Cytokine Networks In Esophageal Squamous Cell Carcinoma

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Cytokine Networks In Esophageal Squamous Cell Carcinoma

Abstract
Esophageal cancer is one of the top reasons for cancer-related deaths worldwide, and esophageal squamous cell carcinoma (ESCC) accounts for up to 90% of all esophageal cancer cases. One of the major reasons for the high mortality rate is the lack of targeted therapies for ESCC, particularly for advanced stage disease. The tumor microenvironment (TME) is a complex system of multiple cell types (fibroblasts, immune cells, blood and lymphatic vessels, etc.) that supports tumor growth in various ways. We aimed to investigate some of the mechanisms of dynamic interaction between tumor cells and TME components and to identify approaches to targeting these interactions therapeutically.

Desmoplasia, or deposition of connective tissue components (fibroblasts and extracellular matrix (ECM)), is a pronounced histological feature of ESCC. Previous research from the Rustgi lab and others has underscored the importance of cancer-associated fibroblasts (CAFs) and ECM components for ESCC progression. We have identified two cytokines that mediate these effects: IL-6 and RANTES. Through in vitro studies we have confirmed the relevance of these cytokines to human ESCC and determined that they function mostly through activation of STAT3 and ERK1/2 signaling pathways. We have also validated IL-6 and RANTES as novel targets for ESCC therapy by using tocilizumab (human IL-6R antibody) and maraviroc (CCR5 inhibitor) to inhibit the growth of subcutaneous ESCC xenotransplants in vivo.

Chronic inflammation and immunosuppressive microenvironment are also essential contributors to ESCC pathogenesis. We have identified the ectoenzyme-receptor CD38 as a driver of expansion of myeloid-derived suppressor cells in several murine models of ESCC. We have also identified six cytokines (IFNγ, TNFα, CXCL16, IGFBP-3, IL-6 and RANTES) as promoters of CD38 expression by these cells in vitro. To determine whether CD38 is a valuable target for therapy, we have utilized a neutralizing antibody to CD38 to suppress tumor growth in a syngeneic murine model of ESCC.

In summary, we have identified novel mechanisms of interaction between tumor cells, CAFs and myeloid-derived suppressor cells in esophageal TME. Furthermore, we have tested three independent in vivo approaches to therapeutic targeting of these mechanisms that can potentially improve the clinical outcomes of ESCC treatment.

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CYTOKINE NETWORKS IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Tatiana Karakasheva

A DISSERTATION

in

Cell and Molecular Biology

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ABSTRACT

CYTOKINE NETWORKS IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Tatiana Karakasheva
Anil K. Rustgi

Esophageal cancer is one of the top reasons for cancer-related deaths worldwide, and esophageal squamous cell carcinoma (ESCC) accounts for up to 90% of all esophageal cancer cases. One of the major reasons for the high mortality rate is the lack of targeted therapies for ESCC, particularly for advanced stage disease. The tumor microenvironment (TME) is a complex system of multiple cell types (fibroblasts, immune cells, blood and lymphatic vessels, etc.) that supports tumor growth in various ways. We aimed to investigate some of the mechanisms of dynamic interaction between tumor cells and TME components and to identify approaches to targeting these interactions therapeutically.

Desmoplasia, or deposition of connective tissue components (fibroblasts and extracellular matrix (ECM)), is a pronounced histological feature of ESCC. Previous research from the Rustgi lab and others has underscored the importance of cancer-associated fibroblasts (CAFs) and ECM components for ESCC progression. We have identified two cytokines that mediate these effects: IL-6 and RANTES. Through in vitro studies we have confirmed the relevance of these cytokines to human ESCC and determined that they function mostly through activation of STAT3 and ERK1/2 signaling pathways. We have also validated IL-6 and RANTES as novel targets for ESCC therapy by using tocilizumab (human IL-6R antibody) and maraviroc (CCR5 inhibitor) to inhibit the growth of subcutaneous ESCC xenotransplants in vivo.
Chronic inflammation and immunosuppressive microenvironment are also essential contributors to ESCC pathogenesis. We have identified the ectoenzyme-receptor CD38 as a driver of expansion of myeloid-derived suppressor cells in several murine models of ESCC. We have also identified six cytokines (IFNγ, TNFα, CXCL16, IGFBP-3, IL-6 and RANTES) as promoters of CD38 expression by these cells in vitro. To determine whether CD38 is a valuable target for therapy, we have utilized a neutralizing antibody to CD38 to suppress tumor growth in a syngeneic murine model of ESCC.

In summary, we have identified novel mechanisms of interaction between tumor cells, CAFs and myeloid-derived suppressor cells in esophageal TME. Furthermore, we have tested three independent in vivo approaches to therapeutic targeting of these mechanisms that can potentially improve the clinical outcomes of ESCC treatment.
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CHAPTER 1: Introduction
**Esophageal squamous cell carcinoma**

Esophageal cancer is a major cause of cancer-related death worldwide: 455,800 new cases were reported and 400,200 deaths occurred from this disease in 2012 (Torre et al., 2015). There are two major subtypes of esophageal cancer: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), and while the latter is more prevalent in North America and Europe, ESCC is the subtype that accounts for 90% of all esophageal cancers worldwide (Rustgi and El-Serag, 2014). According to the SEER database, 16,910 new cases (1% of all cancers) and 15,690 deaths (2.6% of all cancer-related deaths) are predicted to occur in the US in 2016 (Howlader N, et al., 2016).

Over the past three decades, there was a significant increase in overall median survival (from 6 months in 1970s to 10 months in 2000s) and 5-year survival rate (9% to 22%). However, these improvements can be attributed almost exclusively to advances in early detection: increased survival was seen mostly in patients with localized disease and to a lesser extent in patients with regional disease (Njei et al., 2016). The use of surgery and neoadjuvant chemoradiation therapy have also increased, implying that targeted use of these treatment approaches also contributed to improved survival. Importantly, when ESCC and EAC data were analyzed separately, it became clear that the increased survival has been achieved for EAC only. Therefore, despite rising incidence, these patients have better long-term survival prognosis than patients with ESCC (Njei et al., 2016). These data clearly indicate a need for better understanding of the biology of, and novel therapeutic options for, ESCC.
Though both types of cancer arise in the same organ, ESCC and EAC are in fact distinctly different diseases. In addition to different histology and long-term survival statistics described above, they even arise in different parts of the esophagus: ESCC occurs in the lower and mid-esophagus, while EAC is most common in the distal part (Napier et al., 2014). The risk factors for the two subtypes are also distinct: alcohol and tobacco use, particularly in synergy, are most strongly associated with ESCC, while gastroesophageal reflux disease (GERD), obesity (particularly the visceral fat) and alcohol consumption (less strongly than for ESCC) are the major risk factors for EAC (Rustgi and El-Serag, 2014). Finally, genetic risk factors differ as well between the two disease: mutations in the TP53, RB1, CDKN2A, PIK3CA, NOTCH1, NFE2L2, ADAM29 and FAM135B genes are most frequent in ESCC (Song et al., 2014), while TP53, CDKN2A, SMAD4, ARID1A, and PIK3CA mutations occur most often in EAC (Dulak et al., 2013).

The tumor microenvironment

Historically, investigation of cancer biology initially focused on the tumor cell: its physiology, genetic (and later epigenetic) profiles, and signaling pathways. The first generations of cancer therapeutics were developed based on this research. Yet, despite all the breakthrough discoveries in those areas, clinical oncology still faces major obstacles – from the toxicity induced by early chemotherapeutic agents to development of chemo- and radioresistance, resistance to diverse targeted therapeutics, and tumor metastases, all of which stand in the way of developing effective cures for cancer.

The concept of tumor microenvironment (TME) first gained attention in the late XIX century, when Stephen Paget developed his “seed and soil” theory. Dr. Paget’s
observation that “when a plant goes to seed, its seeds are carried in all directions, but they can only live and grow if they fall on congenial soil” (Paget, 1889) prompted the medical community to take a closer look at the “soil” that allowed the tumor to establish and progress. Sadly, this hypothesis and the exciting research that it invited laid dormant for almost a century, possibly due to the fact that Dr. Paget’s views contradicted the then predominant opinion, according to which the cancer cell, once carried by blood or lymph to a distant organ, can remodel that site to suit its needs for successful metastasis.

Only in the 1970s was the “seed and soil” hypothesis revisited, this time gaining the new appreciation (Witz, 2009). In his seminal editorial, Robert Auerbach wrote that “In a time where the primary emphasis in cancer research is at the level of gene expression, of regulation and of epigenetic modification of the "seed," those individuals..., who study the properties of the host environment should not be ignored. Not only are the observations of the 'soil' useful, they provide essential information without which we will not be able to understand the nature of the metastatic process” (Auerbach, 1988). Since then, a plethora of scientific reports have been published that have helped to shape our understanding of the nature of the TME into what it currently is: a complex system consisting of multiple cellular components (fibroblasts, multiple immune cell subpopulations, microvasculature represented by endotheliocytes and pericytes, adipocytes) and the extracellular matrix (ECM), which is a complex system of biopolymers (polysaccharides such as proteoglycans and hyaluronic acid, collagen and elastin fibers, fibronectins), matricellular proteins (non-structural proteins that can modify the ECM, such as osteopontin, periostin and SPARC) and various growth factors and cytokines (EGF, HGF, VEGF, TGFβ) embedded in this matrix. The “official status” of the
TME in the field of cancer research has been sealed, when its components have been incorporated into the famous concept of “hallmarks of cancer” to underscore that “Most of the hallmarks of cancer are enabled and sustained to varying degrees through contributions from repertoires of stromal cell types and distinctive subcell types” (Hanahan and Coussens, 2012).

In the early 1990s it became increasingly clear that a deeper understanding of the TME’s dynamic nature and role in cancer progression was needed. For example, in his keynote address to the attendees of the Third International Conference on the Interaction of Radiation Therapy and Systemic Therapy in 1990, Ralph Durand stated that “that the cellular microenvironment can play a major role in modifying the activity of radiation and chemotherapy”, and he proceeded to suggest “some potential for new treatment strategies designed specifically to exploit the tumor microenvironment” (Durand, 1991). Later research proved Dr. Durand right: for example, increasing evidence exists of chronic inflammation promoting chemoresistance via activation of signaling pathways, most notably STAT3, in the tumor cells (Lee et al., 2014a; Spitzner et al., 2014; Wörmann et al., 2016), while the cancer-associated fibroblasts (CAFs) are also well known to promote resistance to therapy via multiple mechanisms, including secreted factors (Meads et al., 2009), exosomes (Hu et al., 2015) and modifying cancer cell metabolic pathways (Pavlides et al., 2009; Ying et al., 2012). Furthermore, CAFs can affect the delivery of therapeutics to the tumor by affecting angiogenesis (Goel et al., 2012) or the biophysical characteristics of the ECM (Provenzano et al., 2012).

One of the most fascinating features of the TME is its plasticity: unlike the cancer cells, which acquire a whole portfolio of mutations as they evolve, the TME components
generally do not acquire mutations (Lee et al., 2014b; Witz, 2008). This implies that it may be possible to educate the TME and de-differentiate its pro-tumorigenic components back into their normal, anti-tumorigenic state.

**The immune milieu in tumor microenvironment**

The immune part of the TME comprises the cells belonging to the innate immune system (macrophages, neutrophils, dendritic cells, mast cells and myeloid derived suppressor cells), as well as effectors of the adaptive immunity (T and B lymphocytes).

Tumor associated macrophages (TAMs) are possibly the most common immune cell subtype in the TME. Macrophages represent the terminal differentiation state of a monocyte, which becomes adapted to residence within a certain organ. As is often the case with immune cells, macrophages are a very heterogeneous group of cells, and multiple classification schemes exist. The traditional approach is to subdivide the macrophages into M1 and M2 subtypes. M1, or classically activated, macrophages are generally induced by IFNγ and secrete high levels of pro-inflammatory cytokines (e.g. TNFα, IL-6, IL-1, IL-12). By contrast, M2, or alternatively activated, macrophages can be induced by IL-4, IL-10 or IL-13 secrete low levels of IL-12 and high levels of the anti-inflammatory cytokine IL-10 (Grivennikov et al., 2010). However, both M2 and M1 macrophages remain fairly plastic. Most of the TAMs are M2, and can be further subcategorized into angiogenic, immunosuppressive, invasive, metastasis-associated, perivascular, and activated macrophages (Komohara et al., 2016). TAMs have been linked to numerous pro-tumorigenic processes, such as maintenance of chronic inflammation in the TME, immunosuppression (direct or via activation of MDSCs), angiogenesis, tumor cell invasion and metastasis, while in general, the presence of
TAMs in the tumor correlates with poor prognosis (Grivennikov et al., 2010; Komohara et al., 2016). The classically activated M1 macrophages and dendritic cells, however, are essential for antigen presentation and CTL activation, which are the cornerstone of anti-tumor immunity.

Historically, tumor-associated neutrophils (TANs) were considered to be a pro-tumorigenic cell type (Fridlender and Albelda, 2012), and a number of studies demonstrated a correlation between the presence of TANs and poor prognosis in patients with melanoma, renal cancer, head and neck cancer and other cancer types (Swierczak et al., 2015). However, recent studies have shed some light on the temporal relationship between the tumor stage and the anti- or pro-tumorigenic capacity of neutrophils. For example, in early stage human lung cancer TANs are able to activate CTLs, but gradually lose this capacity at later stages (Eruslanov et al., 2014).

Myeloid-derived suppressor cells (MDSCs) originate from alternative differentiation of normal immature myeloid progenitors under the influence of tumor-derived factors. These are a very heterogeneous group of myeloid cells that are united in their ability to suppress immune responses. MDSCs can be sub-divided further into the monocytic (M-MDSC) and perimorphonuclear (PMN-MDSCs, or granulocytic MDSCs) subsets based on their morphology and phenotype (Bronte et al., 2016). M- and PMN-MDSCs employ different mechanisms for immunosuppression, and recent data suggest that each subset’s immunosuppressive capacity also varies based on the location. For example, PMN-MDSCs are the predominant subtype in peripheral lymphoid organs, where they promote the development of tumor antigen-specific T cell tolerance. At the same time, M-MDSCs tend to reside within the tumor and not only exert potent
immunosuppression (antigen-independent) function, but also rapidly differentiate into TAMs (Kumar et al., 2016). In general, the direct association between the presence of MDSCs and poor prognosis in cancer is well appreciated (Gabitass et al., 2011), and the specifics of MDSCs in esophageal cancer will be addressed in detail in Chapter 3 of this thesis.

