# Affinity purification of pbmc Tlr-4 of Intestinal cancer patients

Ali Hasanain Ali

**Abstract**— Toll-like receptors (TLRs) play a critical role in host defense from microbial infection. TLRs recognize conserved molecular structures produced by microorganisms and induce activation of innate and adaptive immune responses. The inflammatory responses induced by TLRs play an important role TLRs not only in host defense from infection, but also in tissue repair and regeneration. This latter function of TLRs can also contribute to tumorigenesis. Recent findings show that functional TLRs are expressed not only on immune cells but also on cancer cells. TLRs play an active role in carcinogenesis and tumor progression during chronic inflammation that involves the tumor microenvironment. Damage-associated molecular patterns (DAMPs) derived from injured normal epithelial cells and necrotic cancer cells appear to be present at significant levels in the tumor microenvironment, and their stimulation of specific TLRs can foster chronic inflammation. These TLRs those are expresses on tumor cells are related to interactions between cancer cells, immune cells, and DAMPs through TLR activation in the tumor microenvironment.
This review discusses how the TLR is responsible for both providing immune response for various disease associated pathogens and how it involves in the carcinogenesis, cancer progression and metastasis

**Index Terms**— - Immune cells, cytokines , DAMPs, pathway , Tumor Angiogenesis , PAMP.

## 1 INTRODUCTION

TLRs are evolutionary conserved from plants to vertebrates. In mammals there are 12 identified TLRs. These receptors undergo homo or hetero dimerization to detect a wide range of PAMPs (Pathogen Associated Molecular Pattern) including lipids, lipoproteins, proteins, glycans, and nucleic acids. These receptors are the prime pathogen sensing gates in the body.

TLRs play essential roles in the innate immune responses to microbial pathogens based on their ability to recognize pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006).

Till date in humans 10 different TLRs have been identified.TLRs1-9 are conserved between humans and mice. In addition, TLR10 is expressed in humans but not in mice, whereas TLR11 is present in mice but not in humans.

TLRs1, 2, 4, 5 and 6 are primarily located on the cell surface and recognize bacterial components. TLRs3, 7, 8 and 9 are generally located in the endocytic compartments and primarily recognize viral products.

This study shows that the immune system, particularly the innate immune system, has a skillful means of detecting invasion by microorganisms. Subsequently, mammalian homologues of Toll receptor were identified one after another, and designated as Toll-Like Receptors (TLRs). Functional analysis of mammalian TLRs has revealed that they recognize specific patterns of microbial components that are conserved among pathogens, but are not found in mammals.

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In signaling pathways via TLRs, a common adaptor, MyD88, was first characterized as an essential component for the activation of innate immunity by all the TLRs. However, accumulating evidence indicates that individual TLRs exhibit specific responses. Furthermore, they have their own signaling molecules to manifest these specific responses.

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Toll-Like Receptors (TLRs) have been established to play an essential role in the activation of innate immunity by recognizing specific patterns of microbial components (Table.1). shows TLR signaling pathways arise from intracytoplasmic TIR domains, which are conserved among all TLRs.

Recent accumulating evidence has demonstrated that TIR domain-containing adaptors, such as MyD88, TIRAP, and TRIF, modulate TLR signaling pathways. MyD88 is essential for the induction of inflammatory cytokines triggered by all TLRs.

TIRAP is specifically involved in the MyD88-dependent pathway via TLR2 and TLR4, whereas TRIF is implicated in the TLR3- and TLR4-mediated MyD88-independent pathway. Thus, TIR domain-containing adaptors provide specificity of TLR signaling.

Infection, inflammation, and injury may converge to increase the risk of cancer in a multitude of ways. Microbial colonization can in some cases promote tumorigenesis. For example, Helicobacter pylori (Schneider et al.,2009) and hepatitis C virus (HCV) ( Ning XU et al.,2008) may occupy host niches that lead to chronic inflammation due either to infection or sterile injury is an important risk factor for cancer. The inflammatory response is well known to play a critical role in all stages of cancer development including initiation, promotion, and progression. In addition, regardless of the origin,

whether it be due to infection, inflammation, irritation, or oncogene or DNA-damage associated apoptosis, there is a great deal of cell death and tissue injury associated with cancer. Indeed there are interesting parallels between tumorigenesis, tissue repair, and regeneration. Parallel to the recognition of the importance of TLRs as sensors and shapers of the overall anti-tumor response, TLRs have emerged as an important application area and focus of basic research and applied to development of cancer therapeutic and vaccine research.

