B cells and antibody production in melanoma

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Abstract

Recent developments in the immuno-oncology field strongly support a role for the immune system in both the prevention and progression of melanoma. Melanoma is a highly immunogenic cancer, including its ability to induce tumour antigen-specific B cell and antibody responses through largely unknown mechanisms. This review considers likely hypothetical mechanisms by which anti-tumour surveillance detects pre-cancerous cells and by which immune (including B cell and antibody) responses may be elicited during malignancy. The review further considers potential pro- and anti-tumour functions of B cells and antibodies (including tertiary lymphoid structures) in both the tumour microenvironment and in circulation. Although the vast majority of studies have focused on T cells, recent evidence highlights the important roles of B cells in response to malignancy. B cells and antibodies are also discussed in the context of their potential utility as clinical biomarkers for various applications (as diagnostic, prognostic, therapeutic efficacy, and toxicity proxies), with a particular focus on protein microarray-based antibody detection and quantitation. Although the role of B cells in melanoma is incompletely understood, the measurement of circulating tumour-specific antibodies represents a promising avenue in the search for melanoma-relevant biomarkers.

Introduction

Melanoma has been suggested as a model for understanding immuno-oncology due to its capacity to evade anti-tumour immunity despite being highly immunogenic (Maio 2012). It is one of the most aggressive and lethal cancers, and its poor prognosis and high mortality necessitate prevention and early detection, with recent public health campaigns significantly reducing mortality rates (Schadendorf et al. 2015). Nonetheless, delayed diagnosis and recurrence are common, and multiple investigated therapeutic modalities (including surgery, chemotherapy, and radiotherapy) have made little impact on the mortality rate of advanced melanoma (Balch et al. 2009). This has led to the development of novel anti-melanoma therapeutics, particularly those leveraging host immunity for improved cancer targeting (immunotherapy). Despite intense interest, however, anti-melanoma therapeutic vaccines (including those comprising tumour antigens) have met with limited success in part due to immune evasion mechanisms and intra-tumoural heterogeneity (Vujanovic and Butterfield 2007; Andrews et al. 2014). On the other hand, immune checkpoint blockade targeting programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) have delivered unmatched clinical success. Immune checkpoints are pivotal in dampening immune responses as part of tolerance and immune homeostasis maintenance, but are exploited by cancer to counteract anti-tumour immunity (Da Gama Duarte et al. 2018b).

Complicating the effort to develop broadly applicable anti-melanoma therapies is the incredible molecular heterogeneity of tumours (Kumar et al. 2015). Because mutations accumulate independently in different tumour cells, sub-clones exhibit varying capacities for growth, immunogenicity, invasion, metastasis, and susceptibility/resistance to therapy. Furthermore, the ratios of anti- and pro-tumour immune cell subsets present in the tumour microenvironment (TME) and in circulation can contribute to highly heterogeneous responses to cancer (Chen and Mellman 2017).

Measuring the extent of immune engagement is of potential diagnostic, prognostic, and therapeutic value, and
warrants investigation. Although the vast majority of studies have focused on T cells, recent evidence highlights the important roles of B cells and antibodies (Tsou et al. 2016). The dynamic balance of B cells and antibodies present in the TME and circulation of melanoma patients influences both anti- and pro-tumour functions (Flynn et al. 2017), accounting for much of the conflicting evidence regarding whether these immune components oppose or favour melanoma progression (Tsou et al. 2016).

This review discusses B cell immunology in melanoma, including the pro- and anti-melanoma roles of B cells and antibodies in the TME and circulation, their detection and quantitation, and their potential for use as diagnostic, prognostic, therapeutic efficacy, and toxicity biomarkers. We focus on evidence deriving from human studies, due to a lack of concordance with murine models.

**B cell immunology**

**Early anti-tumour immune surveillance**

The immune system can efficiently detect and eliminate pre-cancerous cells in a non-inflammatory manner, before malignant transformation occurs (Swann and Smyth 2007). Early surveillance and elimination are performed by a highly specific division of immune effectors, molecules, and pathways (Swann and Smyth 2007), whose identities are dependent on the local tissue type. Cutaneous melanoma—the commonest form of melanoma—is thought to arise from melanocytes of the skin epidermal basal layer (Nishimura 2011; Lo and Fisher 2014) and the human epidermis exhibits a specialised set of humoral and cellular defences that appear to be evolutionarily tuned to the most-commonly encountered abnormalities in this tissue compartment.