T lymphocytes are also commonly found within the tumor, but unlike TAMs, different T cell subtypes bear either pro- or anti-tumorigenic properties. For example, high numbers of the CD8$^+$ cytotoxic T lymphocytes (CTLs) or T helper 1 (Th1) cells can correlate with increased survival (Mao et al., 2016). At the same time, another T cell subset known as regulatory T cells (Treg) has widely appreciated pro-tumorigenic properties. Tregs are a subtype of CD4$^+$ cells that express the transcription factor FoxP3, and their most investigated role in cancer is immunosuppression, which is mediated by the cytokines IL-10, IL-35 and TGF-β (Grivennikov et al., 2010). Multiple reports demonstrate a strong association between Treg infiltration and poor prognosis in cancer patients (Chen et al., 2016; Mao et al., 2016).

The infiltration of B lymphocytes into tumor tissue is in general associated with better prognosis, which can be mediated by more efficient antibody production or antigen presentation (interestingly, B cells can be recruited to the tumor in response to IFNγ production and tend to cluster with the CD8$^+$ CTLs) (Guy et al., 2016; Mao et al., 2016). Yet, similarly to T cells, a subset of B lymphocytes with immunoregulatory capacity (Breg) has also been identified in murine models of cancer. Bregs are activated by TGFβ and express the transcription factor FoxP3 (DiLillo et al., 2010). In human
cancer, these cells are also associated with PD-1 mediated immunosuppression (Guan et al., 2016).

**Cytokines and pro-inflammatory signaling in cancer**

As soluble factors that exist to mediate interactions between cells, the role of various cytokines in cancer is well appreciated. In general, these factors act through activation of numerous downstream effector pathways, such as STAT1/3, NF-κB, Smad and apoptotic signaling. Most of the cell types present in the TME produce cytokines, which can be broadly categorized into anti-tumor (IL-12, IFNγ, TRAIL), pro-tumorigenic (IL-6, IL-23, IL-10, IL-17), and cytokines with direct roles on cancer cells (TGF-β, TNFα, IL-6, FasL). An overview of a few cytokines that have been most extensively studied in carcinogenesis will be provided below.

Transforming growth factor-beta (TGF-β), which normally plays an essential role in development of the tissues of epithelial and neural origin, is also a major tumor-promoting cytokine. TGF-β is a most prominent regulator of angiogenesis, immunosuppression, epithelial-to mesenchymal transition (EMT), ECM remodeling, leading to tumor cell invasion and metastasis. The signaling events initiated by TGF-β include activation of the Smad2/3, p38, JNK, ERK and Akt/mTOR pathways, all of which contribute to tumorigenesis. Clinically, expression of TGF-β in the tumor correlates with poor differentiation, advanced stage, metastases and poor survival (Lin and Zhao, 2015).

Interleukin-1 beta (IL-1β), a major regulator of the acute phase response, is secreted in response to activation of the inflammasome pathway. IL-1β signaling results in production of IL-1R, GM-CSF, G-CSF, TNFα, IL-6, IL-6, cortisol and nitric oxide, which
is accompanied by fever, muscle and joint pain, fatigue, hypotension, neutrophilia and thrombocytosis (Kasza, 2013). IL-1β plays a central role in cross talk between multiple sell types in the TME. For example, it promotes tumor cell invasion by activation of expression of several matrix metalloproteases (MMP-1,3,10 and 14). Furthermore, high levels of IL-1β in serum or tumor tissue correlates with advanced tumor stage in multiple tumor types (Whipple, 2015).

Interleukin 6 (IL-6), also known as “the truly pleiotropic cytokine” due to its numerous roles in the immune system, such as promoting activation and proliferation of T cells, differentiation of B cells, regulation of acute-phase response, as well as hormonal-like ability to regulate vasculogenesis, lipid metabolism, insulin resistance and neuropsychological processes (Hunter and Jones, 2015). The importance of IL-6 in tumor progression via regulation of such essential signaling pathways as STAT3, NF-κB and Wnt has been the subject of researchers’ interest for a while (Jarnicki et al., 2010), yet the detailed mechanisms beyond these events are not always understood. A substantial part of Chapters 2 and 3 of this thesis are dedicated to elucidating some of these mechanisms.

Chemokines as a class were originally defined as cytokines which regulate directional migration of immune cells. However, over time, it became clear that not only can chemokines direct migration of non-immune cells, but that their function is not limited to promoting chemotaxis. A good example is interleukin 8 (IL-8), also known as the CXC chemokine ligand 8 (CXCL8), or “the first chemokine” (Baggiolini et al., 1989). It is a classical chemoattractant for neutrophils, yet it can also activate downstream signaling pathways like p38-MAPK or Erk-MAPK, and due to this outcome IL-8 is
considered a pro-tumorigenic cytokine (Waugh and Wilson, 2008). Among other chemokines implicated in tumorigenesis are GROα/CXCL1, GROβ/CXCL2, GROγ/CXCL3, which affect cell proliferation and tumorigenicity in melanoma, ENA-75/CXCL5, which regulates tumor growth, apoptosis and angiogenesis in melanoma, and macrophage pro-inflammatory chemokine-3α (MIP-3α/CCL20), which promotes tumor cell proliferation in pancreatic cancer (Coussens and Werb, 2002). RANTES/CCL5, another chemokine with pro-tumorigenic properties (Aldinucci and Colombatti, 2014), is a major focus of research described in Chapter 2 of this thesis.

The tumor microenvironment of esophageal squamous cell carcinoma

The pathogenesis of ESCC is tightly linked to chronic inflammation. In fact, serum levels of acute phase response mediators C-reactive protein (CRP) and IL-6 correlate with the stage, depth of invasion and lymph node dissemination in ESCC (Groblewska et al., 2012). Furthermore, positive IL-6 staining of the tumor sections has been linked to distant metastases and poor treatment response in a study of 20 ESCC patients (Chen et al., 2013). In agreement with these findings, a novel inflammation-based prognostic score (based on the CRP/Alb ratio) has been proposed to predict overall survival in a retrospective study of 423 ESCC patients (Wei et al., 2015).

So far, only one study has attempted a more comprehensive analysis of the TME in tumors of 55 ESCC patients (Liu et al., 2012). In addition to overall high proliferative indices (based on Ki-67 staining), Dr. Liu and colleagues observed expansion of myofibroblasts (interestingly, the density of these cells correlated with dissemination to lymph nodes), increased microvessel density (quantified by CD34 staining), high levels of pro-angiogenic factors VEGF and IL-8, as well as notable changes in the distribution
of immune cells throughout the tumors. The majority of T cells (CD3+) and macrophages (CD68+) were localized in the tumor stroma in the periphery and did not penetrate efficiently into the tumor epithelium. These findings imply the existence of an immunosuppressive milieu in these tumors.

**CAFs in ESCC**

One could argue that the cancer-associated fibroblasts represent the best-studied TME compartment in ESCC (Fig. 1), at the level of both clinical prognostic associations and basic biology. For example, Dr. Ha and colleagues have assessed the histopathological profile of CAFs in tissue samples from 116 ESCC patients and found that the expansion of CAFs correlated with poor prognosis, increased microvessel density and epithelial-to mesenchymal transition. Interestingly, expression patterns of the CAF markers used in this study were quite heterogeneous, and only αSMA, FSP1, and PDGFRα could serve as unfavorable prognostic factors for ESCC, while FAP and PDGFRβ offered no significance (Ha et al., 2014). Another study of 97 patients investigated the association between expression of the most commonly used CAF marker (αSMA) and clinico-pathological features of ESCC: positive αSMA staining in the tumor stroma correlated with venous invasion, lymph node involvement, tumor recurrence and predicted poor survival in patients with stage I and II ESCC (Saito et al., 2014). Finally, a study of 95 patients who had undergone esophagectomy for ESCC found that the abundance of CAFs in the original tumor correlated with a low 3-year overall survival rate (Cheng et al., 2015).

A number of studies, including three publications from our lab, have identified mechanisms that contribute to the tumor-promoting properties of CAFs in ESCC. For
example, CAF-derived hepatocyte growth factor has been proved essential for invasive properties of ESCC cells in 3D culture via activation of the Met receptor (Grugan et al., 2010) (Fig. 1B). Direct contact with collagen I fibers (deposited by fibroblasts) stimulates production of the lysosomal enzyme cathepsin B in tumor cells, which, in addition to directly modifying the ECM, also leads to enhanced secretion of TGFβ1 (Andl et al., 2010) (Fig. 1A,B). Another mechanism by which CAFs promote invasion of ESCC cells into the ECM is by promoting expression of the matricellular protein periostin (in an EGFR- and mutant p53-dependent matter), which can only be detected in the invading tumor cells in vitro and in ESCC patient samples (Michaylira et al., 2010). The work described in Chapter 2 of this thesis is aimed to deepen our understanding of the ways in which the CAFs promote ESCC progression.

**Immune microenvironment in ESCC**

Since chronic inflammation plays such a major role in ESCC biology, many groups have focused their studies on the role of immune cells components in this disease. Existing evidence of the role of several such populations (MDSC, Treg, Th17 and TAM) will be summarized in this section (Fig. 2).

The myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myelocytes with one shared property: their ability to suppress activation of both innate and antigen-specific immune response. Chapter 3 of this thesis is dedicated to investigating the detailed mechanisms driving expansion of and acquisition of immunosuppressive capacity by MDSCs in a murine model of ESCC (Karakasheva et al., 2015). In addition to this work, another publication reports on the role of MDSCs in a chemical carcinogenesis-induced model of ESCC, and these data are accompanied by a
survey of an MDSC-like cell population in peripheral blood of ESCC patients, where the presence of these cells was associated with advanced disease, poor prognosis and resistance to therapy (Chen et al., 2014).

The involvement of tumor-associated macrophages (TAMs) has been well appreciated in many types of cancer, but in ESCC this cell subset has not been extensively studied. There are, however, some promising data: secretion of MCP-1 by ESCC cells results in TAM infiltration and production of pro-angiogenic factors, while the presence of TAMs is also associated with poor response to therapy and poor prognosis in ESCC (Shigeoka et al., 2013). Two subsets of T cells – Treg and Th17 – also contribute to formation of an immunosuppressive microenvironment in ESCC (Fig. 2). Tumor cell-derived chemokines CCL17 and CCL22 appear to be responsible for recruitment and expansion of Tregs in this disease. The presence of Tregs also has prognostic significance. Treg infiltration is associated with ESCC invasion, metastases, disease severity and survival post-chemotherapy. Furthermore, in patients receiving neoadjuvant chemoradiotherapy, retention of Tregs in the tumor is associated with poor treatment response and survival (Lin et al., 2016). Some reports demonstrate the relevance of the Th17 subset of T helper cells in ESCC: increased presence of these cells is detected in both peripheral blood and tumor tissue, and the degree of expansion correlates with disease stage (Lin et al., 2016). It is important to keep in mind, however, that the role of Th17 cells in cancer is still a subject to debate and more studies are needed to clarify this issue.
Pro-inflammatory signaling in ESCC

The signal transducer and activator of transcription 3 (STAT3) is among the most-investigated signaling pathways that regulate inflammation in cancer, and ESCC is not an exception: several studies, as well as our own work described in Chapter 2, have reported elevated levels activated STAT3, as well as its major inducer IL-6 and IL-6Rα (Leu et al., 2003; Yan et al., 2008). Furthermore, STAT3 activation correlates with poor prognosis in ESCC patients who have undergone esophagectomy for ESCC (Li et al., 2014). Mechanistically, in vitro studies demonstrated that STAT3 signaling promotes production of anti-apoptotic factors (Leu et al., 2003), while small molecule inhibitor static restores radiosensitivity to ESCC cells in vivo (Zhang et al., 2014a).

The nuclear factor kappa B (NF-κB) pathway is another example of a cancer cell hijacking a normal immune signaling pathway to induce an aberrant state of chronic inflammation. Our lab has previously reported overexpression of the p65 subunit of NF-κB in a genetic model of ESCC (Stairs et al., 2011). Overexpression of NF-κB have also been associated with poor prognosis in patients with ESCC (Hatata et al., 2012). IL-8, one of the major upstream regulators of NF-κB signaling, is also associated with disease progression, inflammation, metastases and poor prognosis in ESCC patients (Ogura et al., 2013). Interestingly, CCL5 (also known as RANTES), which is one of the main players in the mechanism described in Chapter 2, is a downstream target of the NF-κB pathway (Aldinucci and Colombatti, 2014).

It is important to note the existence of a complex interdependent cross-talk between the STAT3 and NF-κB signaling (Jarnicki et al., 2010). One of the most prominent downstream targets shared by the two pathways is the gene encoding
cyclooxygenase-2 (COX-2). Interestingly, a positive correlation exists between COX-2 expression and the degree of dysplasia in esophageal tissue (Zhi et al., 2006), while intense COX-2 staining is associated with poor prognosis and resistance to chemotherapy in patients with ESCC (Akutsu et al., 2011). Mechanistically, COX-2 inhibition leads to decreased proliferation of and lower prostaglandin E2 production by ESCC cells in vitro, as well as to decreased tumor progression in vivo (Zhi et al., 2006).