The tumor microenvironment, which includes cancer cells, stressed normal cells, stromal tissue and extracellular matrix, has recently been implicated as a major factor for progression and metastasis of cancer.

Recent studies show that activated TLRs expressed on cancer cells can dampen the anti-tumor functions of infiltrating immune cells, thereby altering the inflammatory response in a manner that promotes cancer progression.

**Table.1. TLR Recognition of Microbial Components**

| Microbial Components | Species | TLR Usage |
|---|---|---|
| Bacteria | | |
| LPS | Gram-negative bacteria | TLR4 |
| Diacyl lipopeptides | *Mycoplasma* | TLR6/TLR2 |
| Triacyl lipopeptides | Bacteria and mycobacteria | TLR1/TLR2 |
| LTA | Group B *Streptococcus* | TLR6/TLR2 |
| PG | Gram-positive bacteria | TLR2 |
| Porins | *Neisseria* | TLR2 |
| Lipoarabinomannan | Mycobacteria | TLR2 |
| Flagellin | Flagellated bacteria | TLR5 |
| CpG-DNA | Bacteria and mycobacteria | TLR9 |
| ND | Uropathogenic bacteria | TLR11 |
| Fungus | | |
| Zymosan | *Saccharomyces cerevisiae* | TLR6/TLR2 |
| Phospholipomannan | *Candida albicans* | TLR2 |
| Mannan | *Candida albicans* | TLR4 |
| Glucuronoxylomannan | *Cryptococcus neoformans* | TLR2 and TLR |
| Parasites | | |
| tGPI-mutin | *Trypanosoma* | TLR2 |
| Glycoinositolphospholipids | *Trypanosoma* | TLR4 |
| Hemozoin | *Plasmodium* | TLR9 |
| Profilin-like molecule | *Toxoplasma gondii* | TLR11 |
| Viruses | | |
| DNA | Viruses | TLR9 |
| dsRNA | Viruses | TLR3 |
| ssRNA | RNA viruses | TLR7 and TLR |
| Envelope proteins | RSV, MMTV | TLR4 |
| Hemagglutinin protein | Measles virus | TLR2 |
| ND | HCMV, HSV1 | TLR2 |
| Host | | |
| Heat-shock protein 60, 70 | | TLR4 |
| Fibrinogen | | TLR4 |

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### 1.1. General Mechanism of Action Of TLRs In creating Immune Response

The activation of TLR signaling pathways originates from the cytoplasmic TIR domains.

In the signaling pathway downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, is present which plays a crucial role in signal transduction.

Recent studies shows that there are two types of pathway for this TLR mediated signaling. First one is Myd88 dependent Pathway and second one is Myd88 independent pathway (Takeda et al., 2003).

### 1.2. TLRs and Mechanisms of Tumorigenesis

TLR recognize and respond to exogenous and endogenous ligands through signaling pathways leading to inflammatory cascade mediator production which direct the innate and adaptive immune response. It is increasingly recognized that inflammatory processes play a key role in tumorigenesis. TLRs, as in other human diseases, appear to act as double edged swords in tumorigenesis. Overall, research studies suggest that TLRs as a family are involved in both inhibiting and promoting cancer.

### 1.3. How TLR expressed in cancer cells Signals to Carcinogenesis

There is accumulating and steadily growing evidence that cells of several human malignancies express single or more commonly multiple TLRs.There are, however several lines of evidence suggesting biological impact of TLRs expression on tumor cell growth and survival. Different TLRs expressing on cancer cells involve in different tumor progression (Table.2). There is also evidence that bacteria present in the tumor microenvironment are able to promote tumor growth via TLR signaling.

