Disease Models & Mechanisms 2, 107-110 (2009) doi:10.1242/dmm.001842

EDITORIAL

Visualizing anti-tumor immune responses in vivo

Jean Y. Perentes[1,2], Dan G. Duda[1] and Rakesh K. Jain[1,*]

Real-time imaging of stromal and immune cells in tumors is an emerging field that will greatly help us to understand the role of these non-malignant tumor components in tumor progression and therapy

Over the last decade, the host-derived stroma has been shown to be an active and crucial component for tumor growth and progression. Host-derived stromal cells such as immune cells, mesenchymal cells and endothelial cells can comprise more than half of the cellular content of a tumor, especially in breast cancers. Their activation and function is a crucial factor for the regression or, conversely, for the growth of tumors. Experimental evidence and theoretical considerations have suggested that the magnitude of immunological responses generated at the site of the tumor determine the ability of lymphocytes to eradicate an established tumor. However, this goal has yet to be consistently attained in the clinic because the robustness of cellular immunity against tumors appears to be heterogeneous. Further studies should provide a better understanding of the interaction between tumors and the immune system, and this knowledge should be used to optimize cancer therapies.

Multiplexed ex vivo analyses of immune cells in blood/hematopoietic organs or in tumor tissues by flow cytometry or immunohistochemistry have elevated the ability to determine, monitor and measure cellular phenotype and function in growing tumors. Using these approaches, multiple studies have shown the importance of using orthotopic tumor models – i.e. growing tumors in the environment in which they naturally occur – to study cellular immune responses. In addition, it has been shown that effector immune cells might not be distributed appropriately (for example, they are often found in the tumor periphery) or activated adequately to produce a therapeutically effective anti-tumor response. Moreover, it has been demonstrated that some of the hematopoietic cells that often infiltrate tumors have immunosuppressive functions, including $Foxp3^+CD4^+$ regulatory T lymphocytes (Tregs) and $Gr1^+CD11b^+$ myeloid cells. Finally, it has become increasingly apparent that tumor-primed myeloid cells can interact with tumor vessels and modulate their formation. Clearly, new technologies and improved models should be developed to study these intricate phenomena in real time.

Multiple time-point studies necessitate large numbers of mice and are prone to errors owing to the difficulty in accounting for mouse-to-mouse and tumor-to-tumor variability. Thus, capturing the dynamics of in vivo molecular and cellular events in a non-invasive manner is crucial for understanding the mechanisms of successful or failed immunotherapies. Dynamic in vivo studies of cells in tumors have become increasingly feasible by inducing endogenous expression of fluorescent proteins (e.g. the green or red fluorescence proteins, GFP or dsRed) in stromal cells of interest, such as endothelial, myeloid or lymphocytic cells (Fukumura et al., 1998; Stroh et al., 2005; Condeelis and Pollard, 2006; Boissonnas et al., 2007). In addition, this has been further refined by combining transgenic mouse models (OT1-GFP, Foxp3-GFP or -dsRed) with systemic infusion of fluorescently labeled cells or antibodies to track stromal cells within normal and diseased tissues (Boissonnas et al., 2007; Chiang et al., 2007; Breart et al., 2008).

In a recent article in *Disease Models & Mechanisms*, Egeblad et al. report another exciting approach for real-time imaging of the mammary tumor stroma (Egeblad et al., 2008). By combining spinning disk confocal microscopy with a motorized stage and long-term anesthesia in mice, they simultaneously acquire multicolor images in four channels over long periods of time (12 hours on average) in different tumor locations.

[1]Edwin L. Steele Laboratory for Tumor Biology, Department of Radiation Oncology, Massachusetts General Hospital, 100 Blossom Street, Cox-734, Boston, MA 02114, USA
[2]Centre Hospitalier Universitaire Vaudois (CHUV), Division of Thoracic and Vascular Surgery, Rue du Bugnon 46, 1010 Lausanne, Switzerland
*Author for correspondence (e-mail: jain@steele.mgh.harvard.edu)

