CHAPTER I

TLR signaling mediated differential histone modification at IL-10 and IL-12 promoter region leads to functional impairments in tumor associated macrophages.
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 (Bremnes et al., 2011; Lorusso et al., 2008). With tumor progression, tumor-instructed-microenvironment tilts the associated cell populations even host supportive cells towards its own favor (Bremnes et al., 2011; De Wever et al., 2003; Whiteside et al., 2008). One of the most important cell types of this tumor microenvironment is the macrophage. Tumor derived chemokines attract blood-circulating monocytes (Mantovani et al., 1992; Ueno et al., 2000) which appear to be initially respond to acute pro-inflammatory signals, but these macrophages change into the alternative pro-tumor phenotypes in established tumors (Elgert et al., 1998; Mantovani et al., 2004). Classical macrophages produce higher amount of inflammatory mediators like interleukin (IL)-12, tumor necrosis factor (TNF)-α, reactive oxygen species and nitrogen intermediates when activated via lipopolysaccharide (LPS) or interferon (IFN)-γ (Groeneweg et al., 2006; Varesio et al., 1984). Interestingly, these tumor associated macrophages (TAM) exhibited an altered phenotype and produced higher amount of immuno-suppressive cytokine IL-10, however, the IL-12 expression was not significant enough in response to macrophage activators like LPS (Sica et al., 2006; Sica et al., 2000). Moreover, growing evidences from pre-clinical and clinical studies also support abundance of TAM with poor prognosis (Takanami et al., 1999; Leek et al., 1996; Pollard, 2004; Kang et al., 2010).

Toll like receptors (TLR) are very important components of innate immunity (Akira et al., 2003). 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 (Akira et al., 2003; Yu et al., 2010). 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 TIR-domain-containing adapter-inducing
interferon-β (TRIF) dependent signaling pathway (Akira et al., 2003; Zhu et al., 2010). Both of these pathways of TLR signaling ultimately result in the activation of NF-κB and mitogen activated protein kinase (MAPK) to mount a strong pro-inflammatory response against the invaders (Akira et al., 2003; Zhu et al., 2010). The MyD88 dependent pathway activates NF-κB and MAPK in a TRAF (TNF receptor-associated factor)-6 dependent mechanism utilizing MyD88, Interleukin-1 receptor-associated kinase (IRAK)-4 and IRAK-1 signaling molecules (Zhu et al., 2010). TRIF dependent pathway utilizes both of the TRAF-6 dependent and TRAF-6 independent mechanism for the activation of NF-κB and MAPK in macrophages (Zhu et al., 2010). Currently, it is reported that the defective NF-κB activation is responsible for the suppressive phenotype of TAM (Sica et al., 2000; Saccani et al., 2006). 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 pro inflammatory or anti-inflammatory response (Mathur et al., 2004). ERK-1/2 MAPK activation is associated with enhanced IL-10 induction (Xia et al., 2003) and decreased IL-12 induction (Tomczak et al., 2006) in macrophages. Moreover, ERK-1/2 MAPK is also involved in core histone modification at the IL-10 promoter region of macrophages (Lucas et al., 2005). 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 (Zacharioudaki et al., 2009), 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 (Kobayashi et al., 2002). Since, the MyD88-dependent pathway is selectively disrupted in TAM (Saccani et al., 2006; Biswas et al., 2006), 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. Moreover, our findings suggested that ERK-1/2 MAPK activation triggered a biphasic alteration in TAM by enhancing the expression of anti-inflammatory IL-10 and by upregulating the expression of IRAK-M, thereby polarizing the TAM towards a suppressive phenotype. Together, our present study revealed a previously unknown mechanism of defective TLR activation response in TAM which could be further exploited as a target to develop novel therapeutic strategies.

Results

1. Determination of different TLR activities in tumor associated macrophages isolated from B16F10 melanoma

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 MyD88 independent, MyD88 dependent pathway and both of the pathways respectively (Akira et al., 2003; Zhu et al., 2010). Poly (I:C) and LPS treatment induced very high level of IL-10 and TGF-β expression in TAM compared to that of the control peritoneal macrophages (Figure. 1A and 1B). 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 to the control peritoneal macrophages (Figure. 1A and 1B).
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 1B). Interestingly, CpG ODN was also unable to induce significant IL-10 and TGF-β expression in TAM compared to its untreated state (Figure. 1A and 1B).

