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## Visualisation of Myelomonocytic Cells in Tumors

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### 1. Introduction

Emerging evidence suggests that inflammation is one of the major contributing factors for tumor development and progression. In this context, myelomonocytic cells, as key mediators of inflammatory responses, are essential components of the malignant microenvironment. Numerous studies have provided evidence to show that infiltrating tumor-associated macrophages (TAMs) play a critical role in promoting tumor growth. Similarly, human studies also revealed that a high frequency of infiltration by TAMs is associated with poor prognosis in many human cancers (reviewed in (Bingle et al. 2002)). There is now substantial evidence that tumor-associated neutrophils (TANs), like TAMs, have a critical role in tumor development (reviewed in (Gregory and Houghton 2011)). Despite major research efforts in this area, the role of neutrophils in cancer pathogenesis remains controversial. This is likely due to the fact that neutrophils can play a dual role in primary tumors: neutrophils can mediate tumor rejection but also promote angiogenesis and tissue remodelling which favour tumor growth. Furthermore, emerging evidence suggests that neutrophils may also have the ability to promote metastasis. Hence there is great potential for novel neutrophil-based therapies in the treatment of cancer if the contrasting roles of neutrophils in tumor growth are fully elucidated.

In this chapter, we will first briefly introduce the methodology of intravital multi-photon microscopy (MP-IVM), and then provide an overview of the application of MP-IVM for the visualisation of immune cells in tumor models. In addition, we will summarize how this technique has helped to reveal the unique interactive behavior of myelomonocytic cells, in particular neutrophils, during inflammatory responses. Our intention is to discuss the practical aspects of MP-IVM applications, with the aim of highlighting the features of MP microscopy that make it an ideal tool for investigating immune cell-tumor interactions *in vivo*.

### 2. Neutrophils in immunity

The immune system plays a well-established role in protecting the body from a wide variety of infectious diseases and in the elimination of endogenous tumors. In both cases,

immediate but non-specific protection is mediated by the innate immune system, while the adaptive immune system provides more directed 'antigen-specific' responses and immune memory. Neutrophils, traditionally viewed as 'first wave' responders, can perform a diverse array of functions. Despite the central role of neutrophils in the acute inflammatory response, their role in immunity has only recently been "rediscovered". It is possible that neutrophils have been overlooked because they were considered to be too short-lived to play a significant role in the immune response as it evolved over time. Based on *in vitro* assays, neutrophils were thought to have a lifespan <1 day, and only 4-8 hours for activated neutrophils (Dancey et al. 1976). However, more rigorous analysis using *in vivo* deuterium-labeling has shown non-activated neutrophils to have a lifespan of 5.4 days under homeostatic conditions (Pillay et al. 2010). Although the major effector function of neutrophils is to induce rapid destruction of targets by phagocytosis and oxidative burst, emerging evidence suggests that neutrophils also play a crucial role in shaping the subsequent adaptive immune response. Neutrophils have a major effect on the recruitment and activation of additional immune cells via the release of soluble factors such as IL-8 and CXCL10. In certain circumstances, neutrophils were even shown to suppress the adaptive immune response by secreting anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor- $\beta$ ) (reviewed in (Kasama et al. 2005)). Together, these data therefore point to a growing need to reassess the role of neutrophils in immunity.

### 3. Neutrophils in cancer

There is evidence to suggest that neutrophils play an important role in promoting human cancers. Clinical studies have shown that an increase in tumor-infiltrating neutrophils is correlated with a poorer outcome in bronchioalveolar carcinoma (Bellocq et al. 1998) and localized renal cell carcinomas (Jensen et al. 2009). Moreover, CD11b<sup>+</sup> CD15<sup>+</sup> neutrophils and monocytes with immunosuppressive properties were expanded in the peripheral blood of patients with malignant melanoma, and this correlated with disease stage (De Santo et al. 2010). Similarly, elevated numbers of peripheral blood neutrophils and monocytes have been associated with poor prognosis in patients with metastatic melanoma (Schmidt et al. 2005). Despite the clinical associations reported, the precise role of neutrophils in cancer remains controversial (Houghton et al. 2010; Granot et al. 2011).

