Gram-negative bacterial infection increases lung cancer metastasis via Toll-like receptor activation and increased cancer cell proliferation

Marnie Goodwin Wilson
Department of Medicine
Division of Experimental Surgery
McGill University, Montreal
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Dedication

To those who were there in 2016.

Most of all, to MK, DGW and AGW. None of this would have been possible without your time, your support, and your willingness to sit on my couch with me.

To my lovely extended family, especially my grandparents, for cheering me on, even from so far away. Ti amo, Grandpa. I know you're proud.

To the RVH internal medicine residents. Every hug, phone call, post call beer and force-fed muffin has meant so much more than you know.

To my current research and clinical supervisors, for mistaking my insanity as "scrappiness", helping me discover a love of my job, the infinite career advice, and the many glasses of wine.

And to many, many others, who can't possibly know what their kindness has meant.

This would not have been anywhere near possible without all of you. If this work ever saves a life, contributes to a treatment, or helps with ongoing research, that victory is at least as much yours as it is mine.

Thank you.

Abstract

Lung cancer is a leading cause of death in North America, partially due to high rates of recurrence after surgical resection. Clinical data suggests that post-operative infections, a frequent complication of surgery, may increase the risk of both locoregional and distant recurrence. Previous work from our group has indicated that increased adhesion of circulating tumour cells in the context of infection may be partially responsible for this phenotype. However, cancer metastasis is a multi-step process and it is likely that other the events following tumour cell adhesion (mainly increased cancer cell replication and tumour outgrowth) also play a role.

Using intrasplenic injection of tumour cells followed by cecal ligation and puncture (CLP), a highly physiologic model of post-operative gram-negative sepsis, we found a 5-fold increase in hepatic metastases in infected animals. Toll-like receptor 4 (TLR4) knockout mice incapable of mounting a response to gram negative infection had decreased tumour burden compared to wild type mice. This indicated an important mechanistic role for the TLR-initiated host response to gram negative infection, as well as a potential role for direct effect via cancer cell TLR4 signaling.

These results were reproducible *in vitro*. We found that direct stimulation of cancer cells with heat inactivated E. coli, a gram-negative bacterium, resulted in increased proliferation *in vitro* in both two and three-dimensional models. These effects were partially abrogated by tumour cell TLR4 blockade with Eritoran, as well as by tumour cell TLR2 and TLR5 blockade. To model the host response to infection,

conditioned media was made by stimulating normal bronchoalveolar epithelial cells with lipopolysaccharide, the main antigenic component of E. coli. When added to lung cancer cells, conditioned media also resulted in increased cancer cell proliferation. Importantly, these changes were reversed with TLR4 blockade, as well as with blockade of the MAP-kinase cascade downstream of the receptor. This indicated that, while multiple pathways mediate this effect when tumour cells are directly exposed to gram-negative infection, the role of the host response to infection in this phenotype is mainly TLR4 mediated.

Overall, these results imply a more complex mechanistic role of post-operative infection in metastasis. From a clinical standpoint, this evidence strengthens the case for the use of TLR4 blockade as a potential therapeutic target in the prevention of metastasis.

Abstract

Le cancer du poumon est l'une des principales causes de décès en Amérique du Nord, en partie à cause des taux élevés de récurrence après résection chirurgicale. Les données cliniques suggèrent que les infections post-opératoires, une complication fréquente de la chirurgie, peuvent augmenter le risque de récurrence à la fois locorégionale et lointaine. Des travaux antérieurs de notre groupe ont indiqué qu'une augmentation de l'adhésion des cellules tumourales circulantes dans le contexte de l'infection peut être partiellement responsable de ce phénotype. Cependant, la métastase du cancer est un processus en plusieurs étapes et il est probable que d'autres événements consécutifs à l'adhésion des cellules tumourales (principalement la réplication accrue des cellules cancéreuses et la croissance tumourale) jouent également un rôle.

En utilisant l'injection intrasplénique de cellules tumourales suivie d'une ligature et d'une ponction cécales (CLP), un modèle hautement physiologique de septicémie à Gram négatif post-opératoire, nous avons trouvé une augmentation de 5 fois des métastases hépatiques chez les animaux infectés. Les souris knock-out du récepteur Toll-like 4 (TLR4) incapables de monter une réponse à une infection à Gram négatif avaient un fardeau tumoural diminué par rapport aux souris de type sauvage. Ceci a indiqué un rôle mécaniste important pour la réponse de l'hôte initiée par TLR à l'infection gram-négative, ainsi qu'un rôle potentiel pour un effet direct via la signalisation TLR4 des cellules cancéreuses.

Ces résultats étaient reproductibles in vitro. Nous avons constaté que la stimulation directe des cellules cancéreuses avec E. coli inactivé par la chaleur, une bactérie gram-négative, a entraîné une prolifération accrue in vitro dans les modèles à deux et trois dimensions. Ces effets ont été partiellement annulés par le blocage des cellules tumourales TLR4 avec Eritoran, ainsi que par le blocage des cellules tumourales TLR2 et TLR5. Pour modéliser la réponse de l'hôte à l'infection, le milieu conditionné a été fabriqué en stimulant les cellules épithéliales broncho-alvéolaires normales avec le lipopolysaccharide, le principal composant antigénique de E. coli. Lorsqu'il est ajouté aux cellules cancéreuses du poumon, les milieux conditionnés ont également entraîné une augmentation de la prolifération des cellules cancéreuses. Fait important, ces changements ont été inversés avec le blocage de TLR4, ainsi qu'avec le blocage de la cascade MAP-kinase en aval du récepteur. Ceci a indiqué que, bien que de multiples voies médient cet effet lorsque les cellules tumourales sont directement exposées à une infection gram-négative, le rôle de la réponse de l'hôte à l'infection dans ce phénotype est principalement médié par TLR4.

Dans l'ensemble, ces résultats impliquent un rôle mécaniste plus complexe de l'infection post-opératoire dans les métastases. D'un point de vue clinique, cette évidence renforce le cas de l'utilisation du blocage du TLR4 en tant que cible thérapeutique potentielle dans la prévention des metastases.

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Preface and contribution of authors

This thesis is an original work, written its entirety by Marnie Goodwin Wilson.

Concept design for this project was done by Dr. Lorenzo Ferri and Dr. Stephen Gowing, with input from Marnie Goodwin Wilson.

Several members of the Ferri lab provided technical assistance with experiments. Most notably, France Bourdeau was involved in the planning and execution of all *in vivo* experiments, including acquisition and breeding of animals. Betty Giannias provided valuable input regarding *in vitro* experiments, particularly in the areas of flow cytometry and cell culture.

With the exception of certain *in vivo* experiments, which were performed with the assistance of France Bourdeau, all of the data presented herein was acquired through experiments planned and done by Marnie Goodwin Wilson.

Introduction

Lung cancer is the leading cause of cancer related mortality in Canada, accounting for more deaths than breast, colon and prostate cancer combined (1). Prognosis improves with early detection due to the potential for curative surgical resection (2), but therapeutic success in the long-term is hampered by a high rate of metastatic recurrence after surgery (3). Extensive clinical data (4-8) as well as fundamental research from our group and others (9-11) suggest that post-operative infections following tumour resection, a frequent complication or surgery, may contribute to these high recurrence rates.

Two main mechanisms have been proposed to explain the appearance of metastases after surgical resection of cancer with curative intent. Firstly, during surgery, the tumour is manipulated, which may cause introduction of tumour cells into the bloodstream or lymphatic system (3, 12, 13). These cells will then adhere their metastatic sites, resulting in eventual tumour outgrowth and formation of metastases. It is also thought that many patients undergoing cancer surgery already harbor radiologically undetectable micro-metastases, which can serve as a reservoir for future solid organ metastases (14). In some studies, these micro-metastases are estimated to be present in 30-50% of patients undergoing surgery (15).

25-40% of patients undergoing lung cancer resection will have some type of post-operative complication, the majority of which are infectious. Gram-negative organisms account for a significant proportion of post-operative infections in these

cases – in particular, gram-negative pneumonia is a well-known complication following non-small cell lung cancer resection (16, 17). Surgical lobectomy in itself is a risk factor for post-operative pneumonia, while stress, immunocompromised state, comorbid disease, and decreased lung function (for example, due to obstructive pulmonary disease from tobacco use), all increase the risk for post-operative infection (4, 16, 17).

The pro-inflammatory state caused by gram-negative infection is largely due to lipopolysaccharide (LPS). LPS is recognized by Toll-like receptor 4, a highly conserved pattern recognition receptor involved in the initiation of the immune response. Toll-like receptor 4 initiates a cytokine cascade that ultimately results in the activation of transcription factors such as NF- κB and AP1 and host production of factors such as IL-6 and TNF-α. Previous work indicates that these factors can increase mitogenic activity not only in immune cells, but in many types of cancer cells as well (18-21). Similar responses to gram-negative infection are also induced by other Toll-like receptors, including TLR5 (flagellin) and TLR2 (peptidoglycan) (22).

We have previously shown that Toll-like receptor 4 activation by gram-negative pathogens increases solid cancer metastases in murine models (23, 24). Most of this work has shown increased adhesion of circulating tumour cells as a potential mechanism of action. However, it is highly likely that other mechanisms also play a role in this phenotype. The pathogenesis of metastasis is complex and includes both

pre- and post-adhesive factors, including tumour cell survival, adhesion to a metastatic site, cellular replication, angiogenesis to promote tumour growth, and evasion of cell death (25-27). As of now, we can be relatively confident that adhesion plays a role, however, very little work has been done to elucidate the role of the other steps in this pathway.

Though increased cancer cell adhesion to metastatic sites provides an elegant explanation if one assumes that this phenotype is entirely related to circulating tumour cells, it is less likely that adhesion has such a significant role to play in the large proportion of patients already harboring radiologically undetectable distant metastases. Increased efficiency in the events following adhesion in the metastatic pathway, including cell replication and tumour outgrowth, evasion of cell death and increased angiogenic activity, are possible mechanisms by which these micro-metastases could more quickly lead to a clinically detectable recurrence.

We aimed to investigate the role that bacterial infection plays in promoting the formation of lung cancer metastasis by examining the mechanisms of tumour spread that follow cell adhesion. Given that LPS has mitogenic activity in normal tissue (28, 29), we hypothesized that key post-adhesive events - increased cell replication, survival and angiogenesis - are important for the formation of increased solid tumour metastases following post-operative bacterial infection.

Previous work has indicated that both host factors and tumour factors play a role in the increased tumour burden following post-operative infection (10, 21, 23, 24). Many tumour cells are known to express functional Toll-like receptors which allow them to recognize and initiate signaling cascades in response to lipopolysaccharide (30, 31). Meanwhile, gram-negative sepsis also induces significant inflammatory changes in the host. This systemic inflammation, or cytokine storm, in the tumour microenvironment also likely plays a role in increased cancer cell replication (27, 30, 32). We aimed to determine whether tumour cell factors, host factors or both are responsible for any increase in metastases due to post-adhesive events.

Finally, this work has significant therapeutic implications. The inflammatory cascade triggered by Toll-like receptors, in particular Toll-like receptor 4, may be a therapeutic target to reverse this phenomenon. In fact, blockade of Toll-like receptor 4 has previously been investigated and shown to be safe (though not beneficial) in the treatment of gram-negative sepsis (33). If these events can be abrogated via blockade of the inciting factors, this work could have significant implications for patients undergoing surgical resection of cancer.

Literature Review

Impact and treatment methods for Non-Small Cell Lung Cancer (NSCLC)

Lung cancer is the leading cause of cancer related death for both men and women in Canada (1). In 2012, over 1.6 million deaths due to lung cancer were reported globally. In 2017 in the US alone, the American Cancer Society predicts about 222000 new cases of lung cancer, as well as nearly 156000 deaths (34). It is estimated that until 2035, the number of lung cancer deaths will increase globally by 86% (35). Although incidence rates are declining overall in the western world (in part due to awareness regarding the carcinogenic effects of tobacco), on a global scale, an increase in tobacco use has been followed by an ascending trend in lung cancer incidence.

Several subtypes of lung cancer exist, most notably small cell and non-small cell type carcinomas, which together make up the vast majority of lung cancer diagnoses. These two types vary greatly in their response to therapy. Small cell lung cancers are generally very responsive to chemotherapy and are therefore rarely resected surgically. Non-small cell lung cancers (NSCLC), however, have a poor response to chemotherapy (36). Prognosis of NSCLC improves significantly with early detection, mainly due to the potential for curative resection of disease (2, 3). Generally, operative intervention is indicated in the earlier stages of NSCLC. In Stage I and II NSCLC, resection is the primary mode of therapy, while in Stage IIIA NSCLC, it forms an important component of a multimodality approach including management chemotherapy and radiotherapy (37). In addition, the role of peri-operative

evaluation of mediastinal and hilar lymph nodes has become increasingly clear in recent years, particularly when considering patients for adjuvant chemotherapy (38). In recent years, the level of expertise in techniques such as video-assisted thoracoscopic surgery (VATS) and robotic surgery has increased exponentially, making less invasive approaches possible for well-staged, small tumours (39, 40). However, regardless of approach used, lobectomy– a procedure which is invasive and frequently complicated by post-operative events- remains the standard of care for lung cancer resection in cases stage II or higher (5, 7).

Despite significant progress in both the medical and surgical management of lung cancer, mortality remains extremely high, with an overall ratio of incidence to mortality of 0.87 (34). Though treatment availability varies greatly throughout the world, there is very little difference in lung cancer survival between different regions – worldwide, lung cancer mortality data still closely follows incidence data (41).

NSCLC recurrence and distant metastatic disease

Despite appropriate surgical resection of early-stage NSCLC, there is a high rate of metastatic recurrence, which significantly lowers the overall five-year survival rate (5, 38). Within the first five years after surgery, 30 to 55% of NSCLC patients will develop recurrence and die of their disease, despite curative resection (4). Interestingly, recurrences following NSCLC resection are mainly in the form of distant metastases. Various studies indicate that between 40% and 60% of

recurrences are distant or metastatic, while majority of the remainder involve both locoregional and distant involvement (42-44).

While it is clear that complete removal of the primary tumour and removal of any affected nodes needs to be done with negative margins both macroscopically and microscopically, failure to completely remove known affected areas – for example, resections where margins are later found to be positive on pathology – explains an extremely small proportion of NSCLC recurrence (44). Traditionally, the TNM staging of non-small cell lung cancer (NSCLC) is used as an indicator of the level of disease progression and malignant potential of primary lung cancer, and this system is used to determine eligibility for surgical resection. However, patients at the same TNM stage can exhibit wide variability in terms of incidence of recurrence after curative resection (42, 44). In many patients, microscopic disease may exist prior to surgery, undetectable by standard staging methods such as CT imaging (14, 15).

Proposed mechanisms for high prevalence of distant recurrence

Two main mechanisms have been proposed to explain distant tumour recurrences following curative resection. Firstly, as mentioned above, it has been suggested that microscopic disease may exist prior to surgery, undetectable by standard staging methods (14, 15). Therefore, patients who have been "under-staged" and in fact have diffuse microscopic disease frequently undergo surgery. Recurrence, then, in this population, is likely a result of growth of tumour cells that were already disseminated pre-operatively.

