crucial role in cancer stem cell maintenance and help to initiate metastasis by inducing epithelial to mesenchymal transition in cancer cells. Furthermore, TAM inhibit T cell proliferation either directly or via accumulation of regulatory T cells at the tumor microenvironment. Regulatory T cells are essential for tumor progression in a host, since depletion of these cells in a host greatly hampers tumor progression. Regulatory T cells are attracted to the growing tumor along a chemokine gradient produced by the TAM and their precursor monocytic myeloid derived suppressor cells (MDSCs). Accumulation of regulatory T cells at the tumor

Introduction

Most of the solid tumors escape early detection and it is very difficult to recover patients from advanced stage tumors. Modern therapeutic approaches against advanced stage solid tumors include chemotherapy or radiotherapy, which are found to be very much detrimental to the host due to their non-specific action. Moreover, most of the advanced stage solid tumors often metastasize from their site of origin to distant organs, thereby adding further complication to their treatment. In this regard, immunotherapeutic approaches may be helpful due to their specific action on cancer cells. However, the immune effector cells at the tumor microenvironment are alternatively polarized in such a way that they support tumor progression. Among the tumor promoting immune cells, tumor associated macrophages (TAM) and regulatory T cells are found to be crucial due to their profound influence in the progression of most of the solid tumors.
microenvironment attenuates the antitumor T cell responses. Therefore, a possible immunotherapeutic approach would be to redirect the TAM from the pro-tumoral M2 to antitumoral M1 phenotype for the generation of a protective immune response against cancer.

Interestingly, we have recently shown that heat killed *Mycobacterium indicus pranii* (*Mw*) restores pro-inflammatory M1 function in *Leishmania donovani* infected macrophages. Therefore, we examined whether *Mw* could repolarize the TAM in situ and generate a host protective antitumor response. In accordance with other studies, *Mw* failed to generate host protective antitumor responses within the host against advanced stage tumors. However, the molecular mechanism for the failure of *Mw* in advanced tumor setting remains unexplored.

In the present study, we have observed that in advanced stage B16F10 melanoma, *Mw* alone fails to restrict regulatory T cell accumulation within the tumor mass and associated immune alterations in tumor microenvironment. However, combination of an agonistic antibody for GITR, DTA-1, with *Mw* has provided superior antitumor benefits by suppressing intratumoral regulatory T cell populations and by restoration of the compromised pro-inflammatory and antigen presentation function of TAM. Therefore, the combination therapy involving *Mw* and DTA-1 supports its translational utility in the management of advanced stage solid tumors.

### Results

*Mw* induced repolarization of TAM toward M1 phenotype

We first investigated whether the immunomodulator, *Mw*, could re-educate TAM toward immunostimulatory M1 form of macrophages in vitro. We observed that *Mw* induced significantly higher level of IL-12 and NO along with a marked reduction in IL-10 and TGF-β production in TAM compared to that of their untreated counterparts (Fig. 1A). Moreover, *Mw* treatment augmented MHC-II surface expression (Fig. 1B) and restored the MHC-II dependent antigen presentation function in TAM compared to that of the untreated TAM (Fig. 1C). Since, *Mw* induced IFN-γ dependent antitumor function, we checked whether *Mw* could enhance IFN-γ responsiveness in TAM by upregulating the IFN-γ receptor surface expression. Interestingly, *Mw* treatment significantly augmented IFN-γ receptor surface expression in TAM compared to that of the untreated TAM (Fig. 1D).

It has been found that lack of NF-κBp65 nuclear translocation and p38 MAPK activation along with high level of STAT3 and ERK-1/2 MAPK activation with no significant p38 MAPK activation (Fig. 2A–D) and restored the antigen presentation function (Fig. 2E). Therefore, we hypothesized that regulatory T cells induce repolarization of TAM.

