

Secreted tumor antigens – immune biomarkers for diagnosis and therapy

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Abbreviations:

APC: antigen presenting cell

ECM: extracellular matrix

MHC: major histocompatibility complex

CTL: Cytotoxic T cell

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2-DE: 2-dimensional electrophoresis

IEF: isoelectric focusing

SERPA: Serologic Proteome Analysis

TIL: tumor infiltrating lymphocytes

ASC: antibody secreting cell

Keyword: tumor antigens, neoantigens, antibody, autoantibodies, diagnosis, biomarker, cancer vaccine, immunoproteomics, lymph nodes, B cells, antibody secreting cell, ASC probes

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1. Abstract

With the advent of immunotherapies for cancer, there is growing interest in the identification of tumor antigens. Tumor antigens are self-molecules altered through genetic mutations (neoantigens), protein truncation, protein misfolding or abnormal post translational modifications. To induce an immune response, tumor antigens need to be secreted into the tumor environment and presented to the immune system in the draining lymph nodes, resulting in the generation of tumor-specific effector cells and antibodies. Cytotoxic T cells (CTL) are thought to be responsible for killing of tumor cells, and several recent studies have used MS, combined with exome/transcriptome sequencing and bioinformatics, to identify their cognate peptide ligands on tumor MHC class I molecules. Circulating (serum) antibodies have been more widely used to identify tumor antigens in a range of human cancers, using 2-D western blots, immunoaffinity and microarray technologies. More specific antibody probes have been generated by harvesting antibodies directly from antibody secreting cells (ASC) through *in vitro* cultures of lymph node cells (ASC-probes) or B cell immortalization. Further identification and characterization of tumor antigens is likely to have important implications for cancer diagnostic and biomarker discovery, immune profiling, and the development of cancer vaccines and targeted immunotherapies.

2. Introduction

The immune system plays a critical role in oncogenesis [1, 2]. The microenvironment of most solid tumors is characterized by the presence of both innate and adaptive immune cells, and their level infiltration has been shown to be associated with improved survival in some cancer types [3-6]. Infiltration of immune cells is critically dependent on the extracellular matrix (ECM), and altered ECM, in particular fibrosis, is thought to be an important factor in tumor evasion of immune destruction [7]. While the effect of the tumor ECM on immune cell infiltration and activation is increasingly recognized [8, 9], the role of the immune system in establishing an altered ECM is less

clear. Chronic inflammation is well known to result in tissue remodeling in immune-mediated diseases such as allergic asthma and chronic infections [10] and an altered ECM may therefore be the result of chronic immune stimulation with tumor antigens. Other cellular components in the tumor environment such as fibroblasts, macrophage-type cells and vascular endothelial cells contribute to support tumor growth but can also impair host immune responses and contribute to immune cell infiltration [4]. The immune system is therefore an integral component of the tumor microenvironment and a contributor to changes in the ECM. The recent clinical success with immune checkpoint inhibitors has sparked a renewed interest in therapeutically targeting immune system-tumor interactions and identifying the relevant tumor antigens in cancer patients [1, 11-14].

The generation of an immune response in cancer is similar to an infection [2, 15]. The first step is antigen recognition, whereby certain molecules (antigens) are recognized by immune receptors present on the surface of lymphocytes (Figure 1). In infectious diseases, these antigens comprise pathogen molecules exposed to the host's immune system. In the case of cancer, they constitute mutated or altered self-molecules created during oncogenesis [16, 17].

For antigens to be seen by specific T cell receptors, they must be captured by antigen presenting cells (APCs), internally processed, and represented on the surface as peptides in association with major histocompatibility complex (MHC) molecules. B cell antigen-receptors (surface Ig) on the other hand, recognize antigens in their native form and each B cell will only recognize one small part (epitope) of a complex molecule. Most B cells do require help from 'helper' T cells for affinity maturation, isotype switching and transformation into antibody secreting cells or plasmablasts [15], so that antigens have to be presented in both native and processed form for effective antibody generation. MHC class II-restricted helper T cells are also required for the effective generation of cytotoxic T cells (CTL) and the subsequent effector CTL can recognize antigenic peptides bound to MHC class I molecules presented on the surface of tumor cells. These intricate immune interactions take place in the lymph nodes draining the tissue where the antigen is expressed, and the resulting effector cells and antibodies are released into the blood circulation (Figure 1). Factors within the tumor environment will then determine the level of lymphocyte infiltration and activity and are generally suppressive in growing tumors [4, 18].