This cell imaging technology was validated in immune cell-specific transgenic mouse models. The authors detected in vivo Foxp3$^+$ Tregs, CD11c$^+$ dendritic cells, c-fms$^+$ myeloid cells and Fsp1$^+$ carcinoma-associated fibroblasts (CAFs) in spontaneously arising mammary tumors based on endogenous expression of fluorescent proteins. Using their technique, they also show that the stromal cells have different migration patterns and behaviors depending on the microenvironment in which they are located: Tregs, dendritic cells, myeloid cells and CAFs display higher motility patterns at the tumor periphery compared with the tumor center. Interestingly, they show that Tregs, but not myeloid cells, reversibly decrease their motility in low oxygen conditions. Finally, Egeblad et al. identify three distinct myeloid cell subgroups based on the cellular motility, uptake of intravenously infused fluorescently labeled dextran and plasma membrane receptor expression (evaluated ex vivo). In peri-tumoral areas, they detected motile CD68$^+$CD206$^-$ non-phagocytic (dextran$^-$) cells, sessile CD68$^+$CD206$^+$ phagocytic (dextran$^+$) or 'activated' macrophages and sessile CD68$^+$CD206$^-$ non-phagocytic (dextran$^-$) cells within the tumor mass. Using in vivo immunostaining with fluorescently labeled anti-Gr1 antibodies, they established that a subset of these myeloid cells were Gr1$^+$ and preferentially resided in peri-tumoral areas.

This study has numerous important implications. The establishment of real-time microscopy technologies such as the one presented in the article by Egeblad et al. has great potential to enhance our understanding of the interactions of a tumor with its stroma. First, methods that allow the concomitant observation of different stromal compartments in mammary tumors in vivo are crucial for mechanistic studies of cell-cell and cell-matrix interaction. Second, these real-time imaging approaches can be exploited to determine the influence of the tumor microenvironment, and its changes over time, on immune or stromal cell function in cancer. For example, the authors show that a decrease in oxygen tension (a hallmark of most tumors) limits Treg, but not myeloid cell, motility. Finally, if these techniques will permit reliable detection and measurement of the cell surface phenotype in real time, then this will permit unique studies of tumor immunology and immunotherapy.

The study also raises crucial methodological questions. Microscopy imaging technologies are continuously evolving and many of them allow in situ analysis of stromal cells in vivo at high resolution, such as the confocal imaging system reported in this paper or the multiphoton laser scanning systems reported by others (Jain et al., 2002; Condeelis and Pollard, 2006; Mrass et al., 2006). But, as seen in this study and in others, because of overlapping surface marker expression, simultaneous identification of the phenotype and function of multiple different immune and stromal cells can only be accomplished by using panels of positive and negative markers. This cannot be achieved in vivo with standard fluorophores, which have relatively narrow excitation and broad emission spectra. In addition, several excitation wavelengths may be required to excite injected fluorophores and intrinsic signals. Overlapping fluorescence emission may obscure the delineation between multiple probes, resulting in low sensitivity. Moreover, the cells of interest may undergo differentiation and/or changes in phenotype owing to 'activation' in response to tumor-secreted factors such as transforming growth factor-β (TGF-β). Thus, novel probes should be developed for use with confocal and multiphoton microscopy systems to overcome the technical challenges facing this research and to capture the dynamics of multiple molecular and cellular events. One potential solution is the application of fluorescent nanocrystal (NC) technology (also referred to as quantum dots) combined with these microscopy systems. Cell labeling with NCs has seen enormous growth because they are photostable, they can be tuned to a desired narrow emission spectrum, they are relatively insensitive to excitation wavelength, and they are

especially efficient fluorophores. Recent studies have exploited these optical properties for imaging NC-labeled cells in tumor studies and in monitoring the trafficking of bone marrow precursor cells (Stroh et al., 2005). If established and validated, the use of NCs would allow longitudinal studies (across tumor types and mouse strains) of multiple immune cells in tumors without the need for complex transgenic mouse models.

Finally, the study also raises important biological questions. Technologies that allow in vivo multiplexed analyses of cells, molecules and extracellular matrices in real time open the door for studies aimed at deciphering the role of the tumor microenvironment in immune cell dysfunction in tumors and at designing judicious approaches to direct and boost immune responses against tumors. The study by Egeblad et al. reports the consequence of external oxygen tension modulation on immune cell motility in the tumor. These studies could be extended to study the role of intratumoral hypoxia or acidosis, which are frequently seen in tumors and can be measured intratumorally (Helmlinger et al., 1997), on the function of different immune cells. Using time-lapse multiphoton microscopy and GFP-transgenic mice, our group has shown recently that host cells interact with collagen fibers over a long time (Perentes et al., 2009). Similarly, this opens the possibility to study the effects of changes in the tumor microenvironment on the immune response after tumor treatment with various agents. For example, 'normalization' of the tumor vasculature, i.e. improvement in the structure and function of the vasculature, has been achieved transiently (with antiangiogenic therapy) and more persistently (by genetic means such as RGS5 deletion) (Jain, 2005; Hamzah et al., 2008). Vascular normalization has led to a marked improvement in lymphocyte trafficking and function in tumors, opening new avenues for research on combinatorial use of immunotherapy with molecularly targeted