Regulatory T cells abrogated *Mw* induced M2 to M1 transformation of TAM in vitro

Regulatory T cells, in addition to their direct suppressive effect on T cells, may inhibit T cell responses indirectly by preventing the activation of antigen presenting cells. Therefore, we examined regulatory T cell frequency within the tumor mass isolated from *Mw* treated and untreated mice. Surprisingly, we observed very high level of regulatory T cell frequency within the tumor mass of both *Mw* treated and untreated tumors bearing mice (Fig. 2F and K). Therefore, these findings clearly depicted that *Mw* failed to induce repolarization of TAM isolated from advanced stage solid tumors in vitro, which might be the possible reason for the failure of *Mw* in controlling the progression of advanced stage solid tumors.
suppressive effects of regulatory T cells on Mw activated TAM (Fig. 3M–P). Therefore, these findings indicated that regulatory T cells suppressed Mw induced reprogramming of TAM via TGF-β and IL-10 dependent ERK-1/2 MAPK and STAT3 activation.

**Mw** in combination with DTA-1 repolarized TAM toward M1 phenotype *in vivo* and restricted the progression advanced stage tumors

Next, we thought that reduction in regulatory T cell frequency within the tumor mass could restore the
Figure 2. Mw treatment failed to generate protective antitumor response against late stage tumors. (A) $10^5$ B16F10 melanoma cells were injected subcutaneously into the right flank of female C57BL/6 mice and were left untreated or treated with Mw ($10^8$ cells/mouse) on day 9 post-tumor challenge. The tumor volumes were monitored on every two days and the final tumor volume was determined at 21 d post-tumor challenge. The data shown here are representative of three independent experiments with $n = 4$ mice group. TAM ($10^5$ cells) isolated from untreated and Mw treated mice were plated in a 96 well plate were left untreated or treated with LPS (100 ng/mL) or Mw (1:10 ratio to TAM) for 4 h. (B) The cell free supernatant collected at 24 h was subjected to ELISA to detect the presence of IL-10, TGF-β, TNF-α, IL-12 whereas; the cell free supernatant collected at 48 h was subjected to Griess Method assay for the detection of nitrite generation. (C, E) In a separate set of experiments, TAM ($10^5$ cells) isolated from untreated and Mw treated mice were left untreated or treated as mentioned above were subjected to FACS for detection of MHC-II and IFN-γ receptor surface expression. (For continuation of figure legend, see page 5.)
immunomodulatory potential of Mw in vivo. Therefore, we utilized an agonistic GITR antibody, DTA-1, to counteract the suppressive effects of regulatory T cells in vivo. Interestingly, the combination therapy involving Mw and DTA-1 exhibited marked reduction in advanced stage solid tumor progression compared to that of the untreated or only DTA-1 treated tumor bearing mice. However, in accordance with other studies, mice receiving only DTA-1 treatment failed to restrict the progression of advanced stage solid tumor (Fig. 4A). Moreover, TAM isolated from tumors receiving the combination therapy exhibited marked upregulation of IL-12, NO (Fig. 4B), MHC-II dependent antigen presentation function (Fig. 4C), MHC-II (Fig. 4D) and IFN-γ receptor surface expression (Fig. 4E) compared to that of the TAM from untreated or only DTA-1 treated tumors. Furthermore, TAM isolated from the tumor receiving the combination therapy induced significantly higher level of T cell proliferation (Fig. 4F), IFN-γ (Fig. 4G) and IL-2 production (Fig. 4H) compared to that of the TAM isolated from untreated or only DTA-1 treated tumors. Accordingly, we observed significantly higher level of IFN-γ+ T cell frequency (Fig. 4I–K) within the tumor mass of mice receiving the combination therapy compared to that of the untreated tumor bearing mice. Whereas, we observed a moderate increase in intratumoral IFN-γ+ T cell frequency in only DTA-1 treated tumor bearing mice compared to that of the untreated tumor bearing mice. Therefore, these findings suggested that the combination therapy involving Mw and DTA-1 reprogrammed TAM toward M1 phenotype in vivo to generate a host protective antitumor response.