However, anti-tumour surveillance can fail or elimination can be incomplete for various reasons and persistent pre-cancerous cells can become a chronic inflammatory stimulus. Since an ideal inflammatory response acutely clears the inciting stimulus to facilitate healing and restoration of homeostasis, and since inflammation can damage host tissue, chronic inflammation is, by definition, abnormal. Failure to clear pre-cancerous cells leads to a situation in which progressive neoplasia and anti-tumour responses significantly influence each other. The ‘immunoediting’ paradigm describes this unfolding bi-directional relationship, emphasising the paradoxical protective and tumour-sculpting effects of immunity (Dunn et al. 2004). Immunoediting encompasses three possible and sequential (albeit overlapping) phases: elimination, equilibrium, and escape, which have been recently reviewed (Da Gama Duarte et al. 2018b).

Over time, heterogeneity of tumour cells, their microenvironment, and immune responses also increases, becoming progressively less predictable. Relatively predictable anti-tumour surveillance and elimination mechanisms (conducted by pre-existing local sensors and rapid effectors which detect early oncogenic changes) differ significantly from the potential array of immune responses observed in established neoplasia, which can encompass the fuller complexity of systemically available and infiltrating sensors and delayed effectors, including induced adaptive responses. Furthermore, anti-tumour immune responses are only beneficial up to a point, beyond which they can become maladaptive. Thus, depending on the timing and nature of anti-tumour immune responses, they favour either host protection or neoplastic progression (De Visser et al. 2006). B cells and their soluble products (including antibodies) are involved in all stages of these immune responses to oncogenesis/neoplasia, from early surveillance through later adaptive responses, both beneficial and maladaptive.

**Discrimination between normal and tumour cells**

Clearly, immune surveillance and subsequent immune mechanisms must distinguish between normal and malignant cells (Swann and Smyth 2007). Despite central and peripheral tolerance mechanisms protecting host antigens against immunogenicity, even early oncogenic events facilitate immune recognition of pre-cancerous cells, via mechanisms well within normal immune operating parameters.

The pre-cancerous cell itself can respond immunologically to intracellular stress (Chan et al. 2014), for example, undergoing autophagy, inflammasome-mediated production of pro-inflammatory cytokines, or heterogeneous modes of cell death, some of which are more inflammatory than others (Hou et al. 2013; Janssen et al. 2016). Additionally, stress can lead to exposure, presentation, or release of host molecules (‘alarmins’) exhibiting damage/danger-associated molecular patterns: cell death, for example, can release normally intracellular molecules (i.e. molecules ordinarily shielded from the immune system) into the extracellular space. Pre-cancerous cells may even expose or express specific surface markers which facilitate recognition of their status (c.f. phosphatidylserine exposure by apoptotic cells). Furthermore, autoantigens can act immunogenically in various contexts, including increased level of expression/abnormal accumulation, encounter in a highly pro-inflammatory environment, ectopic expression in or accessing of a compartment where the antigen is not normally located (including ‘self’ antigens normally masked from immune encounter), and some form of alteration (including mutation, post-translational modification, misfolding, and aggregation) (Zaenker et al. 2016). These types of immunogenic molecules are all recognisable by innate leukocyte receptors, which recognise a broad array of immunogenic molecules, including glycolipids. Tumour antigens include unique
antigens (generally derived from point mutations) and shared antigens (see Table 1). Specific antigens (proteins, lipids, carbohydrates, or a combination) are also recognizable by innate and adaptive lymphocyte receptors.