There is evidence from animal studies that under some circumstances neutrophils can contribute to tumor rejection whilst under other conditions neutrophils can promote tumor growth. These two opposing phenotypes have been termed N1 and N2 by analogy to M1 and M2 polarised macrophages (Fridlender et al. 2009). Under "N1" conditions, neutrophil recruitment and activation can result in direct tumor cell killing by reactive oxygen species and further recruitment and activation of monocytes, macrophages, dendritic cells, natural killer and cytotoxic T cells (Fridlender et al. 2009). Under "N2" conditions, however, the non-specific tissue destruction and tissue remodelling induced by neutrophil-derived metalloproteases can create space, and angiogenesis induced by neutrophil-derived IL-8 and VEGF can ensure ongoing supply of oxygen and nutrients to support further tumor growth and also promote metastasis (reviewed in (Noonan et al. 2008; Gregory and Houghton 2011)). Immunosuppressive cytokines such as IL-10 and TGF-beta secreted by neutrophils can dampen anti-tumor adaptive immune responses. Furthermore, neutrophil products, such as neutrophil elastase, can directly promote tumor growth (Houghton et al. 2010).

Consistent with this, depletion of Ly6G (Gr-1) positive neutrophils was shown to inhibit tumor growth and angiogenesis in the B16-F10 mouse melanoma model (Jablonska et al. 2010). It is likely that the type of neutrophil response against a tumor is highly dependent on distinct temporal and anatomical factors. Consistent with this view, a study using melanoma cell lines transduced with the neutrophil chemoattractant IL-8 showed a biphasic dose-response curve between the number of infiltrating neutrophils and tumor growth (Schneider et al. 2003). Thus, it is possible that in the early stages of tumor development, neutrophils have an anti-tumor N1 phenotype and that later on neutrophils are conditioned to adopt a pro-tumor N2 phenotype.

Like other cells of the immune system, neutrophils appear to rely on cues from the microenvironment to regulate their functional plasticity, i.e. switching between anti-tumor and tumor-promoting functions. For instance, TGF- $\beta$  can skew the population towards N2 phenotype, whilst blockade of this molecule induces an anti-tumor N1 phenotype (Fridlender et al. 2009). Inhibition of PPAR- $\alpha$  signals was found to switch tumor-promoting neutrophils into neutrophils with anti-tumor activities (Kaipainen et al. 2007). Delineation of neutrophil behaviour in different settings is therefore vital to our understanding of the precise role that these cells play in tumor growth and progression as well as to assess their potential as targets for therapeutic intervention.

#### 4. Neutrophils in tumor metastasis

In addition to their role in primary tumors, neutrophils may also significantly influence tumor metastasis. Tumor metastasis is strongly associated with reduced survival of the host, making this process an important target for therapeutic intervention. Metastasis is a non-random process in which tumor cells acquire the capacity to seed particular organs, a phenomenon first observed more than 100 years ago by Stephen Paget and known as the 'seed' and 'soil' hypothesis (Paget 1889). Evidence that immunological factors (for example, cytokines, chemokines, and proteases) may direct metastasising cells to specific organs by creating permissive microenvironments for the tumor cells, led to the development of the 'pre-metastatic niche' concept (reviewed in (Kaplan et al. 2006)). Neutrophils, together with other bone marrow derived cells, have been implicated in establishing such niches (Yamamoto et al. 2008), although the precise role of neutrophils remains to be elucidated. Neutrophils may play an important role during tumor metastasis by establishing specific microenvironments that promote tumor cell 'seeding' of secondary sites. For instance, neutrophils can release tissue-remodelling factors such as matrix metalloproteases, which may assist in the establishment of a physiological niche, and also secrete chemoattractants that may recruit other cells. Interestingly a recent study by Granot et al. suggests that at least in certain conditions neutrophils 'entrained' by the tumor may actually inhibit metastasis by generating hydrogen peroxide (Granot et al. 2011). This further emphasises the potential complexity of neutrophil functions in cancer and highlights the need for further investigation.

#### 5. Application of multi-photon microscopy in tumor-related research

Imaging has become an important tool in cancer research. Perhaps one of the biggest advancements in recent years is in the field of fluorescence-based imaging. With the availability of a wide array of fluorescent reporter mice and the rapid development of cell- and tissue-specific labeling techniques (reviewed in (Germain et al. 2006; Hickman et al.

2009)), researchers can now address important questions in cancer biology *in vivo*. One fluorescence-based imaging approach that has received a lot of attention in recent years is MP microscopy. This technique has rapidly evolved beyond merely observational to address complex biological questions at a quantitative level. It is now possible to perform dynamic, multi-dimensional imaging to simultaneously track cell populations at the single cell level in living tissues or organs (Cahalan and Parker 2008). Consequently, anatomical, cellular, and molecular information can be obtained through this approach. For more detailed technical information about the general setup of a MP microscope, please refer to these publications (Germain et al. 2006; Phan and Bullen 2010).

