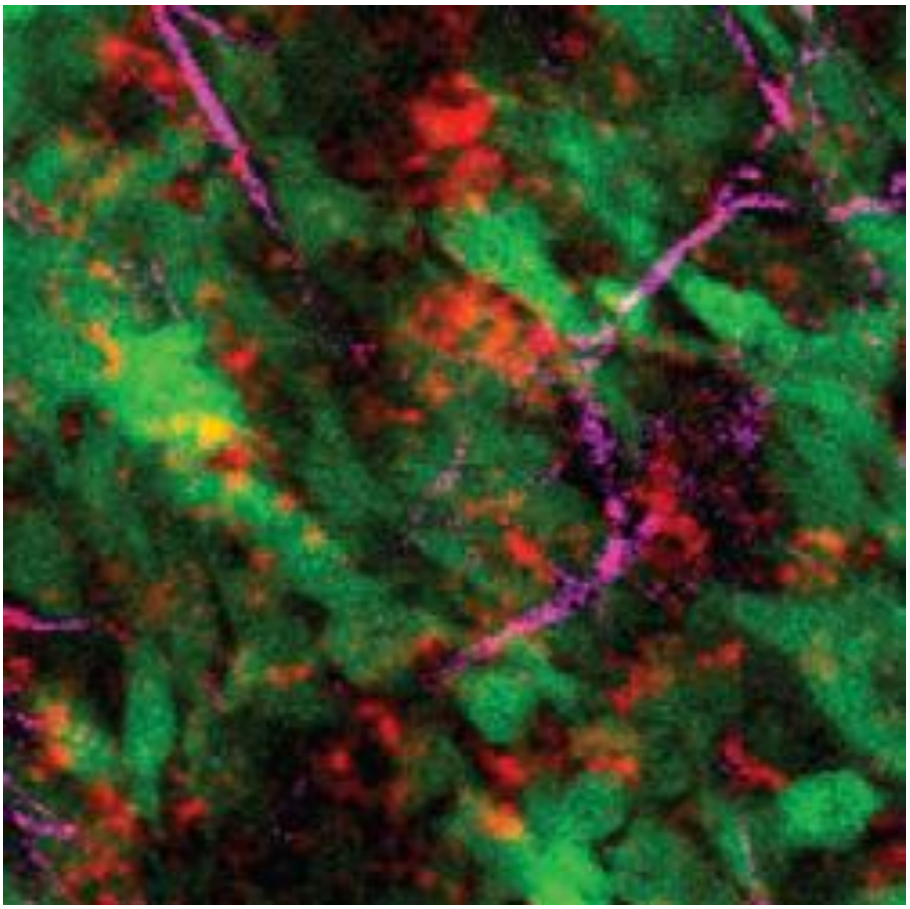
The role of the tumor microenvironment in metastasis studied by intravital imaging



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Master ‘Biology of disease’

Master thesis, March 2011 - April 2011

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* **Intravital image of C26 colorectal tumor cells together with macrophages and collagen I. The merged image visualizes the tumor cells in green, Texas-red labeled macrophages in red and collagen I in purple.** Beerling, Ritsma, Vrisekoop, Derksen and van Rheenen. Intravital microscopy: new insights into metastasis of tumors. *Journal of cell science.* **124**, 299-310(2011).

The role of the tumor microenvironment in metastasis studied by intravital imaging

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Most cancer related deaths are caused by the formation of metastasis, a multi-stage process whereby tumor cells spread from the primary tumor to a secondary site. This process is not only influenced by the tumor cells itself, but also by cells of the host often referred to as the microenvironmental cells. This microenvironment consists of several different cell types together with proteins and extracellular matrix. Although many effort is made in unraveling the process of metastasis and the role of the microenvironment, there are still many questions unanswered. In this review I will focus on a new technique that is used to study tumors and their progression. This technique, termed intravital imaging, is very suitable to study the process of metastasis *in vivo* in real time. I will especially discuss the use of intravital imaging to study the behavior of microenvironmental cells in mammary tumors and their possible role in metastasis. The microenvironmental cells will be limited to three types, macrophages, T-cells and fibroblasts. Several studies already revealed some of their influences and their migration behavior. However, there are still a lot of interesting areas of research that are not yet studied. Especially the possibility to study the effects of cancer treatments on microenvironmental cells will be interesting for the future and might be translated into the clinic. Therefore, intravital imaging is a technique that provides a lot of opportunities for further cancer research in the future.

The study of breast cancer has become an important aspect of cancer research. Breast cancer is one of the most malignant diseases among women, every 1 out of 8 women will develop breast cancer sometime during her lifetime. Not the primary tumor cells cause most of the deaths, but the metastases at a distant site. The process of metastasis itself is a multistage process whereby tumor cells spread from the primary tumor towards a secondary distant organ. Generally, about 40% of the patients with breast cancer will develop these distant metastases.[1] Once a metastatic tumor has developed the 5-year survival is very poor, only 21% of women with metastasis will survive.[2] Therefore, prognosis of an individual with breast cancer is dependent on the presence or absence of metastasis. However, it is often difficult to detect metastasis in the early stages and therefore it is not possible to predict the outcome for every individual. Fortunately, progress in the development of risk predictors for metastasis of the primary breast tumor is being made. For instance, the determination of a gene expression profile can establish a reliable prognosis for the patient.[3] However, the actual process of metastasis is still not completely clear, therefore it is still necessary to put effort in unraveling the mechanism behind metastasis.

The process of metastasis formation can be subdivided as follows: local invasion, intravasation, survival in the circulation, extravasation and colonization. All of those steps are rate limiting and demand different ways of treatment.[4] The first stage of metastasis starts with the invasion of tumor cells in the surrounding tissue of the tumor, by which the tumor cells need to be able to reach a local blood vessel or lymphatic vessel.[4] For this step, cells need to lose the cell-cell contacts and gain motility characteristics.[5] To be able to travel through the body to a distant site, tumor cells need to enter the circulatory system which is called intravasation. The molecular process behind this migration process is still not completely unraveled.[6, 7] Once in the circulatory system, the tumor cells need to survive these different environmental conditions. There is high shear stress and more immune cells are present to attack the tumor cells. After surviving for a while in the blood vessels, tumor cells need to leave the vessels. The timing of this process, called extravasation, differs per tumor.[7] Eventually, when left the blood vessels, tumor cells can form secondary tumors, whereby the preference for certain organs does not happen randomly. In 1980 it was proposed by Paget that tumor cells (seeds) have preferences for a certain organ (soil) to be able to grow out to a new tumor, also known as the seed and soil theory.[8] According to this

theory, two aspects are important in the determination of the organ preference. In first, the target organ needs to have an environment which facilitates the survival of the tumor cells. Second, the tumor cells itself also need to have the appropriate characteristics to colonize at the new target organ.[7] Therefore, every tumor type has its own sites of metastasis. The primary places of breast cancer metastases are the bones, lungs and the liver.[1]
These different stages of metastasis reveal how complex the process of metastasis is, and although a lot of effort is made there are still a lot of questions that remain unanswered. For example, why is the process of metastasis so inefficient; are really all steps rate limiting and how do we treat those different steps? To answer those questions it is important to know the factors involved in stimulating and also inhibiting metastasis. Besides intrinsic factors of the tumor cells themselves, there are also microenvironmental cells, signals and factors that influence metastasis. This so-called tumor microenvironment (TME) consisting of immune cells, extracellular matrix components and cells, is involved in the growth of the primary tumor as well as in the process of metastasis.
To help elucidating the role of the TME on metastasis, most studies so far have used conventional techniques like immunohistochemistry, inspection of tumors and end-stage measurements. However, all these techniques can only give you a static image of one moment in time, while metastases formation is a dynamic process. Furthermore, since all these techniques are being end stage measurements, it is difficult to study the earlier stages of metastasis, like invasion and intravasation. This makes it complex to study metastasis in a suitable way. Therefore, the need for a more elegant way of studying this dynamic process is needed. Already quite some time ago, microscopes were being used to study tissues in living animals (Wagner, 1839). This technique, named intravital imaging, has recently been improved, making it nowadays applicable for studying metastasis. In this report, different intravital imaging options will be discussed together with their contribution to the elucidation of the microenvironmental role in breast cancer metastasis. I will especially focus on the role of macrophages, fibroblasts and T-cells.