Finally, the programmed cell death protein-1 (PD-1) pathway, an increasingly popular target for cancer therapy, has also proven to be important in esophageal carcinogenesis (Fig. 2). Indeed, elevated expression of the pathway ligands PD-L1 and PD-L2 in cancer cells correlates with decreased survival in ESCC patients, while increased PD-L1 expression is associated with greater depth of tumor invasion and poor survival (Lin et al., 2016).
Figures and figure legends
**Figure 1-1: Stromal compartment of the esophageal TME.** (a) Neoplastic cells secrete growth factors to activate quiescent fibroblasts designated as CAFs. CAFs can proliferate to contribute to desmoplasia, secreting ECM components such as fibronectin (FN) to enhance the development of the primary tumor niche. CAFs also secrete cytokines that promote tumor cell survival (anti-apoptosis). (b) Later in tumorigenesis, CAFs remodel the ECM with enzymes like lysyl oxidase (LOX) and MMPs as well as ECM components like dermatan sulfate (DS) and hyaluronan (HA) to promote invasion. CAFs also secrete growth factors that trigger tumor cells to undergo EMT and chemokines that induce tumor cell migration. CAFs can also promote angiogenesis via VEGF secretion. (Modified from Lin E., Karakasheva T., et al., Oncogene 2016).
Figure 1-2: Immune landscape in esophageal cancer. Several immune cell types disrupt antitumor immunity (cytotoxic CD8+ T cells) in the TME. Tregs exert immunosuppressive function via direct contact with effector T cells or by molecules such as adenosine or immunosuppressive cytokines (IL-10 and IL-35). Th17 release adenosine by ectoenzymatic (CD39, CD73) function and have the ability to convert into Tregs (dashed line). MDSCs directly inhibit T-cell activation and NK cell cytotoxicity, while also inducing Tregs. TAMs and tumor cells both express PD-L1/2 to inhibit T-cell activation via the PD-1 receptor. Altogether, these cells suppress antitumor immunity while also promoting tumor growth and progression by various mechanisms. (Modified from Lin E., Karakasheva T., et al., Oncogene 2016).
CHAPTER 2: The impact of interaction between tumor cells and cancer-associated fibroblasts in esophageal squamous cell carcinoma
Abstract

Esophageal cancer is a leading cause of cancer-related death worldwide. The prognosis for these patients remains poor, and targeted therapies are largely absent. Chronic inflammation and the reactive stroma are among the driving forces for esophageal cancer initiation, progression and dissemination. We have identified the cytokines IL-6 and RANTES as prominent mediators of this cross-talk in esophageal carcinogenesis. Loss of IL-6 or RANTES potently suppresses tumorigenesis in 3D models (organotypic and organoid) of esophageal cancer, as well as in subcutaneous xenograft transplant tumors. Furthermore, we found that inhibiting IL-6 or RANTES signaling (with tocilizumab, a neutralizing antibody to IL-6 receptor (IL-6R), or maraviroc, a small molecule CCR5 inhibitor, respectively), suppresses tumor growth in vivo via inhibition of STAT3 and ERK1/2 signaling pathways. We also demonstrate that cotreatment with tocilizumab and maraviroc is more effective than either agent alone in vitro in 3D organotypic culture model of ESCC, providing a rationale for testing combination therapy in vivo. We propose inhibition of IL-6 and RANTES signaling as a novel approach to treatment of esophageal cancer, as well as potentially other types of cancer, particularly squamous cell carcinomas.
Introduction

Esophageal cancer is the 8th most common cancer type and the 6th leading cause of cancer-related death worldwide (Napier et al., 2014). The 5-year survival rate for esophageal cancer patients is estimated to be 15-20%; furthermore, rather than declining, the incidence of esophageal cancer is expected to increase by 140% in the next 10 years (Napier et al., 2014). There are two subtypes of esophageal cancer – esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), which are characterized by distinct histology, location and pathogenesis. ESCC represents up to 90% of all esophageal cases worldwide, but EAC is the prevalent type in the United States and Europe (Rustgi and El-Serag, 2014). The poor prognosis and increasing incidence and mortality indicate a need for improved understanding of mechanisms underlying esophageal cancer initiation and progression. Investigating the interaction between the tumor cells and surrounding microenvironment presents an opportunity to address some of these issues in a thoughtful and targeted way.

Cancer-associated fibroblasts (CAFs) are among the most prominent cell types in the tumor microenvironment. CAFs have been reported to predict outcome (Ha et al., 2014; Saito et al., 2014) and promote tumorigenesis (Zhang et al., 2009) in esophageal cancer. Previous reports from our lab have demonstrated that CAFs mediate ESCC cell invasion via secretion of hepatocellular growth factor (HGF) (Grugan et al., 2010) or cathepsin B (Andl et al., 2010). Furthermore, certain ECM components, secreted by CAFs, such as periostin, dermatan sulfate and fibronectin, are implicated in ESCC progression (Michaylira et al., 2010; Thelin et al., 2012; Wong et al., 2013; Zhang et al., 2005). It is therefore believed that therapeutically disrupting the cross-talk between
CAFs and tumor cells would be beneficial not only in esophageal cancer, but other types of cancer as well.

Interleukin 6: a cytokine involved in the tumor microenvironment

Interleukin 6 (IL-6) is a pleiotropic cytokine, widely appreciated as a major regulator of the acute phase response, yet having numerous functions within the immune system, lipid metabolism, insulin resistance, mitochondrial function and the neuroendocrine system (Hunter and Jones, 2015). IL-6 signaling is mediated by a heterodimeric receptor complex comprised of the ligand-binding subunit (IL-6Rα, or CD126; (Yamasaki et al., 1988)) and the signal-transducing subunit (gp130, or CD130; (Hibi et al., 1990)). The best appreciated signaling event induced by IL-6 is the signal transducer and activator of transcription 3 (STAT3) pathway, which has well characterized functions in promoting tumor progression (Guo et al., 2012). However, recent studies have demonstrated that IL-6 can activate additional signaling pathways, including MEK/ERK and PI3K/AKT (Costa-Pereira, 2014; Guo et al., 2012).

Increased secretion of IL-6 has been detected in multiple epithelial cancers, including, but not limited to, head and neck squamous cell carcinoma (HNSCC), lung cancer, colorectal cancer, gastric cancer, breast cancer, ovarian carcinoma, and other cancers (Guo et al., 2012). In colorectal cancer patients, expression of IL-6 is associated with tumor stage, survival and metastasis, while colorectal cancer cell lines respond to IL-6 with stimulated growth in vitro (Waldner et al., 2012). In a chemical carcinogen-induced model of gastric adenocarcinoma loss of IL-6 was associated with a dramatic
decrease in tumor incidence and reduced tumor number and size, as well as decreased levels of activated STAT3 (Kinoshita et al., 2013).

In ESCC, IL-6 is known to suppress apoptosis (Leu et al., 2003) and cisplatin-induced cytotoxicity (Suchi et al., 2011) in vitro. Furthermore, inhibition of STAT3 promotes radiosensitivity in a subcutaneous xenograft ESCC model in vivo, while in ESCC patients positive IL-6 staining in the tumor is associated with poor prognosis (Chen et al., 2013). Serum levels of IL-6 in esophageal cancer patients correlate with tumor stage, depth of tumor invasion and dissemination to lymph nodes (Groblewska et al., 2012), while presence of soluble IL-6R in the serum is a predictor of poor response to neoadjuvant therapy in patients with ESCC (Makuuchi et al., 2013). In ESCC, IL-6 has been reported to activate STAT3, ERK1/2 and PI3K signaling in vitro, yet the latter pathway was not essential for the antiapoptotic activity of IL-6 (Leu et al., 2003).

Reagents for therapeutic targeting of IL-6 signaling were initially developed for autoimmune diseases. Siltuximab, an IL-6 neutralizing antibody has been approved by the FDA for treatment of Castleman’s disease (Deissher et al., 2015), while tocilizumab, an antibody to IL-6Rα, has been approved by the FDA for treatment of rheumatoid arthritis (Thompson, 2010). Encouraged by favorable preclinical data (Goumas et al., 2015), a number of clinical studies was initiated to investigate the use of siltuximab in cancer, yet, while the therapy was generally well tolerated, no clinical efficacy was detected (Angevin et al., 2014; Rossi et al., 2015). These results have been attributed, in part, to the ability of tumor cells to increase IL-6 production, thus trapping the neutralizing antibody in circulation and preventing its access to the tumor (Hunter
and Jones, 2015). By contrast, neutralization of the IL-6 receptor has the potential to overcome this barrier.

Treatment with tocilizumab resulted in suppressed tumor growth rate, accompanied by reduced STAT3 phosphorylation, in a murine model of oral cancer, the predominant subtype of HNSCC (Shinriki et al., 2009). Furthermore, tocilizumab has been proven to be effective in pre-clinical studies of breast cancer, where its administration resulted not only in stalled tumor growth, but also reduced lung metastases and depletion of CD11b^Gr-1^ myeloid-derived suppressor cells (Chang et al., 2013). A large number of clinical trials for the use of tocilizumab in cancer are ongoing (Table 1), and several novel agents are currently in pre-clinical development (Hunter and Jones, 2015).

**RANTES: a chemokine involved in the tumor microenvironment**

RANTES (regulated on activation, normal T-cell expressed and secreted), also known as CCL5, is a chemokine from the C-C chemokine ligand family. It is produced by activated T cells (but not B cells), platelets, monocytes, synovial fibroblasts and certain types of epithelia (Appay and Rowland-Jones, 2001). Production of RANTES is induced by NF-κB and JNK signaling (Chenoweth et al., 2012). Additionally, acetylated Snail, as well as AP-1 are viewed as transcriptional activators of RANTES, while CREB may be its transcriptional repressor (Hsu et al., 2014; Lv et al., 2013). In addition, adipocytes in the microenvironment and cellular ER stress may stimulate secretion of RANTES (D’Esposito et al., 2016; Zhang et al., 2014b).
RANTES is a potent chemoattractant for eosinophils, monocytes, T cells and basophils (Kameyoshi et al., 1992; Kuna et al., 1992). However, it has other functions, such as promotion of proliferation, invasion and angiogenesis (Aldinucci and Colombatti, 2014). RANTES acts through a number of G-protein-coupled receptors (GPCRs): CCR1 (Neote et al., 1993), CCR3 (Ben-Baruch et al., 1995), CCR5 (Raport et al., 1996) and auxiliary receptors CCR4 (Udi et al., 2013) and CD44 (Roscic-Mrkic et al., 2003). Interestingly, these receptors can be activated by other chemokines, such as MIP-1α (CCR1), eotaxin (CCR3) and MIP-1β (CCR5). Among these GPCRs, CCR5 is by far the best-studied, due to its function as a co-entry receptor for the human immunodeficiency virus (HIV) (Deng et al., 1997).

RANTES expression has been associated with several types of cancers. For example, its levels are elevated in both plasma and tumor tissue of patients with breast and cervical cancers (Niwa et al., 2001). Furthermore, patients with gastric adenocarcinoma have significantly higher levels of RANTES in serum, which are associated with reduced histological differentiation, greater degree of tumor invasion, advanced tumor stage and reduced overall survival (Sima et al., 2014).

Certain functions of RANTES in cancer are related to its activity in the immunosuppressive cell populations. For example, it contributes to recruitment, alternative differentiation and immunosuppressive capacity of myeloid-derived suppressor cells in a murine model of triple-negative breast cancer, as well as in an ex vivo differentiation assay of human patient-derived CD33+ suppressor cells (Zhang et al., 2013). The binding of RANTES to CCR5 leads to phosphorylation of the p38 MAP kinase, while activation of JAK2/3 and subsequent phosphorylation of STAT1/3 (Wong et
al., 2001). Furthermore, RANTES-CCR5 signaling drives homing of regulatory T cells to the tumor and enhances their suppressive capacity in murine models of pancreatic and colorectal cancers (Chang et al., 2012; Tan et al., 2009).

RANTES is essential in mediating the cross-talk between tumor cells and surrounding stromal cells. Treatment of human ovarian cancer cells with cisplatin induces secretion of RANTES by CAFs, which activates the STAT3 and PI3K/AKT signaling pathways in tumor cells, thereby leading to resistance to cisplatin (Zhou et al., 2016). Furthermore, RANTES (along with IL-6) promotes mesenchymal stem cell-induced migration of human breast cancer cells in vitro (Gallo et al., 2012).

Tumor cell-autonomous functions of RANTES have been reported. RANTES-CCR5-induced STAT3 signaling promotes tamoxifen resistance in human breast cancer cells in vitro (Yi et al., 2013). In human lung cancer cells, CCR5 ligation by RANTES promotes migration via activation of the PI3K/AKT and NF-κB signaling pathways (Huang et al., 2009).

Historically, investigation of the pro-tumorigenic functions of RANTES has been focused on its ability to activate CCR5, yet, it is becoming apparent that other GPCRs are quite important as well. Importantly, pharmacological inhibition of CCR5 with TAK-779, while able to suppress the growth of primary tumors, failed to prevent peritoneal dissemination of colorectal cancer cells in vivo, compared to treatment with a RANTES-neutralizing antibody (Cambien et al., 2011). Accordingly, RANTES-CCR1 interaction has been reported to promote invasion of taxane-resistant prostate cancer cells via ERK1/2 and Rac signaling (Kato et al., 2013), while NF-κB signaling induced by
autocrine activation of CCR1 and CCR3 by RANTES drives metastatic dissemination of ovarian cancer cells (Long et al., 2012).

There have been a number of studies to evaluate inhibition of RANTES as an approach to cancer therapy. Several small molecule inhibitors have been developed to target CCR5: maraviroc (Wood and Armour, 2005), vicriviroc (Strizki et al., 2005), TAK-779 (Baba et al., 1999) and anibamine (Zhang et al., 2012). Maraviroc has been approved by the FDA and successfully used for HIV therapy (Van Der Ryst, 2015), resulting in its potential application to target RANTES in cancer. For example, treatment with maraviroc prevents development of tumors in a murine model of hepatocellular carcinoma (Ochoa-Callejero et al., 2013), inhibits growth of orthotopically transplanted colon cancer cells (Tanabe et al., 2016), reduces the capacity of gastric cancer cells to disseminate (Mencarelli et al., 2013) and prevents metastasis of basal breast cancer cells in vivo (Velasco-Velázquez et al., 2012). Finally, a pilot clinical study has been published where maraviroc was used in 11 patients with advanced refractory colorectal cancer. This resulted in central tumor necrosis and a partial remission of lung metastases. A subgroup of patients received maraviroc in combination with chemotherapy, leading to a partial response in 3/5 (60%) cases (Halama et al., 2016).

Other GPCRs may significantly contribute to the pro-tumorigenic effects of RANTES (Cambien et al., 2011). Therefore, the capture of the ligand with a neutralizing antibody or a combination of multiple small molecule inhibitors may be beneficial. A selective CCR1 inhibitor J113863 (Pevida et al., 2014) and its trans-isomer UCB35625 (Sabroe et al., 2000) have been developed and tested in vivo, but no reports of their utility as clinical therapeutic agents are available thus far. In addition to maraviroc,
mogamulizumab, a neutralizing antibody to human CCR4, is being actively studied in the clinic as a novel agent for therapy of hematologic malignancies (Table 1) since CCR4 is one of the receptors utilized by RANTES, mogamulizumab could be tested in solid malignancies as well.

Of importance to our study, there is evidence for cooperation between IL-6 and RANTES in several contexts, including cancer. For instance, RANTES/CCR5 signaling promotes IL-6 secretion by human synovial fibroblasts via the PKCδ/c-Src/c-Jun and AP-1 signaling pathways in a model of osteoarthritis (Tang et al., 2010). In combination with IL-6, RANTES can stimulate proliferation of prostate cancer cells in vitro via ERK1/2 signaling (Colombatti et al., 2009). Furthermore, potent tumor-promoting signaling circuits converging on RANTES and IL-6 in murine models of KRAS-driven lung cancer (Zhu et al., 2014) and IKBKE-driven triple-negative breast cancer (Barbie et al., 2014) have been reported. Interestingly, there may be ways to therapeutically suppress the production of pro-tumorigenic cytokines: zoledronic acid can impair the secretion of RANTES and IL-6 in mesenchymal stem cells, thus blocking their interaction with breast cancer cells (Gallo et al., 2012).

