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Clinical Cancer Research

Immunotherapy of Cancer Visualized by Live Microscopy: Seeing Is Believing

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The success of immunotherapy of cancer depends on several cellular events in the tumors that can be visualized by live microscopy strategies in experimental models. Taking advantage of advanced microscopy techniques, Lehmann and colleagues explore in this issue of *CCR* the mechanism of action of a novel bispecific mAb (TCB-CEA) that targets membrane-bound CEA and CD3ε. *Clin Cancer Res; 22(17); 1–3. ©2016 AACR.*

See related article by Lehmann et al., p. 4417

In this issue of *Clinical Cancer Research*, Lehmann and colleagues (1) provide supporting evidence for the antitumor activity of an immunoglobulin-based bispecific biomolecule that targets carcinoembryonic antigen (CEA)-positive tumor cells and CD3ε (TCB-CEA). Additional data concerning the characterization of this novel antibody-based molecule and its antitumor efficacy have been previously published in this journal (2). The CEA-CD3–bispecific antibody compound (RO6958688) was carefully designed to bind with a high-affinity CEA epitope proximal to the cleavage site of the glycoprotein, allowing therefore to only engage membrane-bound CEA and not the cleaved soluble form. The other specificity of the antibody targets CD3ε, with relatively low affinity. This feature should ensure that the T cells only get activated within the tumors, once getting in touch with target cells.

Several approaches based on microscopy are used to define the activity of the new agent. First, conventional confocal microscopy on living cocultures of T cells and tumor cells was performed to show efficient activation and killing mediated by the bispecific antibody, which also favored the formation of multiple stable cell contacts between T lymphocyte and cancer cells (artificial immune synapses). These data in conjunction with morphological evidence that showed T-cell polarization mediated by TCB-CEA (2) suggest that several mechanisms convey to mediate, at least *in vitro*, effective killing of tumor cells with this compound. However, the outcome of this 2D coculture system reflects defectively the situation *in vivo*. To overcome these limitations, the authors performed whole-body fluorescence imaging and confocal microscopy to confirm that their bispecific antibody effectively accumulates in CEA-positive tumors engrafted in mice.

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Intravital microscopy has rapidly evolved to permit *in vivo* tissue imaging specifically identifying cell subsets by the use of transgenic fluorescent proteins or *ex vivo* labeling with fluorescent probes (Fig. 1). For this purpose, two-photon microscopy is the most commonly used technology. With this technique, tissue can be penetrated up to 200 μm in depth. The physiology of the immune system is one of the research fields that have benefited the most from this microscopy technology advances, mainly through the pioneering work of Cahalan, Germain, Bousso, and Von Adrian (3). Dynamic time-lapse images preserving the physiologic conditions have allowed for important observations regarding cell migration of immune cell subsets and cell-to-cell interactions. Unfortunately, the technique does not yet offer, in general, good subcellular resolution for molecular imaging, although novel complementary and correlative approaches have been recently introduced to overcome these limitations (3, 4).

In the case of tumor immunology, techniques are available to continuously image transplantable mouse tumors under multiphoton confocal microscopy. Using glass chambers on skin flaps over tumors, some groups have shown the kinetics of CTLs and NK cells when dealing with transplanted tumors (5, 6). Several parameters, such as efficient infiltration of T cells in the tumor tissue, numbers and persistence of contacts with tumor cells, and appearance of morphological features of apoptosis on target cancer cells, can be quantified in these studies (Fig. 1). Functional interactions with other components of the complex cellular and extracellular matrix milieu present in tumors have also been studied in the last years, including myeloid cells, blood vasculature, and extracellular matrix constituents. Sufficiently long engraftment of tumors to mimic a well-established lesion is also considered of importance (7). Even if still imperfect, intravital microscopy has allowed us to gain insights into the effects of immunostimulatory mAbs, such as anti-CD137 (8) and anti–PD-1 (9). Those studies have been mainly focused on the CTL behavior within the tumor, although new exciting reports are probably about to unravel the dynamic interplay with other immune cells, such as dendritic cells and CD4 T cells in the context of several strategies of immunotherapy.

To functionally explore TCB-CEA, Lehmann and colleagues (1) use a reductionist but yet relevant strategy, coengrafting PBMCs from a healthy donor stained with a fluorescent probe with red fluorescent CEA^+ transfected tumor cells. Despite the limitations, they are able to show in their imaging data similar results to those

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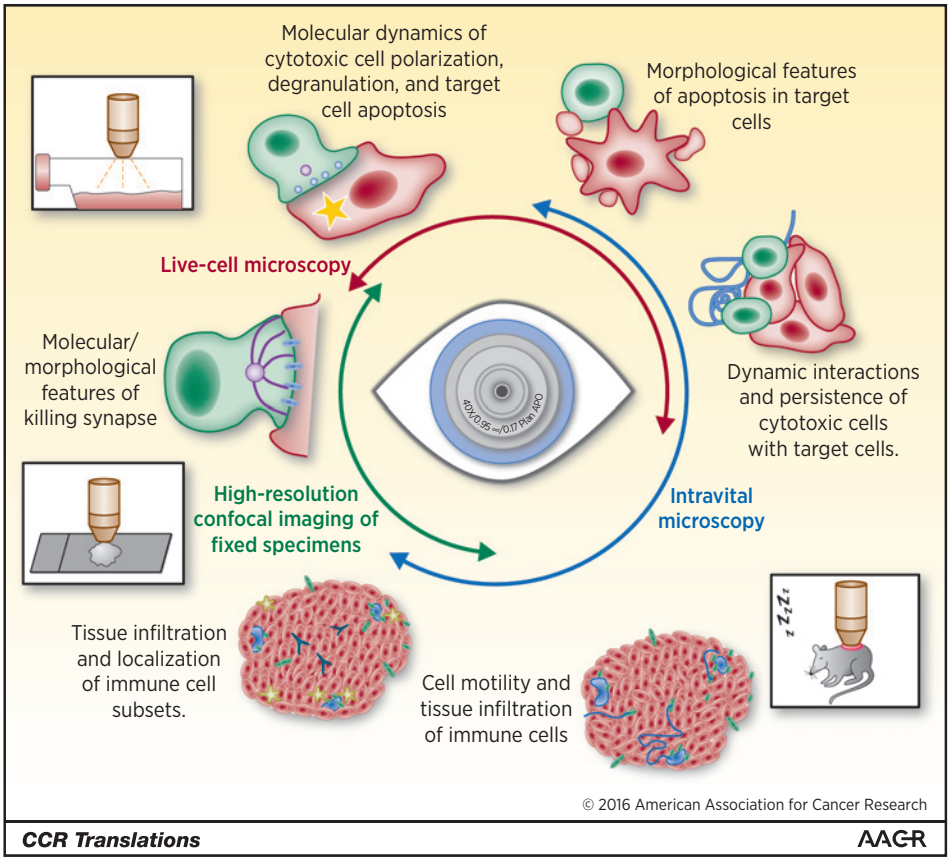