Cells of microenvironment influencing metastasis - macrophages/T-cells/fibroblast
During tumor progression the tumor cells lose the need for cell-cell interactions, whereas interaction with nonmalignant inflammatory cells is maintained.[9] It is suggested that these immune cells function similar as in the case of wound healing.[10] Inhibitory signals are send by these immune cells, however tumor cells often are able to circumvent these signals and manipulate the cells of the microenvironment for their own use, leading to tumor progression. The microenvironment consists of several cell types, among these cells are; pericytes, fibroblasts, macrophages, monocytes, neutrophils, mast cells, myeloid cell-derived suppressor cells, mesenchymal stem cells, endothelial cells, endothelial progenitor cells, T cells, B cells and natural killer cells (Figure 1). I will discuss some of these cells and the current knowledge on how these cells influence metastatic progression.

Macrophages
Macrophages are attracted as monocytes towards the tumor by a range of factors, in the case of breast cancer these include monocyte chemotactic protein-1 (MCP-1).[11] Immunohistochemical stainings revealed that both tumor as well as stromal cells express this MCP-1 factor. In addition, analysis revealed that breast cancer patients with high levels of MCP-1 showed a decreased relapse free survival compared to patients with a low level of MCP-1.[12] It is now well established that the monocytes are formed in the bone marrow and circulate through the body via the blood stream. Once recruited to the tumor site, the monocytes are modified into tumor associated

macrophages (TAMs). As a member of the immune system, the TAMs respond to the tumor cells by providing all kinds of cytokines, like EGF, TNF-α, VEGF, bFGF and IL-8 which can have anti- as well as pro-tumor influences. Moreover, the macrophages are also able to present antigens of the tumor towards other immune cells, causing an immune response against the tumor. Some tumor cells can evade this immune response and thereby have a favorable outcome above others.[10] In addition to evoking an immune response, macrophages can also kill tumor cells directly in two ways, namely via macrophage mediated tumor cytotoxicity (MTC) or via antibody dependent cellular cytotoxicity (ADCC), both causing lysis of tumor cells. MTC is a process whereby the macrophage needs to be in close proximity to the tumor cell, whereby factors are secreted that result in lysis of the tumor cell. ADCC is antibody dependent, in which the antibody binds the tumor cell to the macrophage. This binding also causes lysis of the tumor cells.[10] In addition to the above mentioned anti-tumor actions of the TAMs, these cells also contain functions that have the opposite effect. Many studies have shown that TAMs can promote metastatic outgrowth, however, there is not much known about in which steps of the metastatic cascade they are involved, and what the molecular mechanism is behind these steps. What has been shown is that macrophages promote angiogenesis by secreting several different factors that promote angiogenesis. An example of such a molecule is the vascular endothelial growth factor (VEGF), which is known to be involved in the formation of new vessels during tumor progression. The TAMs are able to release this factor, for instance as reaction to hypoxia in a tumor.[13] This factor together with many others enables the formation of new blood vessels, that are important for the further progression of tumors. Moreover, these vessels provide a route for metastasis of the tumor cells. Besides angiogenesis, TAMs can promote invasion by, for example secretion of cytokines or enzymes like matrix metallo proteases that degrade the extracellular matrix surrounding the tumor. As a result, the tumor cells can move more easily through their surroundings and travel towards the blood vessels. How tumor cells move across the membrane of the bloods vessels is still not completely clear, however, after visualizing the binding of breast cancer cells to the TAMs, it was suggested that the tumor cells bind to the TAMs to help them across.[14]

In conclusion, TAMs can have two roles, an anti- as well as a pro-metastatic effect. Which effect is favored is probably dependent on the tumor type, and other cells present in the microenvironment that influence TAMs

T cells

Besides the TAMs there are many other cells present in the microenvironment of a tumor, for instance, the T cells. Classification of the different T cells, into $CD4^+$, $CD8^+$ and regulatory T cells, provided more insight into the different functions in the microenvironment of the tumor. Moreover, most responses are also organ-specific and give either a pro- or anti-tumor response.[15] The $CD4^+$ T cells consists of a group with different lineages, best known are the helper cells, T_H1 and T_H2. The T_H1 cells are very anti-tumorigenic, whereby release of their cytokines directly kills tumor cells.[15] The T_H2 cells, on the other hand, inhibit apoptosis and induce the proliferation of tumor cells of breast carcinomas. This function was demonstrated *vivo* by the injection of human breast cancer cell lines, whereby the injection of T_H2 cells leads to accelerated growth of the tumors.[16]

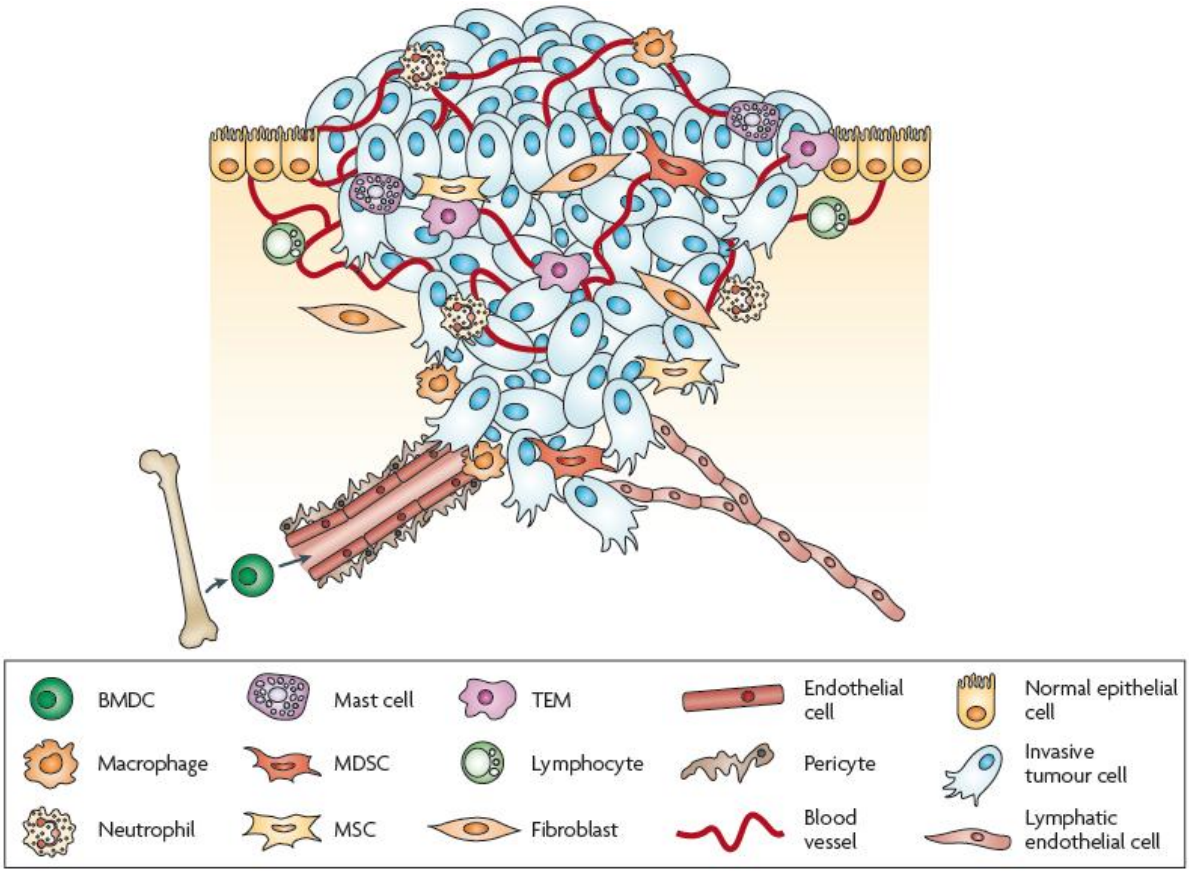

Figure 1: **Tumor with its microenvironment.** The tumor cells, blue, have formed a mass that is invaded by microenvironmental cells. These cells are surrounding the tumor and are also present in the tumor mass. Joyce, J., Pollard, J. Microenvironmental regulation of metastasis. *Nature reviews. Cancer.* **9**, 239-252(2009).