**Figure 1.** Determination of different TLR activation responses in tumor associated macrophages and control macrophages. Peritoneal macrophages (MΦ) (2 x 10^6 cells) or TAM (2 x 10^6 cells) were treated with poly (I:C) (30μg/ml) or CpG ODN (1μM) or LPS respectively (100ng/ml) for 4 h. After which the cells were collected in TRIZOL for RNA extraction and real-time polymerase chain reaction (PCR) analysis was performed to determine the mRNA expression of IL-12, IL-10, iNOS2, TNF-α, IFN-γ and TGF-β. The real-time data presented were the best among the 3 independent experiments that showed similar results, whereas the bar
diagram depicting the fold change in mRNA quantification shows means ± standard deviations of values from triplicate experiments that yielded similar observations (Fig. 1A). In a separate set of experiments, peritoneal macrophages (2 x 10^6 cells) and TAM (2 x 10^6 cells) were stimulated with poly (I:C) (30µg/ml), CpG ODN (1µM) or LPS (100ng/ml) for 24 h 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, TGF-β release (Fig. 1B). 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 as discussed in Materials and Methods. Results were presented as mean values ± standard deviations of results from three independent experiments (Fig. 1B).

2. Different TLR expression in tumor associated macrophages isolated from B16F10 melanoma

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 to 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 to the control macrophages receiving the same treatment (Figure. 2A and 2B). 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 to its resting state (Figure. 2A and 2B). In contrast, the same stimulus induced high level of TLR-9 expression in control macrophages both at the mRNA and protein level (Figure. 2A and 2B). These findings suggested that there was a defect in the MyD88 dependent pathway in TAM and the enhanced IL-10 and TGF-β induction in TAM was not dependent on the MyD88 dependent pathway of TLR signaling.
Figure 2. TLR expression in tumor associated macrophages and control peritoneal macrophages. Control peritoneal macrophages (MΦ) (2 x 10⁶ cells) or TAM (2 x 10⁶ cells) were treated with poly (I:C) (30µg/ml) or CpG ODN (1µM) or LPS respectively (100ng/ml) for 4 h. After which the cells were collected in TRIZOL for RNA extraction and real-time polymerase chain reaction (PCR) analysis was performed to determine the mRNA expression of TLR-3, TLR-4 and TLR-9. The real-time data presented were the best among the 3 independent experiments that showed similar results, whereas the bar diagram depicting the fold change in mRNA quantification shows means ± standard deviations of values from triplicate experiments that yielded similar observations (Fig. 2A). In a separate set of experiments, peritoneal macrophages (2 x 10⁶ cells) and TAM (2 x 10⁶ cells) were stimulated with poly (I:C) (30µg/ml), CpG ODN (1µM) or LPS (100ng/ml) for 24 h and the treated cells were lysed and subjected to western blot analysis for the detection of TLR-3, TLR-4 and TLR-9. The blots shown here are representative of triplicate experiments (Fig. 2B).
3. TAM were unresponsive to TLR-2, TLR-5 and TLR-7 ligands that act through MyD88 dependent pathway

In addition to TLR-9, we examined the expression as well as activities of TLR-2, TLR-5 and TLR-7 in TAM which act via MyD88 dependent pathway. However, stimulation of TAM with TLR-2, TLR-5 or TLR-7 ligands failed to enhance the expression of IL-10, TGF-β, TNF-α, IFN-γ and iNOS2 compared to that of the resting TAM (Figure 3A). Moreover, stimulation of TAM with TLR-2, TLR-5 or TLR-7 ligands failed to augment the expression of their respective TLRs compared to that of the resting TAM (Figure 3B). In contrast, control peritoneal macrophages exhibited high level of IL-12, TNF-α, iNOS2 and IFN-γ expression following TLR-2, TLR-5 and TLR-7 ligand stimulation (Figure 3A). In addition, control peritoneal macrophages exhibited a marked up-regulation of TLR-2, TLR-5 and TLR-7 expression when stimulated with their respective ligands. Therefore, these findings clearly indicated that the MyD88 dependent pathway of TLR signaling was defective in TAM.
Figure 3. Determination of TLR-2, TLR-5 and TLR-7 activities in TAM. Peritoneal macrophages (MΦ) (2 x 10^6 cells) or TAM (2 x 10^6 cells) were treated with Pam or flagellin or ssRNA respectively for 4 h. After which the cells were collected in TRIZOL for RNA extraction and real-time polymerase chain reaction (PCR) analysis was performed to determine the mRNA expression of IL-12, IL-10, iNOS2, TNF-α, IFN-γ and TGF-β (Fig. 3A). Control peritoneal macrophages (MΦ) (2 x 10^6 cells) or TAM (2 x 10^6 cells) were treated as mentioned above. After which the cells were collected in TRIZOL for RNA extraction and real-time polymerase chain reaction (PCR) analysis was performed to determine the mRNA expression of TLR-2, TLR-5 and TLR-7 (Fig. 3B). The real-time data presented were the best among the 3 independent experiments that showed similar results, whereas the bar diagram depicting the fold change in mRNA quantification shows means ± standard deviations of values from triplicate experiments that yielded similar observations.
4. ERK-1/2 MAPK activation is crucial for IL-10 production in TLR stimulated TAM.