### **5.1 *In vivo* imaging of tumor development using multi-photon microscopy**

Over the past few years, a number of laboratories have applied MP microscopy to investigate tumor-related biological questions. In 2001, a seminal study from Jain laboratory showed the applicability of MP imaging for studying tumor cell development *in vivo* for the first time. In this study, MP-IVM was employed to investigate cell behavior in a dorsal skin fold chamber tumor model in mice. Jain and colleagues visualized tumor cell localization, angiogenesis, as well as leukocyte-blood vessel interactions and vessel permeability with high spatial and temporal resolution (Brown et al. 2001). Since then this approach has been adopted by researchers in the field of tumor biology to study tumor cell migration and tissue invasion, as well as angiogenesis, matrix remodeling and metastasis (reviewed in (Zal and Chodaczek 2010)).

### **5.2 *In vivo* imaging of TAMs**

Although macrophages have been implicated in many aspects of tumor development, the spatial and temporal regulation of TAMs in the context of the tumor microenvironment is still poorly understood. In 2007, the Condeelis lab provided detailed information about the localization of macrophages and tumor cells in relation to blood vessels in a three-dimensional (3D) context *in vivo*. TAMs were shown to be predominantly located in the tumor margin. In addition, Condeelis and colleagues reported the presence of a population of perivascular macrophages deep within tumors (Wyckoff et al. 2007). Importantly, this study provided evidence to support the notion that abluminally localized perivascular macrophages are important for the intravasation of tumor cells (Wyckoff et al. 2007). In another study of MP imaging of TAMs, Pittet laboratory demonstrated a novel method to specifically label M2 polarized TAMs with nanoparticles (AMTA680), thereby allowing the tracking of this subset of TAMs *in vivo* by MP-IVM (Leimgruber et al. 2009).

### **5.3 *In vivo* imaging of tumor killing cytotoxic cells**

Arguably, the best-characterised immune imaging model during the early days of MP microscopy was that of B and T cell behaviour in lymph nodes. Most of these studies mainly focused on naïve T cells and their locomotion within the lymph node during priming (Bousso and Robey 2003; Mempel et al. 2004; Miller et al. 2002). Results from these experiments provided important framework for the subsequent studies of effector cells within target tissues. It is now well established that effector cells such as CD8 cytotoxic T cells (CTLs) and NK cells play a crucial role in host defence against malignant cells and viruses. In early MP imaging studies, three independent laboratories analysed the behaviour

of infiltrating cytotoxic CD8<sup>+</sup> T cells (CTLs) in solid tumors (Boissonnas et al. 2007; Breart et al. 2008; Mrass et al. 2006)). These studies revealed for the first time the migratory and interactive behaviour of intratumoral CTLs at different stages of tumor development, for example during progressing or regressing stages. One key observation was that CTL motility and long lasting interactions with tumor cells were dependent on the presence of cognate antigen (Boissonnas et al. 2007; Mrass et al. 2006). In addition, the Weninger lab showed that physical interaction between CTLs and tumor cells preceded the initiation of the killing of the tumor cells (Mrass et al. 2006). A study by the Bousso lab further examined the kinetics of tumor cell killing by CTLs *in vivo*. Surprisingly, this study demonstrated that it required on average 6 hours for a CTL to destroy one tumor cell, which was much longer than expected based on previous *in vitro* results (Breart et al. 2008). A subsequent study from the same lab showed that Natural Killer (NK) cell dissemination and motility within the tumor were highly dependent on the presence of its ligand, Rae-1 $\beta$ . Although it is known that both CTLs and NK cells mediate similar cytotoxic activity (Russell and Ley 2002), this study showed that NK cells had a distinct intratumoral behaviour compared to CTLs. Tumor infiltrating NK cells only formed transient contacts with target cells after initial interaction, whereas CTLs normally establish long lasting contacts that can last for more than 20 minutes (Deguine et al. 2011).