In the case of metastasis, an *in vivo* study demonstrated that depletion of $CD4^+$ T cells led to less development of metastasis. The mice with mammary adenocarcinomas showed reduced metastatic foci in the lungs and decreased numbers of circulating carcinoma cells where present. It appeared that the $CD4^+$ T cells were able to regulate the pro-tumor capacities of the TAMs, where especially T_H2 cells are involved. Thus they stimulate TAMs and their pro-tumor properties, and as a consequence more metastasis occurs.[17]

The other subset of T cells, the $CD8^+$ cells consist of cytotoxic T lymphocytes (CTLs). These CTLs recognize antigens presented on the surface of a tumor cells, and are then subsequently able to kill these antigen-presenting tumor cells. The antigens consists of small peptide sequences presented by the tumor cells using a major histocompatibility complex I (MHCI). In case of antigen recognition, the CTL binds to the target cell and releases granules with several enzymes. These enzymes form pores in the cell membrane of the target cell and attack the DNA of the cell.[18] The pore formation allows other enzymes to enter the cells, which eventually leads to apoptosis of the tumor cell. The antigens presented by the tumor cells differ between distinct tumors, for breast cancer one of the antigens is derived from Her2/neu, a glycoprotein known to be overexpressed in approximately 20% of all breast cancer cases.[19, 20] This antigen consisting of nine amino acids induces a tumor-specific CTL response.[19] So, when an antigen is recognized by CTLs the tumor cells are killed, thereby providing an anti-tumor

capacity. However, evidence arises that metastatic tumors can evade this process of apoptosis. Various mechanisms are used to escape cell death; e.g. the number of MHCI or antigen peptides is decreased. With immunohistochemistry (IHC), Cordon-Cardo *et al.* demonstrated that metastasized breast cancer cells showed no or fewer MHCI complexes in 84% of the cases. Thereby suggesting that these cells evade the immune suppression and could metastasize.[21]

A third group of T cells; the regulatory T cells, are known to regulate the immune response of other immune cells. It is still not exactly known how this regulation is controlled, however, previous research showed two possible mechanisms, either via direct cell-cell contact between the regulatory T cells and the other immune cell, or via the secretion of anti-inflammatory mediators, like IL-10.[22] In 1995, it was already discovered that depletion of this specific subpopulation leads to several kinds of autoimmune diseases. These data show the control possibilities of this subgroup on the other T cells, because in their absence an overactive immune response to self-antigens is induced.[23] Moreover, it is demonstrated that depletion of this subtype of T cells leads to rejection of tumor cells in mice, suggesting that the regulatory T cells in normal circumstances inhibit immune responses. The tumors introduced in the mice with depleted T regulatory cells were rejected rapidly and these mice survived, while the control group died rapidly due to the growing tumors.[24] Focusing on breast cancer, it was shown that these T cells also are involved in the progression of breast cancer. Moreover, researchers were able to demonstrate the presence of regulatory T cells in the microenvironment of the tumor and in peripheral blood samples of the patients. In addition, the inhibition of $CD4^+$ and $CD8^+$ T cells was *ex vivo* confirmed.[25] All these results suggest that the presence of regulatory T cells indicate a poor prognosis for breast cancer patients. Indeed, Bates *et al.* observed a correlation between the presence of regulatory T cells and the more aggressive tumors. Higher number of regulatory T cells predict poor overall survival compared with patients with lower numbers.[26]
In short, there are three subsets of T cells that together have pro- and anti-tumorigenic properties. The regulatory cells control the function of the two other subsets and determine the outcome of mammary tumor progression.

Fibroblasts

Besides immune cells, the microenvironment also contains many other cells that play a role in tumor formation. One of these cell types, the fibroblasts, will also be briefly discussed here in this report. Fibroblasts are embedded in the extracellular matrix (ECM) of tissues, where they synthesize many components of the ECM. Moreover they also regulate inflammation, epithelial differentiation and are involved in wound healing. Besides synthesis, they also regulate the reduction of the ECM by secreting enzymes that regulate this degradation. In other words, the fibroblasts are involved in the homeostasis of the ECM. Furthermore, the fibroblasts also secrete growth factors influencing their neighboring epithelial cells. When a fibroblast becomes activated the shape and the content of the cell will change. This activation occurs as reaction on for instance the process of wound healing.[27]

It is thought that fibroblasts surrounding tumors, also referred to as cancer-associated fibroblasts (CAFs), also have this activated phenotype. It was demonstrated that CAFs of breast cancer patients show a higher migration activity then that of normal controls, probably as consequence of the activation.[28] Moreover, they are present in most of the human breast cancer cases. The continuous activation of the CAFs causes the occurrence of granulation tissue, consisting of the fibroblasts and excessive amounts of ECM.[29]

Orimo *et al.* showed that when tumor cells are mingled with these CAFs there is an increased tumor growth *in vivo*, indicating that fibroblasts are involved in the initiation and

growth of tumors. In this study it also turned out that CAFs secrete growth factors, stimulating tumor growth.[30] Another *in vivo* model demonstrated that injection of CAFs in mice not only accelerated tumor growth, it also stimulated the transition of benign tumors to more invasive variants. Here, injection of CAFs led to invasive carcinomas with an increased proliferation rate.[31] Moreover, inhibition of CAFs suppressed the ability of tumor cells to spontaneously metastasize. Thus besides tumor growth, CAFs are also suggested to be involved in the regulation of metastasis occurrence.[32] A prognostic study revealed that in breast cancer patients with atypical fibroblasts, CAFs have a higher chance for recurrence and cancer-related death. This indicates that the presence of CAFs in tumors is a hallmark of invasive and more aggressive tumors.[33]
Concluding, CAFs have a typical phenotype similar to that of activated fibroblasts, providing them with a higher migration activity. It appears that CAFs stimulate tumor growth, invasiveness and metastasis occurrence.

Taken all together, it has become clear that not only tumor cells, but also the microenvironmental cells play an important role in determining the progression of tumor development. Although much research is already performed to elucidate the complete role of the microenvironment, there are still many questions unanswered. Using intravital imaging, some of these questions have been tackled, which will be discussed below.

Intravital imaging of the TME

As explained above there is need for a technique applicable to study the dynamics of the metastatic process. Using conventional techniques that provide static images or end stage measurements it is difficult to determine the precise role of the TME in the different steps of metastasis. Using intravital imaging, a technique to study tissues and cell behavior *in vivo*, it is possible to image the TME during invasion and intravasation. Multiple adaptations through the years improved this technique at different levels, like microscopy, probes and the location of tumor imaging.

Microscopy

Microscopes are existing for several centuries and two names are usually associated with the first publications about a microscope; Robert Hook and Antonie van Leeuwenhoek.[34] Since the first prototypes many improvements and variants are developed and as a consequence a broad range of microscopes is now available. In the field of intravital imaging many microscopes are applicable to use, however here I will only report about microscopes with a subcellular resolution. Subcellular imaging gives an overview of cells and their internal organization. Three microscopic platforms applicable for subcellular intravital imaging are; (spinning disc) confocal microscopy, multiphoton (MP) microscopy and optical frequency domain imaging (OFDI).