Since, ERK-1/2 MAPK and p38MAPK are associated with TLR mediated cytokine production in macrophages (Akira et al., 2003; Zhu et al., 2010), we investigated their activation in TLR stimulated TAM. LPS and poly (I:C) induced higher level of ERK-1/2 MAPK phosphorylation in TAM compared to the control macrophages (Figure. 4A). Although, CpG ODN treatment failed to induce ERK-1/2 MAPK phosphorylation in TAM, it induced moderate level of ERK-1/2 MAPK phosphorylation in control macrophages (Figure.4A). Interestingly, LPS, poly (I:C) or CpG ODN failed to induce any significant p38MAPK phosphorylation in TAM compared to the control macrophages (Figure. 4A). Since, in many diseases, ERK-1/2 MAPK and p38MAPK reciprocally regulate each other (Mathur et al., 2004), 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-1/2 MAPK by PD98059 or abrogation of p38 MAPK by SB203580 failed to induce significant p38MAPK or ERK-1/2 MAPK phosphorylation in TAM in response to LPS, poly (I:C) or CpG ODN compared to the control macrophages (Figure. 4B, 4C).

We also examined the TLR mediated IL-12, TNF-α, IFN-γ, IL-10, TGF-β production and NO generation in TAM in ERK-1/2 MAPK abrogated condition. Interestingly, inhibition of ERK-1/2 MAPK 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 to the control macrophages in response to LPS, poly (I:C) or CpG ODN (Figure. 4D). Although, abrogation of ERK-1/2 MAPK activation resulted in significant reduction of IL-10 production, but it failed to down-regulate TGF-β expression significantly in both of the TAM and control peritoneal macrophages (Figure. 4D). 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.
Fig. 4: Differential MAPK signaling in TAM from B16F10 melanoma bearing C57BL/6 mice. Peritoneal macrophages (2 x 10^6 cells) and TAM (2 x 10^6 cells) were stimulated with LPS (100ng/ml), poly (I:C) (30µg/ml) or CpG ODN (1µM) for 30 minutes. The cells were then lysed and subjected to western blot analysis with anti-pERK-1/2 MAPK, pp38MAPK, p38MAPK, and ERK-1/2 MAPK antibodies as described in Materials and Methods (Fig. 4A). In a separate
experiment, macrophages or TAM were pretreated with PD98059 (10µM), a specific inhibitor of ERK-1/2 MAPK, for 30 minutes followed by stimulation with LPS (100ng/ml), poly (I:C) (30µg/ml) or CpG ODN (1µM) for 30 minutes. After which the cells were lysed and subjected to western blot analysis with pp38MAPK and p38MAPK antibodies as described in Materials and Methods (Fig. 4B). In another set of experiments, peritoneal macrophages (2 x 10^6 cells) and TAM (2 x 10^6 cells) were pretreated with SB (1µM) for 2 h followed by treatment with poly (I:C) (30µg/ml), CpG ODN (1µM) or LPS (100ng/ml) for 24 h. After which the cells were lysed and subjected to western blot analysis with anti-pERK-1/2 MAPK and ERK-1/2 MAPK antibodies (Fig. 4C). The blots shown here are representative of triplicate experiments. In a separate set of experiments, peritoneal macrophages (2 x 10^6 cells) and TAM (2 x 10^6 cells) were pretreated with PD98059 (10µM) for 2 h followed by treatment with poly (I:C) (30µg/ml), CpG ODN (1µM) or LPS (100ng/ml) for 24 h 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 (Fig. 4D). 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 (Fig. 4D).