**Table.2. Type of Cancer and Involvement of TLR. (Swantek et al.,2000)**

| Type of cancer | TLR |
|---|---|
| Gastric cancer | TLR2,TLR4,TLR5,TLR9 |
| Colorectal cancer | TLR2,TLR3,TLR4,TLR5,TLR9 |
| Ovarian cancer | TLR2,TLR3,TLR4,TLR5 |
| Cervical cancer | TLR3, TLR4, TLR5,TLR9 |
| Lung cancer | TLR2,TLR3,TLR4,TLR9 |
| Prostate cancer | TLR4,TLR9 |
| Melanoma | TLR2,TLR3,TLR4 |
| Brain cancer | TLR2,TLR4 |
| Breast cancer | TLR2,TLR3,TLR4,TLR9 |
| Hepatocellular carcinoma | TLR2,TLR3,TLR4,TLR6,TLR9 |
| Laryngeal cancer | TLR2,TLR3,TLR4 |

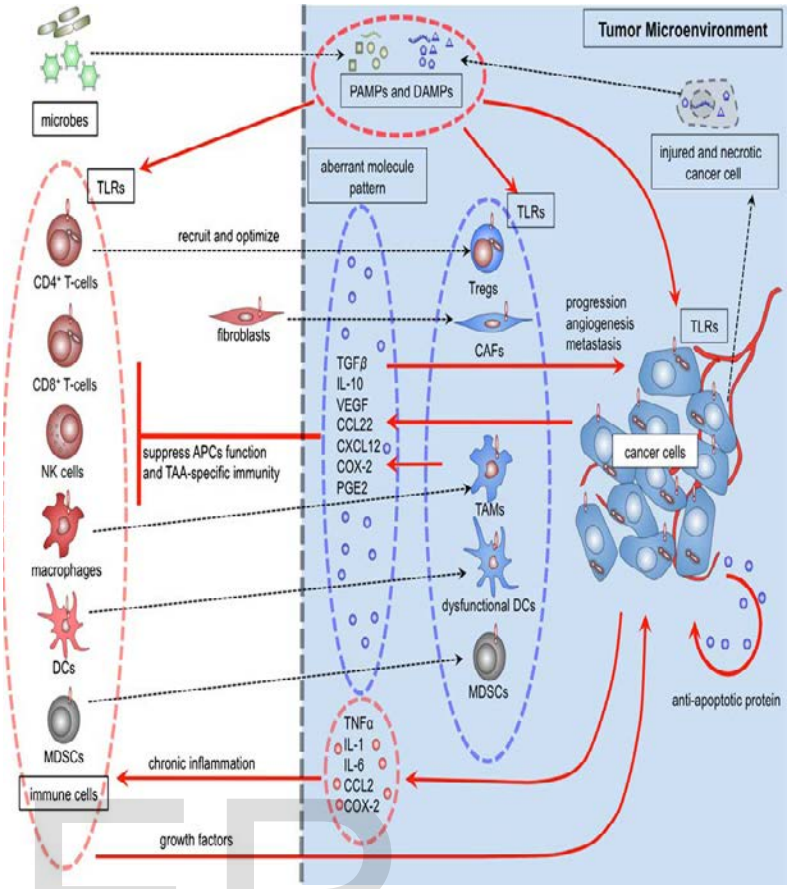
Cancer-associated fibroblasts (CAFs) are important components of the tumor microenvironment, and they are the main

cellular component of the tumor stroma. Unlike normal fibroblasts, CAFs are perpetually activated. (Shimoda et al.,2010).Their origin is not well understood, but they appear to be as important as immune cells in the tumor microenvironment. A recent study proposed that TGFβ has a crucial role in activation of CAFs. Activated CAFs promote the proliferation and progression of cancer through the production of growth factors and metalloproteinases.Therefore, a TLR-related increase in TGFβ might lead to assembly and activation of CAFs in the tumor microenvironment. In summary, during cancer progression in the setting of chronic inflammation, TLR ligands activate, TLRs expressed in cancer cells. Activated cancer cells release cytokines and chemokines that are an important component of the tumor microenvironment. Cytokine-activated infiltrating immune cells subsequently can induce further cytokine release that contributes to activation of CAFs and impairs the function of APCs, effector T-cells and TAA-specific immunity; possibly resulting tumor immunotolerance. The interplay and additive effects of these events facilitate continuous activation of TLR in cancer cells or adjacent normal epithelial cells, thereby maintaining a hostile tumor microenvironment and promoting tumor progression.