Figure 1.
Confocal microscopy technical resources to evaluate cancer immunotherapy. Scheme of microscopy approaches that can be undertaken to observe immune cells performing their functions in malignant and lymphoid tissue. Key measurable parameters by each technique are represented. Coculture systems underestimate the many factors that determine the efficacy of immunotherapy, even though they may outline some of the involved functions. Experimental approaches based on prestaining with fluorescent dyes or transgenic fluorescent proteins are set up to offer more accurate answers. Multiplex fluorescence microscopy of fixed tissue offers much information but misses the kinetics of the relevant immune phenomena only observable by time-lapse microscopy. *In vivo* intravital microscopy takes into account T-cell traffic and migration, which are conceivably key-limiting factors (13) for immunotherapies, such as those based on adoptive T-cell therapy, immunomodulatory mAbs, and tumor-targeted T cell–activating agents, such as TCB-CEA. Intravital microscopy is likely to help discover the unexpected, including immunotherapy combinations (12) and, at the end of the day, seeing is believing.

obtained in the less complex *in vitro* system. They report that efficient killing highly relies on T-cell activation (and subsequent proliferation/survival) within tumors and on an increased T-cell ability to engage several tumor cells. Crucially, T-cell activation in the tumor microenvironment is known to result in local production of chemokines and proinflammatory mediators, these phenomena probably explaining the influx of new coming T cells, following the local activation of preexisting tumor-infiltrating T-lymphocytes by TCB-CEA.

The resulting impressive time-lapse videos offer an important proof of concept with regard to the antitumor effects of the drug. Although the imaging system is not completely physiologic yet, efficient intravital imaging of immunotherapy should benefit in the long run from new models of humanized mice that would to some extent try to recapitulate some of the most important immune features of real patients (10). Some work needs to be done in this direction to improve the visualization of immune populations and tumors, gaining advances in nonlabeled imaging of normal and malignant tissue. Along the same lines, the development of new specific ways to fluorescently stain specific cell subpopulations without altering their functional performance should be accomplished.

The T-cell bispecific compound used by Lehmann and colleagues (1) is already undergoing early-phase clinical trials (NCT02324257). In this regard, the results of Lehmann and colleagues and in conjunction with those by Bacac and colleagues (2) nicely suggest tumor targeting and antitumor efficacy. One of the main advantages of this T cell–engaging bispecific formulation is the intelligent design of the Ab, ensuring effective targeting of the tumor and exclusive activation of T cells within the tumor microenvironment. The key point is to avoid T-cell activation in any other location, thus preventing dangerous systemic inflammation. This depends on the concept that cross-linking of TCR–CD3 complexes is absolutely required for signaling, and monomeric ligands do not trigger T-cell activation. However, in cases with abundant circulating tumor-derived microvesicles with CEA on their membrane, off-target T-cell fire may become a problem.

We will need to wait for the first release of clinical trial data to see whether the design of TCB-CEA accomplishes its intended functions in a safe manner. CEA expression on the luminal side of enterocytes could pose safety problems, but it is likely that such cell location would be out of reach based on the expected the biodistribution of TCB-CEA.

In the clinical arena, at least two combinations encompassing RO6958688 have been started: (i) concomitant combination with an anti–PD-L1 mAb (atezolizumab; NCT02650713) and (ii) with the B-cell anti-CD20 depleting mAb obinutuzumab (NCT02324257). The rationale for the first combination comes from the CD3–IFNγ–PD-L1 adaptive resistance loop. Obinutuzumab presumably aims to avoid antidrug antibodies (ADA) that could be formed against the foreign protein sequences of the bispecific antibody, such as interchain linkers. In addition to the ADAs, there is new evidence that links B cells with a protumorigenic role in gastrointestinal tumors (11) and thus provides another reason to trust the efficacy of the combination of obinutuzumab with the CEA-CD3–bispecific antibody in patients.

In vivo microscopy imaging provides robust results in support of a given immunotherapy strategy and can be especially suitable to

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test and select the most synergistic immunotherapy combinations (12). The article by Lehmann and colleagues (1) clearly adds significance to these experimental approaches and encourages further investigations with more advanced imaging techniques to better understand this and other immunotherapy compounds to define the best opportunities for synergistic combinations.

Disclosure of Potential Conflicts of Interest

I. Melero is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, Incyte, Novartis, and Roche. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: A. Teijeira, I. Etxeberria, M. Ponz-Sarvise, I. Melero

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