DTA-1 induced reduction in regulatory T cell frequency failed to prevent Mw induced reprogramming of TAM toward a M1 phenotype in vivo

Next, we checked regulatory T cell frequency within the tumor mass of untreated mice, DTA-1 treated mice and Mw and DTA-1 treated mice. Interestingly, we observed a very significant reduction in intratumoral regulatory T cell frequency in mice receiving combination therapy compared to that of the untreated tumor bearing mice (Fig. 5A and B). Whereas, only DTA-1 treatment resulted in a moderate decrease in intratumoral regulatory T cell frequency compared to that of the untreated tumor bearing mice. Moreover, co-culture of Mw treated TAM with regulatory T cells with the physiological ratio (Fig. 5S) found in the DTA-1 treated tumor failed to abrogate Mw induced reprogramming of TAM (Fig. 5C–F). In addition, the IFN-γ responsiveness of TAM was found to be significantly higher in mice receiving the combination therapy compared to that of the untreated and only Mw or DTA-1 treated mice (Fig. 5G–J). Therefore, these findings clearly indicated that although DTA-1 treatment reduced intratumoral regulatory T cell frequency, it failed to repolarize TAM in vivo as indicated by the abrogated IFN-γ responsiveness in TAM isolated from DTA-1 treated tumor. However, DTA-1 treatment reduced intratumoral frequency to such an extent where regulatory T cells failed to attenuate the Mw induced reprogramming of TAM toward a M1 phenotype and this was probably the reason for the success of the combination therapy involving Mw and DTA-1 against advanced stage solid tumors.

Discussion

In the present study, we have shown that Mw in combination with an agonistic GITR antibody (DTA-1) regress advanced stage B16F10 tumors. Previously, it was observed that Mw could provide protection against solid tumors via activation of IFN-γ+ T cells when given 3 d post-tumor challenge, however, it failed to do so when given 7 d post-tumor challenge. The activation of T cells is intricately associated with the activation antigen presenting cells, therefore, we thought that TAM, being the most abundant APC at the tumor microenvironment could play a pivotal role in regulating the outcome of Mw mediated anti-tumor T cell responses in vivo.

Therefore, we first examined whether Mw could restore the M1 phenotype of TAM in vitro. Although, Mw restored the M1 phenotype of TAM in vitro, however, it failed to do so in vivo and thereby failed to restrict tumor growth. Therefore, we thought that some tumor microenvironment residing factors might have abrogated the immunomodulatory action of Mw on TAM in vivo. Among the possible factors, regulatory T cells might be a crucial candidate due to their suppressive action on APCs. Accordingly, the regulatory T cell frequency was found to be very much augmented in Mw treated tumor and was very much similar to that of the untreated tumor. Moreover, co-culture of the regulatory T cells with Mw treated TAM according to their physiological frequency found in the tumor mass completely abrogated Mw induced reprogramming of TAM toward M1 phenotype in vitro. In addition, it was observed that...
Figure 3. Regulatory T cells abrogated Mw induced reprogramming of TAM. The tumor from untreated and Mw treated mice were subjected to enzymatic digestion to generate a single cell suspension as described in the Materials and Methods and the tumor infiltrating CD4⁺ T cells were purified by MACS using CD4 magnetic bead. (A, C) The isolated tumor infiltrating CD4⁺ T cells were then subjected to FACS for determination of the proportion of CD4⁺ Foxp3⁺ regulatory T cells. (B, D) In a separate set of experiments, tumors were cryo-sectioned and subjected to immunofluorescence studies with CD4 PE and Foxp3 Alexa Fluor 647 conjugated antibodies for the detection of tumor infiltrating regulatory T cells and DAPI was used for nuclear staining. TAM from Mw treated tumor bearing mice were left untreated or restimulated with Mw (1:10 ratio to TAM) and co-cultured with regulatory T cells isolated from Mw treated tumor bearing mice in a 1:10 (regulatory T cell:TAM) ratio for 24 h. The culture medium was then replace with fresh medium and kept for another 24 h or 48 h. (E) The cell free supernatant was then collected and subjected to ELISA and Griess Method assay for detection of IL-10, TGF-β, TNF-α, IL-12 production and nitrite generation respectively. (For continuation of figure legend, see page 7.)
regulatory T cells neutralized the immunomodulatory action of Mw via TGF-β and IL-10 dependent ERK-1/2 MAPK and STAT3 activation in TAM. Therefore, it was obvious that Mw treatment failed to reduce regulatory T cell accumulation within the tumor mass and the elevated regulatory T cells might have abrogated the Mw induced reprogramming of TAM to the M1 phenotype and that could be the possible reason why Mw failed to induce host-protective antitumor responses in vivo.

