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Novel applications of nanobodies for *in vivo* bio-imaging of inflamed tissues in inflammatory diseases and cancer

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ABSTRACT

In vivo imaging technology holds promise for refined monitoring of inflammation, both in the clinic and in preclinical animal models, with applications including improved diagnosis, prognosis and therapy monitoring. In particular, molecular imaging, aimed at non-invasively studying molecular and cellular processes in intact organisms, can hereby not only provide information about the amount of inflammation, but also on the type of inflammation and on cells and/or receptors involved. Hereto, an important requisite is the availability of the proper biomarkers and specific probes for targeting these biomarkers. In the current review, we focus on a number of markers on inflamed endothelium and infiltrating myeloid cells (including macrophages) as interesting targets for tracking inflammatory reactions and argue that such markers are not only useful in case of inflammatory diseases of infectious or autoimmune origin, but also for monitoring cancer evolution through the associated inflammation. We elaborate on nanobodies as innovative, specific probes to target these inflammation-associated markers for *in vivo* molecular imaging.

Introduction

Inflammation is one of the major signs indicating the presence of either exogenous or endogenous danger signals in one of the body's tissues or organs. Be it infection, external or internal damage or even cancer, immune cells infiltrate the tissue in question in an attempt to remedy the situation. Sometimes this results in resolution of the problem by clearing infectious agents or malignant cells. In other cases, such as tbc or cancer, the problem is not solved and a stand-off between the cause of the inflammation and the immune system ensues. In the worse case, such as auto-immunity, the problem is even caused or aggravated by the involvement of the immune system and the collateral damage to host tissues and organs due to persistent inflammatory insult. The development of new and improved therapeutic modalities and compounds for such inflammatory diseases is dependent on both clinical studies as well as on appropriate animal models. Cutting-edge bio-imaging techniques offer perspectives for refined *in vivo* monitoring of inflammation and the associated biomarker expression, both in the clinic and in preclinical models. Indeed, in the clinic, there is a demand for biomarkers that (i) facilitate early diagnosis and prognosis of disease, (ii) allow more accurate monitoring and even prediction of treatment outcome, including stratification of patients in responders and non-responders and/or (iii) facilitate unveiling of pathogenic mechanisms and pathways, thus paving the way for personalized medicine. In animal models, refined monitoring of inflammatory biomarkers may aid in providing insight in disease pathogenesis and offers perspectives for in-depth preclinical testing and mode-of-action analysis of new disease-modifying compounds.

Inflammation and molecular imaging: homing in on specific targets

Much has changed since the days that inflammation was diagnosed solely by dolor, calor, rubor and tumor. Nowadays, a

Abbreviations: CDR, complementarity determining region; CEA, carcinoembryonic antigen; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; MAbs, monoclonal antibodies; MGL, macrophage galactose-type C-type lectins; MMR, macrophage mannose receptor; PET, positron-emission tomography; PSMA, prostate-specific membrane antigen; SPECT, single photon-emission computed tomography; TAA, tumor associated antigens; TAM, tumor associated macrophage; VCAM-1, vascular cell adhesion molecule-1.

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whole range of sensitive techniques, e.g. serum cytokine ELISAs or immunodetection of biopsy samples, are available. In recent years, non-invasive molecular imaging, aimed at tracking cellular and molecular events in their native environment in the intact living subject, has grown to be a powerful complement to these techniques.

In its broadest sense, molecular imaging entails the administration of a tracer molecule labeled with a contrast reagent for visualization. Primarily, radioactively labeled tracers are used in combination with positron-emission tomography (PET) or single photon-emission computed tomography (SPECT)-based imaging techniques (Pysz et al. 2010). In the clinic, the majority of molecular inflammation and cancer imaging is currently still performed based on detection of enhanced metabolism in activated leukocytes or cancer cells using ^{18}F radiolabeled deoxyglucose (Coenen et al. 2010), while ^{99m}Tc-labeled human serum albumin is used for lymphoscintigraphic mapping of the draining lymph nodes in cancer (Kim et al. 2001). Although useful, these tracers do not target a specific molecule or receptor on the surface of the cells involved in the disease process. Therefore, there is a need for probes that allow a more specific molecular characterization of inflamed or diseased tissue using disease related membrane antigens (Coenen et al. 2010). These specific markers can help to define the phenotype of a disease and can be targeted by specific agents like monoclonal antibodies (MAbs).