Secondly, it has been shown that tumour cells may be present in the bloodstream at the time of surgery, and that surgery itself may result in the "disruption" of tumour and the release of these cells into the blood. If tumour cells are already present in the bloodstream at the time of surgery, even if distant micrometastases are not present per se, the resection is not "complete", and that the cancer stage has been underestimated. These cells have been termed circulating tumour cells (CTCs).

CTCs have previously been detected in peripheral blood and bone marrow using immunological assays directed at tumour specific proteins and PCR-based assays exploiting tumour-specific mRNA (45). Most data to date deals with breast cancer, where CTCs have been directed in the bone marrow and peripheral circulation years after diagnosis and tumour resection (46). High stage of breast cancer pre-treatment, poorly differentiated cells and lymph node involvement were all independent predictors for detection of CTCs (47, 48). Patients with CTCs in bone marrow or peripheral blood had a poor prognosis and lower disease-free survival than patients without detectable CTCs (14).

Although this work is certainly exciting, detection of CTCs before resection of NSCLC is not yet standard of care (49). Therefore, patients who have been "under-staged" and in fact have diffuse microscopic disease frequently undergo surgery. Recurrence, then, in this population, is likely a result of growth of tumour cells that were already disseminated pre-operatively. This theory is not without controversies – in one study, NSCLC patients underwent bone marrow biopsy and detection of

tumour cells. Although disseminated tumour cells were found in 59% of patients, there was no statistically significant difference in relapse-free survival (50).

A second theory explaining recurrence in patients with curative resection deals with the process of surgery itself. During tumour resection, the tumour mass itself is inevitably manipulated. It has been proposed that handling of the tumour may lead to dissemination of tumour cells into the bloodstream. This theory has been supported by data indicating an increase in tumour cells in the pulmonary venous system during surgical manipulation of primary lung cancers (51, 52), as well as reduced intra-operative shedding of tumour cells using a no-touch isolation technique in colorectal cancer (53). Interestingly, the magnitude of this effect may be related to increased tumour vascularization, one of the previously identified risk factors for increased incidence of distant recurrence following curative resection.

Certain studies have accounted for both of these factors in the recurrence of various different tumour types, however, significant variability still exists in time to – or even incidence of – cancer recurrence.

Molecular predictors of risk for NSCLC recurrence

Following of the sequencing of the human genome and an explosion of interest in molecular and personalized medicine, several molecular patterns have been identified in NSCLC that predict recurrence, regardless of pre-operative TNM staging. Most notably, co-expression of KRAS and Ki-67, which are involved in cell

cycle progression, have been shown to be a poor prognostic factor with regards to recurrence (38, 54). Interest in this area increased significantly with advent of monoclonal antibodies targeting factors such as epidermal growth factor (EGFR), which has also been associated with early recurrence (55). These modalities have been shown to be important therapeutic options in patients whose cancers are express these molecules (56, 57). Epigenetics has also been an area of interest - methylation of p16 and CDH13 promoter regions (both involved in cell replication) has been shown to predict early distant tumour recurrence, prompting interest in therapies targeting epigenetic modification in the treatment of cancer (58, 59).

Certain clinical parameters predicting recurrence have been studied as well. CEA, a well-established tumour marker, predicted recurrence, as did low performance status and the presence of symptoms (60, 61). Pathological findings were also of value - one such parameter is intratumoural vascular invasion (62). Invasion of blood vessels within a tumour has been shown to be an independent prognostic factor for poor outcomes, and has also been combined with other factors (for instance, invasion of the pleura or nodes, which also are implicated in the most recent NSCLC staging criteria) to predict earlier recurrences.

These findings are largely intuitive – if a cancerous cell has higher potential to replicate and grow into a larger mass (as do cells expressing KRAS, Ki-67 and EGFR), it is more likely to form larger tumour masses and potentially metastases later on. If cells have decreased propensity to die (the anti-apoptotic molecule Bcl-2, for

instance, has been shown to be a predictor of recurrence) (63), larger masses can be formed faster. If a higher tumour burden is present (as is often the case in patients with poor functional status and high levels of tumour markers), recurrence is more likely (60). However, in the case of curative resection, the tumour and any affected nodes have been completely removed and all cancerous cells have theoretically been eradicated – unless, of course, the patient already has undetected distant disease or circulating tumour cell burden.

NSCLC recurrence rates and post-operative infectious complications

Inflammatory stimuli have also been linked to decreased time to recurrence. While surgery in itself is an inflammatory stimulus, this effect is compounded in patients that have post-operative complications. In one study, complications predicted decreased five-year cancer specific survival (5), while in another, post-operative infection in particular resulted in decreased overall survival (4). Post-operative infection has also been shown to decrease time to local recurrence in colorectal (6), esophageal and gastric cancer (7), and to negatively impact survival in ovarian cancer patients (8). However, limited data exists directly connecting post-operative infectious complications specifically to distant recurrence.

Post-operative pneumonia is the most common infection in patients following surgical resection of NSCLC, with numbers reaching 35-40% in certain series (16, 17). The majority of these pneumonias are caused by gram-negative organisms and frequently result in gram negative sepsis (16), which is widely recognized as a

dangerous and very severe pro-inflammatory state and carries a high mortality rate. The inflammatory cascade induced by these infections trigger activation of various cell lines that can secrete various chemokines and cytokines favouring tumour extravasation, migration, survival, growth and angiogenesis (10, 21, 31, 64, 65). These events in the metastatic pathway are analogous to the genetic tumor cell factors associated with poor outcomes described above.

Toll-like receptors and the initiation of the inflammatory response to infection

Toll-like receptors (TLRs) are one of the body's first lines of defense against bacterial infections – that is to say, they play a key role in the innate immune system and the initiation of the inflammatory cascade induced by infection. They are single, membrane spanning receptors that recognize pathogen associated molecular patterns (PAMPs), which are structurally conserved molecules that are absent in humans and common to large groups of pathogens. Once a microbe has breached the body's physical barrier (in the case of the lung, the bronchial epithelium), its PAMPs are recognized by TLRs, which go on to activate the body's innate and adaptive immune responses (66).

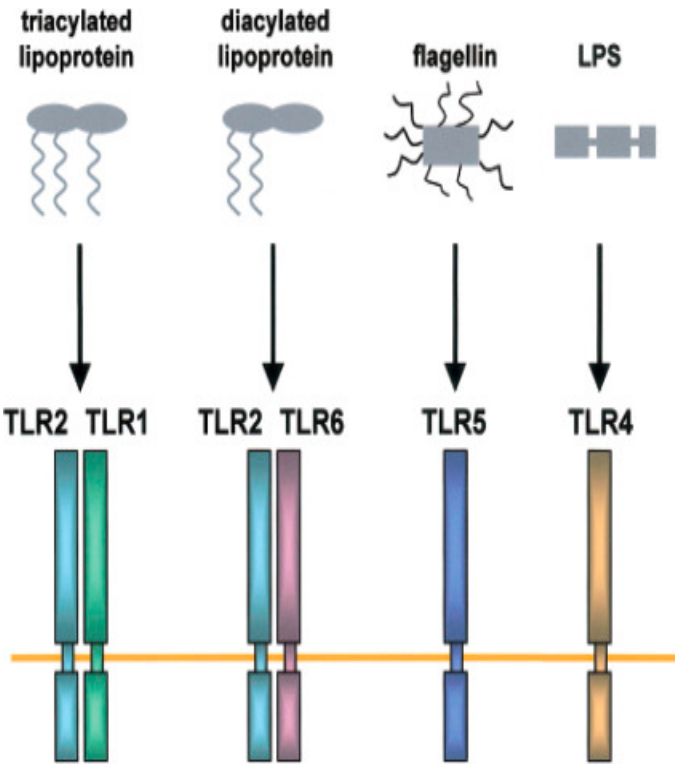


Figure 1: Toll-like receptors 2, 5 and 4 (which heterodimerizes with TLR 2 and 6) are transmembrane receptors involved in the initiation of the host response to gram-negative sepsis via recognition of various pathogen-associated molecular patterns – peptidoglycan, flagellin and LPS respectively.

***Figure 1**: Adapted from McInturff et al, J Invest. Derm. 2005 https://doi.org/10.1111/j.0022-202X.2004.23459.x*

TLRs are mainly expressed on immune cells: predominantly on "surveillance" cells (dendritic cells and macrophages), but also on B, T and NK cells (67). They can also be found on non-immune cells, including bronchial and alveolar epithelial cells (32). NSCLC cells are have been shown to express various functional TLRs, as is consistent with their respiratory epithelial origin (10). When a bacterial pathogen with a corresponding PAMP encounters its TLR, whether on a tumor cell, normal immune surveillance cell or non-immune barrier cell, the PAMP will bind and initiate a signalling cascade inside the cell that recruits protein kinases. These protein kinases lead to amplification of the signal and ultimately lead to upregulation or suppression of genes and the orchestration of the inflammatory response (68). This response can involve cytokine production, migration and

adhesion of cells, cellular proliferation and cellular survival (10, 21, 23, 24). While all of these responses are adaptive in the case of infection (recruitment, proliferation and survival of both innate and adaptive immune cells are necessary components of the body's immune response), these same effects can be deleterious in the case of tumour cells.

Toll-like receptor 4

Lipopolysaccharide (LPS) is the most important gram-negative bacterial antigen. It covers the surface of all gram-negative bacteria and is known to be of the most potent triggers of the systemic inflammatory response in gram-negative sepsis (66). In gram positive sepsis, LPS is also known to have a role: excessive amounts of LPS can be derived from the gut, particularly in cases of hypoperfusion, where bacterial translocation is facilitated (69).

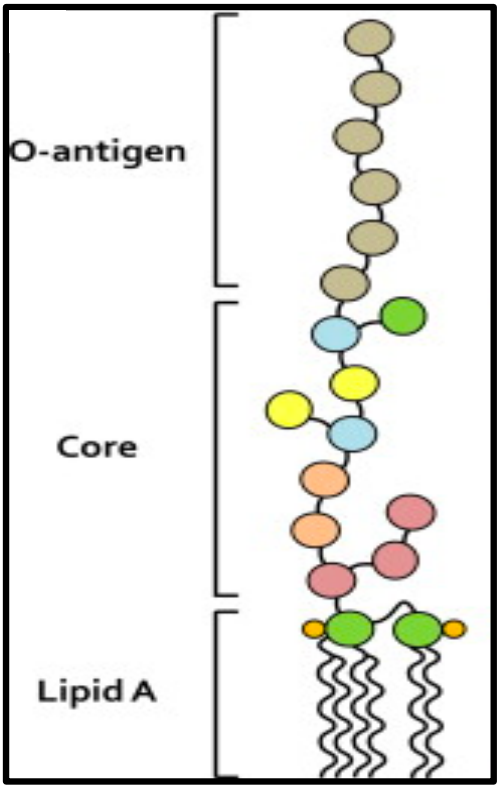


Figure 2: LPS is made up of three components: The O antigen, the core domain, and Lipid A. Lipid A shows minor variations between species, but is largely conserved and allows for the binding of LPS to the gram-negative outer membrane. Lipid A is the pathogen-associated molecular pattern that interacts with the TLR4/MD2 complex and induction of the downstream cascade.

Figure 2: *Adapted from Calabrese et al, Mol. Immunol, 2015. https://doi.org/10.1016/j.molimm.2014.05.011*

Structurally, LPS is made up of three components: the O antigen (a repetitive glycan polymer whose composition varies from strain to strain of bacteria), the core domain (an oligosaccharide component) and Lipid A, a phosphorylated glucosamine disaccharide with multiple hydrophobic fatty acid chains, which allow the LPS molecule to anchor to the bacterial membrane (Figure 2).

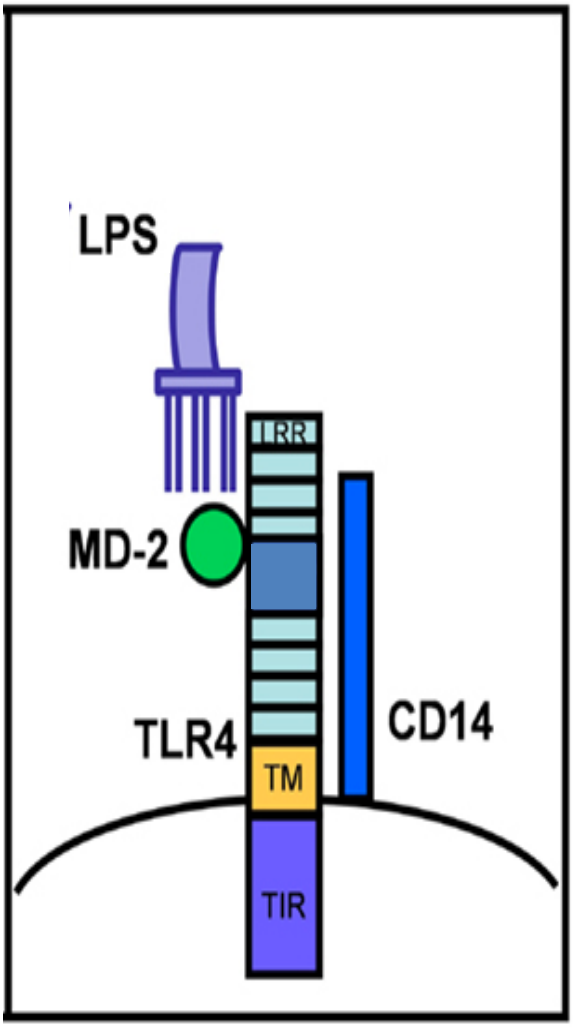


Figure 3: LPS binds to the TLR4/MD2 complex via hydrogen bond formation between the Lipid A component and a hydrophobic pocket formed by interactions between MD2 and TLR4. CD14 is required for stabilization. TLRs are characterized by an extracellular leucine-rich repeat (LRR) domain for ligand recognition, a transmembrane domain (TM) and an intracellular Toll/IL1 receptor-like (TIR) domain, crucial for signal transduction.

Figure 3: Adapted from Vaure et al, Frontiers in Immunology. 2014 https://doi.org/10.3389/fimmu.2014.00316

LPS is recognized by TLR4, which is the receptor responsible for initiating the resultant inflammation in the host. Specifically, the Lipid A component of LPS, which is highly conserved amongst species of gram negative bacteria, is the molecular component that binds to the TLR4/MD2 receptor complex (22, 70). An intracellular signalling cascade is then initiated. MyD88 adaptor-dependent signalling activates mitogen activated protein kinases (MAPKs) including p38, ERK1/ERK2 and JNK, which lead to the production of inflammatory cytokines and the activation of host defense responses (68). TLR4 also activates a MyD88-independent pathway through the protein adaptor TRAM, which leads to the production of type I interferon, a well-known mediator of the immune response (67).