Next, we thought that Mw could activate host protective antitumor immune responses in conditions where regulatory T cell frequency was reduced; however, depletion of regulatory T cells might not be useful, since it could ultimately result in autoimmune condition. Therefore, we selected an agonistic GITR antibody (DTA-1) for the treatment purpose, because it was well known for its ability to induce relative reduction in regulatory T cell frequency by activating the IFN-γ+ T cells within the tumor. Interestingly, the combination therapy involving Mw and DTA-1 prevented tumor growth even after 9 d of tumor challenge, whereas only DTA-1 treated mice failed to do so. Moreover, the combination therapy completely repolarized the TAM to a M1 phenotype, whereas TAM from only DTA-1 treated mice were found to retain their pro-tumoral and anti-inflammatory M2 phenotype. Moreover, regulatory T cell frequency was found to be slightly reduced in the tumor mass of DTA-1 treated mice, whereas DTA-1 and Mw treated mice exhibited marked reduction in regulatory T cell frequency within the tumor mass. Furthermore, co-culture of Mw treated TAM with regulatory T cells according to their physiological frequency found in the only DTA-1 treated tumor failed to attenuate Mw induced restoration of M1 phenotype in TAM. Although, only DTA-1 treatment failed to repolarize TAM and regress tumor growth, it reduced regulatory T cell frequency inside the tumor mass to such an extent where the regulatory T cells could no longer abrogate Mw induced reprogramming of TAM. Therefore, it was quite obvious that DTA-1 induced IFN-γ+ T cell activation was APC independent and the lack of APC activation in DTA-1 treated tumor bearing mice might be the reason why DTA-1 failed to restrict advanced stage tumor progression. Moreover, the IFN-γ responsiveness was found to be very much abrogated in DTA-1 treated mice compared to the mice receiving combination therapy. The abrogated IFN-γ responsiveness was found to be due to the lower IFN-γ receptor expression in TAM from DTA-1 treated mice. Moreover, the combination therapy involving Mw and DTA-1 complemented each other’s function in vivo for the generation of an efficient host protective antitumor immune response to regress advanced stage solid tumors and this immunotherapeutic approach could be utilized for the treatment of other advanced stage murine as well as human tumors.

Materials and Methods

Reagents and chemicals

RPMI-1640 medium (R8758), penicillin and streptomycin (P4333), PD098059 (P2105), SB203580 (S8307), LPS (L2654), collagenase (S5138) and TRI Reagent (15596018) were from Sigma. Fetal calf serum (16140071) was purchased from Gibco BRL. ELISA Assay Kit (Quantikine M) for mouse IL-12 (555256), IL-10 (555252), TNF-α, IFN-γ, IL-2 (555148), CD4 PE (553736), CD8 PerCP (553036) and Foxp3 Alexa Fluor 647 (556401) conjugated antibodies and IMag Anti-Mouse G‐D (551539), C8 (551516) and CD25 (553707) Particles, DTA-1 (558213) antibody and cytokine / cytokine receptor (554714) were obtained from BD Biosciences. Mouse TGF-β ELISA kit (MB100B) and TGF-β neutralizing antibody (MAB240), IL-10 neutralizing antibody (MAB417), control IgG (MAB002) and recombinant IFN-γ (485M100) were obtained from R&D Systems. NF-κB p65 (sc-372), IFN-γ receptor (sc-74449), MHC-II (sc-32247) phosphorylated p38 (sc-9793) and p38 (sc-7972), phospho ERK-1/2 (sc-7383), ERK-1/2 (sc-135900), phospho STAT3 (sc-21876) and STAT3 (sc-482) antibodies and NF-κB pro-preparations