Unique antigens are not exhibited by normal cells and usually result from mutations in ubiquitously expressed genes, but are produced in a modified form or in greater quantities/in ectopic locations by tumours (Vigneron et al. 2013). Unique antigens are not exhibited by normal cells and usually result from mutations in ubiquitously expressed genes, with the resultant products representing neoantigens. Melanomas exhibit the highest amount of somatic mutations due to UV exposure (Alexandrov et al. 2013), and are thereby estimated to have a high neoantigen repertoire (Schumacher and Schreiber 2015). In fact, up to 80% of melanomas have BRAF or NRAS mutations (Akbani et al. 2015). As a result, these and other neoantigens such as cyclin-dependent kinase 4 (CDK4) and β-catenin 1 (CTNNB1) have been explored as attractive cancer immunotherapy targets (Lu and Robbins 2016). However, neoantigen-specific T cells often recognise unique mutations and epitopes that are not shared among patients, leading to costly patient-specific approaches which may not translate into commercially viable therapeutic vaccines (Lu and Robbins 2016). On the other hand, shared antigens are expressed by normal cells, but are produced in a modified form or in greater quantities/in ectopic locations by tumours (Vigneron et al. 2013). Shared antigens can be further sub-divided into tumour-specific, differentiation, and overexpressed antigens (Vigneron et al. 2013). Of particular importance to melanoma, cancer–testis (CT) antigens are categorised as tumour-specific antigens with normal expression usually restricted to the testis, but which are aberrantly expressed by various cancers (Scanlan et al. 2002; Hofmann et al. 2008). As the testis is an immune-privileged site, aberrant expression of such antigens by cancer cells typically triggers spontaneous humoral immune responses (Scanlan et al. 2004). One such well-researched CT antigen is New York esophageal squamous cell carcinoma 1 (NY-ESO-1, also known as CTAG1), with prior studies investigating its use as a therapeutic vaccine for melanoma (Davis et al. 2004). Other CT antigens of interest include melanoma-associated antigens (MAGE) and synovial sarcoma X (SSX) antigens (Barrow et al. 2006). CT antigen expression is, however, heterogeneous among melanoma patients and within tumours, negatively affecting potential therapeutic efficacy and arguing for improved utility of several immunogenic antigens over a single antigen (Barrow et al. 2006). In melanoma, differentiation antigens are derived from proteins involved in melanin or melanosome production (Vigneron 2015), and include tyrosinase, glycoprotein 100 (gp100), and melanoma-associated antigen recognised by T cells (MART-1) (Barrow et al. 2006). However, therapeutically targeting these antigens can lead to skin depigmentation, often referred to as vitiligo (Vigneron 2015). Overexpressed antigens are expressed across a wide variety of normal tissues and overexpressed in tumours, and include preferentially expressed antigen in melanoma (PRAME), p53 protein, and apoptosis protein survivin (Vigneron 2015). Although attractive immunotherapy targets are available for several tumour types, a high risk of autoimmune toxicities is expected due to normal tissue expression of target antigen (Vigneron 2015).

### Elicitation of cellular and humoral B cell immunity

Early on, pre-existing natural (i.e. constitutive, non-elicited) and long-lived antibodies likely play an important role in anti-tumour surveillance and responses. However, over time, the evolving palette of tumour antigens may elicit new antigen-specific B cell responses, with B cells being able to infiltrate into and be influenced by the TME. Although actual mechanisms of antibody elicitation by tumours remain unclear (Tsou et al. 2016), we can hypothesise that B cells should be able to detect and respond to malignant cells in at least four different ways: innate receptor-based detection of stressed cells, pre-existing antigen-specific natural/long-lived antibodies, activation of naïve mature B cells by T-dependent or -independent antigens locally or

### Table 1 List of commonly reported tumour antigens

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Category</th>
<th>Description</th>
<th>Examples</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unique antigens</td>
<td>Mutational neoantigens</td>
<td>Normal genes altered by mutation</td>
<td>BRAF, NRAS, CDK4, CTNNB1</td>
<td>Akbani et al. (2015), Lu and Robbins (2016), and Zaenker et al. (2016)</td>
</tr>
<tr>
<td>Shared antigens</td>
<td>Tumour-specific antigens</td>
<td>Restricted normal expression, but aberrant expression in tumours</td>
<td>Cancer–testis antigens</td>
<td>Scanlan et al. (2002, 2004), Barrow et al. (2006), and Hofmann et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Differentiation antigens</td>
<td>Derived from proteins involved in melanin or melanosome production</td>
<td>Tyrosinase, gp100, MART-1</td>
<td>Vigneron (2015)</td>
</tr>
</tbody>
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This table summarises known unique and shared tumour antigens relevant to melanoma.
in secondary lymphoid organs (SLOs; see Fig. 1), or non-specific activation of memory B cells in a sufficiently inflammatory environment. Regardless of the precise mechanisms, cutaneous melanoma does elicit spontaneous anti-tumour immune responses (Maire et al. 2013; Mukherji 2013), including antibody responses to lineage-specific differentiation antigens, some CT antigens (including NY-ESO-1), and mutational neoantigens (Houghton et al. 2001; Simpson et al. 2005; Maio 2012; Lo and Fisher 2014). Indeed, B cells specific for true autoantigens do exist even in notionally healthy individuals and somatic hypermutation during certain types of B cell responses to inciting stimuli other than the cancer cells can also give rise to cross-reacting autoreactive clones.