(Spinning disc) confocal imaging is a technique that uses visible laser light to excite fluorophores. During intravital imaging, visible light from the microscope excites the fluorescent proteins in the mouse and this light is further emitted to the detector. In this beampath there is a

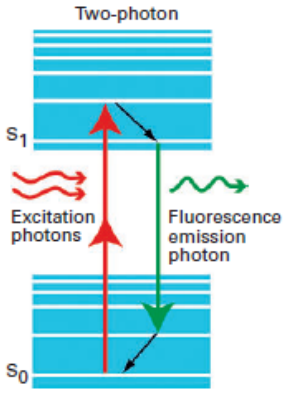

Figure 2: **Basic working mechanism of multiphoton fluorescence microscopy.** Two low-energy photons are used, that together contain enough energy to excite an electron of a fluorphore. This fluorphore then emits a photon that is detected by the microscope. Dunn, K., Young, P. Principles of multiphoton microscopy. *Nephron experimental nephrology.* **103**, 33-40(2006).

pinhole that filters the emitted out-of-focus light, causing improved contrasts and thereby more efficient visualization of subcellular structures. To do long term intravital imaging with low phototoxicity, a spinning disc confocal microscope is preferred over a conventional confocal microscope. In a spinning disc microscope, the pinhole is replaced by a round disc with multiple pinholes that spins. In this way a CCD camera can be used and image acquisition is much faster. A disadvantage of (spinning disc) confocal microscopy is that there is a limited imaging depth of only up to 100µm.[35, 36] Egeblad *et al.* demonstrated that with spinning disc confocal imaging it is possible to perform dynamic imaging for a longer period of time of the microenvironment of a tumor in a living mouse.[37]

MP imaging is based on the absorption of two low-energy photons by a fluorescent molecule, whereby an electron is activated, exciting the fluorescent protein. When the electron falls back, a photon with a lower energy level than the combined energy of the two exciting photons is released, which is called fluorescence emission (Figure 2). For this event to happen, the two exciting photons need to arrive at the same time at the fluorophore. This is accomplished by using an enormous amount of photons or in other words, a very powerful laser. The laser is pulsed, providing a high release of photons only for a short period to avoid tissue damage. Since two photon excitation is a rare event, it will only happen at the focal plane, providing optical tissue sectioning and reducing bleaching of the (out of focus) fluorescent probes. This short period of exposure also has another advantage; there is less toxicity within the tissue. Furthermore, with this method there is more efficient stimulation of fluorescent probes deep in the tissue by use of infrared light. This infrared light is able to stimulate fluorescent deeper into the tissue, when compared to visible light used with confocal imaging. Therefore this method results in a deeper imaging depth compared to confocal microscopy. In other words, for imaging deeper in tissue MP imaging is more effective, however a downside are the high costs associated with this technique.[38]

An example of usage of this technique is that of Wyckoff *et al.*, who used MP imaging to study the role of macrophages during the intravasation of tumor cells into blood vessels.[39]

The last option discussed here is optical frequency domain imaging (OFDI). OFDI was first used for intravital imaging in 2009 by Vakoc *et al.* This technique is capable of rapid *in vivo*

imaging and giving an overview of a wide region of tissue. With OFDI an optical beam is used to focus into the tissue, which scatters the light in three dimensions. After interference with a reference beam the reflected light of the different depths is detected. With this scattered light it is possible to determine structures in depth of the tissue, up to 6 mm.[40] Because OFDI uses the scattering of the optical beam, there is no need for an exogenous contrast dye, thus no phototoxicity of photobleaching.[35] Since OFDI can be used to study a large area of the tumor, showing a more complete image of the vascular network of a tumor compared to other methods, Vakoc *et al.* used this technique to study angiogenesis and lymphangiogenesis. Furthermore, they were also able to study the response of tumors to therapy by making distinction between viable and necrotic tissue.[40]

Overall, it is clear that on the level of microscopy improvements have been made, which contributes to more efficient intravital imaging possibilities, whereby especially resolution, imaging depth and the decrease of toxicity are important areas of improvement. However, also on other levels researcher are constantly trying to develop new advances. With these new techniques it is now possible to study the individual steps of metastasis and especially invasion and intravasation. This makes it possible to study the role of the microenvironmental cells in specific parts of the process.

Probes

Not only the microscopes, but also the use of fluorescent proteins went through a tremendous developmental period. Nowadays, many different fluorescent colors are generated, of which green fluorescent protein (GFP) and its other color derivatives yellow fluorescent protein (YFP), cyan fluorescent protein (CFP) and red fluorescent protein (RFP) are most commonly used. GFP absorbs blue light and can exert this into green light, its derivatives are mutated forms of GFP and therefore reflect other colors of light.[41] The establishment of multiple fluorescent colors enables marking different cell types at once. This enhances the study of the microenvironmental role in tumor metastasis, because tumor cells and stromal cells can now be followed at the same time. In addition to these fluorescent proteins there are also other more recently discovered fluorophores, for instance the photoswitchable proteins. These proteins undergo irreversible changes upon exposure to light of a certain wavelength. The cells can for instance switch from green fluorescent to red fluorescence (Figure 3A), which makes it easier to follow proteins, organelles and cells over long periods of time.[42] The main advantage of using photoswitchable

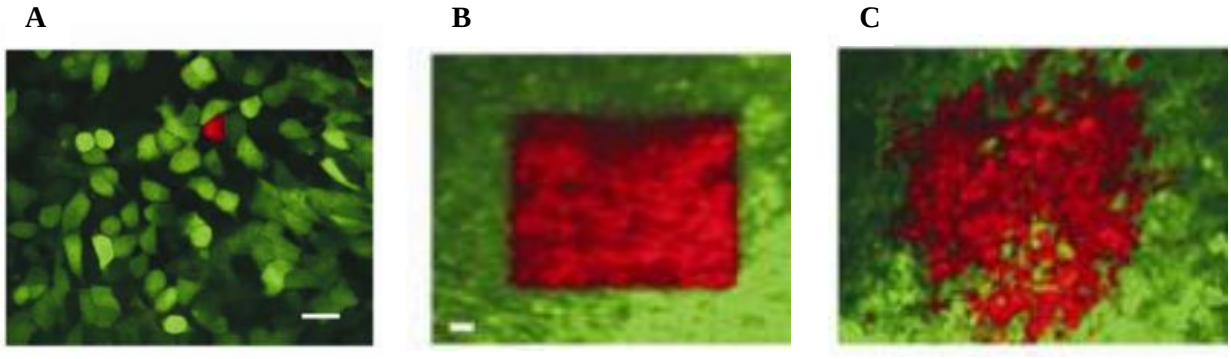

Figure 3: **Photoswitchable protein Dendra2 is converted from green into a red fluorescent protein. A**). Demonstrates the conversion of one single MTLn3 tumor cell from green into a red fluorescent protein. The tumor cells express the photoswitchable protein Dendra, that is here converted *in vivo*. **B**). The same tumor cells, only showing conversion of hundreds of cells at once. **C**). 24 hours after the conversion of the photoswitchable protein. It is visible that the cells invaded the surrounding environment of the tumor. Kedrin, D., Gligorijevic, B., Wyckoff, J., Verkhusha, V., Condeelis, J., Segall, J., van Rheenen, J. Intravital imaging of metastatic behavior through a mammary imaging window. *Nature methods.* **5**, 1019-1021(2008).

proteins is that it is no longer necessary to follow the objects constantly over time, because they can be efficiently localized after long time spans, even after days. This limits bleaching of the fluorescent protein and toxicity of the tissue. Furthermore, with this method it is no longer necessary to use reference points of the tissue itself to relocate an area of interest, which is sometimes problematic in tumors because these are dynamic over time and therefore not always retraceable.[42] Another advantage is that now multiple cells can be traced over time, enabling good quantitative statistical
measurements, and reducing the number of animals needed for an experiment. An example of a photoswitchable protein is Dendra2, a green fluorescent protein that is converted by blue light into a red fluorescent protein. This conversion is irreversible and provides a stable protein, which makes Dendra2 suitable for long term tracking.[43] Kedrin *et al.* used this protein to follow individual and groups of cells in metastatic breast tumors. Dendra2 expression was introduced in a breast cancer cell line and selected cells within a squared region were stimulated to convert (Figure 3B). After a day, this same region could be easily traced back because of its red color, and cell migration was shown by a changed form of the squared region (Figure 3C).[44]