3. Secreted tumor antigens

Tumor cells are derived from normal host cells whose proteins are recognized as 'self' molecules towards which the immune system is normally tolerant. In contrast, tumors are immunogenic to differing degrees, and express a variety of tumor antigens [14]. Tumor antigens have been variably classified according to their genesis and pattern of expression e.g. tumor antigens generated through somatic mutation are tumor-specific and often called neoantigens but tumor antigens can also be non-mutated proteins aberrantly expressed or modified through protein truncation or protein misfolding, or through abnormal post translational modifications such as glycosylation and phosphorylation (reviewed in [19-21]). Tumor antigen classifications are however not always

mutually exclusive and, for this review, the term 'tumor antigens' comprises all molecules produced by tumor cells that induce a detectable immune response in the tumor-bearing host (as distinct from tumor markers such as CEA that are overexpressed on tumor cells but do not generate an immune response in the tumor-bearing host).

As mentioned before, MHC class II-restricted helper T cells are required for the initiation of an immune response in the draining lymph nodes, including the production of antibody responses by B cells and the generation of cytotoxic T lymphocytes (CTL) [21]. Tumor antigens therefore need to be released into the tissue microenvironment so they can reach the lymph nodes via the afferent lymphatic vessels either in solution or captured by APCs [22]. Secretion of proteins from tumor cells can follow several pathways, including specific release through classical secretion pathways, ectodomain shedding of transmembrane proteins, and the active release of extracellular vesicles [23]. Membrane vesicles containing MHC molecules were first shown to be actively shed from lymphocytes [24] and tumor cells [25] and could either induce or suppress CTL responses, respectively [26]. There are different types of extracellular vehicles, such as exosomes, which can function as mediators of the cellular microenvironment [23, 27]. Extracellular vesicles are likely to be important carriers of tumor antigens as their size (30-150 nm) is ideal for uptake by APCs [28]. Tumor-derived exosomes have recently been shown to transfer tumor antigens to dendritic cells, the dominant APC, and induce a potent CTL response [29]. Extracellular vesicles can contain both membrane and cytoplasmic proteins which may explain the predominance of intracellular tumor antigens described in several studies [30, 31].

4. Immunoproteomic approaches towards identification of tumor antigens.

4.1 Cell-based assays

Activated CTLs are thought to be major immune effector cells responsible for killing cancer cells [7, 16]. The CTL T cell receptor recognizes antigens as processed, 8-11 amino acids-long peptides linked to MHC class I molecules on the surface of target cells. A few studies have attempted to identify tumor antigens after elution of peptides from MHC-class I molecules [32]. In one discovery platform (termed XPRESIDENT), Class I MHC molecules were immunoprecipitated from renal cell cancer tissue, the bound peptides eluted followed by mass spectrometry (MS) analysis, gene expression profiling, literature-based functional curation, and *in vitro* human T cell assays [33]. This study identified nine potentially immunogenic peptides that were shared among the patients' tumors, and their source antigens were subsequently identified. Peptides generated from somatic mutations (neoantigens) have also been identified by combining MS with individual exome and transcriptome data [34-36]. A surprising finding in several of these studies was the paucity of predicted neoantigens detected by MS in these 'MHC-ligandomes', which may be due to limitation in sensitivity and/or bioinformatics; alternatively, not all MHC class I-peptide combinations present on tumor cells may be released and presented by APC to generate a CTL response. These types of

assays are complex, generally require large amount of patient's tissues and are limited by the individual, patient-specific MHC molecules with variable binding properties. Recent refinements of procedures have however increased the sensitivity and decreased sample and time requirements which may improve their potential clinical application [34, 37]. Identification of MHC class II binding peptides by MS is more problematic as these molecules are generally only expressed on scarce APCs and not tumor cells.

4.2 Antibody-based assays

Most antigen discovery platforms in infectious diseases and cancer are antibody-based. A general criticism for the use of antibodies to identify tumor antigens, is that they may not be involved in the killing of tumor cells. While this is still debatable [38, 39], bystander antibody responses are still highly relevant to identify tumor antigens even if not functionally important, as the same antigens will also be processed for T cell recognition (Figure 1).