5. Changes in histone modifications at the IL-10 and IL-12 promoter of tumor associated macrophages 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 to the control macrophages (Figure. 5A). However, TAM were unresponsive to CpG ODN for the induction of histone phosphorylation at the IL-10 promoter (Figure. 5A). In contrast, control macrophages...
exhibited moderate amount of histone phosphorylation at the IL-10 promoter in response to the three different TLR ligands (Figure. 5A).

To confirm the involvement of ERK-1/2 MAPK activation in chromatin modification at the IL-10 promoter, we abrogated ERK-1/2 MAPK activation which showed significantly reduced H3 phosphorylation at the IL-10 promoter in both of the TAM and control macrophages (Figure. 5D). Interestingly, LPS, poly (I:C) and CpG ODN failed to induce any detectable histone phosphorylation at the IL-12p40 promoter (Figure. 5B) compared to the control macrophages receiving the same stimuli (Figure. 5B). Interestingly, ERK-1/2 MAPK abrogation resulted in very high level of histone phosphorylation at the IL-12p40 promoter in control peritoneal macrophages, but there was slight increase in the phosphorylation of histones at the IL-12p40 in case of TAM (Figure. 5E). 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-1/2 MAPK 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-1/2 MAPK inhibited condition (Figure. 5C and 5F). Whereas, LPS, poly (I:C) and CpG ODN induced very high level of histone phosphorylation at the IL-12p35 promoter in control peritoneal macrophages (Figure. 5C). Interestingly, abrogation of ERK-1/2 MAPK activation failed to induce further increase in histone phosphorylation at the IL-12p35 promoter of control peritoneal macrophages (Figure. 5F). These findings suggested that ERK-1/2 MAPK activation led to histone phosphorylation specifically at the IL-10 promoter in TAM and the lack of histone phosphorylation at the IL-12p40 as well as at the IL-12p35 promoter might be the reason for the reduced IL-12 induction in TAM.
Fig. 5: TLR ligands mediated changes in histone H3 modifications at the IL-10 and IL-12p40 promoter in TAM. Peritoneal macrophages and TAM (1 $\times$ 10$^6$ cells) were stimulated with medium, LPS (100ng/ml), poly (I:C) (30µg/ml), or CpG ODN (1µM) for 30 minutes. This was followed by ChIP assay which was conducted as described in Materials and Methods. Immunoprecipitations were performed using Abs specific to phosphorylated H3 (IP phospho-H3) and conventional PCR or quantitative real-time PCR was performed using primers specific to the IL-10 or IL-12p40 promoters. For real-time PCR, samples were normalized to input DNA controls (lower lane) (Fig. 5A and 5B). In a separate set of experiments, cells were pretreated with PD98059 (10µM) for 2h and then washed followed by treatment with LPS (100ng/ml), poly
(I:C) (30µg/ml) or CpG ODN (1µM) and ChIP analysis was conducted using a phosphorylated H3-specific Ab. Conventional RT-PCR was conducted using primers specific to the IL-10 or IL-12p40 promoters. Real-time PCR samples were normalized to input DNA controls (lower lane) (Fig. 5D and 5E). The results shown here are representative of triplicate experiments. Peritoneal macrophages and TAM (2 x 10^6 cells) were stimulated with medium, LPS (100ng/ml), poly (I:C) (30µg/ml), or CpG ODN (1µM) for 30 minutes and subjected to ChIP assay (as described in the Materials and Methods) for detection of phosphorylated H3 histones at the IL-12p35 promoter region. Conventional RT-PCR was conducted using primers specific to the IL-12p35 promoters. Real-time PCR samples were normalized to input DNA controls (lower lane) (Fig. 5C). In another set, cells were pretreated with PD98059 (10µM) for 2h and then washed followed by treatment with LPS (100ng/ml), poly (I:C) (30µg/ml) or CpG ODN (1µM ) and ChIP analysis was conducted to detect phosphorylated H3 histones at the IL-12p35 and promoter region. Conventional RT-PCR was conducted using primers specific to the IL-12p35 promoters. Real-time PCR samples were normalized to input DNA controls (lower lane) (Fig. 5F). The results shown here are representative of triplicate experiments.