In the case of a gram-negative post-operative infection, LPS is released into circulation and induces a systemic inflammatory response in the host. In patients with pneumonia, the initial point of contact is TLR4 receptors on alveolar and bronchoepithelial cells in the lung. In patients where CTCs are present, they will interact directly with LPS in the bloodstream (71). NSCLC cells are known to express functional TLR4, as is consistent with their respiratory epithelial origin (recall that bronchial and alveolar epithelial cells are known to express functional TLR4 as well) (10).

In certain cancers, level of TLR4 expression correlates with poor survival (72, 73). In one study, 63% of breast cancer patients were reported to express TLR4 on tumour cells and the level of expression inversely correlated with the survival.

Additionally, low MyD88 expression has been correlated with decreased metastasis to the lung (72). These findings support a potential role for TLR4, and thereby possibly TLR4 activation, in the pathogenesis of cancer cell growth and metastasis. Given the known association between Toll-like receptor activation and production inflammatory factors associated with cell growth and survival, these findings also offer a potential mechanistic explanation for the studies showing worse cancer-related outcomes following post-operative infection or in cancer cells with high levels of TLR4 expression.

Cancer cells treated with LPS have augmented adhesion and migration capacity, through interactions with selectin (in the case of esophageal cancer cells) and with beta1 integrin (in the case of colorectal cancer cells) (10, 21). We have shown that this effect is mediated by the downstream cascade associated with TLR4 activation (10, 21). Similarly, NSCLC cells treated with heat inactivated E. coli, a gram-negative bacterium known to have LPS as a major component of its outer membrane, have enhanced ability to adhere to cellular matrix proteins *in vitro,* as well as adhesion to hepatic sinusoidal epithelium in a murine model (10, 21, 23). Increased migration *in vitro* of NSCLC cells treated with E. coli was also shown. Importantly, all effects were abrogated by pre-treatment of the cells with Eritoran, a small molecule inhibitor of TLR4, prior to stimulation with E. coli, indicating that interaction of the Lipid A moiety of LPS with the TLR4/MD2 receptor complex was responsible for this phenotype (23).

Toll-like receptor 5

Although TLR4 is the PAMP that is thought to elicit the strongest response in gram-negative infection, other Toll-like receptors are also thought to be involved in the initiation of the systemic inflammation. TLR5, which recognizes bacterial flagellin, is also known to be expressed on both immune and non-immune cells. Binding of flagellin induces the dimerization of TLR5, which in turn recruits MyD88 and initiates a MAPKinase cascade (74). This downstream cascade is at least partially redundant with the signalling cascade induced by TLR4.

The involvement of this downstream pathway in increased tumor cell adhesion has previously been shown, therefore, it seems likely that the induction of this pathway by other bacterial antigens could induce a similar response. Importantly, TLR5 has also been previously demonstrated to be expressed on respiratory epithelial cells and function in the inflammatory response to inhaled gram-negative pathogens expressing the flagellin protein for cell motility (75, 76). As with TLR4, this supports the hypothesis that NSCLC cells, which are of respiratory epithelial origin, could also feasibly have surface expression of functional TLR5 and the ability to activate the downstream MAPKinase pathway.

Toll-like receptor 2

Another potential candidate for induction of the immune response to gram-negative pathogens is TLR2, which is also known to be involved in the recognition of respiratory pathogens. Although TLR2 is traditionally associated with the response

to gram-positive sepsis through the recognition of lipoteichoic acid (LTA) and peptidoglycan (PGN), a small body of work shows that it may also be involved in the systemic response to gram-negative sepsis, through the recognition of the small amount of peptidoglycan in the gram-negative cell wall (77, 78) and through interaction with other elements of the immune response, such as human heat shock protein 70 (HSP70) and lipopolysaccharide binding protein (LPSBP) (79, 80). There is also some evidence that at high concentrations, the lipid A portion of LPS can be involved in the activation of TLR2, although it is as of yet unclear whether these interactions are significant in a clinical context (80).

TLR2 is known to be expressed and functional on airway epithelial and alveolar cells, where, like TLR4 and TLR5, it is involved in the innate immune response to respiratory pathogens (81). We have also shown that stimulation treatment of cancer cells with inactivated gram-positive bacteria produces a similar process to that induced by TLR4 and can result in increased cancer cell migration and adhesion. This process is reversed by antibody-mediated blockade of TLR2, and leads to augmentation of NSCLC metastasis in murine models (24). Again, the downstream pathway of TLR2 is redundant with those of TLR4 and TLR5, involving binding of MyD88 and induction of the MAPKinase cascade (30, 81).

Downstream cascades initiated by gram-negative sepsis

The adaptor protein MyD88 is one of the first steps in signal transduction following activation of TLR4, TLR5 and TLR2. MyD88 binds to the intracellular domain of the

Toll-like receptor in question and recruits various signalling protein kinases and adaptor molecules. Ultimately, this leads to the activation of the Mitogen Activated Protein Kinase (MAPKinase) cascade, which leads to the production of the transcription factor AP-1, and the IKK (IκB Kinase) cascade, which leads to the production of the transcription factor NF-κB. Both of these transcription factors result in the expression of genes involved in cell cycle progression, cellular proliferation and the inhibition of apoptosis, as well as the production of pro-inflammatory cytokines (68). This mechanism represents a possible direct role of cell-autonomous TLR signaling in regulation of carcinogenesis; in particular, through increased proliferation of tumor cells and inhibition of apoptosis.

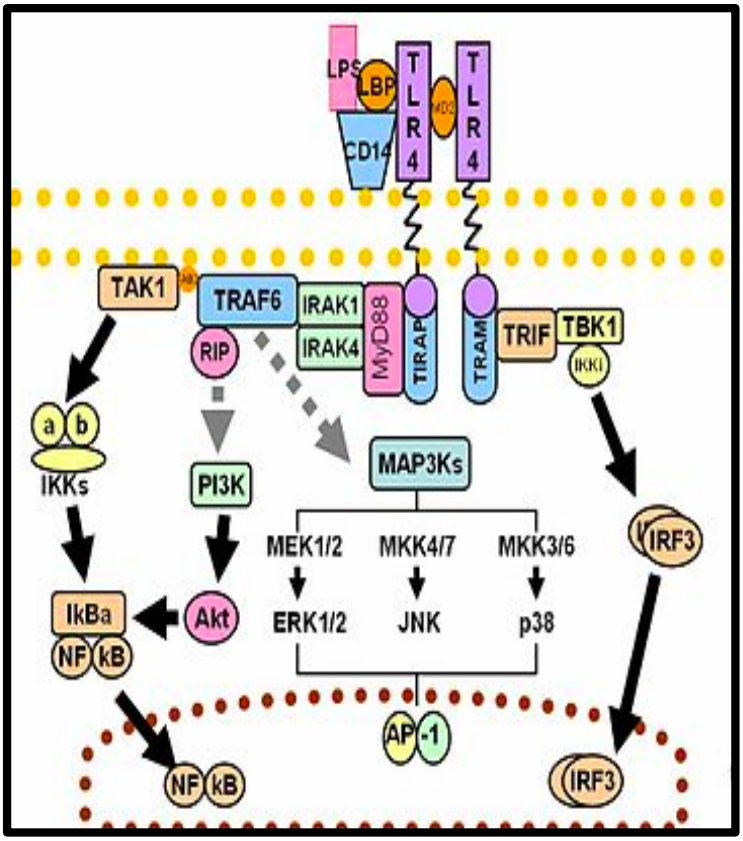


Figure 4: Downstream signalling cascade resulting from the activation of TLR4, and in the case of MyD88-dependent interactions (left) conserved in TLR2 and TLR5 signalling. Adaptor proteins (MyD88, IRAK, TRAF6) result in activation of protein kinase cascades and NF-κB and AP-1 transcription factors.

Figure 4: Adapted from WikiMedia Commons, public domain.

Inhibition of MyD88-dependent pathways in cancer treatment

While inhibition of Toll-like receptors to prevent cancer metastasis is a relatively novel concept, the MAPKinase pathway has been strongly associated with cell replication and malignancy. Small molecule inhibitors of the MAPKinase pathway have been extensively investigated in both preclinical models and clinical studies, with varying results.. PD 184352, an small molecule inhibiting the MEK protein and ultimately resulting in decreased ERK 1/2 phosphorylation, has been tested in vitro and has been shown to cause G1 cell cycle arrest in various cancer cell types, most notably colon cancer cells, which are known to frequently express high levels of phosphorylated MAPKinase pathway proteins. In phase I clinical studies, the treatment was shown to be well tolerated, however, phase 2 clinical studies did not document sufficient responses to therapy to merit ongoing trials (though several patients did achieve stable disease while on treatment).

Another small molecule inhibitor targeting the MAPKinase cascade, BIRB0796, is highly selective for p38 MAPKinase both in vitro and in vivo (84). As of yet, it has mainly been used for research purposes, however, previous work from our group has indicated that pre-incubation with BIRB0796 decreased TLR4-induced cell migration and adhesion (19).

The MyD88-dependent pathway also results in the induction of Phosphoinositide 3-kinase (PI3Kinase), which is also frequently mutated, leading to constitutive activation in aggressive cancers including Glioblastoma (85). Small molecule

inhibitors of this pathway have also been shown to have high anti-tumor activity in vitro in certain NSCLC cell lines (86). In the clinical setting, various small molecule inhibitors of this pathway, including PI 103, TGR-1202 and SAR245408 have been tested in phase I and II clinical trials, as well as in certain ongoing phase III trials, mainly involving hematological malignancies refractory to other therapies (87-89).

Taken together, the success of this work offers further support for the idea that Toll-like receptor dependent signalling could be implicated in the clinical phenomenon of increased cancer metastasis following post-operative infection. Activation of many Toll-like receptors, including TLR2, TLR4 and TLR5, is well known to lead to activation of the PI3Kinase and MAPKinase cascades, both of which have been implicated in cell replication. Additionally, we have previously shown that inhibition of the MAPKinase pathway has an impact on tumor cell adhesion, which certainly helps explain a portion of this phenotype.

Potential roles for post-adhesive events in NSCLC metastasis

Increased tumour cell adhesion and migration following TLR4 stimulation have been proposed as possible mechanisms for increased metastasis following post-operative infection, but this likely does not represent the entire picture. Tumour metastasis is a complex process, involving many steps, the majority of which occur after adhesion. These "post-adhesive events" include cell replication and avoidance of cell death. Escape from dormancy, albeit a controversial topic, combines these two mechanisms and has also been proposed to play a role in metastasis, most

notably in cases of breast cancer, where the presence of circulating tumour cells has been documented in patients years prior to clinical recurrence (82).

Data already exists which demonstrates that surgical manipulation of tumor can cause dissemination of tumor cells into the bloodstream, which of course comes with the potential consequence of hematogenous metastases (9, 42, 51). It is thought that post-operative infection and/or inflammation may exacerbate this phenomenon – in fact, several mechanisms have been proposed to explain increased adhesion of tumor cells to distant metastatic sites in the post-operative period. The time required for tumor cell adhesion is, as of yet, unclear and probably varies greatly with different tumor cell origin and between hosts. However, post-operative infection is rarely an immediate complication of surgery. The majority of deep-seated post-operative infections, including pneumonia, occur between five and seven days after surgery. As such, by the time the inflammatory processes associated with post-operative infection are active, it is likely that some circulating tumor cells have already become adherent to distant metastatic sites – possibly in part due to the inflammatory stimulus of the surgery itself.

Additionally, as previously mentioned, dissemination of circulating tumor cells represents only one theory on post-operative cancer recurrence. Certain data show that radiologically undetectable micrometastases may exist prior to surgery in a high proportion of patients, leading to pre-operative under-staging (15). As these cases likely represent a significant proportion of the patients who have post-

operative recurrence and, as such, a significant proportion of patients who have accelerated recurrence after post-operative infection, we aimed to investigate whether Toll-like receptor activation also has a roll in the acceleration of the events necessary for metastasis after tumor cell adhesion has already occurred. Namely, these events could include cell replication and tumor outgrowth, tumor cell avoidance of apoptosis / cell death, increased angiogenesis and potentially escape from dormancy, a mechanism whereby previously non-replicating tumor cells "awaken" and begin to replicate. We aimed to investigate whether Toll-like receptor activation, both in NSCLC cells themselves and in the host, with resultant changes in the tumour cell microenvironment, also have a roll in the acceleration of the events necessary for metastasis after tumour cell adhesion has already occurred.

Tumour cell replication

Initiation of the cell cycle can be prompted by many different stimuli. In the context of gram negative sepsis, pattern associated molecular patterns are recognized by Toll-like receptors, which results in the initiation of the MyD88-dependent intracellular cascade. Ultimately, this results in the activation of AP-1 and NF-κB transcription factors in both tumour and host cells. In the tumour, activation of NF-κB has been shown to promote cell cycle progression through a mechanism involving Cyclin D1 and the cyclin-dependent kinase system (65). In the tumour microenvironment, accumulation of pro-inflammatory cytokines from the host at the tumour site directly contributes to pro-tumourigenic activity. Factors involved in these interactions include TNF-α, IL-1, and IL-17 (27). IL-6 has also been

implicated and has previously been identified by our group as one of the potential mediators of tumour cell adhesion (23). Furthermore, inflammatory mediators including cytokines, prostaglandin E2, and reactive oxygen species can suppress the DNA mismatch repair machinery through different mechanisms leading to the accumulation of more genetic mutations (27, 29).

Avoidance of cell death

In addition to replication, the tumour cell must also avoid apoptotic stimuli. Generally, tumour cells are recognized by the host, at least to a certain extent, as "non-self", which results in activation of CD8 positive T-cells and cell death. Although a role for the NF-κB pathway has been demonstrated in the initiation of the cellular immunity pathway in response to pathogens, it is thought that the constitutive activation of inflammatory pathways (as is frequently the case in gram-negative sepsis) can in fact result in a paradoxical effect. The anti-apoptotic effect of NF-κB has been attributed to its ability to directly upregulate Bcl-2 and Bcl-XL expression (63). The notion that constitutive activity of NF-κB exerts a pro-tumourigenic effect is underscored by the observation that patients with chronic inflammatory diseases have higher risks for cancer similar to immune-suppressed patients.

Clinical uses of TLR blockade

TLR4 blockade has been investigated in the context of treatment of severe sepsis in the past, due to the well-known phenomenon of LPS triggering inflammation in gram-negative sepsis. Eritoran (E5564), a synthetic analog of lipid A and a potent and specific antagonist of LPS action, inhibits lipid A binding to MD2 and terminates MD2/TLR4-mediated signaling *in vitro*, *ex vivo*, and *in vivo*. In a phase 1 trial, it was shown to block cytokine responses and clinical illness in healthy volunteers, while in a phase 2 trial, Eritoran-treated patients with gram-negative sepsis had a lower mortality, although this was not statistically significant (33).

Ultimately, in phase 3 trials, Eritoran was not shown to have a statistically significant benefit with regards to improved outcomes in gram-negative sepsis. However, during these trials, the molecule was shown not to cause harm in septic patients. As such, in cancer patients with gram-negative sepsis, treatment with Eritoran to decrease activation of both tumor cells and the host response is likely safe (33).