### 1.4. Tumor Angiogenesis and TLR

TLRs also seem to have an important role in tumor angiogenesis, i.e., the formation of new capillary blood vessels from existing vessels outside of the tumor. The developing tumor depends on angiogenesis as a source of more oxygen and nutrients for survival and growth. Vascular endothelial growth factor (VEGF) is the main factor involved in tumor angiogenesis (Reinmuth et al.,2001) and is part of the aberrant molecular pattern associated with TLR signals. VEGF is secreted by cancer cells directly and by immune cells and CAFs. New vessels induced by VEGF are abnormal: they are heterogeneous in distribution, irregular in shape, and not organized into arterioles, venules and capillaries. Their varied permeability leads to high interstitial pressures and further hypoxia, which stimulates additional VEGF production. Hypoxia characterizes solid tumors; it is a stress factor that might cause cells to release DAMPs. These ligands activate TLR signals and contribute to the aberrant molecular pattern in the tumor microenvironment. (Fig.1).

The TLR contribution to tumor angiogenesis has been investigated in H. pylori-associated gastric cancer (Chang et al., 2005).This study reported that H.pylori induced COX-2 expression and PGE2 release enhanced tumor angiogenesis via TLR2and9.




Y. Sato et al., Cancer Microenvironment (2009

**Fig.1. Tumor microenvironment and Angiogenesis**

### 1.5. Disruption of Anti-tumor Response of TLR Expressed in Immune cells

Under normal conditions, scheduled cell death is regulated by adenosine triphosphate (ATP) and related apoptotic pathway factors; this regulation drives fragmentation of cellular macromolecules and the speedy subsequent phagocytosis and clearance of apoptotic debris. However, in cancerous conditions, cells dying by non-apoptotic pathways, principally necrosis, release DAMPs into the extracellular space. DAMPs are nuclear or cytosolic proteins with defined intracellular functions but different extracellular actions after cytolysis.

DAMPs released from injured or dying cells are recognized by TLRs on immune cells; subsequent TLR signals disrupt the anti-tumor immune response and lead to cancer progression (Carta et al,.2009).

Candidate DAMPs include heat shock proteins (HSP 60, 70), ATP and uric acid, the S100 family of calcium modulated proteins, nuclear protein high-mobility group box 1 (HMGB1), and nucleic acids. HMGB1, a DNA binding protein, is one of the best-characterized DAMP. HMGB1 regulates intracellular transcription and mediates extracellular proinflammatory processes. HMGB1 released during unscheduled cell death activates an immune response via TLR signals. During tumor expansion, nucleic acids released from necrotic can-

cer cells or adjacent injured normal epithelial cells also act as DAMPs. The high rate of unscheduled cell death in the tumor microenvironment elevates nucleic acid DAMPs. Elevated levels of nucleic acid DAMPs and other DAMPs might foster chronic inflammation, a hallmark of the tumor microenvironment.

Figure 2 shows how interactions between TLRs and DAMPs could create and maintain a self-perpetuating tumor microenvironment. In this microenvironment, cancer cell death might stimulate cancer progression if nucleic acid fragments released by the dead tumor cells are transfected into normal cells, thereby changing the normal cell's properties. Normal cells in the tumor microenvironment might also be transfected by microRNA released from tumor cells, because these small RNA molecules (20–22 base pairs) are easily taken up by cells. Horizontal mediated transfection of microRNA and mRNA in mammalian cells is an intriguing possibility but has yet to be demonstrated in vivo. This phenomenon could explain the expression of tumor-related proteins by normal cells in the tumor microenvironment.