Since there had been a long-standing clinical experience with radiolabeled human leukocytes for inflammation imaging, anti-granulocyte antibodies have been among the first antibodies to be evaluated for the detection of focal inflammatory lesions (Locher et al. 1986). Specific detection of inflammatory infiltrates in rheumatoid arthritis patients has also been documented with the use of MAbs against CD3 (Marcus et al. 1994) or CD4 (Kinne et al. 1993). In addition to molecules expressed on immune cells, E-selectin, as a marker expressed on vascular endothelium during inflammatory responses, has been documented as a useful target for radiolabeled MAbs to image localized inflammatory tissues, both in preclinical models (Keelan et al. 1994) and in patients (Chapman et al. 1996). As discussed further below, full-sized MAbs have a number of disadvantages that have so far limited their effective use in the clinic. Yet, these early studies with MAbs and follow-up studies using additional targets and tracer formats (Signore et al. 2010) have documented the inherent value of markers on immune cells and inflamed epithelium as targets for molecular imaging of inflammation. They have also offered perspectives for exploiting the full potential of molecular inflammation imaging to provide combined information on the location of inflammatory sites throughout the body, as well as the intensity of inflammation, the type of inflammation and specific cells and receptors involved.

In this context, the choice of the targeted molecular markers will be a critical factor in determining whether it is possible to acquire in-depth molecular information on the underlying disease process. As central players in the inflammatory process, innate immune cells of the myeloid lineage such as macrophages or granulocytes should hereby not be overlooked as source of potential biomarkers. These cells are characterized by a remarkable plasticity and versatility in response to microenvironmental signals to which they are exposed, resulting in a continuum of activation and differentiation states, reflecting different functional phenotypes (Mantovani et al. 2004; Mosser and Edwards 2008). For example, the so-called alternatively activated macrophages (or M2 macrophages) have been associated with limiting collateral tissue damage during excessive infection-associated type 1 inflammation (Raes et al. 2007). As such, mouse and human M2-associated surface markers, such as Macrophage mannose receptor (MMR), Macrophage galactose-type C-type lectins (MGL) and E-cadherin (Ghassabeh et al. 2006; Raes et al. 2005; Stein et al. 1992; Van den Bossche et al. 2009) may represent useful biomarkers for using myeloid cells and the activation states they acquire in response to various triggers as sensitive *in vivo* sensors during inflammation and cancer.

Tumors, more than just cancer cells

Several FDA approved MAbs directed against tumor-associated antigens (TAAs) on malignant cells are being applied for diagnosis and treatment of cancer, with a few of the most commonly used MAbs being human epidermal growth factor receptor 2 (HER2)-specific Trastuzumab (Dijkers et al. 2010), carcinoembryonic antigen (CEA)-specific Arcitumomab (Hong et al. 2008) and prostate-specific membrane antigen (PSMA)-specific Capromab (Aparici et al. 2012). Yet, although the direct targeting of antibody moieties to TAAs on malignant cells is a potent tool that has reached clinical maturity, the non-transformed cells present within the tumor microenvironment can also provide useful biomarkers for molecular imaging, as an alternative or complement to markers on the inherently genetically instable transformed cells. Indeed, tumors should be considered as organ-like structures featuring a complex bidirectional interplay between transformed (cancer) and non-transformed (stromal) cells, whereby stromal cells can critically contribute to tumor initiation, growth and metastasis. Hence, targeting these tumor-associated stroma cells for imaging could provide additional information on the state of the tumor or response to therapy. For example, taking into account that tumors are dependent on angiogenesis for growth beyond 1-2 mm in size, there is a growing interest in techniques for non-invasive visualization of angiogenesis in growing tumors and evaluating the efficacy of angiostatic therapies (Dijkgraaf and Boerman 2009). Potential targets include biomarkers that are preferentially expressed on newly formed blood vessels in tumors, such as alpha(v)beta(3) integrin and vascular endothelial growth factor.

Also tumor-infiltrating immune cells are interesting targets. In this context, accumulating evidence has implicated tumor-associated macrophages (TAMs) in several aspects of tumor biology, orchestrating the inflammatory events during de novo carcinogenesis, participating in tumor immunosurveillance and contributing to the progression of established tumors (Laoui et al. 2011b). Here also, the plasticity of macrophages offers perspectives for using them as *in vivo* sensors for the tumor microenvironment they are exposed to. As a matter of fact, at the tumor site, these cells are confronted with different tumor microenvironments, leading to different TAM subsets with specialized functions and distinct molecular profiles (Laoui et al. 2011a). For example in mammary tumors, at least two distinct TAM subpopulations have been described, based on a differential expression of markers such as MMR or MHC II, differences in pro-angiogenic or immunosuppressive properties and intratumoral localization (normoxic/perivascular tumor areas versus hypoxic regions). In particular, the association of MMR-high TAMs with hypoxic regions in the tumor (Movahedi et al. 2010) offers perspectives for image-guided radiotherapy, as will be discussed below.

Nanobodies: small, high-affinity antigen-binding moieties

MAbs are macromolecules with a relatively poor penetration into solid and isolated tissues such as tumors (Hughes et al. 2000). In addition, complete MAbs feature a long residence time in the body and a potential increase in background signals because of binding to Fc receptors on non-target cells, making them less suitable for molecular imaging applications. Indeed, for imaging the most important properties of a tracer are: rapid interaction with the target, fast clearing of unbound molecules from the body and