In this study, we use 3D culture approaches and murine xenotransplant models to demonstrate a role for tumor cell-derived IL-6 and RANTES in mediating the interaction between tumor cells and CAFs in esophageal squamous cell carcinoma. We define STAT3 and ERK1/2 signaling pathways as the likely predominant downstream mediators of the two cytokines in ESCC. Furthermore, we confirm that IL-6 and RANTES are novel and promising targets for ESCC therapy that can be targeted by tocilizumab (a neutralizing antibody to human IL-6Rα), maraviroc (small molecule CCR5 antagonist), or
a combination of both. Given the existing evidence of involvement of IL-6 and RANTES in breast, lungs and gastric cancer progression, we propose that this treatment strategy can be expanded to other cancer types.
Results

Interaction between tumor cells and CAFs induces changes in cell biology

Desmoplasia is one of the key characteristics of ESCC (Lin et al., 2016), which prompted us to investigate the consequences of interaction between the tumor cells and CAFs. First, we have evaluated the changes in gene expression profiles in TE11 cells grown in mono-culture, compared to co-culture with the FEF3303 esophageal fibroblasts. Upon magnetic bead-based purification from co-culture, RNA was extracted from the tumor cells, followed by qPCR analysis. We have found significant changes in expression of multiple genes encoding matricellular proteins (FBN1, POSTN, SPARC, THBS1), growth factors and their receptors (EGF, HGF, VEGFA, VEGFB, VEGFC, MET, PTK2, FLT1, KDR), EMT-related genes (CDH2, SNAIL2), cytokines, chemokines and their receptors (IL-6, CXCL1, CXCL5, IL-6R, CCR1, CCR4), as well as components of inflammatory signaling pathways (STAT3, SOCS3, SOCS2) (Fig. 1A).

We next decided to investigate the effect of co-culture on cell proliferation. Measured by flow cytometry (dilution of the CFDA-SE dye), three out of four ESCC cell lines (TE1, TE6, HCE7) had increased proliferation rates when co-cultured with fibroblasts (Fig. 1B). Furthermore, proliferation of either FEF3 or FEF3303 esophageal fibroblasts was significantly enhanced by any of the ESCC cells tested (Fig. 1B).

In a 3D organotypic culture model system, addition of FEF3303 esophageal fibroblasts to the epithelial layer (TE11) resulted in a dramatic increase in epithelial layer thickness, as well as enhanced invasion into the extracellular matrix (Fig. 1C, 1D). Importantly, co-injection of TE11 cells with FEF3303 or ESCC-Fb-1 fibroblasts
subcutaneously into flanks of nude mice significantly promoted tumor growth rate, when compared to TE11 cells alone (Fig. 1E). These findings underscore the importance of activated fibroblasts in ESCC initiation and progression.

**IL-6 and RANTES are potential mediators of cross-talk between tumor cells and CAFs in ESCC**

To identify potential mediators of cross-talk between epithelial tumor cells and CAFs in esophageal cancer, we have conducted a cytokine array analysis of conditioned media from co-cultures of ESCC cells with esophageal CAFs. We have identified six cytokines (IL-6, RANTES, ENA-78, GRO-α, IL-8 and MCP3) with enhanced secretion in co-culture, compared to either cell line in mono-culture (Fig. 2A). Secretion of IL-6 was the most dramatically and specifically increased in co-culture, while RANTES was the second most-upregulated in co-culture. We have confirmed the cytokine array results for IL-6 and RANTES by ELISA and observed pronounced upregulation of both cytokines in co-culture of TE11 ESCC cells and FEF3303 fibroblasts (Fig. 2B).

We have immuno-stained sections of paraffin-embedded samples of human ESCC or normal esophagus for IL-6 and RANTES. Interestingly, we have observed increased staining for IL-6 and RANTES in both fibroblasts and epithelial cells in ESCC (Fig. 3A, B). Specifically, in the normal esophagus IL-6 is expressed at low levels in the fibroblasts, as well as the smooth musculature (muscularis mucosae), and is absent in the epithelial layer. By contrast, in ESCC high expression of IL-6 is found in fibroblasts and epithelial cells. RANTES is expressed at low levels in the normal esophageal epithelium and absent in normal stroma, while in ESCC RANTES expression in the
epithelium is intense, and a certain amount is detected in the fibroblasts. These data suggest that IL-6 and RANTES may be relevant to ESCC pathogenesis.

**Tumor cell-derived IL-6 promotes tumorigenic properties of ESCC**

To investigate the role of epithelial cell-derived IL-6 in esophageal cancer, we have used the CRISPR/Cas9 method to knock out IL-6 in TE11 cells, TE11 IL-6KO1 and IL-6KO2 (Fig. 4). We have observed significant differences in two types of 3D culture and *in vivo*. The 3D organotypic cultures formed by TE11 IL-6KO cells reveal decreased epithelial layer thickness and reduced invasion (Fig. 5A). Furthermore, we found that TE11 IL-6KO cells formed smaller 3D organoids with “normalized” morphology, compared to IL-6WT cells (Fig. 5B). Subcutaneous xenograft tumors formed by the TE11 IL-6KO cells are characterized by stalled growth rates (Fig. 5C).

**Targeting IL-6 signaling as a therapeutic approach in ESCC**

Since we found that IL-6 signaling drives ESCC progression, we conducted a therapeutic study using tocilizumab, a neutralizing antibody against the human IL-6Rα. Nude mice bearing subcutaneous xenograft tumors formed by TE11 and FEF3303 cells were treated with tocilizumab or a human IgG isotype control. Interestingly, tocilizumab treatment resulted in stalled tumor growth (Fig. 6A), which was accompanied by reduced STAT3 and ERK1/2 signaling (Fig. 6B).

**Tumor cell-derived RANTES promotes tumorigenic properties of ESCC**

We have next generated single cell-derived subclones of TE11 cells with CRISPR/Cas9-mediated knockout of RANTES: TE11 RantesKO1 and RantesKO2 (Fig.
In the 3D organotypic cultures formed by TE11 RantesKO cells there were fewer nucleated epithelial layers with reduced invasion (Fig. 7A). In addition, we found that RANTES-deficient 3D organoids were smaller and had “normalized” morphology (Fig. 7B). Finally, subcutaneous xenograft tumors formed by the TE11 RantesKO cells had decreased growth rates, compared to RantesWT tumors (Fig. 7C).

Targeting RANTES signaling as a therapeutic approach in ESCC

To target RANTES signaling in vivo, we selected maraviroc, a small-molecule antagonist of CCR5. We found that treatment with maraviroc suppressed growth of subcutaneous xenograft tumors formed by TE11 and FEF3303 cell lines co-implanted into hind flanks of nude mice (Fig. 8A), compared to treatment with the vehicle (DMSO). Surprisingly, tumors from the maraviroc-treated animals had significantly reduced ERK1/2 signaling in both epithelial cells and fibroblasts, which was accompanied by a trend of reduced STAT3 signaling (Fig. 8B).

Co-targeting of IL-6 and RANTES is a preferential strategy to ESCC therapy in vitro

Our results indicate that IL-6 and RANTES play an important role in ESCC pathogenesis and that at least part of their functions are mediated through the STAT3 and ERK1/2 signaling pathways (Fig. 6,8). This led us to investigate whether simultaneous inhibition of IL-6 and RANTES signaling may provide a further therapeutic benefit. To that end, we treated 3D organotypic ESCC cultures with tocilizumab, maraviroc or a combination of both. Interestingly, we have found that though either drug
alone or in combination suppressed tumor cell invasion, only the combination of tocilizumab and maraviroc produced a significant reduction in the number of nucleated epithelial layers (Fig. 9). These data indicate that simultaneous inhibition of both IL-6 and RANTES-induced signaling may indeed be a better approach for the treatment of ESCC, although in vivo testing of this hypothesis is needed.

**STAT3 and ERK1/2 signaling promotes ESCC tumorigenesis**

We have next treated 3D organotypic cultures of ESCC with small molecule inhibitors of STAT3 and MEK/ERK signaling pathways (stattic and trametinib, respectively). Surprisingly, while treatment with either drug individually suppressed tumor cell invasion, the combination of stattic and trametinib inhibited invasion even more efficiently than either drug alone (Fig. 10A,B). Only STAT3 inhibition led to reduced number of nucleated cells in the epithelial layer, while only MEK/ERK inhibition efficiently suppressed cell proliferation, measured by Ki-67 staining (Fig. 10A,B). Interestingly, only combination of stattic and trametinib promoted apoptosis, while mono-therapy with either drug induced no change, measured by immune-staining for cleaved caspase 3 (Fig.10A,B). These data suggest that the signaling pathways induced by IL-6 and RANTES promote tumor cell invasion, proliferation and evasion of apoptosis in ESCC.

**IL-6 signaling as a therapeutic target in multiple types of cancer**

To determine the potential of targeting IL-6 signaling in other cancers besides esophageal, we performed in silico survival analysis using IL-6 and IL-6R expression (RNAseq) data acquired from TCGA. Remarkably, we found that low relative expression
of IL-6 and IL-6R was correlated with improved survival, not only in esophageal cancer but in the TCGA pan-cancer dataset (Fig. 11A). In addition, immunohistochemical staining for IL-6 in head and neck squamous cell carcinoma (HNSCC) and gastric adenocarcinoma showed overexpression in tumor sections compared to matched normal oral and gastric mucosa, respectively (Fig. 11B,C). (All data in this section are used with permission from Eric Lin).
Discussion

The importance of the tumor microenvironment in cancer in general, and in esophageal cancer specifically, is well appreciated (Lin et al., 2016). Yet, the precise mechanisms of interaction between different components of the TME and the tumor cells remain to be elucidated. We focused our attention on fibroblasts, because of existing evidence linking CAFs to prognosis and therapeutic outcome in ESCC (Ha et al., 2014; Saito et al., 2014). Specifically, we wanted to identify novel mediators of interaction between CAFs and esophageal tumor cells.

Based on the cytokine array results, IL-6 and RANTES were selected as likely candidates, since they were specifically upregulated in co-cultures of ESCC and esophageal CAFs, compared to mono-cultures (Fig. 2). This was strengthened by the IHC staining of human ESCC biopsies, where either IL-6 or RANTES staining was significantly stronger in both epithelial cells and fibroblasts, compared to normal samples. Previous studies have demonstrated the role of ESCC cell-derived IL-6 in evasion of apoptosis (Leu et al., 2003), and our findings support this premise. Importantly, we show herein that expression of IL-6 by tumor cells is induced through interaction with CAFs. Yet, our data from 3D organoid cultures suggest an autocrine IL-6 signaling mechanism (Fig. 5B), in addition to paracrine signaling induced by fibroblast-derived IL-6. To our knowledge, no reports describing the function of RANTES in ESCC have been published. Our observations regarding the role of RANTES in the growth and invasiveness of 3D tumor cultures (Fig. 7), are noteworthy and in agreement with current perspectives on the importance of RANTES in other cancers (Aldinucci and Colombatti, 2014).
Tocilizumab is widely used in clinic to inhibit IL-6 signaling in autoimmune diseases, and a number of pre-clinical studies have indicated its efficacy in cancer. Since our data demonstrate that IL-6 promotes esophageal carcinogenesis, we have tested tocilizumab in a xenograft model of ESCC and observed a potent tumor-suppressive effect, which was accompanied by decreased STAT3 and ERK1/2 signaling in both epithelial and fibroblast compartments (Fig. 6). Furthermore, we provide evidence of IL-6 involvement in pathogenesis of multiple types of cancer, such as head and neck squamous cell carcinoma and gastric adenocarcinoma (Fig. 11). Similarly, prompted by our finding that RANTES is also important for ESCC progression, we sought to pharmacologically inhibit its signaling (at least partially) in vivo. In our xenograft model, maraviroc suppressed ESCC tumor growth, likely via inhibition of STAT3 and ERK1/2 signaling pathways (Fig. 8).

Importantly, we demonstrate that co-inhibition of signaling induced by IL-6 and RANTES provides a better tumor-suppressive effect than inhibition of either cytokine alone in vitro (Fig. 9). Interestingly, a synergistic interaction between IL-6 and RANTES has been previously described in aggressive subtypes of lung and breast cancer (Barbie et al., 2014; Zhu et al., 2014). Along with our data, this suggests that co-targeting IL-6 and RANTES could provide for a superior treatment strategy not only in ESCC, but in other types of cancer.

In agreement with previous publications (Aldinucci and Colombatti, 2014; Leu et al., 2003), we find that the pro-tumorigenic role of IL-6 and RANTES in ESCC is mediated via STAT3 and ERK1/2 signaling pathways. In addition to suppression of apoptosis, we also found that these pathways mediate tumor cell proliferation and
invasion (Fig. 10). These findings provide a rationale for potential future studies of combining downstream inhibitors of these pathways for therapeutic application, which could provide a better outcome, compared to “upstream intervention”, such as receptor blockade with tocilizumab and/or maraviroc.

Since the key role of activated stroma in the establishment and progression of cancer in general, and ESCC in particular, is no longer under question, the next logical step should be to use this knowledge in designing the new generation of cancer therapeutics. In addition to promoting tumorigenesis, CAFs are known to contribute to resistance to multiple treatments, including neoadjuvant chemotherapy, anti-angiogenic therapy, and receptor tyrosine kinase inhibitors (Pietras and Östman, 2010). We have identified two cytokines that mediate the interaction between tumor cells and CAFs in the esophageal cancer microenvironment, as well as the key signaling pathways utilized by these cytokines. These data shall be used to develop novel targeted approaches to cancer therapy, as well as to improve the currently used strategies. For example, combining inhibition of IL-6 and RANTES with chemo- or radiotherapy may produce superior results.
Materials and methods

Cell lines and reagents

The TE and HCE cell lines were cultured in DMEM supplemented with 10% FBS (Sigma) and 1x penicillin/streptomycin (Invitrogen) in 5% CO₂ as described previously (Okano et al., 2000). FEF3 and FEF3303 cell lines were cultured in DMEM supplemented with 15% FBS (HyClone) and 1x penicillin/streptomycin in 5% CO₂ as described previously (Okawa et al., 2007). The ESCC-Fb-1 and ESCC-CAF-J1 cell lines have been generated from esophageal biopsies. The tissues were incubated in dispase/collagenase/Y27632 solution for 45 min at 37°C (vortexed every 10 minutes and mechanically disrupted with a P1000 pipette tip). Preparations were incubated for 10 minutes at 37°C in 0.25% trypsin/EDTA. Soybean trypsin inhibitor (Sigma) was used to inactivate trypsin. The resulting cell suspension and tissue fragments were forced through a 70 μm strainer, centrifuged, resuspended in fibroblast media supplemented with fungizone (Gibco) and gentamicin (Gibco) and seeded into one well of a 6-well plate. Culture media was replaced every 48 hours or as needed.