Antibodies have the distinct advantage that they recognize the whole, unprocessed antigen, are stable, highly specific, and biochemically well characterized. There are also many reagents and techniques available for detection and assay development. As anti-tumor antibodies can recognize (altered) self-proteins in the cancer patient, they are often referred to as 'autoantibodies'. It is still disputed if most autoantibodies truly recognize antigens specific to the tumor or are directed against unmodified self-proteins associated with tumor development [30]. While the proximal biological fluids around tumors would be the ideal source for the discovery of secreted tumor antigens [40, 41], collection is problematic and the use of tumor extracts, tumor cell lines or proteins released during cell culture are generally used in a variety of immunological assays to identify tumor antigens.

4.2.1 2-dimensional electrophoresis (2-DE) and western blotting (2-D western blots)

2-D western blots are the classical proteomic approach to antigen identification and consist of an isoelectric focusing (IEF) gel run (first dimension) combined with an SDS-PAGE run in the second dimension. 2-DE allows thousands of completely processed and post-translationally modified proteins to be resolved on one gel. Transfer of proteins from the gel to a carrier membrane makes these proteins available for screening with antibodies to identify antigenic protein spots [19]. Reactive spots are then excised from duplicate gels and identified by MS. The sensitivity of this technology has been improved over the years by automation of 2-DE techniques and improvements in the sensitivity of MS. A 2-D western blot approach, also known as Serologic Proteome analysis (SERPA), has been used to identify tumor antigens in a variety of cancers by using protein extract from tumor tissue or cultured tumor cells probed with control or patient serum to identify antigen spots uniquely present in the cancer patients [42-44].

Several problems are inherent to the first dimensional IEF run which poorly resolves very hydrophobic (most membrane proteins) or very acid or basic proteins, as well as proteins with high post-translational modification. In addition, the second dimension SDS-PAGE does not work well for very large (>200 kD) or small (<15 kD) proteins and denatures conformational epitopes [20], although partial renaturation occurs during the blotting procedure [45]. Some of the IEF limitations can be alleviated by 1-D western blotting of highly fractionated and selected protein mixes. Despite these limitations, 2-D western blots remain an efficient method for separation of complex protein mixtures and allows for global screening with antibodies of variable affinity to identify relevant antigens.

4.2.2 Immunoaffinity methods

A range of affinity methodologies have used patient serum antibodies to capture tumor antigens present in tumor cell or tissue lysate and subjecting the bound protein mix to laser desorption/ionization, TOF or LC-MS/MS [19, 20]. The antibodies can be immunoprecipitated through incubation with protein A/G beads, or immobilized to sepharose beads or immunoaffinity columns [46-48]. Generally, lysates are pre-absorbed with serum from healthy individuals to reduce non-specific reactivity or non-tumor-specific autoantibodies. Serum from cancer patients pre- and post-surgery has also been used to identify tumor antigens that decline after tumor removal [49]. A serum-based immunoprecipitation approach has been used to identify potential pancreatic cancer biomarker antigens by subtracting pre-vaccination from post-vaccination serum reactivity [48].

The advantage of the immunoaffinity approach is that it can be adapted for high throughput analysis and is sensitive to low molecular weight antigens. Disadvantages of this technique include low sensitivity for antigens > 20 kD and the difficulty of simultaneous identification of protein mixes as compared to single spots on 2-D western blots. Analysis of bound proteins by differential gel electrophoresis (DIGE) analysis has been used to resolve individual antigens after differential immunoaffinity capture [49] but adds an extra layer of complexity to the assay. Anti-tumor or autoantibodies are generally also of low affinity which diminishes their usefulness as affinity capture reagents which generally require selective, high affinity (monoclonal) antibodies to be effective.

4.2.3 Protein microarrays

Microarray technology has allowed thousands of analytical reagents to be immobilized on solid surfaces for screening of biological samples [50, 51]. It has the advantage of requiring small sample size and being amenable to high throughput and automation [40, 52]. Proteomic microarrays are comprised of protein fractions extracted from cancer cell lines using multi-dimensional fractionation techniques and LC, and have been successfully used to identify tumor antigens after screening with serum antibodies and further fractionation [6, 19, 53].