Importantly, downstream inhibitors of the PI3Kinase and p38 MAPKinase cascades, which result in activation of AP-1 and NF-κB, have previously been targeted as chemotherapeutic agents (83-85). This underscores the importance of these signalling cascades in tumour cell replication, furthering the evidence that blockade of these pathways could abrogate tumour cell replication and thereby decrease metastatic potential. Although, to date, none of these inhibitors have been approved for clinical use, trials have focused on their utility in treatment of existing tumours,

rather than on their potential effects abrogating the mechanisms of increased tumour prevalence in patients with infection following curative resection of cancer. As discussed above, the majority of these treatments have been investigated in the context of tumor cell replication and cell death, which again indicates a potential role for these pathways in the events following tumor cell migration and adhesion in the metastatic pathway.

Specific aims and potential clinical relevance of this work

In this work, we aim to demonstrate that post-adhesive events in metastasis have a significant effect on the well-known phenotype of increased metastatic recurrence in patients with post-operative gram-negative infection following surgical resection of NSCLC. Moreover, we hope to demonstrate that these events are related to activation of the Toll-like receptor cascade, both within tumor cells themselves and within the host.

Should increases in cancer metastasis be related to activation of Toll-like receptors following gram-negative infection, this opens the door for a wide variety of potential therapeutic modalities. Blockade of this mechanism by small molecule inhibitors, such as Eritoran, could result in a decreased prevalence of post-operative recurrence via distant metastases, or alternatively, of an increased time to recurrence in such cases.

Metastatic recurrence of non-small cell lung cancer following surgical resection is a significant burden on the health care system and, perhaps more importantly, on patients hoping to obtain cure through definitive surgical treatment. If increased cancer metastasis is related to TLR activation, this opens the door for a wide variety of potential therapeutic modalities. Blockade of this mechanism by small molecule inhibitors could result in a decreased prevalence of post-operative recurrence via distant metastases, or alternatively, of an increased time to recurrence in such cases.

Materials and Methods

Animals

Seven-week-old C57BL/6 mice (Charles River, St. Constant, Canada) of approximately 20–25 grams were maintained in the Royal Victoria Hospital Animal Facility and used for all experiments. Mice were either anesthetized with a mixture of 200 mg/kg ketamine (Wyeth-Ayerst Canada, Guelph, Ontario) and 10 mg/kg xylazine (Bayer Canada, Etobicoke, Ontario) injected intraperitoneally or via inhaled anesthetic isoflurane (Baxter, Mississauga, Canada). All experiments were approved and supervised by the McGill University Animal Care Committee and conducted in accordance with local institutional guidelines.

TLR4 -/- animals

TLR4 -/- mice were a kind gift from the lab of Dr. Salman Qureshi, but originated from the lab of Dr. Michel Chignard at the Pasteur Institute in France. The mice were maintained and bred in facilities at the MUHC. Mice of approximately 7 to 9 weeks old and 20-25 grams in weight were used for experiments. All experiments, as well as breeding practices and protocols, were approved and supervised by the McGill University Animal Care Committee and conducted in accordance with local institutional guidelines.

Reagents

LPS derived from Escherichia coli strain 055:B5 was purchased from Sigma-Aldrich and aliqoted and stored at -20°C according to the manufacturer's instructions. E. coli

(NCTC strain 9001) were grown in culture in-lab, aliquoted into 1 mL Cryovials and maintained at -80°C. Prior to experiments, bacteria were thawed, heat-inactivated by boiling at 95°C for 10 min and cooled to room temperature before use.

Eritoran (E5564) is a second-generation lipid A analog that acts as a competitive inhibitor and antagonist to TLR4 and was a kind gift from Eisai. BIRB0796 was kindly provided by Professor Sir Philip Cohen (MRC PPU, University of Dundee, UK). PD184352 was purchased from US Biological (Swampscott, MA). PI103 was purchased from Cayman Chemical (Ann Arbor, MI). All inhibitors were aliquoted in quantities appropriate for single experiments and stored according to the manufacturer's instructions.

Cell culture methods

Lewis lung adenocarcinoma subline H59, stably expressing GFP after plasmid transfection, were a kind gift from Pnina Brodt's laboratory (McGill University, Montreal, Canada). They have been tested and authenticated in accordance with our institutional policies for contaminants using PCR within the past year. These cells were maintained in a sub-confluent state using modified Roswell Park Memorial Institute media with 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin and 1% streptomycin. At the time of use, confluent monolayers of H59 were detached using a PBS-EDTA solution and re-suspended in PBS for injection.

Of note, the Lewis lung adenocarcinoma cell line has been selected and cultured as a highly metastatic liver-colonizing subpopulation (86). It has previously been demonstrated to metastasize to liver following intrasplenic injection in immunocompetent C57Bl/6 mice.

A549, a human lung adenocarcinoma cell line was obtained from the American Type and Culture Collection via Cedarlane Labs, Canada. Cells were grown and maintained in DMEM/F12 media supplemented with 10% FBS and 1% Penicillin-Streptomycin. BEAS-2B, a human lung bronchoepithelial cell line (epithelial virus-transformed), was obtained from the American Type and Culture Collection via Sigma-Aldrich. These cells were grown and maintained using modified Roswell Park Memorial Institute media with 10% fetal bovine serum (FBS), 1% glutamine, 1% Penicillin-Streptomycin.

Standard testing for contaminants for both A549 and BEAS-2B cells was performed per institutional protocols, as above. Detatching and manipulation of both cell lines was performed using 0.5% Trypsin solution (Wisent). All cells were incubated in standard eukaryotic cell culture conditions at 37°C with 5% CO2. Cell culture and manipulation was done using sterile technique. All cell culture reagents were purchased from Wisent.

A549 NSCLC cells, as well as human bronchoepithelial cell line BEAS-2B, are known to express functional Toll-like receptor 4 (30, 31, 65).

Cell culture in three-dimensional media

Technique for overlay three-dimensional culture of C59-GFP cells on Matrigel was done as described in Debnath et al, 2003 (87). Briefly, Matrigel from BD Biosciences (BD No.354230) was aliquoted and stored at -20°C. Matrigel was thawed at overnight at 4°C to achieve liquid phase, then 40 uL was added to each well of a precooled eight well glass chamber slide and spread evenly. Chamber slides were placed in incubator immediately to solidify for 15 minutes. Meanwhile, H59-GFP cells stained with CellTracker Red (Invitrogen) at a concentration of 0.5 uM as described below. Cells were then detached and re-suspended to achieve a final concentration of 25000 cells/mL. Assay medium, containing 4% Matrigel, was also prepared. 200 uL of cell mixture and 200 uL of assay medium were added to each Matrigel-coated well, for a final concentration of 5000 cells/well in medium containing 2% Matrigel.

Cells were allowed to grow in a 5% CO_2 humidified incubator at 37°C. Overlying media was changed 1 day after plating to allow for treatments (200 uL of double concentration Eritoran + 2% Matrigel in Eritoran-containing conditions, followed by 200 uL of double concentration E. coli + 2% Matrigel in E. coli containing conditions).

Cell culture incubation conditions

H59 and A549 cells were incubated in RPMI media plus 10% FBS for all experiments. Prior to experiments involving cell division, cells were pre-incubated

for 24 hours in serum free media to synchronize cell cycle. For certain experiments, LPS was added at a concentration of 100 ng/mL with or without Eritoran at a concentration of 100 nmol/L. In other experiments, heat-inactivated E. coli was used at a final concentration of 1×10^8 colony-forming units (CFU)/mL with or without 100 nmol/L of Eritoran.

When Eritoran was used, cells were pre-incubated with Eritoran for 30 minutes prior to adding LPS or heat-inactivated E. coli. The MEK1/2 inhibitor PD184352, the p38 MAPK inhibitor BIRB0796, and the PI3Kinase inhibitor PI103 were used at 2, 0.1 and 0.1 nmol/L concentrations, respectively. These inhibitors were added 1 hr prior to incubation with LPS or heat-inactivated E. coli and remained in the media during stimulation.

Antibodies: For Toll-like receptor blockade

In human cells: Neutralizing recombinant IgA2 monoclonal antibody to human TLR5 was purchased from Invitrogen (San Diego, CA) and used at a concentration of 0.1 μg/mL. Neutralizing recombinant IgA2 monoclonal antibody to human TLR2 was also purchased from Invitrogen and used at a concentration of 0.1 μg/mL. All concentrations were derived from the recommendations of the manufacturer. Isotype controls were used at identical concentrations. Human IgA2 isotype control was used for both TLR2 and TLR5 and was also purchased from Invitrogen.

In murine cells: Neutralizing IgG2a rat monoclonal antibody to murine TLR5 was purchased from Invitrogen (San Diego, CA) and used at a concentration of 0.1 μg/mL. Neutralizing IgG2a recombinant monoclonal antibody to murine TLR2 was also purchased from Invitrogen and was used at a concentration of 1 μg/mL. All concentrations were derived from the recommendations of the manufacturer. Isotype controls were used at identical concentrations. Rat IgG2a isotype control (0.1 μg/mL) and mouse IgG2a (1 μg/mL) isotype controls were also purchased from Invitrogen.

Antibodies: For detection of Toll-like receptors via flow cytometry

Fluorescein-conjugated monoclonal rat anti-mouse TLR2 IgG2a was purchased from R&D systems (Minneapolis, MN) and was used at a concentration of 50 μg/mL. Fluorescein-conjugated monoclonal mouse anti-mouse TLR5 IgG2a and fluorescein-conjugated monoclonal rat anti-mouse TLR4 IgG2b were purchased from Novus Bio (Oakville, ON) and were both used at a concentration of 500 μg/mL. All concentrations were determined based on recommendations from the manufacturer. All antibodies were incubated for one hour with cells in flow cytometry buffer (FBS + 0.1 M TRIS-HCl + 10% FBS) at 4°C.

Fluorescein-conjugated monoclonal mouse IgG2a (R&D systems), fluorescein-conjugated monoclonal rat IgG2a (R&D systems) and fluorescein-conjugated monoclonal rat IgG2b (R&D systems) were used at identical concentrations as isotype controls.

Conditioned media with LPS

BEAS-2B cells were maintained in a sub-confluent state using DMEM-Hi media + 10% FBS (Wisent) prior to experiments. Cells were then stimulated with LPS at a concentration of 100 ng/mL, with or without a 30-minute pre-incubation with Eritoran, a small molecule inhibitor of TLR4, for two hours. Cells were then washed twice with PBS and serum-free DMEM high glucose media was added. Twenty-four hours later, conditioned media was collected, supplemented with 10% FBS, and frozen in 1mL cryovials (Corning) at -80° Celsius until needed. In samples treated with Eritoran, Eritoran was added to serum-free media 30 minutes prior to incubation with LPS and remained in the media during stimulation.

Staining with CellTracker Red

Prior to proliferation assays, H59 cells were stained with CellTracker Red (Invitrogen) according to the manufacturer's instructions at concentrations of 1 uM, 0.5 uM, 0.25 uM and 0.125 uM. Cells were detached, washed and then immediately analyzed with a flow cytometer FACScan and CellQuest Software immediately after staining. Analysis was repeated at 24 and 48 hours post staining, in order to determine baseline detection of CellTracker red staining levels following 1 and 2 cell cycles, respectively (we have previously demonstrated that doubling time is approximately 24 hours in H59 cells). Unstained cells were used as control.

Cell proliferation assay – Confluence in GFP expressing cells

Images were taken of H59-GFP cells under various conditions. Three random fields were captured with a 20× microscope objective of an inverted fluorescent microscope (Nikon TE300) and a digital SLR camera (Nikon D90). The adherent cells per high power field (hpf) were counted using a macro function on the ImageJ software (version 1.43U, downloaded from the NIH). Three technical replicates were performed for each condition.

Cell proliferation assay - MTT

Cells (count of 5 x 10^3) with 100 µL of serum free media were seeded into each well of 96-well flat bottom plates. Cells were incubated 24 hours to synchronize cell cycle and allow for adhesion, then treated according to the specifications above – either with E. coli with or without Eritoran, or with pre-made conditioned media from BEAS cells (see above). Cell proliferation was assessed by MTT assay kit as described in the manufacturer's protocol (R&D Systems) at 48 hours.

Flow cytometry – detection of cell death markers

Cells were plated in 6 well plates in media supplemented with 10% FBS and allowed to adhere for 24 hours. Media was then aspirated and cells were treated according to the specifications above – either with E. coli with or without Eritoran, or with pre-made conditioned media from BEAS cells (see above). Cells were allowed to incubate for 24 and 48 hours after treatment.

Apoptosis and cell membrane integrity was assessed using FITC conjugated Annexin-V (BD Biosciences) and Propidium Iodide (Sigma-Aldrich) double staining method at 24 and 48 hours. Annexin-V was staining was done according to manufacturer's instructions. Propidium Iodide was added for a final concentration of 2 μg/mL after completion of Annexin-V staining and kept on ice for an additional 15 minutes. Cells were then immediately analyzed with a flow cytometer FACScan and CellQuest Software.

Cell proliferation assay in 3D media

Cells were pre-stained with CellTracker Red (Invitrogen) at a concentration of 0.5 uM and plated in 8-well slides containing Matrigel base and media as described above. Cells were allowed to incubate for 24 and 48 hours. Representative images were captured at both time points using a Zeiss LSM 780 laser scanning confocal microscope using a ×40/1.20 W corr C-Apochromat air objective. Two laser excitation wavelengths (514, and 633 nm; Argon and HeNe) were used. 3-dimensional reconstruction of confocal z-stacks (10- to 20-μm thickness,1-μm intervals) was done.

Liver metastasis assay

H59 cells were maintained in vitro as above in a sub-confluent state. C57BL/6 male mice received one intrasplenic injection of 5×10^5 H59 or H59-GFP cells suspended in 100 μL of PBS followed by splenectomy 1 minute post-injection.

24 hours after intrasplenic injection, peritonitis was induced by cecal ligation and puncture (CLP) as previously described (88-90). Briefly, a midline laparotomy was performed under inhaled anesthesia, and the cecum was exteriorized. The distal 1 cm of the cecum was ligated with a 4-0 silk suture, and the tip of the cecum was perforated on the anti-mesenteric aspect with a 25-gauge needle. The cecum was returned to the abdominal cavity, and the abdomen was closed in 2 layers with a 4-0 silk suture. Animals received 0.2 mg/kg buprenorphine subcutaneously every 12 to 24 hours or as required. Sham animals had their cecums exteriorized without CLP. Mice were monitored postoperatively for signs of systemic sepsis, including changes in posture or activity, abdominal tenderness, rubor, and calor.

Mice were sacrificed 2 weeks later and the livers were harvested. Gross liver metastases were counted. Representative images of harvested livers were captured immediately after removal using a digital camera (Nikon, Mississauga ON).

Staining for Ki-67

Murine livers were immediately preserved in formalin for histological analysis. Following sectioning, all were pretreated with DIVA reagent (Nobel BioCare, Richmond Hill ON) and were both enzyme and protein blocked (DAKO, Mississauga ON). Ki-67 was detected using monoclonal rabbit anti-Ki-67 antibody (Cell Signaling, Boston MA) at a 1:400 dilution.