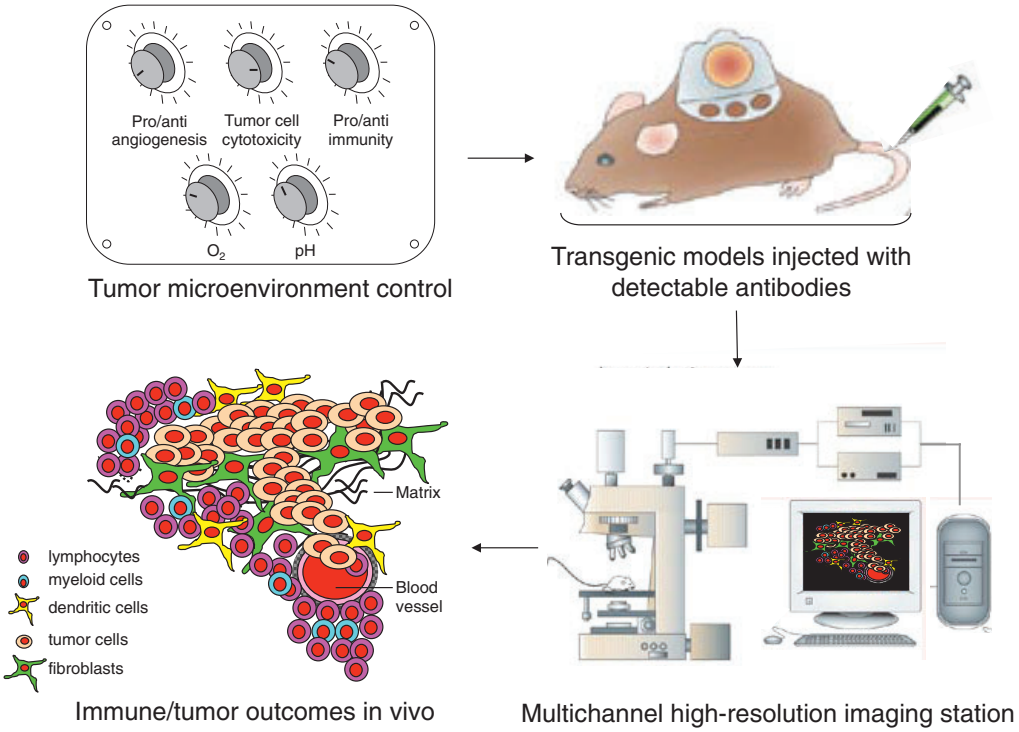

Fig. 1. Multiplexed imaging of cells in the tumor stroma using high-resolution multichannel microscopy. This technique enables studies of the tumor microenvironment in real time, and might allow an evaluation of how changes in the microenvironment during treatment affect cellular immune responses. Figure adapted with permission from Nature Publishing Group (Jain et al., 2002).

therapies (Manning et al., 2007; Hamzah et al., 2008). However, prolonged antiangiogenic treatment or the use of other anti-cancer agents, such as less specific cytotoxics, could result in significant inflammatory cell infiltration in tumors, for example with myeloid suppressor cells. This has been shown to cause tumors to become resistant to therapy and might promote tumor re-growth after treatment (Shojaei et al., 2007; Ahn and Brown, 2008). Imaging the interactions between these cells and the tumor during re-growth would be important for the development of strategies aimed at preventing tumor re-growth after treatment (Fig. 1). Finally, the lack of lymphatic function in most tumors might limit the trafficking of antigen-presenting cells to lymph nodes and thus inhibit immune responses against the tumors (Jain et al., 2002). Since intravital microscopy techniques are available to study the function of lymphatics and cellular trafficking to lymph nodes, future studies of immune cells should address this important issue.