Microarrays can also be prepared using highly purified proteins or by 'reverse capture' using high affinity monoclonal antibodies (mAb) to immobilize tumor proteins on the array surface, thereby avoiding complex fractionation and preserving native configuration [6, 54]. These arrays are however limited by the availability of purified proteins and mAb.

The advantage of protein microarrays compared to the more commonly recombinant or synthetic protein and peptide arrays is that the immobilized antigens are present with their (semi)native form and post-translational modifications. The main disadvantages of this technology are the critical need for bioinformatics to process the large dataset, large-scale protein and/or antibody production and the relatively high cost. Results also need to be validated by other technologies such as ELISA.

4.3 Source of antibody probes used for immune-screening.

Most antibody screening platforms rely on the use of whole serum as a source of antibody probes for identifying tumor antigens. However, serum is a very crude source of tumor-specific antibodies, since it contains mostly antibodies induced by infections and unrelated pathological processes as well as high levels of polyreactive antibodies [55, 56]. In addition, autoantibodies may be found in the serum in the absence of overt disease and may differ among individuals [57, 58]. This makes it difficult to separate tumor antigen-specific responses from background. While proteomic technologies have been refined, this major limitation of the antibody-based discovery approach has mostly been ignored. Removal of background reactivity is therefore a major issue in serum-dependent immune assays, but attempts at background reduction can also eliminate the minor tumor-specific antibody response.

As mentioned previously, antigen-specific B cells are activated in the lymph nodes that drain the tissue site or organ from which antigens are released (Figure 1). This results in the generation of highly productive antibody secreting cells (ASCs) and the eventual release of antigen-specific antibodies into the blood circulation where they are vastly diluted with non-tumor related and cross-reactive antibodies. To bypass the problem with serum, several studies have attempted to harvest the anti-tumor antibody response directly from the B cells in the draining lymph nodes. The 'sentinel' lymph nodes draining a tumor are the first organs of metastasis in several types of cancers, such as melanoma and breast cancer, and are therefore often removed for routine evaluation. These lymph nodes can be enlarged even in the absence of tumor metastases and have been shown to contain increased IgG-positive B cells and plasma cells [30]. This is consistent with an immune response against the tumor, generally involving germinal center formation and isotype switching [15]. Tumor-draining lymph nodes therefore present a unique opportunity to access a concentrated source of tumor-specific immune cells [59]. Attempts to capture this response for identification of tumor antigens have mostly involved the generation of mAb from lymph node B cells through fusion with immortalized cell lines or Epstein–Barr virus transformation (reviewed in [30]). However, these

are complex and time-consuming procedures and the resulting antibody producing clones are not representative of the full anti-tumor repertoire [60]. More recently, recombinant antibody libraries have been derived from B cells isolated from tumor tissue, however, their selection depends on panning with known tumor antigens [61].

A simple technique for capturing the full immune profile of draining lymph node B cells, initially developed for the identification of stage-specific parasite antigens [62, 63], has been adapted for the identification of tumor antigens [64]. This technology is based on the dramatic increase of Ig protein synthesis by *in vivo* induced and fully differentiated ASCs, characterized by their ability to secrete thousands of antibody molecules/sec [65], and their ability to keep secreting these antibodies in tissue culture [66]. The 'ASC-probe' technology involves culturing cells from lymph nodes draining a tissue containing an antigenic stimulus, and harvesting the supernatant containing the antibodies secreted by the *in vivo* induced ASCs, termed ASC-probes. ASC-probes can be harvested from 1-5 days after culture and used directly to screen for antigen identification [63, 67]. ASC-probes only contain antibodies specific to the antigenic stimulus present in the target tissue at the time of collection and have a much more restricted profile than serum antibodies with minimal non-specific reactivity [63, 67]. As lymph node-derived ASC-probes contain the non-manipulated, *in vivo* immune response, they provide a rapid and valuable tool for defining the complete cancer immune profile at different stages of tumorigenesis, and for identifying the relevant tumor antigens.

Once specific tumor antigens have been identified at their point of origin, the next stage for biomarker discovery would be to develop sensitive assays to detect these antigens or their respective autoantibodies in peripheral blood or in other sources that can be non-invasively sampled. Antibodies produced in the lymph nodes will be secreted into, and should be detectable, in the bloodstream; however, not all tumor antigens will be released into the bloodstream as some may be retained in lymph nodes or rapidly degraded.