Imaging site

Besides the probes and the microscope it is also essential to use an appropriate location to actually visualize the tumor cells in real time in the animal model. Since the beginning of intravital imaging several imaging sites have been proposed and used. One of the first models was the ear chamber, used in rabbits, which is a special surgical implanted chamber (Figure 4A). In this chamber small pieces of granulation tissues are able to grow and after about 6 weeks it is possible to introduce tumor cells into this chamber. About halve of the cases show a successful invasion of the tumor cells into the granulation tissue. It is then possible to study the tumor growth through the coverglass of the chamber.[45]

In addition to the ear chamber, there is also a dorsal skinfold chamber, which is suitable to use in smaller rodents like mice (Figure 4B). This chamber is implanted surgically around a double layer of skin of the back of the mouse. One side is covered with a glass coverslip to enable microscopy of the underlying tissue.[46] Also in this chamber the tumor cells are able to grow under the coverglass. When the chamber is placed in close proximity of a vessel it is possible to study the tumor and the associated microcirculation.[47] Although the chamber models are suitable for long-term imaging, they have similar limitations. In both cases, only tumor cell lines can be studied, not genetic tumor models. Moreover, the environment for most tumors (like mammary tumors) is not orthotopic, thereby it is not possible to study the microenvironmental role in tumor progression.[44]

Another more temporary imaging model is the skin flap, which is basically an incision through the skin surrounding the tumor, leaving the blood supply intact (Figure 4C). The skin is lift up, the flap is elevated and a piece of glass is placed on top of the tumor allowing microscopy of the tumor. The advantage of this method is that tumors can grow in their natural environment, because this skin flap can also be performed at the site of the mammary glands.[48] However, this method is not really suitable for long term imaging, because the tissue can dehydrate and the animals die usually within 10 hours.[44] Only with an indwelling IP line to compensate for the loss of fluid, and heating of the mouse this can be extended to 40 hours.[49] Moreover, the animals have to be under anesthesia all the time which might influence cell behavior.[44]

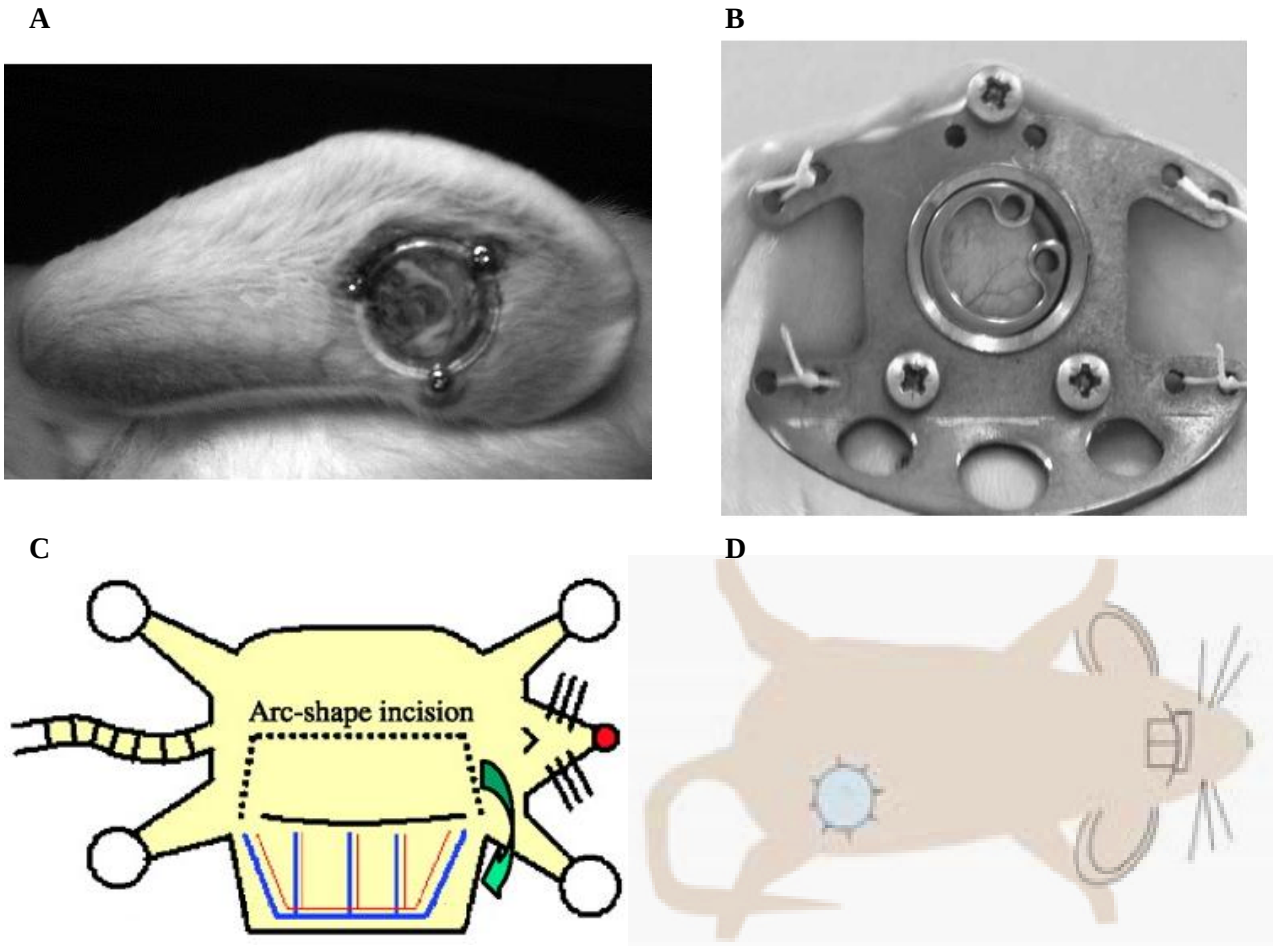

Figure 4: **Several imaging methods that can be used for intravital imaging. A**) The ear chamber implanted in a rabbit ear. The chamber consists of a ring with at one site a coverslip, that enables microscopy. Tumor can be injected into the chamber and are able to grow. The growing and invading tumors cells can then be studied in real time. Komori, M., Takada, K., Tomizawa, Y., Nishiyama, K., Kondo, I., Kawamata, M., Ozaki, M. Microcirculatory responses to acupuncture stimulation and phototherapy. *Anathesia & Analgesia.***108**, 635-640(2009). **B)** The dorsal skinfold chamber surrounding the double layer of skin on the back of a mouse. In this model it is also possible to simulate tumor grow and image it *in vivo*. Bingle, L., Lewis, C., Corke, K., Reed, M., Brown, N. Macrophages promote angiogenesis in human breast tumour spheroids *in vivo*. *British journal of cancer.* **94**, 101-107(2006). **C**) Illustration of a surgical made skinflap, an incision of the skin that surrounds the tumor. The blood vessels stay intact while the skin is lift up and a coverslip is placed. Through this glass microscopy can take place. Yamauchi, K., Yang, M., Jiang, P., Xu, M., Yamamoto, N., Tsuchiya, H., Tomita, K., Moossa, A., Bouvet, M., Hoffman, R. Development of real-time subcellular dynamic multicolor imaging of cancer-cell trafficking in live mice with a variable-magnification whole mouse imaging system. *Cancer research.* **66**, 4208-4214(2006). **D**) Illustration of the mammary imaging window (MIW) placed over the mammary gland of a mouse. This window is surgically placed and stays in place during several days. This window enables imaging of a mammary tumor at its natural environment. Kedrin, D., Gligorijevic, B., Wyckoff, J., Verkhusha, V., Condeelis, J., Segall, J., van Rheenen, J. Intravital imaging of metastatic behavior through a mammary imaging window. *Nature methods.* **5**, 1019-1021(2008).