Real-time imaging of stromal and immune cells in tumors is an emerging field that will greatly help us to understand the role of these non-malignant tumor components in tumor progression and therapy. In particular, the imaging of lymphocyte interactions with cancer cells (indicative of cell killing), and of myeloid cell interactions with lymphocytes (indicative of lymphocyte activation or suppression) or with blood vessels (suggestive of new vessel formation) will greatly enhance our understanding of the immune response and angiogenesis in tumors. Finally, the real-time imaging technology of the immune responses in orthotopic tumor models might help guide the optimal use of immunotherapies.

ACKNOWLEGEMENTS

The research of the authors is supported by grants P01-CA80124, R01CA115767, R01CA85140 and R01CA126642 from the National Cancer Institute of the USA.

REFERENCES

Ahn, G. O. and Brown, J. M. (2008). Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. *Cancer Cell* **13**, 193-205.

Boissonnas, A., Fetler, L., Zeelenberg, I. S., Hugues, S. and Amigorena, S. (2007). In vivo imaging of cytotoxic T cell infiltration and elimination of a solid tumor. *J. Exp. Med.* **204**, 345-356.

Breart, B., Lemaitre, F., Celli, S. and Bousso, P. (2008). Two-photon imaging of intratumoral CD8+ T cell cytotoxic activity during adoptive T cell therapy in mice. *J. Clin. Invest.* **118**, 1390-1397.

Chiang, E. Y., Hidalgo, A., Chang, J. and Frenette, P. S. (2007). Imaging receptor microdomains on leukocyte subsets in live mice. *Nat. Methods* **4**, 219-222.

Condeelis, J. and Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263-266.

Egeblad, M., Ewald, A. J., Askautrud, H. A., Truitt, M. L., Welm, B. E., Bainbridge, E., Peeters, G., Krummel, M. F. and Werb, Z. (2008). Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. *Dis. Model. Mech.* **1**, 155-167.

Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E. C., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R. K. et al. (1998). Tumor induction of VEGF promoter activity in stromal cells. *Cell* **94**, 715-725.

Hamzah, J., Jugold, M., Kiessling, F., Rigby, P., Manzur, M., Marti, H. H., Rabie, T., Kaden, S., Grone, H. J., Hammerling, G. J. et al. (2008). Vascular normalization in Rgs5-deficient tumours promotes immune destruction. *Nature* **453**, 410-414.

Helmlinger, G., Yuan, F., Dellian, M. and Jain, R. K. (1997). Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nat. Med.* **3**, 177-182.

Jain, R. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307**, 58-62.

Jain, R. K., Munn, L. L. and Fukumura, D. (2002). Dissecting tumour pathophysiology using intravital microscopy. *Nat. Rev. Cancer* **2**, 266-276.

Manning, E. A., Ullman, J. G., Leatherman, J. M., Asquith, J. M., Hansen, T. R., Armstrong, T. D., Hicklin, D. J., Jaffee, E. M. and Emens, L. A. (2007). A vascular endothelial growth factor receptor-2 inhibitor enhances antitumor immunity through an immune-based mechanism. *Clin. Cancer Res.* **13**, 3951-3959.

Mrass, P., Takano, H., Ng, L. G., Daxini, S., Lasaro, M. O., Iparraguirre, A., Cavanagh, L. L., von Andrian, U. H., Ertl, H. C., Haydon, P. G. et al. (2006). Random migration precedes stable target cell interactions of tumor-infiltrating T cells. *J. Exp. Med.* **203**, 2749-2761.

Perentes, J. Y., McKee, T. D., Ley, C. D., Mathiew, H., Dawson, M., Padera, T. P., Munn, L. L., Jain, R. K. and Boucher, Y. (2009). In vivo imaging of extracellular matrix remodeling by tumor-associated fibroblasts. *Nat. Methods* **6**, 143-145.

Shojaei, F., Wu, X., Malik, A. K., Zhong, C., Baldwin, M. E., Schanz, S., Fuh, G., Gerber, H. P. and Ferrara, N. (2007). Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat. Biotechnol.* **25**, 911-920.

Stroh, M., Zimmer, J. P., Duda, D. G., Levchenko, T. S., Cohen, K. S., Brown, E. B., Scadden, D. T., Torchilin, V. P., Bawendi, M. G., Fukumura, D. et al. (2005). Quantum dots spectrally distinguish multiple species within the tumor milieu in vivo. *Nat. Med.* **11**, 678-682.