All antibodies were detected using a secondary goat anti-rabbit antibody at a 1:500 dilution (Jackson Lab, Bar Harbor ME), and the chromogene Dako Dab (DAKO, Mississauga ON). All samples were counterstained with Hematoxylin Harris.

Rabbit anti-mouse tumor IgG (Cell Signaling) was used as a positive control for Ki-67 following the same process as above. Dako Rabbit Negative Control (DAKO) was used for negative control samples. All samples were visualized and photographed using an inverted light microscope with 10X, 20X and 100X microscope objective (Nikon TE300) and a digital SLR camera system (Nikon D90).

Statistical analysis

Graphs and statistical analysis were performed using SPSS Statistics software (IBM). Numerical data was analyzed using the analyzed using the Student's t-test or one-way ANOVA with Tukey's HSD post-hoc analysis. Data points greater than three standard deviations from their group mean were excluded from analysis. Statistical significance was defined as $p<0.05$. Data are graphically represented as their group mean +/- standard deviation for all bar graphs and metastasis experiments. All cell replication experiments were performed a minimum of three times independently.

Results

Sepsis induced by cecal ligation and puncture increases liver metastatic burden following intrasplenic injection of tumour cells

Given the significant clinical association between pneumonia and NSCLC cancer metastases, as well as previous work by our group indicating TLR4 as a potential mechanism for this phenotype via increased cell adhesion and migration, first investigated whether the events following adhesion were also playing a role. Previous work has shown that in mice with active infection, intrasplenic injection of tumor cells results in increased liver metastatic burden. In order to better replicate the clinical model of post-operative infection and to determine whether post-adhesive events were contributory to this phenotype, we intrasplenically injected C57BL/6 mice with H59 murine lung adenocarcinoma cells. Cecal ligation and puncture (CLP) to induce gram-negative bacterial sepsis or sham surgery was performed 24 hours after tumor cell injection. The time between intrasplenic injection of tumor cells and CLP allowed cells to migrate to the liver and become adherent, meaning that any increases in metastases observed were due to post-adhesive events in the metastatic process, such as escape from cell death, increased replication, or increased escape from dormancy. C57BL/6 mice that underwent CLP demonstrated an approximately 5-fold increase in hepatic metastases compared to those that underwent sham surgery ($p<0.05$).

To investigate whether TLR4 activation contributed to this increase in H59 hepatic metastases, the experiment was repeated with TLR4 knockout mice. In knockout

mice that underwent CLP, a two-fold increase in hepatic metastases was observed compared to controls that received sham surgery. This change was not statistically significant from mice that underwent sham surgery. A statistically significant difference in hepatic metastases was also observed between wild type and TLR4 knockout mice that underwent intrasplenic injection of tumor cells followed by CLP.

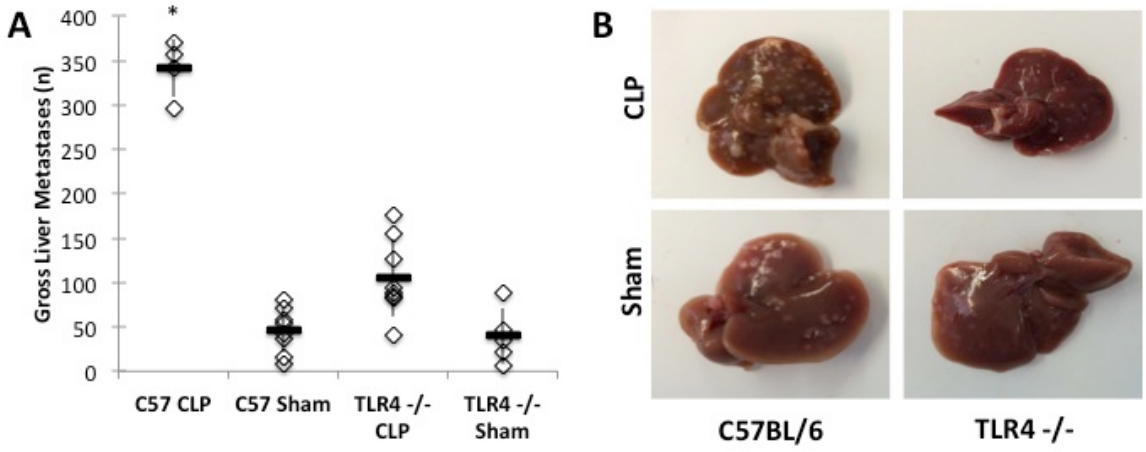


Figure 1: Murine H59 liver metastasis is augmented *in vivo* by cecal ligation and puncture and resultant gram-negative sepsis 24 hours post-injection of tumor cells. C57BL/6 (wild type) (C57) and TLR4 knockout mice (TLR4 -/-) were injected intrasplenically with 30000 H59 cells. Twenty-four hours following tumor cell injection, mice were subjected to either CLP (denoted CLP) or sham surgery (denoted Sham). Two weeks after tumor cell injection, surviving mice were euthanized and gross hepatic metastases were counted. Cecal ligation and puncture and the resultant gram-negative sepsis increased the formation of H59 liver metastases in wild type mice compared with TLR4 knockout mice. *$p<0.05$

compared to sham surgery in wild type and CLP or sham surgery in TLR4 knockout mice as determined by one-way ANOVA with Tukey's HSD post-hoc analysis. Data are represented as mean +/- standard deviation. Results represent pooled data from three independent experiments. C57 + CLP (n=4), C57 + sham surgery (n=7), TLR4 -/- + CLP (n=8), TLR4 -/- + sham surgery (n=5). **A)** Numbers of gross liver metastases in all animals surviving to two weeks post tumor cell injection **B)** Representative images of livers from animals surviving to two weeks post tumor cell injection.

TLR4 activated BEAS conditioned media and direct stimulation of TLR4 do not affect cell death in vitro

Previous work in our lab, as well as the significantly decreased metastatic burden seen in TLR4 knockout mice compared to wild type mice (Figure 1), indicates that the host response to infection likely plays a significant role in augmenting the metastatic capability of NSCLC cells. After identifying the important contribution of gram negative sepsis-induced cecal ligation and puncture to increased metastatic burden in the liver in mice that had previously undergone intrasplenic injection of NSCLC, we hypothesized that both direct stimulation of the tumor cells with gram-negative organisms and host response to gram-negative sepsis likely both augmented the metastatic capability of NSCLC cells. To investigate both of these mechanisms, we used both direct stimulation of H59 cells with heat inactivated E. coli and stimulation of A549 cells with BEAS-2B cell conditioned media, an *in vitro* model of the host response.

To model direct stimulation of TLR4 on cancer cells, media containing either TLR4 activating LPS or heat inactivated E. coli was added directly to a subconfluent layer of H59 murine NSCLC cells. Cells were then incubated for 24 hours, stained with Annexin-V (a well described marker for cellular apoptosis) and analyzed by flow cytometry. To model the host response, human bronchoepithelial cells (BEAS), which represent the body's first line of defense against pathogens in the lung and are known to express TLR4, were stimulated *in vitro* with either TLR4-activating LPS or heat inactivated E. coli for two hours. The cells were then washed with PBS and incubated for 24 hours in serum free media to create conditioned media, containing factors secreted by BEAS cells in response to TLR4 activation. This media was then supplemented with FBS and added to A549 cells, a human non-small cell lung cancer line. Human cells were used due to the availability and efficacy in the production of conditioned media of BEAS cells, which are also a human cell line and, as such, secrete human factors in response to infection. Cells were incubated for 24 hours with conditioned media and then similarly stained with Annexin-V and analyzed by flow cytometry.

No differences in Annexin-V staining were observed between cells that underwent stimulation; either directly via treatment with LPS or heat inactivated E. coli (Figure 2A) or via conditioned media from BEAS cells previously treated with LPS or heat inactivated E. coli (Figure 2B). As there was no decrease in Annexin-V staining in stimulated cells, which could have indicated a role for decreased activation of the

apoptotic pathway in the increased metastatic burden seen in mice with gram-negative infection, it was concluded that decreased activation of apoptosis likely did not play a role.

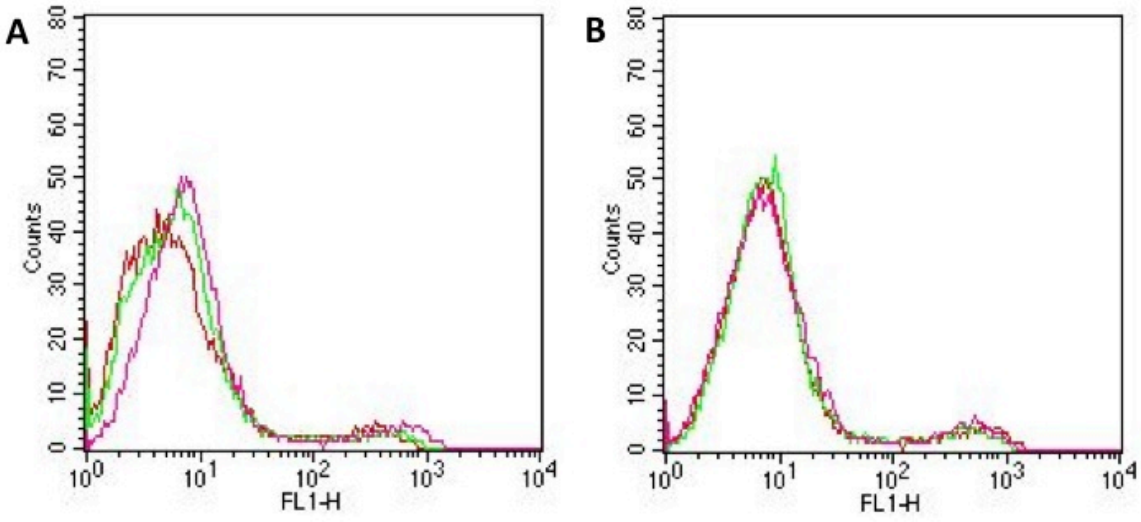


Figure 2A: H59 cells, stimulated for 24 hours with LPS (green), E. coli (pink), or unstimulated (red), were assessed by flow cytometry for binding of Annexin-V, a well established marker for apoptosis. Annexin-V bound to H59 cells was detected via FL1-H laser. A minimum of three technical replicates were performed for each condition.

Figure 2B: Conditioned media was obtained via stimulation of BEAS cells treated with LPS (green), E. coli (pink), or untreated (red), and allowed to secrete factors involved in the response to gram-negative infection for 24 hours prior to collection. A549 cells were then stimulated for 24 hours with conditioned media. Flow

cytometry was used to detect binding of Annexin-V to A549 cells via FL1-H laser. Ten thousand cells were counted for each condition. A minimum of three technical replicates were performed for each condition.

Sepsis induced by cecal ligation and puncture increases proliferation of tumor cell micrometastases in vivo

As above, wild type and TLR4 knockout mice were intrasplenically injected with H59 NSCLC cells, then underwent either cecal ligation and puncture or sham surgery 24 hours later. Two weeks after tumor cell injection, the surviving mice were euthanized. Livers were removed and Ki-67, a marker of cell replication, was detected via immunohistochemistry. Livers were also stained with hematoxylin and eosin. All samples were reviewed with a pathologist to confirm findings.

a. Ki-67 analysis of murine liver metastases and detection of micrometastases

Tumor metastases uniformly showed 90-100% Ki-67 positivity compared to background normal liver parenchyma, likely due to increased tumor cell growth compared to normal tissue. Signs of necrosis were observed centrally in all macrometastases, consistent with normal tumor cell necrosis (likely secondary to replication of tumor cells outstripping angiogenic activity, a known phenomenon in tumors with rapid growth). Slides were reviewed with a pathologist, which showed increased lymphocytic liver infiltration in samples where CLP had been performed. Within obvious tumor metastases, Ki-67 expression was high in both CLP and Sham mice.

Importantly, C57BL/6 mice that underwent CLP, previously shown to have increased metastatic burden (see Figure 1), had qualitatively increased levels of Ki-67 in macroscopically normal liver parenchyma, indicating that tumor cell replication in the context of gram-negative sepsis and increased tumor burden may be increased compared to conditions where mice underwent sham surgery rather than CLP. In mice that had undergone cecal ligation and puncture, microscopic deposits of tumor cells were also observed, consistent with rapidly replicating micrometastatic deposits in the liver parenchyma. Positive and negative control samples were also reviewed (not shown).