## 6. Dynamic view of neutrophil responses

As outlined in the previous section, there is now a wealth of knowledge about the behaviour of adaptive immune cells in primary tumors. Neutrophil dynamics in tumors and other inflammatory settings remain largely unexplored. However, recent studies utilizing intravital microscopy have provided some insight into the mechanisms of neutrophil adhesion and transmigration across the endothelium into the tissues, as well as the dynamics of neutrophil interstitial migration to or within their target sites (McDonald and Kubes 2011). Specifically intravital imaging studies including infection models in the skin, lymph nodes, and lungs have provided us with the first clues as to how neutrophil interstitial migration is regulated *in vivo* (Chtanova et al. 2008; Kreisel et al. 2010; Peters et al. 2008; Zinselmeyer et al. 2008). A seminal study by Sacks and colleagues provided the first *in vivo* observations of neutrophil recruitment from the blood vessels into the skin in response to *Leishmania Major* transmitted by sand bite (Peters et al. 2008). Interestingly, neutrophils readily internalised the parasites but failed to destroy them, thus aiding parasite dissemination by acting as Trojan horses.

Another important step in characterising neutrophil function *in vivo* came from a study of the immune response to *Toxoplasma gondii* infection in mice. Analysis of neutrophil behaviour in toxoplasma-infected lymph nodes revealed a strikingly coordinated pattern of migration with multiple cells simultaneously responding to an external signal to coalesce into dynamic swarms (Chtanova et al. 2008). Interestingly, neutrophil swarm formation coincided in space and time with the removal of the underlying tissue suggesting that swarm formation may present a mechanism for large scale tissue remodelling. This process could in turn provide a means for early and rapid removal of infected tissues preventing pathogen dissemination. Furthermore, these neutrophils also destroyed infected CD169<sup>+</sup> subcapsular sinus (SCS) macrophages found in the lymph nodes. These SCS macrophages play a key role in sampling antigen from the periphery drained by the lymph, and are responsible for antigen presentation to B cells (Phan et al. 2007), CD8<sup>+</sup> T cells (Chtanova et

al. 2009) and NK T cells (Barral et al. 2010). Thus, the results of this study imply that not only can neutrophils clear infected cells but may also indiscriminately destroy cells that are important for the initiation of anti-pathogen immune responses. Dynamic clustering (or swarming) of neutrophils was also observed in lung tissues in response to bacterial challenge (Kreisel et al. 2010)

These findings suggest that specific molecular cues guide neutrophils through the compacted interstitial tissues towards the foci of infection/inflammation. Indeed, a recent study by Kuberski lab using *in vivo* confocal microscopy to examine sterile liver injury has uncovered several molecular pathways involved in neutrophil sensing of injury. The authors showed that the exit of neutrophils from the circulation depended on ATP from the necrotic cells, and the Nlrp3 inflammasome, whereas migration towards the site of injury required a chemokine gradient and formyl-peptide signals as guidance cues (McDonald et al. 2011).

Using MP-IVM, we have recently characterised the cascade of events and molecular cues that guide neutrophils through the extravascular space within skin (Figure 1). In this study, we demonstrated that following confined physical injury, neutrophil accumulation at the injury site takes place in three distinct sequential steps (Ng et al. 2011). Initially, rare scouting neutrophils migrate in a directional manner towards the injury site. This is followed by the amplified attraction of additional waves of neutrophils in a highly