A more recently developed imaging model is the mammary imaging window, which can be placed on top of the mammary gland (Figure 4D). The window consists of a glass coverslip that is surrounded by two plastic rings. The rings contain holes enabling suturing into the skin, which allows establishment on top of a gland that contains a growing tumor. Tumor cells can be injected into the fat pad of the gland before or after situating the window on top of the gland. Placement of the window do not affect the tumor growth or behavior and there are no signs of inflammation after the surgery.[44] Therefore, it seems to be the most reliable imaging method of this moment for the study of breast tumors in real time.

The combination of better microscopes, other fluorescent probes and the new mammary imaging window makes it easier to study metastasis of breast tumors in real time. Intravital imaging can give insights in the action of primary tumors in living animals, can provide us with observations about migration and invasion of tumor cells into the microenvironment and in addition demonstrate the role of the microenvironment in the process of metastasis. In other words, intravital imaging can reveal tumor behavior *in vivo* in real time at a subcellular resolution. With this technique it is possible to study every step of metastasis individually, which is not possible with the conventional techniques. In the future this will provide more insight into the multi-stage process of metastasis, whereby especially invasion and intravasation can be studied. Different combinations of microscopic techniques with probes have unraveled already some of the questions of the microenvironment in metastasis. In the next section of this report I will discuss several new insights in the role of microenvironmental cells on metastasis progression revealed by intravital imaging.

Intravital imaging of microenvironmental cells

One of the microenvironmental cells, the tumors-associated macrophage (TAM) is known to play a dual role in metastasis of a tumor; pro- and anti-metastatic. TAMs promote metastatic outgrowth via induction of angiogenesis and perhaps invasion, but there is not much known about whether or not macrophages play a role in the other steps of the metastatic cascade, and what the mechanism is behind it. Using intravital imaging, the involvement of macrophages in the different steps of the metastatic cascade can be examined. To study macrophages *in vivo*, Wyckoff *et al.* used TexasRed-dextran, a fluorescent dye that can be ingested by macrophages.[39] With intravital imaging it was demonstrated that perivascular TAMs in mammary tumors are located adluminal in the stroma of the tumor. Moreover, it appeared that most macrophages are located at the edge of a tumor.[39, 50] In the center most TAMs are located near the blood vessels that provide blood and nutrients to the tumor. It turned out that TAMs stimulate movement of the tumor cells; about 80% of the tumor cells move only when they are in close proximity of a macrophage. The tumor cells migrate directly to the macrophages, enabling invasion into normal tissue (macrophages at the edge of the tumor) and intravasation into the blood vessels (macrophages in close proximity of a vessel). Intravasation only occurred near macrophages, tumor cells become associated or need to be close to the macrophages, with a maximum of 20 µm. In mice lacking CSF-1, a cytokine that regulates differentiation into macrophages, there were less TAMs found, however the tumors showed a normal growth pattern.[39] Interestingly, another study of Wyckoff *et al.* demonstrated the presence of a paracrine loop between the tumor cells and the TAMs. They showed that tumor cells secrete CSF-1 which attracts TAMs, these are stimulated and release EGF. This growth factor at his turn stimulates the tumor cells to migrate.[51] Although the growth was normal, the progression of the tumors was slower and the tumors showed less invasion and metastasis. Which can be explained by the paracrine loop, when CSF-1

is removed there is no stimulation anymore of the TAMs and therefore no migration of the tumor cells. In addition to decreased TAMs there were also less circulating tumor cells in the blood of the mice. These results demonstrate that the macrophages around the blood vessels are involved in the intravasation of the tumor cells. With intravital imaging they demonstrated the location of the TAMs and the close proximity of the tumor cells before they enter the vessel. Moreover, in real time they were able to visualize the migration of the tumor cells towards the macrophages around the vessels.[39]

Interestingly, what was shown in another study is that there are differences in the migration behavior of macrophages within the same tumor. What they found is that enhanced green fluorescent protein (EGFP) labeled myeloid cells, among which are TAMs as well as myeloid-derived suppressor cells (MDSCs), at the margins of the tumor mass were migrating more than myeloid cells present within the tumor mass of mammary tumors. This suggests that there are multiple microenvironments present in which the stromal cells behave differently. It was established that the differences in migration are not caused by the different levels of oxygen present in the different areas of a tumor.[37] Therefore, it was hypothesized that different subtypes of myeloid cells have different migratory velocities. To make a distinction between the different subtypes of cells, macrophages were first marked with Texas red-dextran, the fluorescent dye that was also used in the Wyckoff study.[39] Differences were observed in the periphery of the tumor, macrophages that ingested the dextran dye did not migrate. On the other hand, the macrophages in the same area that did not ingest the dye were migrating. This result indicates that there are different subtypes of macrophages that behave differently once infiltrated into the tumor.[37] This is in agreement with the literature that already described two subtypes of macrophages, M1 and M2. M1 are classical activated macrophages, these are activated by microbial agents and cytokines. The M2 macrophages are activated by an alternative mechanism stimulated through anti-inflammatory molecules, like IL-10. M1 macrophages are known to have pro-inflammatory effects and are thought to be anti-tumorigenic. The M2 macrophages have the opposite effect and are thought to be pro-tumorigenic.[52, 53] To determine which macrophages ingested dextran and migrated, positive dextran cells were isolated and macrophage markers were identified. The cells positive for dextran appeared to be the M2 subtype of macrophages, whereas the negative dextran cells did not express the M2 specific marker, CD206.[37] This study suggest that the pro-tumorigenic M2 macrophages are not migration much. This could indicate that these macrophages are in close proximity of blood vessels and help tumor cells to invade into the circulation.

In conclusion, it was shown using intravital imaging that there are two subtypes of macrophages that show differences in migration; the M2 negative and the M2 positive macrophages. The M2 macrophages do not migrate a lot, but were shown to facilitate intravasation and induces migration in a paracrine fashion involving CSF-1 and EGF. Thus, macrophages can help facilitate metastasis not only via angiogenesis, but also by inducing tumor cell migration and intravasation.

In case of the T cells it is known that there are several subtypes present in tumors which have anti- and pro- tumorigenic properties. The influence of T cells on the progression of a tumor is already shown, however the influence on metastasis is still not identified. To elucidate the role of the T cells in one or more steps of metastasis, intravital imaging is a suitable technique. There is research performed to study the behavior of T cells in tumors in real-time, however the role of T cells in metastasis is still open for more research. The conducted research did give suggestions for possible roles of the T cells in metastasis. In 2008, Egeblad *et al.* investigated aside from the

macrophages also the migration behavior of the regulatory T cells. Using a mouse model of mammary carcinoma they studied regulatory T cells by labeling this subtype of T cells with EGFP. Most of the labeled cells were moving, about 55%. There were also regulatory T cells that moved for a while and then paused for some time, with an average of 18 minutes. This last group was especially moving around blood vessels. A small percentage was integrated in the tumor mass, however showed no migration. Interesting was the fact that upon induced hypoxia the regulatory T cells stopped moving, which was restored when oxygen was available again (Figure 5). This demonstrates that T cell migration is sensitive for the oxygen levels of the tissue, which might explain the movement of the cells near blood vessels.[37] However, another explanation of the movement of the regulatory T cells can be the migration behavior of the CTLs. As mentioned in the introduction T regulatory cells are able to inhibit the functions of $CD4^+$ and $CD8^+$, the CTLs, cells and these CTLs were also detected in close proximity of blood vessels. A real-time observation showed migration of CTLs along blood vessels, some moved from one vessel to another.[54] It appears that the CTLs migrate in the same area as the regulatory cells, which suggests that the regulatory cells follow the CTLs to inhibit their function. This could have consequences for the metastatic properties of a tumor, since CTLs are able to kill tumor cells, which was also demonstrated with an *in vivo* intravital imaging study. This study provided insight into the model of tumor cell killing, whereby the CTLs kill tumor cells and then move further into the tumor to encounter other tumor cells. In some cases the CTLs moved along the blood vessels towards new areas with tumor cells.[54] Although, this is not yet confirmed in a solid tumor model, CTLs killing inhibits metastasis of the tumor cells. The migration of regulatory T cells towards the area of the CTLs could inhibit this function, leading to increased metastasis. Therefore, it would be interesting to study the migration stops of the regulatory cells, if they are interacting whit the CTLs in those periods.