Figure 3: In a separate set of experiments, TAM (106 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to FACs for detection of MHC-II or IFN-γ receptor surface expression. (G) In another set of experiments, TAM (106 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to MHC-II dependent antigen presentation assay. (H) In another set of experiments, TAM (106 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to FACs for detection of MHC-II and IFN-γ receptor surface expression. (I) The cell free supernatant was then collected and subjected to ELISA and Griess Method assay for detection of IL-10, TGF-β, TNF-α, IL-12 production and nitrite generation respectively. (J, L, K) In a separate set of experiments, TAM (106 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to FACs for detection of MHC-II dependent antigen presentation assay. In another set of experiments, TAM (106 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to FACs for detection of MHC-II dependent antigen presentation assay. In another set of experiments, TAM (106 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to FACs for detection of MHC-II and IFN-γ receptor surface expression. (O) In another set of experiments, TAM (106 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to FACs for detection of MHC-II dependent antigen presentation assay. The immunofluorescence and FACs data shown here are the best representatives among three independent experiments. The data shown here are mean ± standard deviation of three independent experiments, a value of p < 0.05 (*) was considered to be significant.
Figure 4. The combination therapy involving Mw and DTA-1 restricted the progression of advanced stage tumors. (A) 10^5 B16F10 melanoma cells were injected subcutaneously into the right flank of female C57BL/6 mice and were left untreated or treated with only DTA-1 (1 mg/mouse) or Mw (10^8 cells/mouse) and DTA-1 (1 mg/mouse) on day 9 post-tumor challenge. The tumor volumes were monitored on every 2 d and the final tumor volume was determined at 21 d post-tumor challenge. The data shown here represent three independent experiments with n = 4/mice group. TAM (10^6 cells) isolated from untreated and only DTA-1 or Mw and DTA-1 treated mice were plated in a 96 well plate and were left untreated or treated with LPS (100 ng/mL) for 4 h. (B) The cell free supernatant collected at 24 h was subjected to ELISA to detect the presence of IL-10, TGF-β, TNF-α, IL-12 whereas; the cell free supernatant collected at 48 h was subjected to Griess Method assay for the detection of nitrite generation. (C) In a separate set of experiments, TAM (10^6 cells) isolated from untreated and only DTA-1 or Mw and DTA-1 treated mice were left untreated or treated as mentioned above were subjected to MHC-II dependent antigen presentation assay as described in the Materials and Methods. (D, E) In another set of experiments, TAM (10^6 cells) isolated from untreated and only DTA-1 or Mw and DTA-1 treated mice were left untreated or treated as mentioned above were subjected to FACS for detection of MHC-II and IFN-γ receptor surface expression. (F) In a separate set of experiments, CD25+ T cell isolated from both the untreated and only DTA-1 or Mw and DTA-1 treated tumor bearing mice were co-cultured with DC pre-activated with whole tumor lysate pulsed and TAM isolated from untreated and DTA-1 or Mw and DTA-1 treated tumor bearing mice were added to the culture at 1:1 ratio (DC:TAM) and T cell proliferation was determined. (G, H) The cell free supernatant was collected and subjected to ELISA for detection of IFN-γ and IL-2. In a separate experimental set, tumors from untreated and DTA-1 or Mw and DTA-1 treated mice were subjected to enzymatic digestion to generate a single cell suspension as described in the Materials and Methods and the tumor infiltrating CD4+ and CD8+ T cells were purified by MACS using CD4 and CD8 magnetic beads. (I–K) The isolated tumor infiltrating CD4+ and CD8+ T cells were then subjected to FACS for determination of the proportion of CD4+ IFN-γ+ and CD8+ IFN-γ+ T cells. The data shown here are mean ± standard deviation of three independent experiments, a value of p < 0.05 (*, † or ‡) was considered to be significant, whereas the FACS data shown here are the best representative among three independent experiments.

p65 (sc-29411), STAT3 (sc-29494) small interfering RNA (siRNA) and control siRNA (sc-35449) were obtained from Santa Cruz Biotechnology.
Mice

Six- to eight-week-old female C57BL/6 mice were purchased from National Institute of Nutrition (Hyderabad, India). These mice were housed in specific pathogen-free conditions and all experiments were conducted in compliance with Institutional Animal Ethics Committee regulations.