Stattic, trametinib and Maraviroc were purchased from Selleckchem, and tocilizumab - from the pharmacy of the Hospital of the University of Pennsylvania. Human IgG control was from Sigma.
3D organoid and organotypic culture

The cells were trypsinized, mixed with matrigel (BD) and seeded at 3000 cells/well. After matrices solidified, growth media were added (CnT Prime 3D Barrier Media for Epidermal Models, ZenBio) and replaced every 48-72 hours. For IHC, organoids were recovered on day 12 from matrigel with dispase digestion, fixed overnight in 4% paraformaldehyde and embedded in 2% Bacto-Agar:2.5% gelatin before dehydration and embedding in paraffin.

TE11 cells were grown in organotypic culture as described previously (Kalabis et al., 2012), with the following modifications. Transwell inserts (30mm, PET, 0.4μm pore; Millipore) were used to support the 3D collagen/matrigel matrices, containing 27% Nutragen bovine collagen solution type I (Advanced Biomatrixx), 18% matrigel (BD), 1× minimal essential medium with Earle’s salts (BioWhittaker), 1.68 mM L-glutamine (Cell- gro), 10% fetal bovine serum (HyClone), 0.15% sodium bicarbonate (BioWhittaker), and 2×10⁴ FEF3303 fibroblasts. On day 7 of culture, 2.5x10⁵ epithelial cells were seeded on top of the matrices. On day 15, the cultures were fixed in Zn-buffered formalin (Thermo) for 2 hours at 4ºC before dehydration and embedding in paraffin.

Real-time qPCR

RNA was isolated using the RNAqueous-4PCR kit (Ambion). cDNA was generated using oligo-dT primers and Superscript II Reverse Transcriptase (Life
Technologies). Real-time PCR was performed using validated SYBR Green primers and ABI7000 and ABI StepOne instruments (Applied Biosystems).

FACS

Cells were labeled with CFDA-SE (Invitrogen; 1 or 0.1 μM for epithelial cells or fibroblasts, respectively) for 30 minutes at 37°C, quenched for 30 minutes at 37°C, trypsinized and seeded into co- or mono-cultures. After 72 hours, the cells were trypsinized, resuspended in PBS with 1% BSA and 0.01% sodium azide. Samples were analyzed on a FACScalibur (BD) or Accuri (BD). Data were analyzed using FlowJo (Treestar).

Cytokine array

3x10^5 cells were seeded per 6 cm dish and grown in 5 mL DMEM with 10% FBS (HyClone) and 1x penicillin/streptomycin. Each culture condition was carried out in duplicate. After 72 hours, conditioned media were collected, frozen and stored at -80°C. The relative concentration of 42 cytokines was measured using the Human cytokine array G3 (Raybiotech).

ELISA

Cytokine levels in culture supernatants were quantified using the Human IL-6 ELISA MAX and Human CCL5 (RANTES) ELISA MAX kits (BioLegend)
according to the manufacturer’s instructions. Absorbance was measured on the Sunrise Microplate Reader (TECAN).

**Histology**

ESCC tissue samples were obtained as surgical biopsies from Kagoshima University Hospital, as described previously (Natsuizaka et al., 2014). The clinical materials were obtained from informed-consent patients according to the Institutional Review Board standards and guidelines. IHC and immunofluorescence were performed as described previously (Karakasheva et al., 2015).

**Xenograft tumor growth studies**

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. A total of 6x10^6 cells (all epithelial, or 5x10^6 epithelial cells with 10^6 fibroblasts) resuspended in 50 μl matrigel were injected subcutaneously into rear flanks of female athymic nude mice (6-10 weeks old, Taconic). Tumors were measured twice a week after becoming palpable. For therapeutic studies, tocilizumab or human IgG control were delivered intraperitoneally at 10mg/kg three times a week; maraviroc or vehicle control (DMSO) were delivered intraperitoneally at 10mg/kg daily.
Statistical analysis

Student’s t-test was used to determine significance. ANOVA analysis was used to determine significance for multi-parameter comparisons. Unless noted otherwise, the p-values are listed on graphs. All analyses were performed in R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria).
Figures and figure legends
A. Gene expression, relative to mono-culture

B. ESCC cell lines

C. TE11

D. Epithelial layer and Invasive area

E. Tumor volume, mm³

Days post-implantation
Figure 2-1: Direct interaction between tumor cells and CAFs induces changes in cell biology. (A) qPCR analysis of gene expression in ESCC cells (TE11) co-cultured with CAFs (FEF3303), compared to mono-culture (n=2/group; *p<0.05). (B) Relative proliferation rates of ESCC cells (TE1, TE6, TE11, HCE7) and CAFs (FEF3, FEF3303) in mono- or co-culture, measured by CFDA-SE fluorescence dilution (n=3/group; *p=10^{-4}, 10^{-6}, 10^{-5}, 10^{-6}, 0.007; **p<10^{-3}; ***p<10^{-2}). (C) Representative images of 3D organotypic cultures where epithelial cells have been seeded alone (TE11) or mixed directly with CAFs (TE11+FEF3303) (n=3/group). Number of nucleated epithelial layers and relative invasive area are quantified. (D) Growth kinetics of subcutaneous xenograft tumors formed by ESCC cells alone or co-injected with CAFs (n=4/cohort; *p<0.05). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 2
Figure 2-2: IL-6 and RANTES are potential mediators of cross-talk between tumor cells and CAFs in ESCC. (A) Representative results of the cytokine array conducted on conditioned media from mono- and co-cultures of ESCC cells (TE1, TE6, TE11, HCE7) and CAFs (FEF3, FEF3303) (n=2/group). (B) Concentration of IL-6 and RANTES in conditioned media from mono- and co-culture of ESCC cells and CAFs, measured by ELISA (n=3/group). P-values calculated via Student's t-test, p≤0.05 considered statistically significant.
Figure 2-3: IL-6 and RANTES are overexpressed in epithelial and stromal compartments of ESCC. (A, B) Representative images of normal esophagus and ESCC stained immunohistochemically for IL-6 or RANTES (n=40). Histopathological scoring results are shown on the right. P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 2-4: Quality-control of the IL-6 and RANTES-knockout subclones of TE11 ESCC cell line. Levels of corresponding cytokines in cell culture conditioned media were measured by ELISA and compared to wild-type TE11 conditioned medium, as well as to DMEM (negative control). Results shown are for the subclones at passage 3 post-transfection with the CRISPR/Cas9 construct. The stability of knockout was confirmed by ELISA for up to passage 12 post-transfection (n=3 wells/clone). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 5

A  TE11 IL-6WT  TE11 IL-6KO-1  TE11 IL-6KO-2

Bar=50μm

B  TE11 IL-6WT  TE11 IL-6KO-1  TE11 IL-6KO-2

Bar=10μm

C

Tumor volume, mm³

Days post implantation

52
Figure 2-5: Tumor cell-derived IL-6 promotes tumorigenic properties in ESCC. (A) Representative images of 3D organotypic cultures (Hematoxylin-Eosin stained paraffin-embedded sections) formed by wild-type or IL-6 knockout TE11 cells (n=2/clone). (B) Representative images of 3D ESCC organoids (brightfield and Hematoxylin-Eosin staining of paraffin-embedded sections) formed by wild-type or IL-6 knockout TE11 cells. Organoid size is quantified as relative cross-section area, organoid shape quantified as deviation from elliptical shape (n=4/clone). (C) Growth kinetics of subcutaneous xenograft tumors formed by wild-type or IL-6 knockout TE11 cells (n=6/clone; *p≤0.01). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 6: Targeting IL-6 is a promising novel approach for ESCC therapy. (A) Growth kinetics of subcutaneous ESCC xenograft tumors (TE11+FEF3303) treated with Tocilizumab or isotype control (i.c.) antibody (n=10/cohort, results from one representative study out of three shown; *p=0.009, 0.004, 0.0002). (B) Representative images of tumor sections from (A) stained for pSTAT3 and pERK. (C) Quantification of staining from (B). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 2-7: Tumor cell-derived RANTES promotes tumorigenic properties in ESCC.
(A) Representative images of 3D organotypic cultures (Hematoxylin-Eosin stained paraffin-embedded sections) formed by wild-type or RANTES knockout TE11 cells (n=2/clone). (B) Representative images of 3D ESCC organoids (brightfield and Hematoxylin-Eosin staining of paraffin-embedded sections) formed by wild-type or RANTES knockout TE11 cells. Organoid size is quantified as relative cross-section area, organoid shape quantified as deviation from elliptical shape (n=4/clone). (C) Growth kinetics of subcutaneous xenograft tumors formed by wild-type or RANTES knockout TE11 cells (n=6/clone; *p≤0.05). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 2-8: Targeting RANTES is a promising novel approach for ESCC therapy. 
(A) Growth kinetics of subcutaneous ESCC xenograft tumors (TE11+FEF3303) treated with maraviroc or DMSO (n=4/cohort; *p=0.02). (B) Representative images of tumor sections from (A) stained for pERK1/2 and pSTAT3. (C) Quantification of staining from (B). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 2-9: Co-inhibition of IL-6 and RANTES signaling may be superior to mono-therapy. (A) Representative images of 3D organotypic ESCC cultures treated with tocilizumab, maraviroc or a combination of both (Hematoxylin-Eosin staining; n=2/condition). (B) Quantification of images from (A). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 10

A

<table>
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<td>cl. caspase 3</td>
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Bar=50 µm

B

- **Invasive area**
  - DMSO: 0.0005
  - static: 0.02
  - trametinib: 0.03
  - static + trametinib: 0.02

- **Epithelial layer**
  - # nucleated layers
  - DMSO: 0.0007
  - static: 0.005
  - trametinib: 0.003
  - static + trametinib: 0.002

- **Ki-67**
  - % positive
  - DMSO: 0.002
  - static: 0.008
  - trametinib: 0.002
  - static + trametinib: 0.001

- **Cl. caspase 3**
  - % positive
  - DMSO: 0.0007
  - static: 0.0007
  - trametinib: 0.0002
  - static + trametinib: 0.0002
Figure 2-10: STAT3 and ERK1/2 signaling mediate ESCC tumorigenesis *in vitro*. 
(A) Representative images of 3D organotypic ESCC cultures (Hematoxylin-Eosin, 
cleaved caspase 3 and Ki-67 staining; arrows indicate cleaved caspase 3-positive cells; 
n=4/condition). (B) Quantification of images from (A). P-values calculated via Student’s t- 
test, p≤0.05 considered statistically significant.
Figure 2-11: Potential of targeting IL-6 in multiple types of cancer. (A) Kaplan-Meier survival analysis based on differential expression of IL-6 and IL-6R in esophageal cancer and pan-cancer (RNAseq data, TCGA; p=0.0065 (esophageal), 3x10^{-11} (pan-cancer)). (B) Representative images of normal oral mucosa and head and neck cancer stained for IL-6 by immunohistochemistry, with quantification. (C) Representative images of normal gastric mucosa gastric adenocarcinoma stained for IL-6 by immunohistochemistry, with quantification. P-values calculated via Student’s t-test, p≤0.05 considered statistically significant. (Data used with permission from Eric Lin).
Tables and table legends
Table 1

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Table 2-1: Clinical trials targeting IL-6 or RANTES signaling in cancer.
CHAPTER 3: Cytokine regulation of the immunosuppressive microenvironment in ESCC

The data in chapter 3 have been published in Cancer Research.

Abstract

Myeloid derived suppressor cells (MDSCs) are an immunosuppressive population of immature myeloid cells found in advanced stage cancer patients and mouse tumor models. Production of inducible nitric oxide synthase (iNOS) and arginase, as well as other suppressive mechanisms, allow MDSCs to suppress T cell-mediated tumor clearance and foster tumor progression, in addition to other mechanisms such as fostering angiogenesis and inducing activation of fibroblasts. We have identified CD38 as playing a vital role in MDSCs biology in a murine model of ESCC. CD38 belongs to the ADP-ribosyl cyclase family and possesses both ectoenzyme and receptor functions. It has been described to function in early myeloid cell differentiation, cell activation and neutrophil chemotaxis. We found that CD38 expression in MDSCs is evident in several mouse tumor models, and CD38$^{\text{high}}$ MDSCs are more immature than CD38$^{\text{low}}$ or CD38$^{\text{neg}}$ MDSCs, suggesting a potential role for CD38 in the maturation halt found in MDSC populations. CD38$^{\text{high}}$ MDSCs also possess a greater capacity to suppress activated T cells, and promote tumor growth to a greater degree than CD38$^{\text{low}}$ MDSCs, likely as a result of increased iNOS production. Tumor-derived factors, including IL-6, IGFBP-3 and CXCL16 (identified by us), play a role in the induction of CD38 expression. This work may have implications for use of FDA-approved anti-CD38 therapy in patients with solid tumors where MDSCs are upregulated.
Introduction

The immune system (both innate and adaptive) plays an essential role in limiting tumor growth; therefore, tumor progression requires escape from immune surveillance. One of the mechanisms that allows for tumor escape is the activation and expansion of immunosuppressive cell populations, including but not limited to, regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) (Schreiber et al., 2011), the latter also referred to as immature myeloid cells. Certain therapeutics have been demonstrated to have potential efficacy against MDSCs (Waldron, T J, Quatromoni G J, Karakasheva, T A, Singhal, S, Rustgi, 2013); however, the need for more selective anti-MDSC therapeutics remains.

MDSCs have been observed in a number of mouse tumor models and represent a heterogeneous population of immature monocytes, macrophages, granulocytes, and dendritic cells that are identified by their CD11b+Gr-1+ phenotype in mice. The first human MDSC population was identified in head and neck cancer (Pak et al., 1995), and has since been documented in cancers of the esophagus (Gabitass et al., 2011), stomach (Gabitass et al., 2011), pancreas (Gabitass et al., 2011; Schmielau and Finn, 2001), lung (Liu et al., 2010), kidney (Zea et al., 2005), colon (Schmielau and Finn, 2001), skin (melanoma) (Filipazzi et al., 2007), prostate (Vuk-Pavlović et al., 2010), and breast (Schmielau and Finn, 2001). The immunophenotype of human MDSCs varies (Greten et al., 2011), however, their immunosuppressive mechanisms match those found in murine CD11b+Gr-1+ MDSC populations.