The interaction of the CTLs with tumor cells is already established by intravital imaging together with the consequences for the viability of the tumor cells in the presence of CTLs. After incubation with CTLs most tumor cells were dead, showing less fluorescence. Moreover, it was established that the amount of collagen fibers was increased, indicating the presence of fibrosis as a consequence of the dying tumor cells (Figure 6). The CTLs showed interaction with the tumor cells and when the tumor cell died the CTL is released again and migrates away from this area.[54]

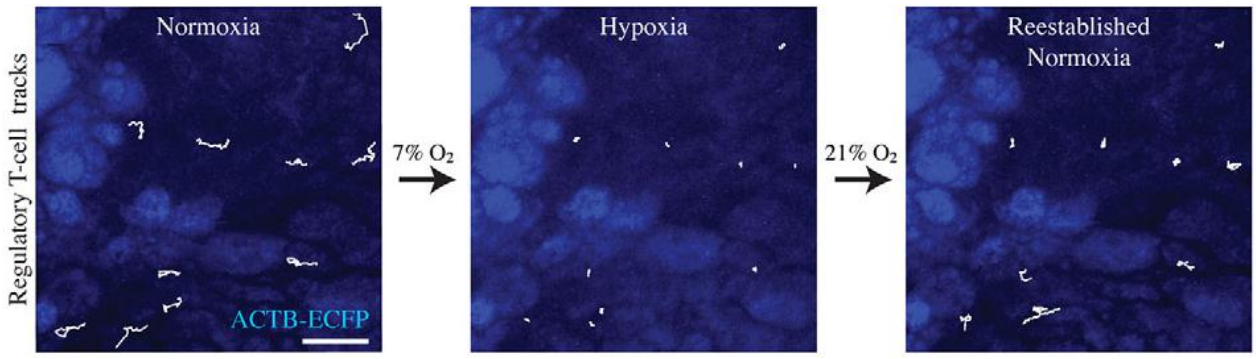

Figure 5: **Migration behavior of Tregs in MMTV-pyMT tumors upon oxygen changes.** In the normoxia situation the Tregs are normally migrating, visualized with the white lines. Upon lowering of the oxygen levels to 7% the T cells stop migration, only white dots are now visible. Interestingly when oxygen levels are normalized again, the T cells are migrating again.
Egeblad, M., Ewald, A., Askautrud, H., Truitt, M., Welm, B., Bainbridge, E., Peeters, G., Krummel, M., Werb, Z. Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. *Disease models & mechanisms.* **1**, 155-167(2008).

The interactions of these T cells and tumor cells was long lasting in almost all cases, lasting mostly more than 20 minutes.[55] From these studies it has become clear that interaction of CTLs with tumor cells leads to the death of the tumor cell, thereby prohibiting progression but also metastasis.

A possible keyplayer of T cell migration is CD44, an adhesion molecule that is suggested to be involved in T cell adhesion to the wall of a blood vessel. MP imaging demonstrated that removal of CD44 disturbed the polarity of CTLs, causing the cells to migrate less efficiently. However, the interactions with the tumor cells were still stable. When migration was decreased, through removal of CD44, tumor rejection was decreased. Without CD44 the CTLs were able to find the tumor, however the migration through the tumor stroma was disturbed.[56] This indicates that tumor killing is dependent of signal molecules and inhibition of these signals can be a way for the tumor to evade the responses of the immune system.

In conclusion, intravital imaging demonstrated that the regulatory T cells and the CTLs showed similar migration behavior. Both cell types are migrating around the blood vessels, it can be suggested that the regulatory T cells follow the CTLs to inhibit their function. In case of the the T cells intravital imaging was especially effective to demonstrate the tumor cell killing by CTLs *in vivo* in real-time, indicating that CTLs are inhibitors of metastasis by tumor cells. For the future there are a lot of possibilities for intravital imaging and the study of T cells in metastasis regulation.

The third group of cells, the cancer associated fibroblasts (CAFs) gain an activated phenotype that stimulates their migration behavior. Conventional research provided some evidence for the involvement of CAFs in tumor growth, invasion and metastasis. However, the exact role of CAFs in metastasis is still unknown, providing an opportunity for intravital imaging. With intravital

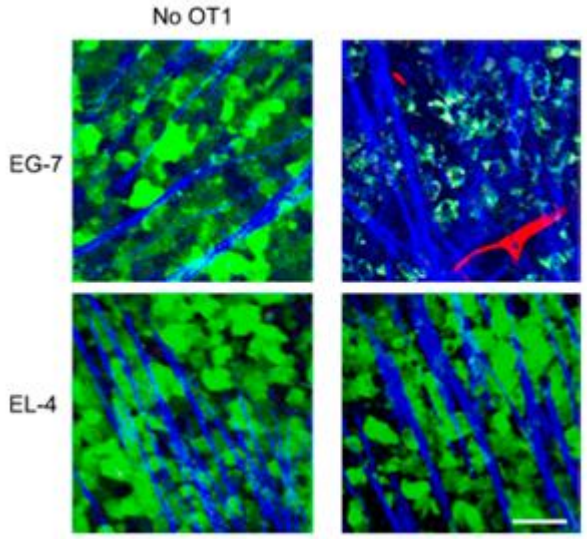

Figure 6: **CTL killing of tumor cells expression specific antigens.** Two different thymus tumors were used, EG-7 and EL-4, whereby the first one expresses exogenous antigens that can be recognized by CTLs. The second tumor type, EL-4 does not express these antigens. The left pictures represent the begin stage of both tumors, whereby tumor cells are marked in green and collagen fibers in blue. Upon CTL administration (OT1), pictures on the right, there is tumor rejection of the EG-7 tumor, while the EL-4 shows no reaction. The EG-7 tumor cells were killed and an increase in collagen fibers is visible. Boissonnas, A.., Fetler, L., Zeelenberg, I., Hugues, S., Amigorena, S. *In vivo* imaging of cytotoxic T cell infiltration and elimination of a solid tumor. *Journal of experimental medicine.* **204**, 345-356(2007).

imaging it can be possible to study the role of CAFs in the different steps of metastasis. In the elaborated study of Egeblad *et al.* fibroblasts were detected at the border, within the tumor and in the stroma of the tumor.[37] To elucidate the role of fibroblasts in ECM remodeling, MP intravital imaging was used to study real time movement of the CAFs. Human sarcomas were grown in dorsal skinfold chamber and with GFP fibroblasts were marked. Since the remodeling is a slow process, it was induced with the hormone relaxin to increase it and to enable tumor cell invasion. After relaxin administration, the recruitment of CAFs to the tumor site was not affected. On the other hand the migration of CAFs once at tumor site was significantly decreased, while the speed of the cells that did migrate was increased. When fibroblasts attach to fibers of the ECM, these fibers moved. After removal of the CAFs the fibers are either going back to their old position or they are remodeled into a new location. Moreover, the CAFs were causing changes in the morphology of single fibers, for instance making gaps. The decrease of migration is probably enabling more interactions between CAFs and the fibers. This study showed the possibilities of following a certain cell type and determine interactions with ECM.[57] The remodeling of the ECM provides opportunities for the tumor cells to invade into the surrounding tissue through the gaps made by the fibroblast. In this case intravital imaging showed one of the role of the CAFs in stimulation invasion. The tumor cells are able to invade the tissue and migrate towards blood vessels to intravasate into the circulation. However, more research into the CAFs is necessary to determine their exact role in metastasis formation of mammary tumors.