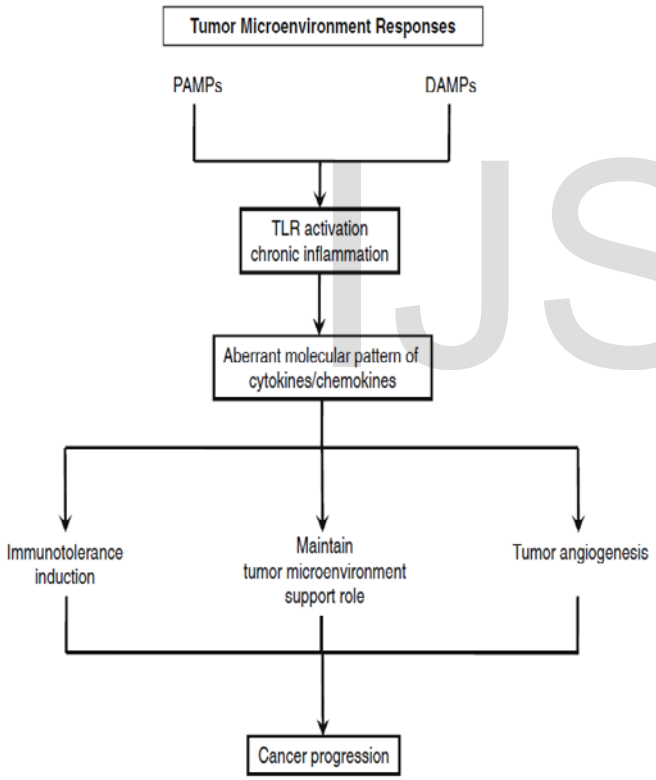



**Fig.2. Interaction between TLR, DAMP and PAMP**

## 2 The objectives of our work is_

1 . Collection of blood from healthy and cancerous patients.
2. Density gradient centrifugation for Separation of PBMC (Peripheral Blood Mononuclear Cells) from blood by using ficoll.
3. Extraction of protein from PBMC.
4. Affinity Chromatography
SDS PAGE of protein and comparison with protein molecular weight marker for the determination of the protein bands responsible for the TLR expression in cancer patients

## 3. Material and Methods

TLR Protein Isolation Buffer Isolation from Blood Sample Preparation of HBSS Solution (Hank's balanced salt solution)

**3.1 Requirements**:
- Blood
- HBSS solution
- Ficoll

**3.2 Affinity Chromatography:**
Two phases of Affinity Chromatography
- Stationary Phase
- Mobile Phase

**3.3 SDS PAGE**
PREPARATION OF ACRYLAMIDE

**3.4 PROCEDURE:**
- All the vertical gel apparatus was washed by spirit.
- The gel casting tray was prepared by using the 3 spacer and join the glass by silica gel.
- After casting the vertical gel apparatus, separating gel was loaded (up to 5cm long) between the glass plates and allowed to solidified.
- Stacking gel was poured on the top of the separating gel.
- The comb was inserted immediately into the stacking gel and allowed the gel to set.
- Protein sample with the sample buffer was taken in the ratio 1:1.
- The sample was boiled for 2minutes in the water bath.
- After settling of the stacking gel the comb was removed slowly.
- Water molecules were dried from the wells by whatmann filter paper.
- The bottom spacer was removed and the gel slab was placed in the buffer in the vertical gel apparatus.
- The sample was loaded into the wells.
- Powers of 50 volts was supplied to the apparatus and allows the sample to run in the gel.

- The tracking dye bromophenol blue when reaches at the bottom of the gel the current was turned off.
- The gel was removed slowly from between the glass plates and put in coomassieve blue staining solution for overnight.
- The gel was washed in the destaining solution till a clear back ground comes.
- Only the stained proteins were visible as blue colour bands.

## 4 DISCUSSION& RESULT

The complex formed between Toll receptor TLR4 and myeloid differentiation factor MD2 defines a major cell surface receptor for lipopolysaccharide (LPS), a gram-negative bacterial antigen that has been implicated in infectious complications. In our present study we have isolated the PBMC (peripheral blood mononuclear cells) from normal and Intestinal cancer blood samples. We extracted the total proteins from PBMC through ultrasonication by dissolving the cells in appropriate protein extraction buffer.SDS PAGE has done for the qualitative analysis of the protein. We compared the cancerous blood sample with the protein molecular weight marker.

Then, from the total protein, purification has done by affinity chromatography for TLR-4 by fixing E.coli cells in the stationary phase. As E.coli cells outer membrane consists of Lipopolysachcharides(LPS).As it can be used for TLR-4 to bind to the stationary phase. Collection of TLR-4 from the stationary phase has done by using buffer Tris Nacl. In this buffer the concentration of Tris is 10mM with a pH 8 and the concentration of Nacl is 200mM.