MDSCs induce immune suppression primarily through the inhibition of T cell-mediated tumor clearance (Gabrilovich and Nagaraj, 2009), but can also promote
inhibition of NK cells (Li et al., 2009) and activation of Tregs (Huang et al., 2006; Serafini et al., 2008). Arginase-1 (ARG1) and inducible nitric oxide synthase-2 (iNOS) provide the bulk of the enzymatic activity required for MDSCs to suppress proliferation and activation of T cells (Gabrilovich and Nagaraj, 2009). ARG1 deprives T cells of arginine by converting L-arginine into urea and L-ornithine, thereby leading to reduced expression of CD3ζ chain, which renders T cells unable to respond to activation signals (Rodriguez et al., 2003, 2004). iNOS inhibits T cell function by a variety of mechanisms, including the inhibition of JAK3/STAT5 signaling (Bingisser et al., 1998), MHC Class II expression (Harari and Liao, 2004) and induction of apoptosis (Rivoltini et al., 2002).

CD38 expression is a common characteristic to several immunosuppressive cell types. Foxp3⁺CD25⁺CD4⁺ Tregs expressing high levels of CD38 possess greater immunosuppressive activity than CD38low Tregs (Patton et al., 2011). CD38⁺CD8⁺ T cells suppress proliferation of CD4⁺ effector T cells, which requires secretion of IFNγ and cell-to-cell contact (Bahri et al., 2012). Similarly, CD19⁺CD24hiCD38hi B cells inhibit the differentiation of T helper 1 cells through an IL-10-dependent mechanism, and their dysfunction may play a role in autoimmune disorders such as systemic lupus erythematosus (Blair et al., 2010).

CD38 is a member of the ribosyl cyclase family and is expressed on the surface of diverse immune cells, including B cells, T cells, NK cells and myeloid cells (Malavasi et al., 2008a). CD38 possesses independent ectoenzyme and receptor functions. As an ectozyme, CD38 catalyzes the synthesis and hydrolysis of cyclic ADP-ribose (cADPR), converting NAD⁺ to ADP-ribose (ADPR), as well as cADPR into ADPR (Lee, 2012; Malavasi et al., 2008a). Furthermore, at acidic pH, CD38 catalyzes the synthesis and
hydrolysis of nicotinic acid adenine dinucleotide phosphate (NAADP) (Lee, 2012; Malavasi et al., 2008a). Both reactions are important for calcium signaling, specifically for mobilization of intracellular Ca$^{2+}$ (Lee, 2012). Receptor activity of CD38 has been documented in several immune cell types, where it is dependent on localization to the lipid rafts and association with professional signaling complexes (Malavasi et al., 2008a). In both mouse and human myeloid cells, ligation of CD38 leads to suppressed growth and survival leading to loss of the most differentiated immune populations (Todisco et al., 2000).

We have identified CD38 as a novel marker for MDSCs that possesses greater immunosuppressive capacity, thereby promoting tumor growth in vivo. Mechanistically, CD38 promotes expansion of the monocytic MDSC population and regulates expression of the effector molecule iNOS by these cells. Importantly, we have established for the first time that several cytokines, specifically IFNg, TNFa, IGFBP-3, CXCL16, and IL-6, are capable of inducing CD38 expression in MDSCs. Finally, we have demonstrated that administration of an anti-CD38 monoclonal antibody slows disease progression in tumor-bearing mice.
Results

**CD38\textsuperscript{high} MDSCs possess greater immunosuppressive and tumor-promoting capacity than CD38\textsuperscript{low} MDSCs**

We have previously demonstrated that MDSCs play a fundamental role in tumor initiation and progression in a spontaneous genetic mouse model of ESCC (\(L2-Cre;p120^{f/f}\)) (Stairs et al., 2011), and our next goal was to identify genes associated with an immature myeloid phenotype that contribute to the tumor promoting activities of MDSCs. To that end, we performed microarray analysis of splenic MDSCs from tumor-bearing \(L2-Cre;p120^{f/f}\) mice and age-matched littermate controls and identified *Cd38* (ranked fifth highest among all genes tested as a candidate gene of interest), as it has roles in both innate and adaptive immunity in mice and humans, including, but not limited to chemotaxis of murine and human neutrophils, early myeloid differentiation and lymphoid cell activation (Karakasheva et al., 2015).

These findings led us to the hypothesis that CD38\textsuperscript{high} MDSCs possess greater immunosuppressive potential than CD38\textsuperscript{low} MDSCs. To test our hypothesis, we FACS-sorted both CD38\textsuperscript{high} and CD38\textsuperscript{low} populations from mice bearing ESCC tumors generated by flank injections of the HNM007 cell line in syngeneic C57BL/6 recipients and tested their capacity to suppress OT-1 T cell growth following stimulation with cognate antigen. The capacity of total CD11b\textsuperscript{+}Gr-1\textsuperscript{+}, CD11b\textsuperscript{+}Gr-1\textsuperscript{+}CD38\textsuperscript{low}, and CD11b\textsuperscript{+}Gr-1\textsuperscript{+}CD38\textsuperscript{high} cells to suppress T cell proliferation after stimulation was tested. CD38\textsuperscript{high} MDSCs demonstrated significantly greater T cell suppressive capacity, compared to their CD38\textsuperscript{low} counterparts (Fig. 1A), at 2:1 OT-1 to MDSC ratio, while a trend of increased suppression was observed at 1:1 and 4:1 ratios.
Next, we evaluated the impact of coinjection of CD38\textsuperscript{high} MDSCs with HNM007 cells on tumor growth. Tumor volumes in the CD38\textsuperscript{high} group were significantly larger than the CD38\textsuperscript{Low} tumors when measured on days 6 and 10 (Fig. 1B), and larger than control HNM007 tumors at days 8, 10 and 13 (Fig. 1B). No differences in size were detected between the CD38\textsuperscript{Low} and control HNM007 tumors. These results suggest that CD38\textsuperscript{high} MDSCs possess greater tumor-promoting capacity than CD38\textsuperscript{low} MDSCs \textit{in vivo}.

We next investigated whether CD38 is required for immunosuppressive function of MDSCs. We therefore tested the capacity of $Cd38^{-/-}$ and $Cd38^{+/+}$ (wt) CD11b\textsuperscript{*}Gr-1\textsuperscript{*} cells sorted from mice bearing HNM007 tumors to suppress growth of OT-1 T cells following antigen stimulation and found that $Cd38^{-/-}$ CD11b\textsuperscript{*}Gr-1\textsuperscript{*} cells exhibited significantly reduced immunosuppressive capacity at 1:1 and 4:1 OT-1 to MDSC ratio (Fig. 1C).

\textbf{IFN\gamma, TNF\alpha, CXCL16, IGFBP-3 and IL-6 induce the expression of CD38}

We have observed that unlike the HNM007 ESCC cell line, the tumors generated by flank injection of the AKR ESCC line do not induce high expression of CD38 on the surface of MDSCs (Fig. 2). This prompted us to employ these two cell lines to better understand the tumor cell-specific signaling pathways that result in increased expression of CD38. We performed \textit{ex vivo} differentiation of bone marrow using GM-CSF, IL-4 (the cytokines required for generation of CD11b\textsuperscript{*}Gr-1\textsuperscript{*} cells from bone marrow progenitors (Youn et al., 2008)) and either HNM007 or AKR conditioned media. HNM007 conditioned medium induced cells to express CD38 while AKR conditioned medium did
not (Fig. 3B). Since IFN\(\gamma\) and TNF\(\alpha\) are key components of the pro-inflammatory milieu and both are known activators of CD38 transcription (Malavasi et al., 2008a), we performed an \textit{ex vivo} differentiation assay with the addition of IFN\(\gamma\), TNF\(\alpha\), or both and found that both factors induced CD38 expression in the CD11b\(^+\)Gr-1\(^+\) population, while the combination of IFN\(\gamma\) and TNF\(\alpha\) induced the most profound effect (Fig. 3B). We subsequently performed a cytokine array using media collected from the \textit{ex vivo} differentiation experiments and compared the levels of 62 cytokines present in the culture medium derived from HNM007 conditioned medium to culture medium derived from AKR conditioned medium. Several factors including CXCL16, IGFBP-3 and RANTES were present at higher levels in the HNM007 conditioned medium samples after 24 hours in culture (Table 1). CXCL16 and IGFBP-3 levels were maintained at higher levels in HNM007 conditioned media-containing cultures after 120 hours (Table 1), suggesting that either of these two factors, or both, may play a role in induction of CD38. In addition, the pro-inflammatory cytokine IL-6, a predicted activator of CD38 transcription (Malavasi et al., 2008b), was elevated in HNM007 conditioned media-containing cultures after 24 and 120 hours, albeit not as dramatically as CXCL16 or IGFBP-3 (Table 1). We tested next the capacity of recombinant IL-6, CXCL16 and IGFBP3 alone or in combination to increase CD38 expression in our \textit{ex vivo} differentiation experiments. Addition of the combination of IL-6, CXCL16 and IGFBP3 was able to induce CD38 expression in MDSC cultured with AKR conditioned medium (Fig. 4B). Nevertheless, the most potent induction of CD38 expression was observed when IFN\(\gamma\) and TNF\(\alpha\) were added to the culture. The importance of IL-6 in ESCC pathogenesis is elucidated in Chapter 2 of this thesis.
Cross-linking of CD38 by an agonistic antibody impairs expansion and survival of CD11b+Gr1+ cells in vitro and suppresses tumor growth in vivo

In order to test whether cross-linking of CD38 with a monoclonal antibody has an effect on MDSC function(s), we performed a colony-formation assay. CD11b+Gr-1+ cells were isolated from the spleens of diseased L2-cre;p120−/− mice and cultured in methylcellulose-based medium in the presence of an anti-CD38 monoclonal antibody (NIM-R5) or an isotype control. Addition of an anti-CD38 antibody inhibited the growth of colonies from sorted splenic CD11b+Gr-1+ cells (Fig. 5A and 5B). The effect of the anti-CD38 antibody remained the same regardless of whether splenocytes were pre-sorted (Fig. 5A and 5B). These data demonstrate that anti-CD38 treatment inhibits MDSC proliferation and survival in vitro independent of stromal support. We similarly tested the effect of anti-CD38 treatment in suspension culture. FACS-sorted CD11b+Gr-1+ cells survive only a few days in culture, but their survival is significantly reduced in the presence of anti-CD38 antibody (Fig. 5C). We have also tested whether cross-linking of CD38 inhibits the accumulation of CD11b+Gr-1+CD38high cells produced by our ex vivo culture of bone marrow in the presence of tumor cell conditioned medium, IL-4 and GM-CSF. Using a CD38 antibody (clone 90) different from NIM-R5, we observed a dose-dependent decrease in the CD38 expression within the CD11b+Gr-1+ population (Fig. 5D). Given that the proportion of CD11b+Gr-1+ cells within the bone marrow culture remained consistent (25-30%; data not shown) the CD38 expression data demonstrate that the CD11b+Gr-1+CD38high population is reduced as a result of CD38 cross-linking. Lastly, we have attempted a pilot study investigating the effect of CD38 antibody treatment on tumor growth rate in vivo in a subcutaneous transplant model of ESCC.
HNM007 tumor-bearing mice were treated every 48 hours with anti-CD38 (NIM-R5) or isotype control (IgG2a) antibody, starting on day 5 post-injection. We have observed a significant decrease in tumor volume in the NIM-R5-treated cohort, compared to the IgG2a-treated cohort (Fig. 5E). These data demonstrate the importance of CD38 for MDSC-mediated tumor progression, as well as suggest targeting CD38 as an experimental approach to ESCC therapy.
**Discussion**

We have identified CD38 as a functional marker of MDSCs with higher immunosuppressive capacity. Yet, we did not know what factors are responsible for induction of CD38 expression in this population. Interestingly, two different ESCC cell lines used in our studies exhibited differential capacities to induce expansion of a CD11b⁺Gr-1⁻CD38⁺⁺ MDSC population, thereby suggesting that the tumor cells are responsible for initiating the signaling cascade that results in the increased CD38 expression on MDSCs. Based upon our ex vivo studies, the tumor-derived signals may act directly on immature myeloid cell populations present in hematopoietic tissues to promote CD38 expression.

Several factors are likely to be responsible for activating CD38 expression, including IFNγ, TNFα, IL-6, IGFBP-3 and CXCL16. Transcriptional regulation of CD38 expression by both IFNγ and TNFα has been reported (Malavasi et al., 2008a), and we demonstrated that both are capable of inducing cultured bone marrow derived CD11b⁺Gr-1⁺ cells to express CD38. As both IFNγ and TNFα are often produced during chronic inflammation, they may be primary inducers of CD38 expression and the resulting changes in immature myeloid cell populations (Fig. 6). In fact, use of a TNFα inhibitor (etanercept) has been demonstrated to inhibit suppressive capacity of MDSCs and induce differentiation in a murine model of chronic inflammation (Sade-Feldman et al., 2013). Coupled with our observation that TNFα potently induces CD38 expression, the finding that MDSCs from both *Cd38⁻/⁻* and *Tnf⁻/⁻* mice exhibit reduced iNOS levels (Levy et al., 2012; Mayo et al., 2008) suggests that TNFα likely activates CD38 expression in MDSCs, thereby leading to the induction of iNOS expression in vivo.
IL-6 is a major regulator of activation of STAT3 and a key transcriptional factor for establishment of immunosuppressive microenvironment within the tumor (Kortylewski et al., 2005). Importantly, a recent report demonstrated correlation between increased serum levels of IL-6 and poor prognosis, as well as with the expansion of MDSC-like population (CD11b⁺CD14⁺HLA-DR⁺) in peripheral blood of ESCC patients (Chen et al., 2014). It has been shown that overabundance of activated STAT3 stalls differentiation of myeloid cells, resulting in expansion of the immature myeloid cell pool (Nefedova et al., 2004). In MDSCs, STAT3 activation enhances production of the S100A8/A9 pro-inflammatory factors, which also contribute to maintenance of a reduced differentiation state (Cheng et al., 2008; Sinha et al., 2008). These data are in agreement with our observation that IL-6 can promote CD38 expression on MDSCs generated ex vivo, since CD38high MDSCs are less differentiated than their CD38low counterpart (Karakasheva et al., 2015). At the same time, in lymph nodes, B cell-derived IL-6 prompts resident macrophages to secrete CXCL10, which promotes differentiation of B cells into CD138⁺CD38⁺ plasma cells (Xu et al., 2012). This report supports our observation that IL-6 can induce CD38 expression; it also implies that in our ex vivo differentiation system this process may also be mediated by bone marrow-derived macrophages.

Our finding of a CXCL16-mediated response in MDSC has not been described previously. However, it is interesting to note that CXCL16 is induced by IFNγ and TNFα (Abel et al., 2004). Dendritic cells express CXCL16, which in turn is found in T cell replete areas of the spleen and lymph nodes (Matloubian et al., 2000). CXCL16 binds its cognate receptor, CXCR6, and induces the migration of T and NK cell populations (Matloubian et al., 2000). Thus, CXCL16 is found in tissues where MDSCs and T cells
may interact and may serve to recruit T cells to these regions. If CXCL16 can also induce MDSCs to adopt a more immunosuppressive phenotype, as we suggest, it could greatly contribute to T cell suppression.