5. Clinical utility of tumor antigens and anti-tumor antibodies

5.1 Early diagnosis of cancer

Many cancers are curable if they are detected sufficiently early, however most existing blood-based cancer screening tests, such as PSA and CA-125, lack sensitivity and reliability for the early diagnosis of cancer. Many studies have attempted to identify diagnostic biomarkers in the blood using a variety of sophisticated proteomic technologies [68-70]. However, the biochemical makeup of blood is very complex and variable, making it extremely difficult to identify low abundance tumor-specific

proteins, including tumor antigens, and none of the protein biomarkers identified so far are currently in clinical use for detection of early cancer.

The production of antibodies against tumor antigens results in a very effective and specific biological amplification of a tumor biomarker as thousands of antibodies are produced against each antigenic epitope present on the tumor protein. This amplification has been shown to begin at an early stage in carcinogenesis when the tumor is not clinically detected [20, 71-73]. Assays based on detecting autoantibodies against selected tumor antigens therefore provide a magnified and indirect measurement of very small amounts of tumor proteins that are easier to detect than the protein itself, making them ideal for the detection of disease at an early stage [74-76]. In addition, while most proteins released from tumors are rapidly degraded or cleared from the circulation, antibodies are highly stable in serum, not subject to proteolysis and have a long half-life (7-30 day depending on isotype) [19, 20]. Antibody-based assays have been a hallmark for detection of infections for decades but their potential use in cancer diagnosis has not yet been fully exploited.

Steps involved in the development of a diagnostic assay based on antibodies involve (1) the discovery and identification of target-specific antigen(s), (2) the development of a sensitive *in vitro* assay for the detection of antigen-specific antibodies using native, recombinant or synthetic antigen(s), (3) differential detection of antibodies in accessible body fluids (blood, urine, saliva) of a small cohort of patients compared to healthy controls, and finally, (4) the validation of the assay for clinical use.

Most of the tumor antigens evaluated for early cancer diagnosis have been identified using serum antibodies, but, as mentioned previously, more targeted antibody sources such as lymph node-derived antibodies may be more appropriate for this first step. Several immunodiagnostic assays have been developed for disease diagnosis ranging from laboratory-based ELISA and individual point-of-care assays to fully automated multiplex immunoassay analysis [77, 78]. Several studies have now successfully tested for the presence of autoantibodies in cancer patients and a few of these have, or are in the process of being, validated in clinical trials [79-81].

5.2 Prognostic and predictive biomarkers for immunotherapy

At present, only a minority of patients responds to immunotherapy (10-40% depending on the type of cancer), but there are no accurate predictive biomarkers in routine use and it is unclear which

subgroup of patients will respond to immunotherapy. The identification of predictive immune biomarkers could dramatically increase the response rates of immunotherapy and reduce cost and unnecessary toxicity in subgroups of patients. Immunotherapy, by definition, involves immunological events and it is therefore highly likely that immune parameters or patient-specific immune signatures will be the best candidates for distinguishing responders from non-responders [82]. Immune biomarkers do not necessarily have to have a direct association with immune protection and may even indicate the opposite (e.g. markers of immune suppression).

Several studies have looked for biomarkers that indicate a general activation or suppression of an immune response, such as the infiltration of tumor tissue with lymphocytes (TIL), production of cytokines, increase in activated immune cells and expression of the immune inhibitor, PD-L1, on tumor cells [14, 83-85]. More specific biomarker studies have included profiling the patient's T- and B-cell receptor repertoire [13]. Global gene expression analysis has identified B-cell gene expression signatures associated with a favorable prognosis for several subtypes in breast and ovarian cancer suggesting that subtype-specific immunogenic epitopes could promote development of subtype-specific antibody responses [38, 86]. Despite the rapid improvements in sequencing technologies, the isolation of TILs and global gene expression profiling of individual patients is still expensive and time-consuming and does not provide a direct pathway to identification of the relevant tumor antigens. Both B and T cell receptor repertoires are shaped by the tumor antigens that induce the immune response in the tumor-draining lymph nodes resulting in proliferation and differentiation of antigen-specific T and B cells. The resulting ASCs produce antibodies that are representative of the full antigenic repertoire of the tumor. Capturing this lymph node antibody response through the ASC-probe procedure can therefore provide a specific and individual immune signature as well as a reagent for identifying relevant tumor antigens. These tumor antigens can then also be further developed into a blood-based assay for monitoring treatment responses.