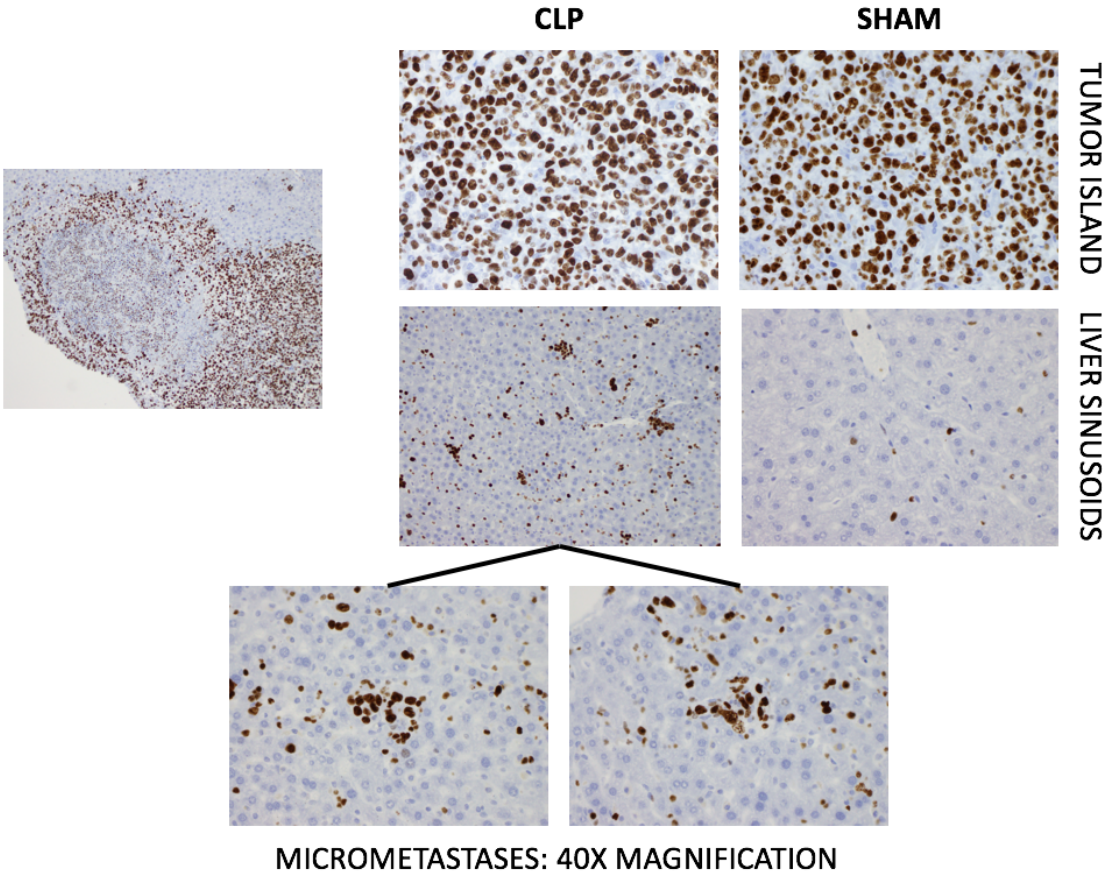


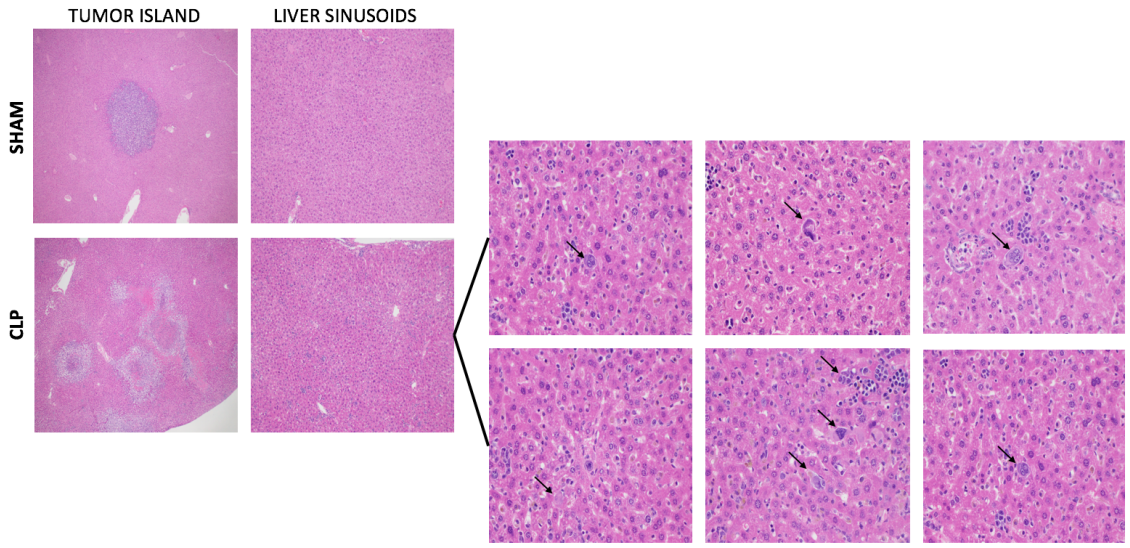
Figure 3A: C57BL/6 and TLR4 KO mice were injected intrasplenically with H59 NSCLC cells, then underwent either CLP or sham surgery, as above. Two weeks post tumor cell injection, livers were removed, preserved in formalin, sectioned and stained for presence of Ki-67, a marker of cell replication Representative images were taken using an inverted light microscope with 20x and 40x microscope objectives. Tumor islands (top row, indicated by "tumor island") showed increased expression of Ki-67 compared to normal liver parenchyma (bottom row, indicated by "liver sinusoids") in all conditions. A representative image of a tumor cell macrometastasis, with central necrosis, is shown on the top left of the figure.

b. Histological detection of sinusoidal micrometastases

Tumor metastases were detected in samples derived from mice that had undergone both CLP and sham surgery. In both cases, macroscopic tumor metastases showed similar characteristics (large clusters of tumor cells with a high percentage of mitotic figures and prominent central necrosis). However, in samples derived from mice that had undergone CLP, we observed presents of microscopic clusters of cells consistent with replicating tumor micrometastases within the liver sinusoids, consistent with the increased Ki-67 positivity previously observed (see Figure 3A). These clusters were counted over 20 high powered fields in samples from mice that had undergone both CLP and Sham surgery. In mice subjected to CLP, 20 clusters were seen over 20 high powered fields (representative images shown below), while

in mice that underwent sham surgery, no micrometastatic deposits of tumor cells were observed.

As expected, livers from mice that had undergone CLP showed increased lymphocytic infiltration, consistent with the systemic response to gram-negative infection. These small basophilic cells were also detectable in samples from CLP mice, however, they were morphologically different from tumor cell deposits, allowing for clear identification of metastatic disease.



Micrometastases count (20 consecutive high power fields)	
CLP	SHAM
20	0

Figure 3B: Murine livers were obtained as above, preserved in formalin and stained with hematoxylin and eosin. 20 consecutive high power fields were observed in each sample under supervision of a pathologist for assistance with detection of tumor cell deposits. 20 total tumor cell micrometastases (indicated by arrows) were

observed on 40X magnification in samples from mice that underwent CLP, while no micrometastases were found in mice that underwent sham surgery.

Increased murine H59-GFP NSCLC cell proliferation following stimulation with E. coli in two and three-dimensional media

When investigating whether increased cancer cell proliferation is implicated in the mechanism for increased tumor burden found in wild type mice undergoing CLP, we again hypothesized that there were likely two mechanisms at play; the effect of direct stimulation of the cancer cells with LPS and the effect of the tumor microenvironment, as modified by the presence of gram-negative sepsis. To test this hypothesis, we first focused on the effects of TLR4 stimulation on the cancer cell directly. GFP expressing H59 NSCLC cells plated and stimulated with heat inactivated E. coli, with or without the small-molecule TLR4 inhibitor Eritoran. At 24, 48 and 72 hours, cells were observed using the 20x objective of an inverted fluorescent microscope, photographed, and analyzed for confluence.

At all observed time points, increased confluence was shown in cells treated with E. coli, although this difference did not become statistically significant ($p<0.05$) until 48 hours post treatment. At all observed time points, partial abrogation of this effect was seen in samples treated with Eritoran, however, samples treated with Eritoran only showed a statistically significant difference ($p<0.05$) from samples treated with E. coli alone 72 hours following the initiation of treatment.

To investigate this effect under more physiologic conditions, the experiment was repeated using Matrigel, a three-dimensional medium that allows for the formation of tumor islands. H59-GFP cells were first counterstained with CellTracker Red, a cell marker that gradually becomes diluted with cell replication and therefore less visible under fluorescent microscopy. Cells were then plated in a thin Matrigel layer with a 4% Matrigel liquid media overlay and allowed to adhere for 12 hours. 4% Matrigel liquid media overlay was then removed and replaced with either fresh non-treated media or media containing heat inactivated E. coli and left to incubate for a further 48 hours. Due to technical constraints of this technique, it was not possible to pre-treat cells with Eritoran. 48 hours after treatment, cells were visualized with a laser scanning confocal microscope using a 40x objective and a digital zoom function. Due to the three-dimensional nature of the samples, reconstruction of confocal z-stacks was done. Images were acquired using wavelengths of 514 nm (to detect CellTracker Red) and 633 nm (to detect expression of GFP by cells).

Increased tumor cell proliferation in cells treated with E. coli was seen using the 40x objective alone, along with decreased staining with CellTracker Red, consistent with cell replication. Using the microscope's digital zoom function, tumor islands were visualized. E. coli treated samples showed larger tumor islands, again along with decreased staining with CellTracker Red. These results indicated increased tumor cell proliferation and tumor island formation in three-dimensional media in samples treated with heat inactivated E. coli.

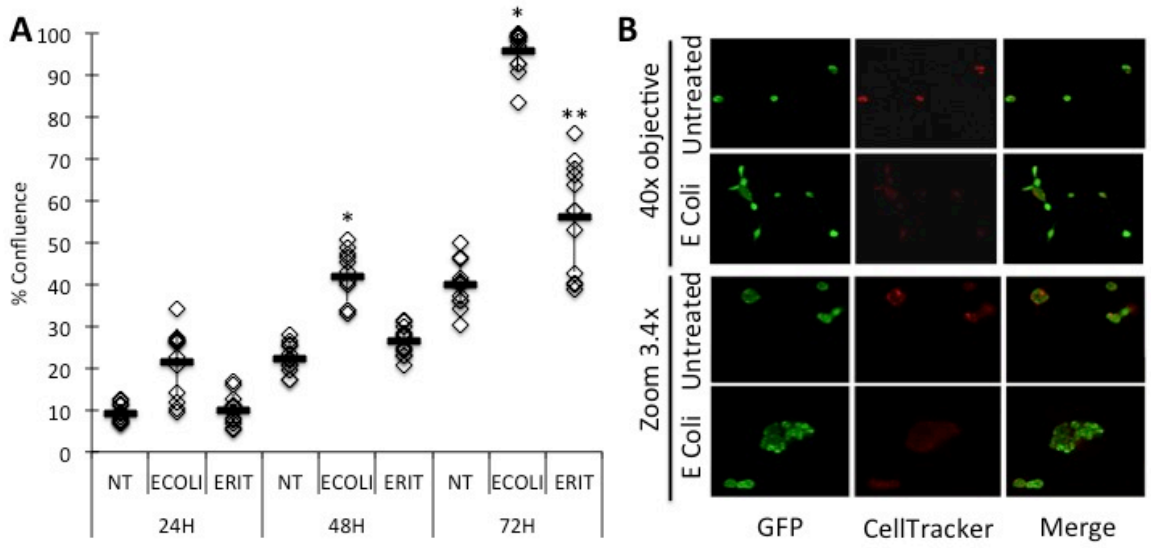


Figure 4A: Increased proliferation of GFP-expressing H59 NSCLC cells in two-dimensional media. Cells were stimulated with either heat inactivated E. coli (denoted ECOLI) or a combination of heat inactivated E. coli and Eritoran (denoted ERIT). Untreated cells (denoted NT) were used as controls. Three representative images of each condition were assessed at 24, 48 and 72 hours post stimulation using fluorescent microscopy and assessed for percent of image showing expression of GFP (used as a surrogate for % confluence of cells) using ImageJ software. *p<0.05 compared to untreated cells at the same time point **p<0.05 compared to cells stimulated with heat inactivated E. coli at the same time point. All p values were determined by one-way ANOVA with Tukey's HSD post-hoc analysis. Data are represented as mean +/- standard deviation. Results represent pooled data from three independent experiments, resulting in n=9 for all time points and conditions.

Figure 4B: Increased proliferation of GFP-expressing H59 NSCLC cells counterstained with CellTracker Red in three-dimensional media (Matrigel). Images were taken 48 hours post-stimulation with E. coli. Untreated cells were used as controls. Images were obtained with confocal microscopy with a 40x objective, and reconstructed using Z-stacking due to the three-dimensional nature of cell growth in Matrigel. 3.4x digital zoom was also used with the 40x objective (third and fourth row, as indicated) for detection of tumor islands.

H59 cells express cell surface TLR2, TLR4 and TLR5

Although significant decreases in cell replication were observed in cells pre-treated with Eritoran (Figure 4), growth of cells stimulated with heat inactivated E. coli consistently remained above growth of non-treated controls despite TLR4 blockade. To determine the cause of this phenomenon, we chose to pursue experiments to determine whether other TLRs are implicated in this mechanism. Heat inactivated E. coli NCTC strain 9001, the strain used in all of the above experiments, is known to express Flagellin, a pathogen associated molecular pattern (PAMP) detected by TLR5. Moreover, although TLR2 is most known for the detection of lipoteichoic acid, a cell surface component present only on the cell surface of gram-positive bacteria, is also known to initiate responses to peptidoglycan, which is a component of both the gram-positive and gram-negative cell membranes. Previous work by our group has implicated TLR2 in increased tumor cell adhesion and migration following gram-positive infection, increasing the possibility that TLR2 is implicated in post-adhesive events as well. Moreover, downstream signaling pathways of these three

receptors, while distinct in many ways, converge around the production of IL-6 and TNF-α, both of which have been associated with cancer cell replication in the past.

To test this hypothesis, we first sought to determine whether TLR2 and TLR5 are present on the surface of H59 NSCLC cells. We also detected the presence of TLR4 cell surface expression, which has not previously been documented, despite its evident activation and downstream effects, which have been previously documented in several studies.

H59 cells were detached, immediately placed on ice to maintain surface expression of relevant molecules, re-suspended in cold flow cytometry buffer, and stained with fluorescein-conjugated anti-mouse TLR2, TLR4 and TLR5 antibodies. Appropriate fluorescein-conjugated isotype antibodies and non-treated cells were used as controls. Samples were analyzed by flow cytometry where fluorescein was detected using the FL1-H laser. Analysis of these results showed expression of TLR2, TLR4 and, possibly to a lesser extent, TLR5 on the cell surface of H59 cells.

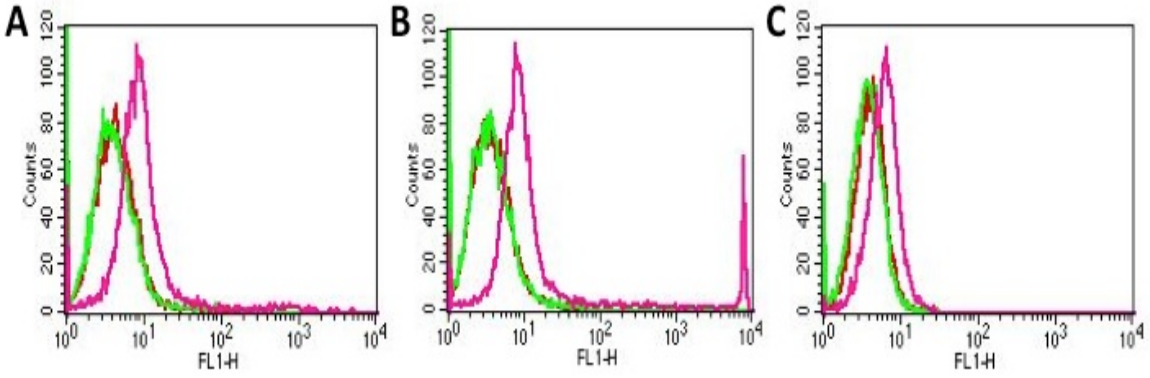


Figure 5: Cell surface expression of **A)** TLR2, **B)** TLR4, and **C)** TLR5 via flow cytometry. Non-treated cells are indicated with green, isotype control with red, and antibody against surface TLR molecule with pink. 10000 cells were counted for each sample.

Augmentation of H59 cell proliferation following direct stimulation with heat inactivated E. coli is partially abrogated by TLR blockade

Given previous results indicating a large increase in proliferation of cancer cells following direct stimulation with heat inactivated E. coli (Figure 4), along with only a partial abrogation of this effect following treatment with the small molecule inhibitor of TLR4 Eritoran, we decided to investigate whether increased cell replication following E. coli stimulation could also be related to detection of PAMPs by other Toll-like receptors.

Given that E. coli NCTC strain 9001 is known to contain peptidoglycan and flagellin, as well as LPS, and given that H59 cells have surface expression of both PAMP receptors (TLR2 and TLR5), and given the known association of both receptors with activation of pro-inflammatory cytokines previously implicated in increased cancer metastasis (in particular, IL-6), we investigated whether inhibition of these receptors could also abrogate the increased proliferation of H59 cancer cells seen after stimulation with E. coli.