Figure 5. DTA-1 treatment restored Mw induced reprogramming of TAM inside advanced stage tumors. Tumors from untreated, DTA-1 or Mw and DTA-1 treated mice were subjected to enzymatic digestion to generate a single cell suspension as described in the Materials and Methods and the tumor infiltrating CD4^+ T cells were purified by MACS using CD4 magnetic bead. (A) The isolated tumor infiltrating CD4^+ T cells were then subjected to FACS for determination of the proportion of CD4^+ Foxp3^+ regulatory T cells. (B) In a separate set of experiments, tumors were cryo-sectioned and subjected to immunofluorescence studies with CD4 PE and Foxp3 Alexa Fluor 647 conjugated antibodies for the detection of tumor infiltrating regulatory T cells and DAPI was used for nuclear staining. TAM from DTA-1 treated tumor bearing mice were left untreated or stimulated with Mw and co-cultured with regulatory T cells isolated from DTA-1 treated tumor bearing mice in a 1:20 (regulatory T cell:TAM) ratio for 24 h. The culture medium was then replaced with fresh medium and kept for another 24 h or 48 h. (C) The cell free supernatant was then collected and subjected to EUSA and Griess Method assay for detection of IL-10, TGF-β, TNF-α, IL-12 production and nitrite generation respectively. (D, F) In a separate set of experiments, TAM (10^5 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to FACS for detection of MHC-II and IFN-γ receptor surface expression. (E) In another set of experiments, TAM (10^5 cells) from Mw treated tumor bearing mice were left untreated or treated as mentioned above were subjected to MHC-II dependent antigen presentation assay as described in the Materials and Methods. In another set of experiments, TAM (10^5 cells) isolated from untreated, only Mw treated, only DTA-1 treated or Mw and DTA-1 treated mice were stimulated with recombinant IFN-γ (50 ng/mL). (G) The cell free supernatant collected at 24 h was subjected to EUSA to detect the presence of IL-10, TGF-β, TNF-α, IL-12 whereas, the cell free supernatant collected at 48 h was subjected to Griess Method assay for the detection of nitrite generation. (H, J) In another set of experiments, TAM (10^5 cells) from Mw treated tumor bearing mice were left untreated or treated with recombinant IFN-γ (50 ng/mL) were subjected to FACS for detection of MHC-II and IFN-γ receptor surface expression. (M) In another set of experiments, TAM (10^5 cells) from Mw treated tumor bearing mice were left untreated or treated with recombinant IFN-γ (50 ng/mL) were subjected to MHC-II antigen presentation assay. The data shown here are mean ± standard deviation of three independent experiments, a value of p < 0.05 (*) was considered to be significant, whereas the immunofluorescence and FACS data shown here are the best representative among three independent experiments.
Tumor induction and treatment
For tumor development, mice were inoculated subcutaneously with $10^5$ B16F10 melanoma cells (purchased from National Centre for Cell Sciences, Pune, India) as described elsewhere.\textsuperscript{31} Tumor size was measured bi-dimensionally with calipers every 2 d, and tumor volume was calculated using the formula: $V = \frac{a \times b^2}{2}$, where $a$ is the largest diameter and $b$ is its perpendicular. In general, mice were euthanized 21 d after tumors were surgically excised.\textsuperscript{25} Mice showing discomfort or difficulty in walking were sacrificed irrespective of the tumor size. In some experiments, tumor bearing mice were treated with PBS, $M_{aw}$ (10$^8$ cells/mice), DTA-1 (1 mg/mice)\textsuperscript{32} or a single dose of DTA-1 and $M_{aw}$ at 9 d post-tumor challenge respectively.

Isolation of tumor associated macrophages and peritoneal macrophages
Tumor associated macrophages were isolated from B16F10 tumors as described elsewhere.\textsuperscript{33,34} Peritoneal macrophages were isolated on the same day under similar treatment conditions from control C57BL/6 mice as described elsewhere.\textsuperscript{35}

Isolation of tumor infiltrating CD4$^+$, CD8$^+$ T cells, and regulatory T cells
For isolation of tumor infiltrating CD4$^+$ and CD8$^+$ T cells, tumors were subjected to enzymatic digestion to generate a single cell suspension and the CD4$^+$ and CD8$^+$ T cells were positively selected from using BD IMag Anti-Mouse CD4 or CD8 Particles. Flow cytometric analysis confirmed the purity of cells to be > 95%. For isolation of tumor infiltrating regulatory T cells, CD4$^+$ T cells isolated from tumors as mentioned above were positively selected from using BD IMag Anti-Mouse CD25 Particles.

Cell culture
TAM and peritoneal macrophages were cultured in complete RPMI-1640 medium containing 10% FCS (v/v), 2 mmol/L glucose and 100 units/mL penicillin and streptomycin. TAM or control macrophages were stimulated with LPS (100 ng/mL) or $M_{aw}$ (10$^8$ cells/10$^5$ TAM or control macrophages) (Fig. S1) in complete RPMI-1640 medium. For different treatments, PD98059 (a specific pharmacological inhibitor of ERK-1/2 MAPK) (10 $\mu$M) or SB203580 (a specific pharmacological inhibitor of p38MAPK) (1 $\mu$M), NF-$\kappa$B p65 siRNA, STAT3 siRNA or control siRNA were used.