IGFBP-3 is most widely known as the main binding partner for the insulin-like growth factors (IGFs), and as a part of this complex IGFBP-3 can stimulate cell proliferation, differentiation, cell survival and enhanced metabolic activity. However, the same signaling complex can suppress proliferation and induce apoptosis. Furthermore, IGFBP-3 can act independently of IGF either by entering the nucleus and directly binding DNA or by binding to several receptors on the cell surface, for example TGFβRV (Jogie-Brahim et al., 2009). The potential effects of IGFBP-3 on hematopoietic cells have not been investigated, however, there are several reports addressing its role in cancer. For example, IGFBP-3-induced increase in intracellular Ca^{2+} levels has been reported in breast cancer and myoblast cell lines (Seurin et al., 2013). This area certainly requires additional investigation, but it is plausible to hypothesize that since Ca^{2+} signaling is important for multiple immunomodulatory processes (Sochorová et al., 2009; Vellenga et al., 1993; Vukcevic et al., 2010), IGFBP-3 may potentiate expansion of the more immunosuppressive CD38^{High} MDSCs.

The chemokine RANTES was also associated with conditions promoting CD38 expression in the ex vivo differentiation culture (Table 1). We have yet to validate these results with recombinant RANTES, however, there is published evidence supporting its role in immunosuppressive capacity of MDSCs. Interestingly, monocytic MDSCs from tumor-bearing Rantes^{-/} mice were shown to be less immunosuppressive than MDSCs from Rantes^{+/} mice (Zhang et al., 2013). Furthermore, this phenotype was attributed to
reduced production of iNOS by these cells (Zhang et al., 2013). These data seem to correlate with our findings of monocytic MDSCs relying on iNOS for their immunosuppressive capacity (Karokasheva et al., 2015).

We also demonstrate here that targeting CD38 with a monoclonal antibody resulted in suppressed tumor growth rate in vivo; therefore, anti-CD38 therapy may represent a novel approach to targeting this immunosuppressive population in cancer treatment strategies. Recently, an anti-CD38 monoclonal antibody (daratumumab) was shown to be efficient in treatment of multiple myeloma (MM) in pre-clinical studies (de Weers et al., 2011). Moreover, in early-stage clinical trials daratumumab was found to be effective in patients with relapsed MM both as a single agent and in combination therapy, which resulted in its approval for MM and designation of the drug as “breakthrough therapy” by the US FDA (Laubach et al., 2014). A similar approach may allow ablation of MDSCs in patients with advanced stage solid cancer, and thus, may be suitable as an adjuvant to conventional therapies.

The expression pattern of CD38 in a broad range of cell types can raise a concern about potential adverse effects of anti-CD38 therapy (discussed in (Stevenson, 2006)), however, early clinical studies of daratumumab in MM have demonstrated an acceptable safety profile, suggesting that an appropriate dosage and treatment schedule allow for minimizing of the effects of targeting CD38 in normal tissue (Laubach et al., 2014). In addition, it is known that daratumumab induces killing of CD38-expressing cells through antibody-dependent cell-mediated cytotoxicity (ADCC) (Phipps et al., 2015). The main caveat of such a mechanism is that it only works on cells expressing high copy numbers of the target antigen, therefore, certain populations may be left unharmed. This
emphasizes the need for development of a new generation of CD38-targeting therapeutics. One of the options would be to conjugate the antibody to a cytotoxic drug that would be cleaved upon the internalization of CD38. Another promising avenue is development of bispecific antibodies, which will allow for a more selective targeting of immunosuppressive populations. Finally, the efficacy and specificity of CD38 binding-induced ADCC can be fine-tuned via modifications in the Fc domain of the antibody (Liu et al., 2014).

Like the robust immunosuppressive characteristics of CD38\textsuperscript{high} MDSCs that we describe in this study, CD38\textsuperscript{high} regulatory T cells possess enhanced suppressive potential compared to CD38\textsuperscript{low} counterparts (Bahri et al., 2012; Patton et al., 2011). This may indicate that the evolution of suppressive immune populations has selected for the cells that are capable of expressing CD38. Furthermore, this suggests that anti-CD38 therapy may present the advantage of targeting several immunosuppressive cell types at the same time.

We tested the effects of anti-CD38 monoclonal antibody (mAb) treatment \textit{in vitro} and observed a reduction in survival of CD11b\textsuperscript{+}Gr-1\textsuperscript{-} cells sorted from the spleens of tumor-bearing \textit{L2-cre;p120\textsuperscript{-/-}}. Furthermore, we demonstrated that \textit{ex vivo} production of CD11b\textsuperscript{+}Gr-1\textsuperscript{-}CD38\textsuperscript{high} cells was inhibited by treatment with anti-CD38 mAb. While the overall production of CD11b\textsuperscript{+}Gr-1\textsuperscript{-} cells was unaffected, the reduction of CD11b\textsuperscript{+}Gr-1\textsuperscript{-}CD38\textsuperscript{high} population may provide the benefit of reducing numbers of the most immunosuppressive MDSC population. In addition, our data suggest that the CD11b\textsuperscript{+}Gr-1\textsuperscript{-}CD38\textsuperscript{high} population likely possesses survival and self-renewal capacity absent in the CD11b\textsuperscript{+}Gr-1\textsuperscript{-}CD38\textsuperscript{low} populations. Anti-CD38 mAb treatment of sorted and
unsorted splenocytes led to a loss of all CD11b^Gr-1^ cells, whereas control antibody treated cultures were capable of colony formation and exhibited greater survival potential. However, when bone marrow cultures were treated with anti-CD38 mAb, only the CD11b^Gr-1^CD38^{high} cells were lost, suggesting that a progenitor population present in the bone marrow, but likely absent in the spleen, may be capable of replenishing the CD11b^Gr-1^CD38^{low} cell population.

The efficacy of immunotherapy has been demonstrated in several tumor types (Brahmer et al., 2010, 2012; Hodi et al., 2010; Robert et al., 2013; Topalian et al., 2012). Several of the most promising approaches function through impairment of T cell suppression mediated by antigen-presenting cells, as in the case of anti-CTLA-4 therapy (Ipilimumab) (Hodi et al., 2010; Robert et al., 2013), or suppression mediated by tumor cells, as in anti-PD-1 therapy (Brahmer et al., 2010; Topalian et al., 2012). MDSCs represent another facet of the T cell suppression repertoire found in cancer that merits further investigation as a potential therapeutic target (Marigo et al., 2008). In this study, we have identified CD38 as being suitable for potential MDSC targeting, and useful in the identification of potently immunosuppressive MDSC populations. Thus, anti-CD38 monoclonal antibody therapy (de Weers et al., 2011) may hold potential for targeting CD38-expressing MDSCs (Chillemi et al., 2013) in patients with solid tumors.
Materials and methods

Cell lines and reagents

AKR and HNM007 mouse ESCC tumor lines have been described previously (Opitz and Harada, 2002; Takaoka et al., 2004). Cells were maintained in DMEM + 10% FBS and passaged or harvested at ~80% confluency. Recombinant mouse CXCL16, GM-CSF, IGFBP-3, IL-4, IL-6 were purchased from Peprotech. TNFα was from R&D. 2-mercaptoethanol was purchased from Sigma. Anti-CD38 monoclonal antibody (clone NIM-R5) for in vitro and ex vivo studies was purchased from Abcam. Anti-CD38 monoclonal antibody (clone NIM-R5) for in vivo studies was a kind gift from Dr. Fabio Malavasi. The isotype control antibody (rat IgG2a) was purchased from BioXcell.

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Flow cytometry and cell sorting

Single cell suspensions were prepared from mouse bone marrow or spleen by mechanical disruption. Red blood cells were lysed, and the remaining leukocytes were washed with PBS, and resuspended in PBS + 2% FBS. Samples were analyzed on a FACSscalibur (BD) or LSRII (BD). Cell sorting for multiple markers was performed on a FACSAriaII (BD). Data were analyzed using FlowJo (Treestar).
Generation of MDSCs

The Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania approved all animal studies. Mice were housed under a 12-hour light/dark cycle and fed ad libitum. Three mouse ESCC models were used for production of MDSCs. The first is the L2-Cre:p120ctnﻭ mouse model, which has been described previously by us and involves the conditional knockout of p120ctn in the squamous oral cavity, esophagus and forestomach, resulting in invasive squamous cell cancer with desmoplasia and the specific recruitment of MDSCs (Stairs et al., 2011). We used also two syngeneic transplantation models utilizing the HNM007 and AKR esophageal squamous cell carcinoma (ESCC) cell lines. HNM007 or AKR cells were subcutaneously injected into C57BL/6J (Jackson Labs) and Cd38ﻭ mice (gift from Dr. Eduardo Chini). Mice were aged until tumors reached a volume of 0.8cm³. Spleens and bone marrow were harvested at euthanasia for MDSC isolation.

Colony formation and cell recovery assays

200,000 cells were seeded into 35 mm plates containing 1 ml MethoCult medium (M3534; Stem Cell Technologies). Anti-CD38 monoclonal antibody and IgG2a isotype control were used at 10ug/mL. Colonies were counted after 7 days. For recovery assays, 5x10⁵ MDSCs were seeded in complete RPMI 1640 medium supplemented with antibodies; cells were quantified by Trypan Blue exclusion using a Countess automated cell counter (Invitrogen).

T cell suppression

CD11b+Gr-1+, CD11b+Gr-1+CD38low, and CD11b+Gr-1+CD38high cell populations
were sorted by flow cytometry. Antigen-specific CD8$^+$ T cell suppression was tested as described previously (Stairs et al., 2011). Splenocytes from OT-1 mice were activated by addition of the OVA peptide to culture medium. The cultures were pulsed with $^3$H-labeled thymidine, and $^3$H incorporation was assessed 24 hours later to quantify cell proliferation rates.

**Ex vivo MDSC differentiation**

The generation of MDSCs from bone marrow has been described previously (Youn et al., 2008). Bone marrow cells were isolated from femurs and tibias of C57BL/6J mice, and the red blood cells were lysed. The remaining cells were seeded into 24-well plates (1×10$^6$ per well) in RPMI + 10% FBS supplemented with 0.1 ng/ml GM-CSF, 0.1 ng/ml IL-4, 50 μM 2-mercaptoethanol (Sigma), 10 ng/ml TNFα, 10 ng/ml IFNγ, 100 ng/ml IL-6 100 ng/ml CXCL16, 100 ng/ml IGFBP-3, and 50% v/v of either DMEM + 10% FBS or conditioned medium (CM) from HNM007 or AKR cells. For antibody treatment experiments, anti-CD38 monoclonal antibody and IgG2a isotype control antibody were added at 10μg/ml. The cells were cultured at 37°C in 5% CO$_2$ humidified atmosphere. On day 3 of culture the spent media were replaced with fresh media supplemented with cytokines and CM. The cells were harvested on day 5 and analyzed by flow cytometry.

**Cytokine Array**

Media from ex vivo differentiation cultures was collected and snap frozen after 1 or 5 days of culture. Array analysis was performed using the mouse cytokine array C3 (Raybiotech) according to the manufacturer’s protocol. Cytokines tested included Axl,
BLC, CD30 Ligand, CD30, CD40, CRG-2, CTACK, CXCL16, Eotaxin-1, Eotaxin-2, Fas Ligand, Fractalkine, G-CSF, GM-CSF, IFN-gamma, IGFBP-3, IGFBP-5, IGFBP-6, IL-1 beta, IL-10, IL-12 p40/p70, IL-12 p70, IL-13, IL-17, IL-1 alpha, IL-2, IL-3, IL-3 Rb, IL-4, IL-5, IL-6, IL-9, KC/CXCL1, Leptin/OB, Leptin R, LIX, L-Selectin, Lymphotactin, MCP-1, MCP-5, M-CSF, MIG, MIP-1 alpha, MIP-1 gamma, MIP-2, MIP-3 beta, MIP-3 alpha, PF-4, P-Selectin, RANTES, SCF, SDF-1 alpha, sTNFRI, sTNFRII, TARC, TCA-3, TECK, TIMP-1, TNF alpha, Thrombopoietin, VCAM-1, and VEGF-A. Quantification of results was performed using the ImageJ protein array analyzer. Results were normalized to positive controls to allow for comparison of relative expression levels.

**ESCC/MDSC Co-transplantation**

C57BL/6J recipient mice from Jackson Labs were injected subcutaneously with a mixture of 2.5x10^5 syngeneic HNM007 tumor cells with either 2.5x10^5 CD38<sub>low</sub> or CD38<sub>hi</sub> MDSCs obtained from HNM007 tumor-bearing C57BL/6J mice. Recipient mice injected with 2.5x10^5 syngeneic HNM007 tumor cells alone served as controls. Measurements were taken every 2-3 days once tumors became palpable.

**Statistical analysis**

The Student t-test was used to determine whether there is significant difference between two experimental groups (P≤0.05 was considered statistically significant).
Figures and figure legends
Figure 1

(A) CD38<sup>high</sup> and CD38<sup>low</sup> MDSCs from tumor-bearing p120<sup>−/−</sup> mice were used in a T-cell suppression assay (n=3; *, P=0.0007). (B) C57BL/6 mice were injected with HNM007 cells in combination with MDSCs (CD38<sup>high</sup> or CD38<sup>low</sup>) or alone (n= 5 per group). Tumor volumes were compared between the CD38<sup>high</sup> and CD38<sup>low</sup> groups (*, P=0.004 and 0.03), and between CD38<sup>high</sup> and control HNM007 tumors (**, P= 0.01, 0.003, and 0.01). (C) Splenic MDSCs from HNM007 tumor-bearing Cd38<sup>−/−</sup> or wt mice were used in a T-cell suppression assay (*, P=0.003 and 0.04). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.