H59 cells were plated in serum free media for 24 hours to synchronize cell cycle. Prior to the addition of E. coli, the cells were pre-treated with Eritoran, function blocking antibody to TLR2, or function blocking antibody to TLR5. Appropriate isotype controls were used for conditions involving antibodies. Heat inactivated E. coli was added to all conditions, along with 10% FBS to allow for cell growth. 48 hours after treatment, cell proliferation was analyzed using MTT assay. Cell counts were determined using standard curves. Untreated cells were used for comparison and statistical analysis.

As previously described (Figure 4), cells treated with heat inactivated E. coli without Toll-like receptor blockade showed an important increase in cell proliferation. Blockade of TLR4, TLR2 and TLR5 individually all resulted in a slight abrogation of H59 cell proliferation; however, none of these effects were statistically significant. Co-blockade of all three receptors resulted in a decrease in proliferation compared to single receptor blockade. This difference was found to be statistically significant when compared to cells stimulated without Toll-like receptor blockade ($p<0.05$), although again, it did not represent a complete return to the baseline rate of cell proliferation associated with untreated cells. All isotype control conditions resulted in no significant difference compared to cells treated with heat inactivated E. coli alone.

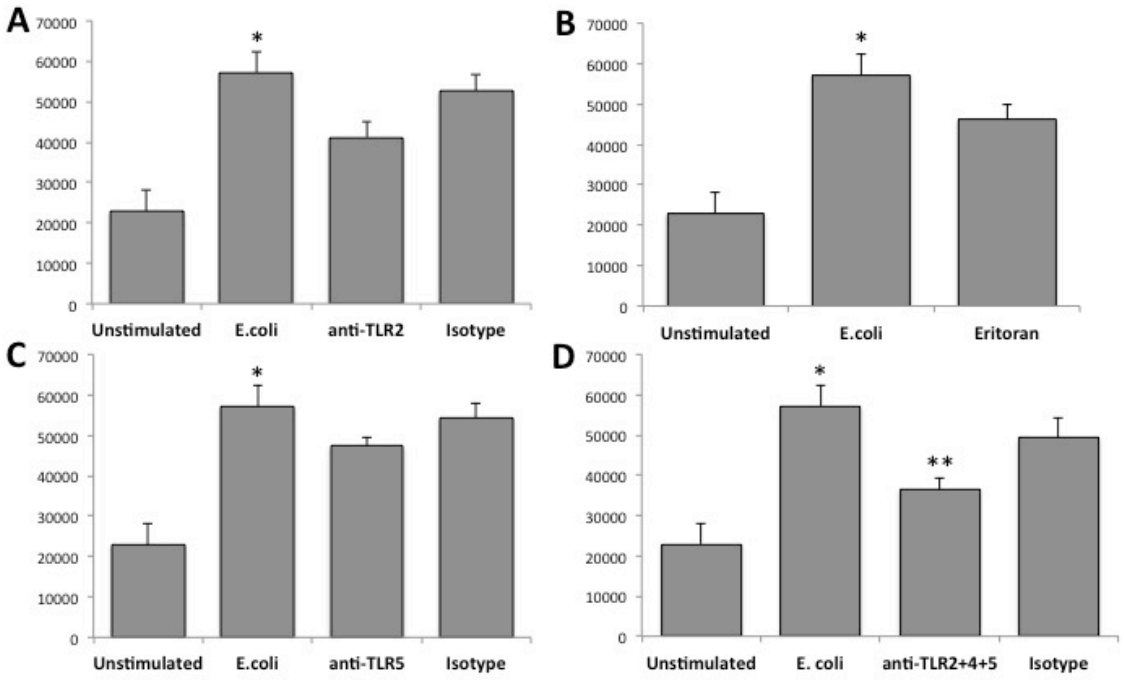


Figure 6: Results of MTT assay 48 hours post stimulation with heat inactivated E. coli. Standard cell growth curves were used to determine cell number (y axis). Appropriate isotype controls were used in conditions requiring the use of function blocking antibodies (TLR5 and TLR2) (denoted Isotype). **A)** Proliferation in H59 cells following blockade of TLR2 with function blocking TLR2 antibody **B)** Proliferation in H59 cells following blockade of TLR4 with Eritoran **C)** Proliferation in H59 cells following blockade of TLR5 with function blocking TLR5 antibody **D)** Proliferation in H59 cells following blockade of TLR2, TLR4 and TLR5 simultaneously. * $p<0.05$ compared to unstimulated cells, ** $p<0.05$ compared to cells stimulated with heat inactivated E. coli. All p values were determined by one-way ANOVA with Tukey's HSD post-hoc analysis. Data are represented as mean + standard deviation. A minimum of three technical replicates were performed per

condition. Results represent pooled data from three separately performed experiments.

A549 cancer cell proliferation is augmented by conditioned media collected from cells stimulated with LPS

After investigation of the effect of direct stimulation of tumor cells with heat inactivated E. coli, we turned our attention to our in vitro model of the effect of the host immune response on tumor cell proliferation. For these studies, BEAS-2B cells were treated with LPS and allowed to secrete infection response factors into serum free media for 24 hours. Media was then supplemented with 10% FBS to allow the necessary materials for cell replication. A549 cells were serum starved for 24 hours to synchronize cell cycle, after which conditioned media was added. Cells were then incubated with conditioned media for 48 hours and allowed to replicate. After 48 hours, MTT assay was done to assess for cell replication and cell numbers were determined using a standard curve.

Cells treated with conditioned media from BEAS cells stimulated with LPS (denoted LPS), showed increased replication compared to cells treated with media from untreated BEAS cells (denoted NT) or BEAS cells pre-treated with Eritoran (denoted Eritoran) prior to stimulation with LPS ($p < 0.05$). In addition, in this case, the increased replication was completely abrogated by TLR4 blockade. No significant difference was seen between cells treated with conditioned media with untreated cells and cells treated with conditioned media from cells pre-treated with Eritoran.

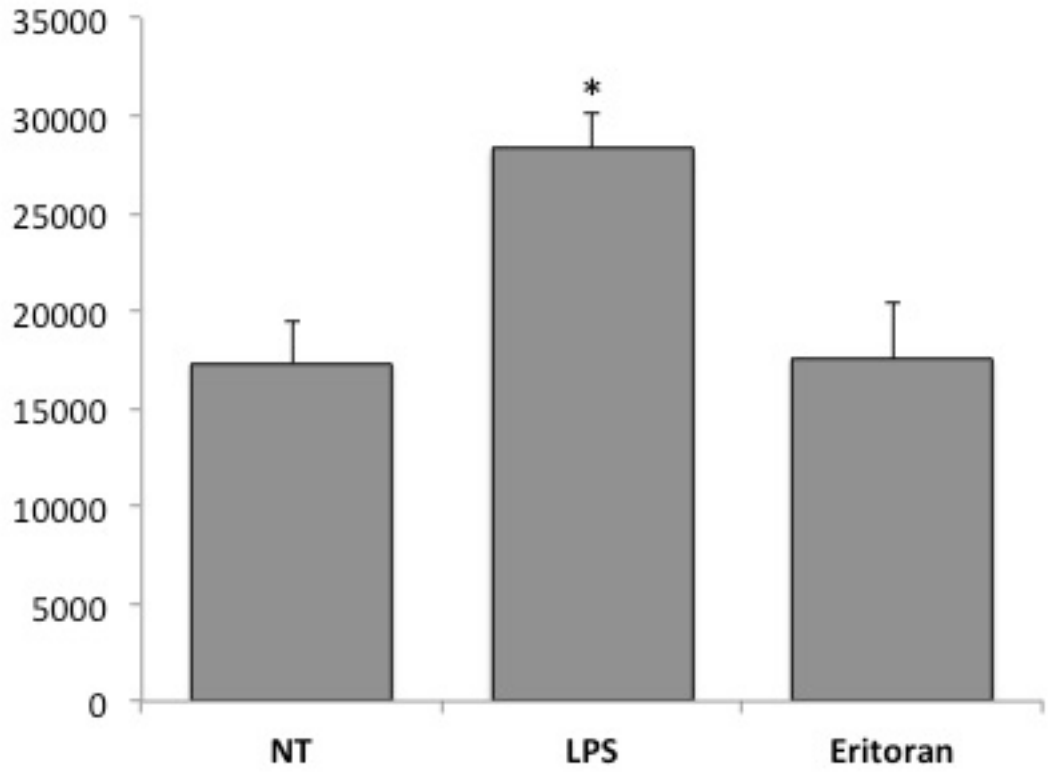


Figure 7: Results of MTT assay 48 hours post stimulation with conditioned media from untreated BEAS-2B cells (denoted NT), BEAS-2B cells stimulated with LPS (denoted LPS) and BEAS-2B cells stimulated with LPS following treatment with Eritoran. Standard cell growth curves were used to determine cell number (y axis). *$p<0.05$ compared to both cells treated with conditioned media from untreated BEAS-2B cells and BEAS-2B cells pre-treated with Eritoran prior to stimulation with heat inactivated E. coli. All p values were determined by one-way ANOVA with Tukey's HSD post-hoc analysis. Data are represented as mean + standard deviation. A minimum of three technical replicates were performed per condition. Results represent pooled data from three separately performed experiments.

Mechanism for increased cell proliferation using conditioned media is ERK and p38 MapKinase dependent

Given that stimulation of BEAS-2B cells with LPS enhanced cell replication (Figure 7), we sought to determine whether this mechanism was in fact dependent on activation of the downstream cascade of TLR4. To investigate the involvement of factors downstream from TLR4, a variety of small molecule inhibitors were used. PD184352, a small molecule inhibitor of ERK 1 / ERK 2, BIRB0796, a small molecule inhibitor of p38 MapKinase, and PI103, a small molecule inhibitor of PI3Kinase, were all added to BEAS cells one hour prior to exposure to LPS. Cells were then incubated, as done previously, and allowed to secrete factors in response to LPS stimulation. Control conditioned media was made using PD184352, BIRB0796 and PI103 alone, without the addition of LPS, to rule out an effect of the small molecule inhibitors alone on BEAS-2B cells. Human A549 cells were serum starved for 24 hours to synchronize cell cycle then treated with conditioned media for 48 hours. MTT assay was done to assess for cell replication. Cell numbers were determined based on standard curves.

As previously, conditioned media from BEAS-2B cells treated with LPS resulted in a significant increase in cell replication ($p<0.05$). However, when BEAS-2B cells were pre-treated with either PD184352 (ERK1/ERK2 inhibition) or BIRB0796 (p38 MapKinase inhibition), prior to addition of LPS, cell replication returned to levels seen in non-treated cells. This effect was not seen when using conditioned media from cells pre-treated with PI103 (inhibition of PI3Kinase) prior to the addition of

LPS. This implicates a role for the ERK1/ERK2 pathway and the p38 MapKinase pathway, both of which are activated downstream of TLR4, as well as for the production of pro-inflammatory factors downstream of these two pathways, in the increased replication seen in A549 cells treated with conditioned media from cells stimulated with LPS.

Conversely, a return to normal levels of A549 cell replication was not seen in cells treated with conditioned media from cells pre-treated with PI103 – in this case, A549 cells also showed significantly increased replication compared to non-treated controls ($p<0.05$). Since PI103 is a specific blocker if the PI3Kinase pathway, this implies less of a role for this pathway in the mechanism behind increased cancer cell replication in the inflamed environment. Importantly, conditioned media from cells treated with with PD184352, BIRB0796 and PI103 alone (with no addition of LPS) did not result in a significant decrease in A549 cell replication. This implies that the activity of these small molecules is interrelated with LPS stimulation and TLR4 activation, and does not decrease cell replication in the absence of inflammatory stimuli.

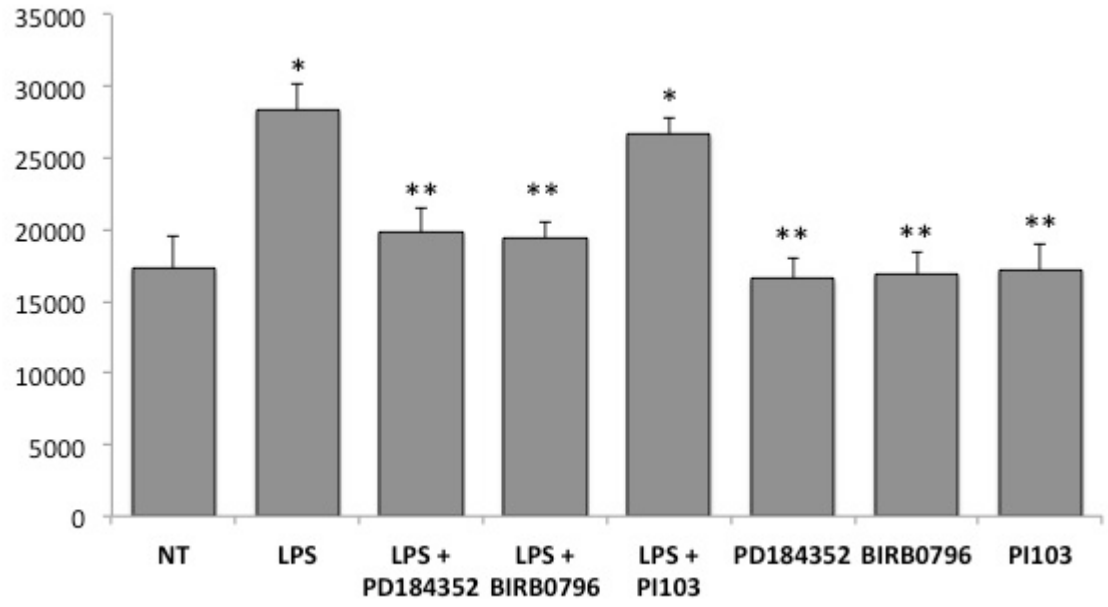


Figure 8: Results of MTT assay 48 hours post stimulation with conditioned media from untreated BEAS-2B cells (denoted NT), BEAS-2B cells stimulated with LPS (denoted LPS) and BEAS-2B cells stimulated with LPS following treatment with small molecule inhibitors PD184352, BIRB0796 and PI103 (denoted as LPS +). Conditions where treatment with inhibitors was done without addition of LPS were used as controls. Standard cell growth curves were used to determine cell number (y axis). *$p<0.05$ compared to both cells treated with conditioned media from untreated BEAS-2B cells, **$p<0.05$ compared to cells treated with conditioned media from BEAS-2B cells exposed to LPS alone. All p values were determined by one-way ANOVA with Tukey's HSD post-hoc analysis. Data are represented as mean + standard deviation. A minimum of three technical replicates were performed per condition. Results represent pooled data from three separately performed experiments.

Discussion

Despite advances in treatment, lung cancer remains the leading cause of cancer-related death in North America. Surgery remains the cornerstone of treatment for early stage NSCLC, and comprises resection of the primary tumor and draining lymph nodes. However, despite optimal surgical management, the majority of NSCLC patients will have recurrence with metastatic disease, which carries a high mortality rate despite chemotherapy, radiotherapy, and further surgical intervention.