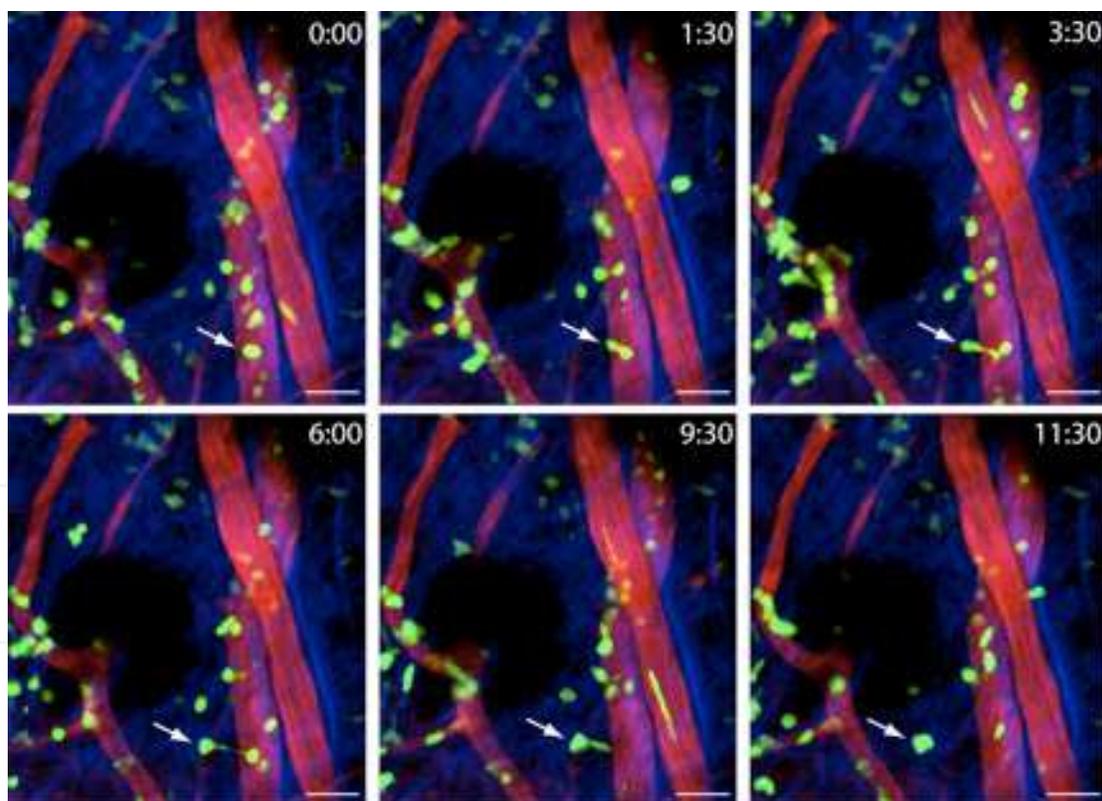


Fig. 1. Multiphoton imaging of neutrophil responses in Lysozyme-GFP mice

Time-lapse images showing the applicability of Lysozyme-GFP mice for studying neutrophil dynamic *in vivo* (skin), allowing co-visualization of neutrophils (green, tagged by green fluorescent protein), blood vessels (red, highlighted by Evans blue injection) and dermal collagen fibers (blue, detected through second harmonic generation signals). White arrows indicate the migratory path of an extravasating neutrophil over time. Time: min:sec. Scale bar: 30  $\mu$ m.

synchronized manner, and finally there is a stabilisation of the neutrophil cluster around the injury. Interestingly, while neutrophil migration during steady-state conditions and during the scouting phase depended on G protein-coupled receptor signaling, the amplification phase was sensitive to interference with the cyclic adenosine diphosphate ribose pathway (Ng et al. 2011).

Together, these studies show that neutrophil responses to pathogen or injury are regulated by complex mechanisms. These observations also clearly demonstrate that neutrophil responses are highly dependent on the nature of the initial stimuli, injury versus pathogenic. Understanding the molecular cues governing the distinct neutrophil behaviour in response to different stimuli may lead to new knowledge about neutrophil function.

## 7. Mouse models and experimental considerations

As outlined above are heavily involved in immune responses to pathogens and injury, and that their recruitment and behaviour are intricately controlled. Although neutrophil dynamics in the context of tumors remain to be visualised, it is likely that a similarly complex picture will emerge for neutrophils inside primary tumors and at potential sites of metastasis. Here we will provide information about the tools available for *in vivo* microscopic examination of neutrophil-tumor interactions. In addition, we will also several experimental considerations in applying MP microscopy to visualise intratumoral cellular activities.

### 7.1 Visualizing neutrophils, blood vessels and extracellular matrix structures in tumors

Currently, there is a wide array of fluorescent transgenic mice suitable for MP microscopy. Lysozyme-GFP (Faust et al. 2000) and MacGreen-GFP mice (MacDonald et al. 2005) are the most widely used mice for direct visualisation of neutrophils *in vivo*. Although all myeloid cells in these mice express GFP, neutrophils express the highest amount of the fluorescent protein (Ng et al. 2011), which makes them distinguishable from monocytes or macrophages based on the fluorescent intensity and cell morphology. To visualize blood vessels, quantum dots, fluorescent dextrans and lectins or Evans blue dye can be injected intravenously (Ng et al. 2011; Hickman et al. 2009; Germain et al. 2006; Li et al. 2012). Furthermore, changes in the extracellular matrix within tumors can be monitored by second and third harmonic generation signals (Friedl et al. 2007). Alternatively cells of implanted tumors can be labeled by genetically expressing a fluorescent protein of choice (Mrass et al. 2006; Wyckoff et al. 2007).