6. **Defective p38MAPK activation is due to the down regulation of MyD88 dependent signaling molecules in tumor associated macrophages.**

As TRAF-6 is an essential molecule for the activation of MAPK and NF-κB following TLR stimulation (Akira et al., 2003; Zhu et al., 2010), we analyzed the TRAF-6 expression in TAM. TRAF-6 expression was very much lower in TAM compared to the control macrophages (Figure. 6A). We also observed that IRAK-1 expression level was significantly down regulated in TAM compared to the control macrophages (Figure. 6A). However, the MyD88, TRIF and TRAF-3 expression level in TAM were almost similar to that of the control macrophages (Figure. 6A).
Since, TLR-MyD88 and MyD88-IRAK-1 interactions are essential prerequisites for the activation of the MyD88 dependent pathway of TLR signaling (Akira et al., 2003; Zhu et al., 2010), 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 6B and 6C) and the MyD88 and IRAK-1 interaction was also significantly down regulated in comparison to the control macrophages (Figure 6D). 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.

**Fig. 6: Abrogation of MyD88 dependent signaling molecules in TAM.** Peritoneal macrophages (2 x 10^6 cells) or TAM (2 x 10^6 cells) were cultured and then lysed. The cell lysates were subjected to western blot analysis to determine the expression of MyD88, IRAK-1, TRAF-6,
TRIF and TRAF-3 (Fig. 6A). In another experiment, peritoneal macrophages (6 x 10^6 cells) or TAM (6 x 10^6 cells) treated with LPS (100ng/ml), poly (I:C) (30µg/ml) or CpG ODN (1µM) for 24h. After which, the cell lysates were subjected to immunoprecipitation with anti-TLR-4 and anti TLR 9 antibodies, and the blots were probed with anti-MyD88 antibody to detect the TLR-4-MyD88 and TLR-9-MyD88 interaction (Fig. 6B and 6C). In a separate experiment, peritoneal macrophages (6 x 10^6 cells) or TAM (6 x 10^6 cells) were cultured and treated as described above. After 24 h of incubation, the cells were lysed and subjected to coimmunoprecipitation to analyze the association between MyD88–interleukin 1 receptor–associated kinase-1 (IRAK-1) (Fig. 6D). The blots shown here are representative of triplicate experiments.

7. Elevated IRAK-M expression in tumor associated macrophages is associated with TLR mediated ERK-1/2 MAPK 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 (Standiford et al., 2011), unstimulated TAM showed high level of IRAK-M expression (Figure. 7A) however, when stimulated with LPS and poly (I:C), the IRAK-M expression level was significantly enhanced compared to the control macrophages (Figure. 7A). However, CpG ODN treatment failed to induce any significant induction of IRAK-M expression in TAM compared to its resting state (Figure. 7A).

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 to the control macrophages (Figure. 7A). However, CpG ODN treatment failed to induce IRAK-1-IRAK-M interaction in TAM (Figure. 7A). Recent findings suggest that ERK-1/2 MAPK activation is associated with enhanced IRAK-M induction in macrophages (Zacharioudaki et al., 2009). To investigate the role
of ERK-1/2 MAPK in IRAK-M induction, we inhibited ERK-1/2 MAPK 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. 7B). These results indicated that TLR mediated ERK-1/2 MAPK 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 to that of the control siRNA treated TAM (Figure. 7C and 7D) 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.
Fig. 7: ERK-1/2 MAPK signaling pathway is responsible for enhanced IRAK-M induction in TAM. Peritoneal macrophages (6 x 10⁶ cells) or TAM (6 x 10⁶ cells) were stimulated with LPS (100ng/ml), poly (I:C) (30µg/ml) or CpG ODN (1µM) for 24 h. The cells were then lysed and subjected to western blot analysis using IRAK-M antibody (Fig.7A). The cell lysates were also subjected to immunoprecipitation with anti-IRAK-1 and the blots were probed with anti-
IRAK-M antibody to detect the interaction between IRAK-1 and IRAK-M (Fig. 7A). In a separate experiment, peritoneal macrophages (6 x 10^6 cells) or TAM (6 x 10^6 cells) were either kept untreated or were pretreated with PD98059 (10 µM) for 2 h followed by stimulation with LPS, poly (I:C) or CpG ODN as described above. The cell lysates were subjected to immunoprecipitation with anti-IRAK-1 and the blots were probed with anti-IRAK-M antibody to detect the interaction between IRAK-1 and IRAK-M (Fig. 7B). The cells lysates were also subjected to western blot analysis using IRAK-M antibody (Fig. 7B). In another experiment, cells were transfected with MyD88 specific small interfering RNA or control si-RNA for 18h in RPMI containing low quantity of FBS, washed and then stimulated with LPS, poly (I:C) or CpG as described above. The treated cells were lysed and co-immunoprecipitation was performed to detect the IRAK-1-IRAK-M association (Fig. 7C and 7D). The lysates were also subjected to western blot analysis to detect IRAK-M expression (Fig. 7C and 7D). The blots shown here are representative of triplicate experiments.
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 (Saccani et al., 2006; Biswas et al., 2006), CpG ODN failed to induce any significant increase in the IL-10, IL-12, TNF-α, IFN-γ, TGF-β and NO production compared to the control macrophages (Figure. 1A and 1B). Interestingly, poly (I:C) treatment led to increased IL-10 and TGF-β production (Figure. 1A and 1B), but there was no detectable IL-12, TNF-α, IFN-γ expression or NO generation in TAM compared to the control macrophages (Figure. 1A and 1B). 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 (Akira et al., 2003; Zhu et al., 2010), 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 to the control macrophages (Figure. 4A). Whereas, there was no significant p38 MAPK phosphorylation in TAM in response to poly (I:C) and LPS compared to the control macrophages (Figure. 4A). 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 to the control macrophages (Figure. 4A), which supported further the presence of a defective MyD88 dependent pathway in TAM.