It is well documented that post-operative pneumonias, which can occur in as many as 25-40% of patients following lobectomy for NSCLC, increases rates of cancer recurrence. Previous work has shown that the induction of a systemic inflammatory response may create a favorable environment for adhesion of circulating tumor cells, which may be already present in the bloodstream before surgery or inoculated into the bloodstream during surgical manipulation of the tumor and lymph nodes. Cells then adhere to metastatic sites and form stable metastases. Our group has shown the implication of various Toll-like receptors in this process, most notably TLR4 in the case of gram-negative infections.

Previous work has focused on the adhesion of circulating tumor cells to distant metastatic sites as a mechanism for this phenomenon. However, metastasis is a complex process, comprising several events that occur downstream from adhesion, namely the evasion of cell death, tumor outgrowth and the induction of angiogenesis

in metastatic sites, as well as more disputed processes such as tumor cell escape from dormancy. As of yet, no work has been done showing whether these mechanisms also play a role in the clinical phenotype of increased metastatic burden following post-operative infection.

In this work, gram-negative sepsis was induced 24 hours after tumor cell inoculation, thus providing a more clinically relevant model for post-operative infection and bypassing the majority of tumor cell adhesion processes previously documented to be involved in this phenotype. A robust increase in metastases was seen in wild type C57Bl/6 mice injected with NSCLC cells prior to the induction of gram-negative sepsis via cecal ligation and puncture, as compared to those that underwent sham surgery (Figure 1), lending support to the hypothesis that post-adhesive mechanisms play an important role in this phenotype.

Previous work has indicated that cell surface TLRs on malignant cells can be implicated in this phenotype. However, there is a paucity of data around the role of the host immune response in this phenomenon. The inflammatory response in response to gram-negative infection is mediated by healthy tissue, which in turn releases a “cytokine storm”, which mediates the inflammatory response. Here, we induced gram-negative infection in both wild type and TLR4 knockout mice, both of which were previously inoculated with H59 NSCLC cells, which are known to express TLR4. While gram-negative sepsis resulted in an increase in tumor burden in both wild type and knockout mice, increase in tumor burden in wild type mice

was much more profound (Figure 1). This indicates a significant role for the host response to infection, over and above the direct effect of gram-negative sepsis on tumor cells, in the mediation of increased tumor burden via post-adhesive mechanisms. This also indicates the important role of TLR4 activation in this process.

Several significant events occur post-adhesively in the process of metastasis formation. To determine which of these events were affected by gram-negative sepsis and activation of TLR4, we identified two key post-adhesive components of the metastatic process; namely, tumor cell avoidance of cell death and tumor cell replication.

To determine the role of direct stimulation of tumor cells in this phenotype, H59 cells were incubated *in vitro* with heat inactivated E. coli. Lung carcinoma cells are derived from native respiratory epithelial cells, which represent a first line of defense against inhaled pathogens and as such are well equipped to recognize a variety of pathogen-associated molecular patterns. As part of this mechanism, these cells constitutively express TLRs including TLR4, which are most often conserved throughout the process of cell transformation. This suggests that cancer cells expressing TLR4 may be able to detect postoperative gram-negative infections that drive NSCLC cancer metastasis.

In terms of the role of the host response to infection, we focused on the respiratory epithelium, which has been shown to play a significant role in the first line of detection of pathogens in the airways and subsequent airway inflammation. Human bronchoepithelial BEAS-2B cells (which, as detailed above, are known to constitutively express TLR4) were incubated with either heat inactivated E. coli or lipopolysaccharide, the main antigenic component of gram-negative bacteria and a key inflammatory mediator in gram-negative sepsis. The resultant media was used to create a clinically relevant *in vitro* model of the host response to infection. Following incubation, the "conditioned" media was removed and incubated with A549 human NSCLC cells. This was intended to simulate the tumor microenvironment in an inflamed host *in vitro.*

Cellular apoptosis seemed to be unaffected by stimulation of NSCLC cells with both E. coli and conditioned media (Figure 2), indicating that decreases in cell death processes may not play a large role in this phenotype. This data was further supported by histological detection of cleaved Caspase-3, a molecule involved in the common pathway of the apoptotic cascade, in murine liver samples, which did not show increased staining in livers from mice that underwent cecal ligation and puncture (Figure 3).

However, NSCLC cell replication was increased following direct stimulation with heat inactivated E. coli (Figure 3) and with stimulation with conditioned media from BEAS-2B cells treated with LPS (Figure 7). This indicates that cell replication is a

likely mechanistic target for the increased metastatic burden seen in mice with gram-negative sepsis.

In terms of direct stimulation of tumor cells with heat inactivated E. coli, an increase in proliferation was seen both in two-dimensional and three-dimensional cell culture models (Figure 4), the latter of which provides a more physiologically relevant model for tumor island outgrowth through the extracellular matrix. Interestingly, however, these results were only partially abrogated by blockade of TLR4 with the small molecule inhibitor Eritoran, indicating a role for other mechanisms in this phenotype.

With this in mind, we determined that H59 NSCLC cells express not only TLR4, but also TLR2 and TLR5 (Figure 5), which are involved in the detection of gram-negative bacteria via peptidoglycan and flagellin respectively. While there is some controversy around the role for TLR2 in the detection of gram-negative bacteria, TLR2 is known to detect peptidoglycan, which is forms a layer between the outer and inner membrane of E. coli. Although this layer is structurally much thinner in gram-negative bacteria than in gram-positive bacteria, it is antigenic and known to induce expression of interleukin-6, which has previously been associated with the mechanism of increased cancer metastasis via increased adherence of circulating tumor cells following both gram-positive and gram-negative sepsis. Meanwhile, E. coli NCTC strain 9001, which was used in these experiments, is known to express Flagellin, which, while generally considered to be not as antigenically significant as

LPS, also induces a robust immune response in both *in vitro* and *in vivo* models. Importantly, there is significant overlap in the downstream signaling pathways of all three of these receptors, indicating that the downstream effects of TLR2 and TLR5 likely result in a similar phenotype to that of TLR4.

In subsequent experiments, MTT assay was used as a measure of cell replication. *In vitro* blockade of TLR4 yielded similar results when MTT assay was used; a significant increase in cell replication was seen in cancer cells treated with heat inactivated E. coli. Though pre-treatment with Eritoran decreased cell replication overall, it did not bring replication down to levels seen in untreated cells (Figure 6). *In vitro* blockade of TLR2 and TLR5 also resulted in only a partial abrogation of the increase in replication. With blockade of all three receptors, cell replication was further decreased, but still did not return to baseline levels seen in unstimulated cells (Figure 6).

Overall, this seems to indicate a role of the detection of and response to E. coli infection by several different mechanisms within cancer cells themselves. It stands to reason that induction of the inflammatory cascade could also be induced by other mechanisms that we did not explore, which could account for the incomplete decrease in cell replication that we observed. Nod-like receptors, for example, have been previously shown by our group to play a role in these types of events, and may be implicated in this process. However, overall, as indicated by the murine experiments detailed in Figure 1, it is likely that the host response to infection,

rather than the response of the cancer cell itself, plays a more significant role in producing the increased metastatic burden seen with post-operative infection.

As such, we then turned our attention to the mechanism of increased cancer cell replication in cells treated with conditioned media from BEAS-2B cells stimulated with LPS. Human A549 NSCLC cells (which were used due to the fact that BEAS-2B cells are of human origin and therefore secrete factors that are likely more compatible with human cancer cells) were incubated with conditioned media and similarly assessed for cell replication using MTT assay. Cells treated with conditioned media from LPS-treated BEAS cells showed a significant increase in replication compared to untreated controls. Moreover, unlike our experiments centered on direct stimulation of the cancer cell, this increased replication was completely reversed when BEAS cells were incubated with Eritoran prior to treatment with LPS. This indicates an extremely important role for activation of TLR4 in increased cancer cell replication – one that, importantly, seems to be more or less completely reversible using a single agent.

Downstream signaling cascades originating from TLR4 have also been implicated in cancer cell metastasis and proliferation. Among others, activation of p38 MAP kinase, ERK1/ERK2 and PI3Kinase have all been implicated in cell cycle regulation in cancer cells via various mechanisms, lending support to the hypothesis that TLR4 activation, which similarly activates these same molecules, could increase cancer cell replication via a similar mechanism. Previous studies have shown the effects of

these inhibitors on the cancer cell directly, with a proposed mechanism that involves blockade of constitutively activated pathways that cause cell replication. In our study, downstream blockade of TLR4-induced signaling pathways in BEAS cells prior to stimulation and collection of conditioned media provided important insight into the mechanisms by which conditioned media (and, by extension, the tumor microenvironment *in vivo*) contributes to increased cancer cell proliferation.

Inhibition of downstream TLR4 kinases p38 MapKinase with BIRB0796 or ERK1/2 with PD184352 in BEAS-2B cells prior to stimulation with LPS and collection of conditioned media significantly decreased TLR4 activated cancer cell replication in our *in vitro* model of the host immune response (Figure 8). Inhibition of PI3Kinase with PI103 did not result in decreased cancer cell replication. PI3Kinase activation in cancer cells has previously been associated with increased cell survival more so than increased replication, although there are varying data to support both mechanisms. Meanwhile, constitutively activated p38 MapKinase or ERK1/ERK2 are related more predominantly to increased rates of cell replication, invasion and migration.

Interestingly, activation of p38 MapKinase and ERK1 / ERK2 has been implicated in the production of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), both of which are potent pro-inflammatory cytokines that are induced by TLR4 activation and have been linked to cancer progression. In a clinical context, IL-6 is

prognostically relevant in NSCLC. Elevated serum levels of IL-6 have been associated with decreased survival and increased risk of post-operative metastatic recurrence.

TNF-α, meanwhile, has a role that is more difficult to elucidate, and seems to have both pro- and anti-metastatic functions depending on the circumstances. It has been shown that TNF-α levels increase with increasing burden of disease and that certain TNF-α promoter polymorphisms can be associated with more or less aggressive cancer phenotypes. Most recently, researchers from our group have shown that stimulation of gastric cancer cells via either LPS or heat inactivated E. coli results in a TNF receptor-dependent increase in cancer cell adhesion. Although the data surrounding TNF-α and cancer metastasis are still difficult to reconcile with known biologic mechanisms, it remains a possibility that TLR4 induced induction of TNF-α could be implicated in TLR4 mediated cancer cell metastasis.

Given the previously discussed burden of lung cancer mortality due to post-operative recurrence, the potential clinical implications of this work are extremely significant. This study shows the potential utility of inhibiting the TLR4 pathway with the goal of diminishing NSCLC metastasis following post-operative infection. Although the role of TLR4 in cancer cell adhesion has already been described, cancer cell adhesion is one of many steps in the sequence of metastasis. Additionally, the timing of cancer cell adhesion in these cases is unclear – while it is probable that circulating tumor cells, whether already present or induced due to tumor manipulation, are present for some time following surgery, it is unclear whether by

the time a post-operative pneumonia develops, adhesion of circulating tumor cells will have already taken place.

Perhaps more importantly, radiologically undetectable micrometastases are often thought to be present at the time of surgery, and represent another mechanism for post-operative recurrence in metastatic sites. These data indicate that post-operative gram-negative sepsis could result in an increased rate of cell proliferation in these micrometastases, thereby shortening the time to recurrence and thereby the overall survival of the patient. While it is of course probable that these micrometastasis-harboring patients will recur at some point, increased time to recurrence would be an extremely meaningful outcome in these cases. The TLR4 inhibitor Eritoran has been utilized in a stage III randomized control trial of severe sepsis patients and did not augment mortality risk. This medication could be an exciting avenue for future clinical investigations aiming at decreasing recurrence.

Additionally, TLR2 function blocking antibodies are currently in stage I/II clinical trials. While the effect of TLR2 in gram-negative sepsis is likely very small, previous work from our group indicates that post-operative gram-positive infection may also influence this phenotype via similar mechanisms. Specifically, adhesion has been studied and has shown to be similarly increased in models of both gram-positive and gram-negative post-operative infection. While work on post-adhesive events has not been done using gram-positive organisms, the common inflammatory pathways induced by stimulation of these two toll-like receptors (namely, induction

of interleukin-6 and tumor necrosis factor alpha), gives weight to the hypothesis that similar post-adhesive mechanisms may be involved in gram-positive infection.

Overall, this suggests that anti-inflammatory therapies, in combination with concurrent standard medical treatments for gram-negative infection, could help reduce the cancer metastasis that is augmented by post-operative infectious complications.

Conclusion

Recurrence of non-small cell lung cancer after surgical resection with curative intent is a significant problem, both in terms of public health and with regards to the individual patient. Epidemiologically, it is clear that post-operative infection increases risk of early metastatic recurrence in NSCLC patients. At this time, no therapeutic options are available that target this phenomenon.

We have previously shown that the initiation of the innate immune response through the stimulation of Toll-like receptor 4 results in increased tumor cell adhesion, offering a partial explanation for this phenomenon in patients with circulating tumor cells. However, this mechanism does not account for the significant proportion of patients thought to have radiologically undetectable metastatic disease prior to surgery. These patients are at high risk for early recurrence, and it seems likely that post-operative infection could have deleterious effects with regards to recurrence rate on this population as well.

In this work, we show that stimulation of various TLRs expressed on tumor cells, as well as the stimulation of TLR4 in the host, results in increased replication of NSCLC tumor cells, both *in vitro* (in both two and three-dimensional models) and *in vivo*, in a highly physiologic model of gram-negative infection. Moreover, we show that this result is at least partially abrogated in TLR4 knockout mice, indicating a potential role for therapeutic blockade of TLR4 to inhibit this phenomenon. *In vitro,* the small molecule inhibitor Eritoran, which targets TLR4, shows significant potential in the

abrogation of the host response and resultant increased tumor cell proliferation, while blockade of TLR4, TLR2 and TLR5 all showed a potential effect in decreasing proliferation of tumor cells directly stimulated with heat inactivated E. Coli.

These results are biologically feasible in that the NF-κB pathway, which is initiated by Toll-like receptor activation, has been previously shown to have a role in cell mitosis and has been implicated in oncogenesis. In addition, the PI3Kinase cascade, which is one of the initiators of the NF-κB-mediated response to infection, has been targeted in the past for cancer-cell directed small molecule inhibitors of replication.

With regards to the role of the host response to infection, which is thought to be the major driver of the tumor cell microenvironment, both our *in vitro* and *in vivo* experiments have demonstrated that TLR4 activation may be one of the key components in driving increased tumor cell replication. Importantly, blockade of this pathway has already been investigated and has been shown to be safe in patients with gram-negative sepsis outside of the context of cancer. The small molecule inhibitor Eritoran has been trialed extensively in gram negative sepsis. While blockade of TLR4 has not been shown to be beneficial in the gram-negative sepsis population, these results indicate a potentially important role for TLR4 blockade as a therapeutic modality to decrease post-operative recurrence of NSCLC.

While these results are still preliminary, they represent an exciting avenue for further research directed at decreasing post-operative recurrence of NSCLC. More

work will be required to determine whether these results are able to translate into clinical benefit at the bedside.

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