### 7.2 *In vivo* versus explanted tumor imaging

Most tumor imaging studies have been performed *in vivo*, either through skin flaps or the skin window chamber approach (Wyckoff et al. 2007; Boissonnas et al. 2007). Although these approaches are useful, they only allow a limited field of view of the tumor cells due to physical constraints. Furthermore, some tumors are inaccessible. These limitations can be circumvented by using tumor explant models (Mrass et al. 2008; Mrass et al. 2006), which allow imaging of multiple fields of view per explanted tumor. The ability to image multiple regions is important, as tumor tissues are highly heterogeneous in composition in terms of tumor cell density, extracellular matrix remodeling and angiogenesis, as well as cellular infiltration. However, important drawbacks of this approach include lack of blood circulation and lymphatic flow, as well as innervations.

### 7.3 Choice of tumor models

When selecting an appropriate tumor model for MP imaging in addition to the biological question being asked, it is important to review several technical considerations. For instance, we have observed that pigmented tissues and cells are highly sensitive to MP illumination due to their high MP absorption, and contribute to the generation of non-specific high intensity signals (“speckling”) (Ng et al. 2011; Li et al. 2012). This speckling not only compromises the image quality, but also induces heat injury that can trigger an inflammatory response and severely damage the tissue. We therefore recommend the use of non-pigmented tumor models in order to minimise artifacts caused by photodamage during imaging.

## 8. Neutrophil dynamics in tumors – A prospective

Great strides have been made in understanding the function of different immune cell subsets in cancer. Intravital MP imaging has played an important role in this process, and has allowed us to view CD8 T cells and NK cells killing tumor cells, and TAMs aiding tumor intravasation *in vivo*. However, many more questions remain unanswered. This is particularly true with regard to neutrophils. Recent technological advances in MP microscopy together with an ever-growing array of biological tools now make it possible to unravel the role that neutrophils may play in tumor growth and metastasis by directing visualising their activities within their native environment.

Intravital imaging experiments have already demonstrated neutrophil recruitment and subsequent swarming in inflamed tissues during immune responses to injury and infection. We anticipate that a similarly complex picture will emerge for neutrophils inside primary tumors and also at potential sites of metastasis. We expect that N1 and N2 neutrophils might exhibit different patterns of behaviour. For instance, neutrophils of the anti-tumor phenotype might function as they do in infection, that is, by initiating cell recruitment followed by a coordinated response to external signals with formation of dynamic swarms, which remodel underlying tissue. On the other hand a change in neutrophil behaviour might reflect a conversion to a pro-tumor phenotype. Pro-tumor neutrophils maybe less motile reflecting their angiogenesis-promoting role. Thus, by directly observing the behaviour of these cells in tumors, we will be able to gain unique insight into their role in tumor growth.

## 9. Conclusion

In conclusion, although neutrophils are frequently dismissed as short-lived ‘foot-soldiers’ of immunity, recent studies point to an important contribution of these cells to cancer. Furthermore, the apparent functional plasticity of neutrophils makes them a great target for therapeutic intervention and further highlights the need to unravel the role of neutrophils in cancer pathogenesis. We envision that MP imaging will not only contribute to the basic knowledge related to tumor development, but also will gradually become an important tool for preclinical studies for assessing drug delivery to tumor cells as well as the effects of therapeutic agents on immune and tumor cells.

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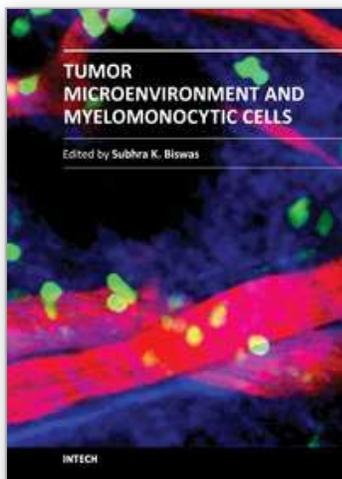
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## **Tumor Microenvironment and Myelomonocytic Cells**

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Tumor microenvironment represents an extremely dynamic niche shaped by the interplay of different cell types (e.g. tumor cells, stromal cells), their soluble products (e.g. cytokines, chemokines and growth factors) and varied physico-chemical conditions (e.g. low oxygen concentration or hypoxia). Recent studies have identified myelomonocytic cells as key players in regulating the tumor microenvironment and hence, tumor progression in a variety of cancers. In view of these findings, the present book attempts to provide a comprehensive account of the diversity of tumor microenvironment across different cancers and how myelomonocytic cells have taken the center-stage in regulating this niche to direct cancer progression. A better understanding of the myelomonocytic cells and the mechanisms by which they regulate cancer progression will open new vistas in cancer therapeutics.

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