Biomarker-driven patient selection has the potential to distinguish patients most likely to benefit from different immunotherapy approaches and define progression and resistance [1, 4, 14, 84]. Development of any biomarkers for clinical use can be more difficult than discovery and includes several steps in the validation process [83, 84]. The Society for Immunotherapy of Cancer (SITC) has established an 'Immune Biomarker Taskforce' to facilitate and coordinate biomarker discovery and validation (<https://www.sitcancer.org/membership/volunteer/task-forces/biomarkers>). Tumor antigens that provide the initial stimulus and specific template for immune responses are likely to have an important place in immune biomarker development.

5.3 Cancer vaccines

The discovery of tumor-specific antigens was the first indication that tumors could indeed invoke an immune response although this does not lead to protection in growing tumors [4]. Recent findings have led to the 'immunoediting' hypothesis that highlights the initial role of the immune system in destroying nascent cancer cells (immune surveillance) and the subsequent selection of immunological variants with low immunogenicity preventing rejection of established tumors (tumor escape) [2]. Recent clinical results targeting inhibitory immune checkpoints support the role of immunosuppression within the tumor microenvironment, which may explain why cancer vaccination studies have largely been unsuccessful, as they were administered in the setting of decreased immunogenicity and/or active immune suppression. Consequently, there is current interest in combining vaccination with checkpoint inhibitors as an immunotherapy strategy for cancer [1, 11, 87]. Tumor antigens are the likely targets of immune checkpoint therapy, and vaccination with appropriate tumor antigens has the potential to enhance therapeutic efficacy and expand the number of patients responding to checkpoint inhibitor therapy [12, 16, 88]. In addition, specific targeting the immune response with tumor antigen vaccines may remove the need to further increase non-specific immune activation through combining several immune checkpoint inhibitors, thereby reducing non-specific adverse effects.

6. Conclusion

With the emergence of newer, more effective immunotherapy strategies, there has never been a time where interest in cancer immunology has been as high as it is today. Tumor antigens initiate and shape the immune response and the tumor microenvironment. Emerging proteomic technologies allow for the study of antigenic changes related to conformational and posttranslational modifications that are not evident with genomic studies. It is estimated that approximately 90% of antibody recognition is through conformational epitopes [89]. Combined with more specific immunological reagents, such as tumor-specific T cell receptors and lymph node-derived ASC-probes, immunoproteomic techniques represent a valuable tool for further characterizing the cancer immune profile. Tumor antigens have the potential to distinguish patients most likely to benefit from different immunotherapy approaches and define progression and resistance. Improved approaches to identifying and characterizing tumor antigens would also have an impact on cancer vaccine development. In addition, antigen-specific antibodies may have a role in cancer diagnosis and the specific targeting of chimeric antigen receptor (CAR)-T cells and antibody-drug conjugates (ADC). It is increasingly clear that immunoproteomic approaches are vital to the successful characterization of tumor antigens and the development of immune-based therapies for cancer.

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Conflict of interest

EM is a co-founder and director of CancerProbe Pty Ltd, a company involved in identification of tumor antigens.

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Figure Legends

Figure 1

Generation and use of tumor-specific antibodies.

Tumor antigens drain to the local lymph nodes where they are taken up and processed into peptides by antigen presenting cells (APC) and presented on the APC surface in association with MHC class II molecules. Helper ($CD4^+$) T cells (Th) with complimentary T cell receptors that recognize peptide-MHC class II complexes are then activated to provide help to cognate B cells (B). B cells carry antigen-specific surface Ig that recognize antigens in their unprocessed form. These interactions take place in the lymph node germinal centers and result in B cell proliferation, isotype switching and differentiation into highly productive antibody secreting plasma cells (ASC). Tumor-specific antibodies secreted by the ASC are released into the bloodstream from where they can reach the tumor tissue. Tumor-specific antibodies can be used to identify tumor antigens and are usually collected from the blood (serum probes), where they are vastly diluted with non-specific antibodies. A more concentrated source of tumor-specific antibodies can be recovered directly from the lymph node ASC through the production of monoclonal antibodies (mAb) from ASC or after secretion of antibodies in culture (ASC-probe).