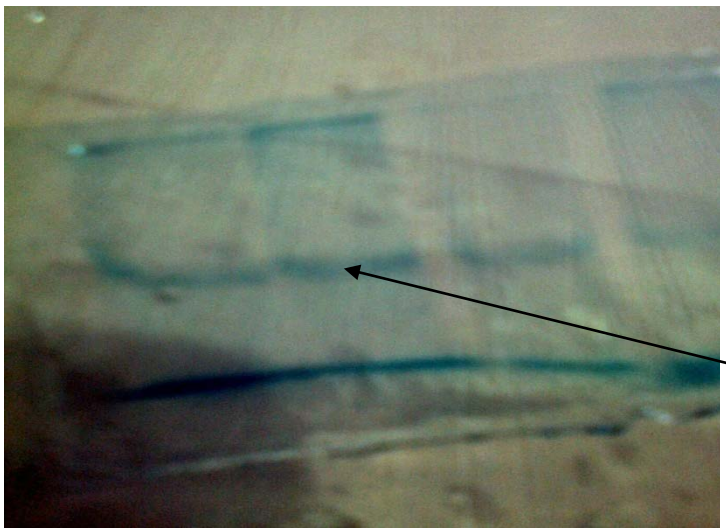



**Fig: 2 TLR-4 Protein Purified Affinity chromatography**

From the SDS PAGE of eluted sample it has found the bands are detected near to 90 KDa, in between 97and 66 KDa. We compared these results with the data of the molecular weight of TLR of a normal person. So as per the data it

shows___

| | |
|---|---|
| TLR-1 | 84KDa |
| TLR-2 | 84KDa |
| TLR-3 | 97KDa |
| TLR-4 | 90KDa |
| TLR-5 | 91KDa |
| TLR-6 | 91KDa |
| TLR-7 | 21KDa |
| TLR-8 | 120KDa |
| TLR-9 | 116KDa |
| TLR-10 | 95KDa |

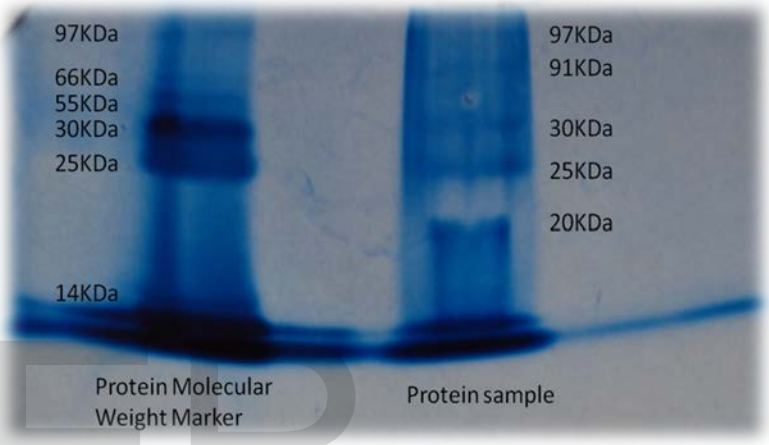



**Fig: 3 Comparison of Cancerous protein sample with Protein Marker**

According to the above data for human TLR family, we didn't get any protein band above 97KDa.The bands for 90KDa may be of the presence of TLR 4 and Bands coming near 91KDa
the presence of TLR 5/6.

From the results we got as TLR 3, 4, 5 and 6 are expressing at same level both in normal and intestinal patients.As it is expressing in both normal and cancerous blood, for further studies we have to study whether the protein is coming in its native structure by native gel electrophoresis in cancerous blood sample. Further analysis of TLR-4 in cancer can be done by Western blotting.

**Fig:4 TLR Protein Isolated from Normal Blood**

## 4 CONCLUSION

TLRs are expressed on many types of cancer cells, tumor stromal cells and infiltrating immune cells. TLR activation during inflammation and injury plays an active role in the surrounding microenvironment. Similarly, in carcinogenesis and tumor progression TLRs play an active role in the tumor microenvironment. During chronic inflammation, abnormal activation of TLRs in normal fibroblasts and epithelial cells might facilitate neoplastic transformation and carcinogenesis. Cancer cells activated by TLR signals can release cytokines and chemokines that recruit and optimize immune cells to release further cytokines and chemokines. The result is an aberrant cytokine profile associated with immune tolerance, cancer progression and propagation of the tumor microenvironment. DAMPs derived from injured normal epithelial cells and necrotic cancer cells appear to be present at significant levels in the tumor microenvironment, and their stimulation of specific TLRs might foster chronic inflammation.

This mechanism is complex and thus far not well understood; however, it is clear that carcinogenesis, cancer progression, and site specific metastasis are related to interactions between cancer cells, immune cells, DAMPs and PAMPs through TLR signals in the tumor microenvironment. Better understanding of these signals and pathways will lead to development of novel therapeutic approaches to a wide variety of cancers.

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