In conclusion, intravital imaging revealed the presence of CAFs throughout the whole tumor. An important property of the CAFs is the remodeling of the ECM producing for instance gaps into the normally dense structure. These gaps provide routes for the tumor cells to invade into the normal tissue, thereby spread of the tumor is induced. This suggests that the CAFs play an important role in stimulating invasion and metastasis.

As outlined above several intravital imaging studies revealed some information about microenvironmental cells and their behavior in tumors. One example of a more general study is that of Kedrin *et al*. where they looked at different microenvironments within the same tumor. Tumor cells in close proximity of a blood vessel showed more invasion and the tumor cells were mostly moving towards a vessel. Some of the tumor cells already invaded the vessel and migrated to other organs, like the lungs. When looking a tumor cells in a different area, without detectable vessels, there was almost no migration visible. This suggests that the microenvironment is important for the stimulation of migration and metastasis.[44] Upon comparison of two mammary adenocarcinomas, MTLn3 a metastatic cell line and MTC a non-metastatic one, difference in host cell numbers were detected. Tumor cells were labeled with GFP and visualized with intravital imaging. Besides the tumor cells there were also a lot of non-fluorescent cells, most likely these are host cells. It appeared that within MTLn3 tumors there are increased numbers of these host cells present when compared to the MTC tumors. Where MTLn3 tumors had an average of 11 cells per observation site, MTC tumors had only 2 host cells. It is suggested that these increased numbers enables the tumor cells of the metastatic tumor to migrate towards the blood vessels. Besides, these cells might also be involved in inducing pores into the vessel wall through which tumor cells can intravasate into the circulation.[58]

Altogether, the above discussed studies demonstrate the usefulness of intravital imaging in determining the behavior of microenvironmental cells. Some of these new insights into the role of tumor progression are directly linkable to metastasis, like the TAMs that enable tumor cells to

invade into blood vessels. Other new insights need to be studied more to exactly determine their importance for the process of metastasis. One thing is inarguable, the fact that microenvironmental cells play an important role in the progression and metastasis of tumors.

Discussion and future perspectives

In this review I focused on intravital imaging of the microenvironmental cells and their behavior within the tumor stroma of mammary tumors. With conventional techniques it was already shown that the TME is important for metastasis. However, their exact role in the different steps of this dynamic process was lacking. Therefore, intravital imaging can be used to study the individual steps of metastasis, especially the first two invasion and intravasation. This provided some new insights into the role of the microenvironmental cells. The TAMs induce cell invasion and thereby metastasis via a paracrine loop. The role of the T cells is less clear, however it is demonstrated that the $CD8^+$ and the regulatory T cells migrate in the same area of the tumor. Moreover, the CTLs are able to kill tumor cells through a stable interaction between both cells. These CTLs are thereby inhibiting tumor progression and metastasis. With intravital imaging it was shown that the CAFs are able to remodel the ECM, providing a way for tumor cells invasion and metastasis. In conclusion, intravital imaging demonstrated that the microenvironment has a great influence on the first steps of metastasis. This could not be studied without intravital imaging.

Intravital imaging already proved its value in studying the first steps of metastasis. However, the last stage of metastasis, extravasation into a secondary organ and subsequent outgrowth, is also an area of investigation that would be interesting to study. This research is still in its infancy, just a few studies tried to visualize tumor cells in these organs, like the lungs. The lung is an organ that is very hard to image *in vivo*, however recently there was a study that succeed. With use of dual-colored mammary cancer cells, GFP in the nucleus and RFP in the cytoplasm, metastasis into the lung was observed.[59] More studies into the last stages of metastasis are necessary to create a complete picture of the process. Therefore, new imaging windows need to be developed to study other organs as well, without surgical interventions. In the future these might give answers to open questions like, why tumor cells have a preference for certain organs. This could complete the picture of the individual steps of metastasis.

Besides studying metastasis at the cellular level, intravital imaging can also be used to study metastasis at the molecular level. For instance, several signaling pathways in tumors have been the subject of investigation. By combining fluorescent proteins to other proteins, it is possible to track those proteins intracellular. An example is Philippar *et al.* who combined EGFP to Mena, a protein that is upregulated in some breast cancer cells that show invasion.[60] More of these studies are necessary to complete the picture of metastasis.

From the above discussed studies it has become clear that not all new insights are already confirmed in mammary tumors, providing an interesting direction of future research. Intravital imaging can be used to visualize the interactions of CTLs with tumor cells of a mammary tumor. It would be interesting to see if these CTLs are also able to kill mammary carcinoma cells. Moreover, the degradation of ECM by CAFs is still not visualized in mammary tumors. Altogether, there are many unsolved questions that can be studied with intravital imaging.

Intravital imaging is not only useful for the study of the natural situation of the process of metastasis. Another interesting aspect that should be implemented more often in the future in intravital imaging studies, is the possibility to study the consequences of certain manipulations. For instance, as discussed above, it would be interesting to determine the consequences of low

oxygen levels on the migration behavior of microenvironmental cells. These manipulations could provide even more insight into the functions of the microenvironmental cells in metastasis.
Other fields that might benefit from intravital imaging are the clinical and drug treatment fields. For example, intravital imaging can also be used to study the effects of for instance cancer treatments or induced genetic adjustments on specific microenvironmental cells. When the role of a specific microenvironmental cell becomes clearer it might be possible to develop a treatment that inhibits or stimulates the host cell from interacting with the tumor cells. The effects of such a treatment can eventually be studied with intravital imaging before any pre-clinical track is started. Furthermore, with intravital imaging it will become possible to visualize the specific effects of a treatment on the individual steps of metastasis. This will give more information about the exact mechanism of metastatic inhibition and provides opportunities for improving drugs by changing their effect on a certain processes. The advances made in intravital imaging improved the options of these kind of studies. With the present possibilities it is achievable to study single cells in real time *in vivo* and the effects of drug administration. This in combination with *in vitro* experiments will lead to a strong base for new medications. A combination of targeting both tumors cells as well as microenvironmental cells opens up new strategies for the fight against cancer. However, this is still a future plan and will take time before enough knowledge is gained to perform this kind of research.
Another clinical application of intravital imaging in the future might be the use of it in humans. In endoscopy there is already a technique developed called confocal laser endomicroscopy, which makes it possible to perform immediate *in vivo* imaging. Direct microscopical examination of the mucosal layer provides images of vessels, connective tissue and other subcellular structures. This technique is applicable for the upper as well as the lower gastrointestinal tract.[61] However, at this moment this is the only option of using intravital imaging in humans. Long-term tumor imaging is not possible and probably will be very hard to perform. Therefore, other clinical approaches of intravital imaging are limited and moreover it is hard to compare the data obtained from mice with the human situation. Nevertheless, intravital imaging in humans is still in its infancy so maybe in the future it will be possible to study effects in humans in real time *in vivo*.

Altogether intravital imaging studies provided great new insights in how tumor cells leave the primary tumor. The *in vivo* studies show a dynamic picture of metastasizing tumor cells that invade the blood vessels or lymph nodes. In contrast to the conventional techniques intravital imaging is able to study the individual steps of metastasis, in particular invasion and intravasation. The advances of intravital imaging have made it possible to study single tumor cells and at the same time look at the host cells. This review focused on the microenvironmental cells that play an important role in the metastasis of tumor cells to a secondary site. Intravital imaging demonstrated new insight into their behavior and influences on tumor cells, both anti- as pro-tumorigenic actions were visualized. Although there have been great advances made during the last years there are still many areas open for investigation, making intravital imaging an interesting research tool for the future.

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