Figure 3-1: CD38<sup>high</sup> MDSCs are more immunosuppressive and promote tumor growth more efficiently than the CD38<sup>low</sup> MDSCs. (A) CD38<sup>high</sup> and CD38<sup>low</sup> MDSCs from tumor-bearing p120<sup>−/−</sup> mice were used in a T-cell suppression assay (n=3; *, P=0.0007). (B) C57BL/6 mice were injected with HNM007 cells in combination with MDSCs (CD38<sup>high</sup> or CD38<sup>low</sup>) or alone (n= 5 per group). Tumor volumes were compared between the CD38<sup>high</sup> and CD38<sup>low</sup> groups (*, P=0.004 and 0.03), and between CD38<sup>high</sup> and control HNM007 tumors (**, P= 0.01, 0.003, and 0.01). (C) Splenic MDSCs from HNM007 tumor-bearing Cd38<sup>−/−</sup> or wt mice were used in a T-cell suppression assay (*, P=0.003 and 0.04). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 3-2: HNM007, but not AKR ESCC cell line promotes expansion of CD38$^{\text{high}}$ MDSCs. Splenocytes from AKR or HNM007 syngeneic subcutaneous transplant tumor-bearing mice were analyzed by flow cytometry. CD38 expression levels on the CD11b$^+$Gr-1$^+$ subpopulation are shown as histograms.
Figure 3-3: HNM007 conditioned medium, IFNγ and TNFα induce CD38 expression in an *ex vivo* differentiation model. (A) Scheme of the *ex vivo* differentiation assay: bone marrow (BM) extracted from naïve C57BL/6 mice is cultured for 120 hours in the presence of conditioned media (CM) from either AKR or HNM007 cell lines, supplemented with a cocktail of cytokines. (B) CD38 expression in CD11b*Gr-1* cells from *ex vivo* differentiation cultures was tested by FACS (MFI = mean fluorescence intensity; n=3; *P=10^-4; **P=6x10^-4; ***P=2.5x10^-5*). P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
Figure 3-4: IL-6, IGFBP3 and CXCL16 are novel regulators of CD38 expression in an *ex vivo* differentiation model. (A) Scheme of the *ex vivo* differentiation model assay, followed by a cytokine array. (B) CD38 expression in CD11b*Gr-1* cells from *ex vivo* differentiation cultures was tested by FACS (MFI = mean fluorescence intensity; n=3; *P=0.05; **P=0.005). P-values calculated via Student's t-test, p≤0.05 considered statistically significant.
Figure 5

A

B

C

D

E

Figure 5

A

B

C

D

E
Figure 3-5: Anti-CD38 monoclonal antibody inhibits Gr-1$^{+}$CD11b$^{+}$ expansion and survival. (A) Colony formation of sorted Gr-1$^{+}$CD11b$^{+}$ from 6-8 month old p120$^{-/-}$ mice grown in methylcellulose-based medium containing cytokines for the detection of granulocyte-macrophage progenitors (CFU-GM, CFU-G, CFU-M). Colonies were counted following treatment with anti-CD38 monoclonal antibody (NIMR-5) or isotype (IgG2a) control (10ug/ml) for 7 days. Results are representative of two different experiments performed in triplicate. (* p=4x10$^{-5}$) (B) Colonies observed after 7 days of culture with anti-CD38 or isotype control. (C) Cell recovery of sorted Gr-1$^{+}$CD11b$^{+}$ from p120$^{-/-}$ mice. Gr-1$^{+}$CD11b$^{+}$ cells were isolated, cultured in RPMI with anti-CD38 or isotype control antibody, and quantified at indicated time points. (* p=0.0005, **p=5x10$^{-7}$) (D) Ex vivo differentiation of wt bone marrow in the presence of anti-CD38 or isotype control antibody. Cells were cultured for 5 days, and CD38 expression (detected using anti-CD38 clone 90) on the surface of CD45$^{+}$7-AAD Gr-1$^{+}$CD11b$^{+}$ was measured by flow cytometry (MFI = mean fluorescence intensity; * p=0.005, ** p=0.0005). Each treatment was performed in triplicate. P-values calculated via Student’s t-test, p≤0.05 considered statistically significant.
**Figure 3-6: Model.** In mice, tumor progression leads to MDSC expansion. Tumor progression leads to amplified signals (such as cytokines) reaching MDSCs, which induces a differentiation halt and expansion of CD38\textsuperscript{high} MDSCs with enhanced immunosuppressive capacity [mediated by iNOS, which produces nitric oxide (NO)].
Tables and table legends
Table 1

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<td>VEGF</td>
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**Table 3-1:** Cytokines differentially secreted in ex vivo bone marrow differentiation cultures associated with high CD38 expression. Cytokine array was performed with media from ex vivo differentiation cultures (24 or 120 hours). Each cytokine tested in duplicate. Difference in normalized expression between HNM007 and AKR groups is shown.
The focus of this thesis was on the mechanisms of dynamic interaction between the tumor cells and certain components of the tumor microenvironment (CAFs, immune cells) in esophageal cancer. This was prompted by previous research performed by the Rustgi lab members, as well as other publications.

The tumor cell – CAF cross-talk

Our lab has a long-standing interest in the role of stroma, particularly CAFs, in esophageal carcinogenesis. For example, our lab reported a pro-invasive induction of cathepsin B in ESCC cells, which was induced by a fibroblast-secreted factor (Andl et al., 2010). Another report from our lab elucidated the mechanism where fibroblast-derived HGF promotes invasion of ESCC cells into the ECM via activation of Met receptor signaling within the ESCC cells (Grugan et al., 2010). Furthermore, research from our lab identified the matricellular protein periostin as a stroma-derived factor essential for invasion of ESCC cells into the ECM (Michaylira et al., 2010). Finally, a recent report from our lab has focused on the function of another matricellular protein – SPARC – in stromal expansion and metastasis in a murine model of pancreatic cancer (Heeg et al., 2016). Not surprisingly, when we investigated the impact of co-culture with CAFs on gene expression in ESCC cells, we found the genes encoding periostin, SPARC and HGF to be strongly upregulated in response to interaction with fibroblasts (Chapter 2, Figure 1a).

As described in detail in Chapter 2, we chose to study the dynamic interaction between CAFs and tumor cells in the esophageal cancer microenvironment, focusing on IL-6 and RANTES, the cytokines that mediate (at least partially) this interaction. While in this thesis we focus mainly on the role of tumor cell-derived cytokines, it is essential to
keep in mind that, in addition to the mechanisms described herein (regulation of tumor cell invasion, proliferation and apoptosis), IL-6 and RANTES are likely to be promoting tumorigenesis in many other ways. Such mechanisms can include resistance to chemotherapeutic and radiotherapy, expansion of reactive stroma, lympho- and angiogenesis, induction and maintenance of chronic inflammation, as well as formation of an immunosuppressive microenvironment (addressed, in part, in Chapter 3). Accompanied by their roles in such quality-of-life-affecting conditions as cancer cachexia (Narsale and Carson, 2014) and cancer-related pain (Hang et al., 2013), these observations make IL-6 and RANTES attractive targets for cancer therapy. We provide in this thesis evidence of the anti-tumorigenic effects of treatment with tocilizumab or maraviroc in vivo (Chapter 2, Fig. 6,8) and the superiority of combination treatment, compared to either drug alone, in vitro (Chapter 2, Fig. 9). We believe that this approach would bring improved survival and quality of life to esophageal cancer patients.

Cross-talk between MDSCs and tumor cells or CAFs

The interest in myeloid-derived suppressor cells and their role in ESCC was sparked by the fairly unexpected results of investigating the changes in immune cell populations in response to tumor progression in the L2-cre:p120^{"f/f"} mouse model of ESCC (Stairs et al., 2011). One of the most pronounced features of tumor-bearing L2-cre:p120^{"f/f"} mice was splenomegaly, and upon immunophenotyping of these splenocytes, the only population that significantly expanded in response to tumor progression were the CD11b^{+}Gr-1^{+} MDSCs. We set out to determine the genes that could be mechanistically linked to alternative differentiation of normal immature myeloid cells into MDSCs in tumor-bearing animals, and identified Cd38, a gene encoding a cell surface-
expressed ectoenzyme receptor (Karakasheva et al., 2015). As described in detail in Chapter 3, we proceeded to identify a panel of cytokines, specifically IFNγ, TNFα, IL-6, CXCL16, IGFBP-3 and RANTES, that are responsible for the induction of CD38 expression by MDSCs in ESCC. Importantly, these cytokines can be produced by multiple components of the TME. For example, IL-6 and RANTES, which are a major focus of this thesis, are known to be secreted by the tumor cells, CAFs, immune cells, as well as endothelial cells and adipocytes. CXCL16 can also be produced by CAFs (Allaoui et al., 2016) and dendritic cells (Matloubian et al., 2000), while IGFBP-3 can be produced in the liver, kidney and smooth muscle (Ranke, 2015). These facts suggest the existence of intricate signaling networks that regulate the processes contributing to the maintenance of immunosuppressive microenvironment, including the expansion of CD38+ MDSC population.

**Future perspectives**

The findings presented in this thesis demonstrate the potential of disrupting the interaction between tumor cells and stromal components (CAF, MDSC) for cancer therapy. Furthermore, in many cases this can be achieved by repurposing of drugs already in the clinic: tocilizumab, maraviroc and daratumomab have already been extensively tested and approved by the FDA, which should dramatically simplify the translation of our findings from the bench to the bedside.

Although the therapeutic approaches investigated herein resulted in significant decreases in tumor growth rates, neither was capable of completely eliminating the tumor. Therefore, future breakthroughs in cancer therapy will likely depend on improvements in drug design and combination therapies. In addition to the combination
of tocilizumab and maraviroc proposed in Chapter 2, it is reasonable to expect that addition of conventional chemo- or radiotherapy to this treatment scheme could provide improved outcomes. At the moment, a new generation of IL-6Rα targeting antibodies is being rationally developed in such a way so as to specifically block IL-6 trans-signaling, which is considered to be more relevant to carcinogenesis (Hunter and Jones, 2015). In contrast to targeting the receptors, it could be beneficial to target downstream components of the signaling pathways these receptors activate, such as combining small molecule inhibitors of STAT3 and MEK/ERK as described in Chapter 2. This approach can decrease the opportunities of cancer cells to acquire resistance to targeted therapies, as well as potential compensation by other cytokines in the TME. Finally, CD38-targeted therapy could also benefit from a combination with cytotoxic drugs or radiation: these approaches are known to activate CD8+ T cells, which would in this case cease being suppressed by MDSCs and proceed to clear tumor cells.

We are also looking forward to expanding our findings from Chapter 2 onto other types of cancer. Based on published research and in silico analyses of gene expression profiles from the TCGA database, it is evident that the relevance of both IL-6 and RANTES spans beyond esophageal cancer: breast cancer (including the most aggressive triple-negative subset), KRAS-mutant lung cancer, and colon cancer are taking advantage of signaling induced by these cytokines. Therefore, we plan to test the targeted therapies evaluated in this thesis in murine models of non-GI cancers.
In regard to the basic science aspect of the research presented herein, many things remain to be investigated. For example, while in Chapter 3 we defined a subset of MDSCs (CD38\textsuperscript{high} monocytic MDSC) that is more immunosuppressive than the total MDSC population; this does not diminish the functional impact of granulocytic MDSCs on cancer progression. We are working on developing a murine model that would allow us to distinguish more accurately between the roles that each MSDC subset plays in carcinogenesis. This model utilizes the “suicide gene” methodology by driving expression of the herpes simplex virus thymidine kinase under either the Ly-6C or Ly-6G promotor. Administration of ganciclovir to these animals will result in specific depletion of monocytic or granulocytic MSDCs, respectively. In addition, given the differences between murine and human immune systems in general, and the myeloid compartment in particular, it would be extremely interesting to investigate the role of CD38 in the biology of MDSCs from human cancer patients.

So far, our research described in Chapter 2 has been focused on the function of tumor cell-derived cytokines. Yet, the mechanism through which CAFs in the TME induce the tumor cells to secrete those factors (Fig. 1) remains to be elucidated. It is likely that multiple factors, both soluble and membrane-bound, regulate this process. Identifying these factors would be of utmost importance, since they can also be utilized as targets for cancer therapy.

In conclusion, this thesis provides novel insight into the mechanisms of dynamic interaction, or cross-talk, between the tumor cells and two components of the TME (CAFs and MDSCs) in esophageal cancer. Interestingly, the findings from both Chapters 2 and 3 demonstrated the importance of the same two cytokines – IL-6 and RANTES –
in these processes. We have also evaluated a number of novel therapeutic approaches, all of which proved to be effective in our murine models of ESCC. We propose that these approaches, individually or in combination, might improve existing approaches to cancer therapy by disrupting the complex signaling networks in the tumor microenvironment.
Figures and figure legends
Figure 1

- **Chronic inflammation**
- **↑ proliferation**
- **↓ apoptosis**
- **↑ invasion**
- **↑ tumor growth**

- **IL-6 RANTES**
- **Immunosuppressive microenvironment**
- **↑ tumor growth**

**Tumor cell**  **CAF**  **MDSC**  **IMC**  **CD38**  **CTL**  **IL-6**  **RANTES**
**Figure 4-1: Model.** Chronic inflammation leads to activation of normal fibroblasts and their conversion into cancer-associated fibroblasts (CAFs). CAFs acquire a new secretory profile, producing pro-tumorigenic cytokines, including IL-6 and RANTES. CAFs also interact with the tumor cells directly and alter their gene expression profile, enabling tumor cells to secrete high levels of IL-6 and RANTES. These two cytokines activate corresponding receptors (IL-6Rα and CCR5) on both tumor cells and CAFs in an autocrine-paracrine manner, which results in activation (to a different extent) of the STAT3 and ERK signaling pathways. These events result in increased tumor cell proliferation, decreased apoptosis, increased invasion and promotes overall tumor growth. IL-6 and RANTES also contribute (via induction of CD38 expression) to alternative differentiation of normal immature myeloid cells (IMCs) into functional myeloid-derived suppressor cells (MDSCs), which establishes an immunosuppressive microenvironment within the tumor by suppressing the cytotoxic T cells (CTLs), and therefore contributes to increased tumor growth.
### APPENDIX: list of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADPR</td>
<td>Adenosine diphosphate ribose</td>
</tr>
<tr>
<td>ARG1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>Breg</td>
<td>Regulatory B cell</td>
</tr>
<tr>
<td>cADPR</td>
<td>Cyclic adenosine diphosphate ribose</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer-associated fibroblast</td>
</tr>
<tr>
<td>CCL5</td>
<td>C-C motif chemokine ligand 5</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C motif chemokine receptor 5</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCL16</td>
<td>C-X-C motif chemokine ligand 16</td>
</tr>
<tr>
<td>CXCR6</td>
<td>C-X-C motif chemokine receptor 6</td>
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<tr>
<td>EAC</td>
<td>Esophageal adenocarcinoma</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ESCC</td>
<td>Esophageal squamous cell carcinoma</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>GERD</td>
<td>Gastroesophageal reflux disease</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-6R</td>
<td>Interleukin-6 receptor</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>M-MDSC</td>
<td>Monocytic myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>NAD</td>
<td>Nocotinamide adenine nucleotide</td>
</tr>
<tr>
<td>PMN-MDSC</td>
<td>Perimorphonuclear myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
</tr>
<tr>
<td>TAN</td>
<td>Tumor-associated neutrophil</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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