Flow cytometric staining
Flow cytometric analysis for cell-surface phenotypic markers was performed after labeling of 1 $\times$ 10$^6$ cells with different fluorescently labeled anti-mouse Abs and their corresponding isotype controls as per the manufacturer’s recommendations. Intracellular IFN-$\gamma$, Foxp3 was stained with anti-mouse fluorescence-labeled Abs using Cytocfix/Cytoperm reagents per the manufacturer's instructions. For all immunofluorescence analyses, cells were fixed with 1% paraformaldehyde in PBS and screening was performed using a FACS VERSE (Becton Dickinson) and suitable negative isotypecontrols were used to establish background staining profiles.

Western blot
Cell lysates and nuclear extract were prepared as described elsewhere.\textsuperscript{36,37} Equal amounts of protein (40 $\mu$g) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and were subsequently transferred to a nitrocellulose membrane. The membrane was blocked overnight with 3% bovine serum albumin in Tris-saline buffer (pH 7.5), and immunoblotting was performed to detect NF-$\kappa$B, phosphorylated or dephosphorylated forms of p38MAPK, ERK-1/2 and STAT3 as described elsewhere.\textsuperscript{38}

Antigen presentation assay
TAM or control peritoneal macrophages were incubated with anti-Ova T-cell hybridoma 13.8 (a kind gift from Prof. Syamal Roy, IICB, Kolkata, India), with for 16–20 h at a fixed ratio of 1:10 APC:T cell in complete RPMI 1640 medium. The culture supernatants were analyzed for the presence of IL-2 by ELISA as described elsewhere.\textsuperscript{39}

T cell immunosuppression assay
Dendritic cells (DCs) were generated from bone marrow (BM) precursors isolated from the tibias/femurs of C57BL/6 mice, as previously described.\textsuperscript{39} At days 7–10 of culture, BM derived DCs were stimulated to mature by addition of 200 ng/mL LPS (Sigma-Aldrich) for 48 h.\textsuperscript{40} DCs were loaded with freeze-thawed whole tumor lysate and were used to activate tumor infiltrating CD25$^+$ T cells (10$^3$ cells/mL). Tumor associated macrophages were then added to the culture at 0:1 or 1:1 ratio to tumor antigen pulsed DC and T cell proliferation, IFN-$\gamma$ and IL-2 production were determined as described elsewhere.\textsuperscript{41}

Enzyme-linked immunosorbent assay (ELISA)
The cell free supernatant from control peritoneal macrophages and TAM were assayed for mouse IL-12, IL-10, TNF-$\alpha$, IFN-$\gamma$, TGF-$\beta$ and IL-2 cytokines with use of the sandwich ELISA kit. The assay was performed according to the manufacturer’s instructions.

Nitrite generation
Nitrite level in culture was measured using the Nitric Oxide (NO) Colorimetric Assay kit (Boehringer Mannheim Biochemicals).\textsuperscript{42} Cell-free supernatants were collected from different experimental sets and nitrite levels were estimated in accordance with the manufacturer’s instructions. Data were expressed in micromoles of nitrite.

Fluorescence imaging of tumor sections
Tumor tissue samples were prepared and 6-$\mu$m sections were stained as previously reported\textsuperscript{43} and CD4 PE and Foxp3 Alexa conjugated antibodies were used for determination of tumor infiltrating regulatory T' cells. Imaging was performed under...
fluorescence microscope (Leica DM4000B; Leica, Wetzlar, Germany).

Statistics
All reported results represent the mean ± SD of data obtained from three independent experiments. For in vivo studies, we repeated the experiments three times with n = 4 per group. Significance was established by one way ANOVA using GraphPad prism software, with differences between groups attaining a value of p < 0.05 (*, *, or †) was considered to be 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 procedures were approved by the Institutional Animal Ethical Committee (Bose Institute, Registration Number: 95/99/CPSCSEA).

References

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental data for this article can be accessed on the publisher’s website.


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