Although in disease condition, ERK-1/2 MAPK and p38 MAPK are known to be reciprocally regulated in macrophages (Mathur et al., 2004; Feng et al., 1999), however, this did not hold true in case of TAM. Abrogation of either of the ERK-1/2 MAPK or p38MAPK activation by their pharmacological inhibitors could not induce any significant enhancement of p38 MAPK or ERK-
1/2 MAPK activation in TAM upon stimulation with LPS, CpG ODN or poly (I:C) compared to the control macrophages (Figure. 4B and 4C). Although, abrogation of ERK-1/2 MAPK activation significantly reduced IL-10 production in both the TAM and control macrophages in response to LPS and poly (I:C) (Figure. 4D), 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-1/2 MAPK 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-1/2 MAPK inhibited condition. Therefore, ERK-1/2 MAPK 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-1/2 MAPK 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. 5D-5F). 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 down regulated in TAM compared to the control macrophages (Figure. 6A). Furthermore, there was no significant TLR-MyD88 and MyD88-IRAK-1 interactions in TAM compared to the control macrophages (Figure. 6B and 6C). Moreover, unstimulated TAM showed significantly higher level of IRAK-M expression compared to the control macrophages (Figure.
Chapter I…

7A), which was consistent with other studies (Standiford et al., 2011). LPS and poly (I:C) treatment induced further enhancement of the IRAK-M expression and IRAK-1-IRAK-M interaction in TAM (Figure 7A). The enhancement of IRAK-M induction in TAM was found to be ERK-1/2 MAPK mediated, since inhibition of ERK-1/2 MAPK activation by PD98059 resulted in significant reduction of IRAK-M expression and IRAK-1-IRAK-M interaction in TAM (Figure 7B). 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-κB in macrophages following TLR stimulation (Akira et al., 2003; Zhu et al., 2010) and it is utilized in both the MyD88 and MyD88 independent pathways (Akira et al., 2003; Zhu et al., 2010). Interestingly, TRAF-6 knockdown macrophages upon stimulation with LPS exhibited significant induction of the ERK-1/2 MAPK phosphorylation by activation of the IKK dependent NF-κB1 p105-TPL2 pathway (Loniewski et al., 2007). TAM were defective in TRAF-6 expression, however, the expression of the TRIF adaptor protein and TRAF-3 in TAM was similar to that of the control macrophages (Figure 6A). Since, both of the LPS and poly (I:C) act via the TRIF dependent pathway, therefore, the enhanced ERK-1/2 MAPK 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 (Zhu et al., 2010). 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 (Loniewski et al., 2007). 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-κB1 p105-TPL2-ERK-1/2 MAPK 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 MAPK 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 (Yu et al., 2010; Amarante et al., 2010; Erridge, 2010). Possibly, these endogenous TLR ligands at the tumor microenvironment constitutively activate TAM to maintain their pro-tumor 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 targets of cancer therapies that can restrain tumor angiogenesis, immunosuppression and metastasis.
Chapter I…

References

- Bremnes RM, Dønnem T, Al-Saadi S, Al-Shibli K, Andersen S, Sirera R, Camps C,
- Bremnes RM, Al-Shibli K, Donnem T, Sirera R, Al-Saadi S, Andersen S, Stenvold H,
70.