While T cells are classically believed to be restricted to peptide antigens presented in the context of a major histocompatibility complex (MHC), B cells can respond to both peptide and carbohydrate antigens via differently timed routes to produce differential antibody responses. B cells can respond to the same MHC-presented peptides which activate T cells (T-dependent antigens): interaction of activated CD4+ T cells with cognate B cells within SLO is required for full activation of each (Breitfeld et al. 2000). In addition, B cells can also respond to soluble peptide antigens in their native conformation, as well as to soluble non-peptide (T-independent) antigens, albeit B cell receptors (BCRs) more efficiently recognise membrane-bound than soluble antigens in vivo (Heesters et al. 2016). Activation by T-independent soluble antigens requires either a single signal derived from cross-linking of multiple BCR by a high-avidity molecule, or two signals derived from simultaneous BCR and pattern recognition receptor (PRR) ligation (Berkowska et al. 2011). T-independent responses are known to occur in the mucosae, splenic marginal zone (Cerutti and Rescigno 2008; Weill et al. 2009) and marrow. Activation by T-dependent membrane-bound antigens requires two signals: BCR interaction with antigen-presenting cells (APCs) displaying the target antigen (which is then internalised by B cells for processing and display as an MHC-II complex), followed by interaction with pre-activated cognate CD4+ T cells (Garside et al. 1998; Cyster 1999). The nature of—and BCR affinity for—the antigen determines the outcome of B cell activation: Only APC-displayed peptide antigens facilitate B cell interaction with activated cognate CD4+ T cells and thus enable full activation of the former (including affinity maturation) (Heesters et al. 2016). A third signal (PRR agonisation or provision of APC-derived cytokines) (Ruprecht and Lanzavecchia 2006), while not strictly necessary, is often present, and influences the outcome of B cell activation: like other lymphocytes, functionally plastic B cells are influenced by microenvironmental cues and can adopt B cell effector 1 (Be-1), B cell effector 2 (Be-2), or regulatory B cell (Breg) (Zhang et al. 2015) phenotypes (Egbuniwe et al. 2015).

![Fig. 1](image-url) Mechanisms of B cell activation via antigen recognition. B cells may be activated by T-independent or -dependent antigens locally or in secondary lymphoid organs. Antigens targeted by B cells/antibodies are usually either free or present on cell surfaces (i.e. the targeted epitopes are usually hydrophilic and discontinuous). Cognate CD4+ T cells are capable of targeting the same antigen, via either distinct or shared epitopes. Various local stimuli (including other activated leukocyte types) determine the outcome of B cell activation. For example, stimulation in the absence of sufficient activating signals can lead to B cells becoming regulatory, or undergoing tolerisation, anergy, or clonal deletion. Successful activation produces different B cell phenotypes (e.g. IFNγ-secreting Be-1, IL-4-secreting Be-2, or IL-10- and TGFβ-secreting Breg), and plasmablasts which produce different isotypes and subclasses of antibody, with differential post-translational modification patterns and functions.
As a peripheral tolerance mechanism, not receiving all required activating signals leads to the specific lymphocyte clone becoming tolerogenic/suppressive or anergic, or undergoing clonal deletion. Successful adaptive lymphocyte activation, on the other hand, leads to clonal expansion and differentiation into effector and memory cells. Activated B cells have various functions, including acting as APC, secreting cytokines/chemokines, and producing antibodies (Egbuniwe et al. 2015). Different B cell sub-populations exhibit vastly differing functions: Be-1 produce interferon (IFN)-γ, Be-2 produce interleukin (IL)-4, and Breg produce IL-10 and transforming growth factor β (TGFβ) (Fremd et al. 2013). Antibody functions relevant to cancer include agglutination, receptor neutralisation, complement activation, opsonisation, and antibody-dependent cell-mediated cytotoxicity. Specific effects are dependent on antibody isotype, subclass, post-translational modification (e.g., Fc region glycosylation) profile, and the activating/inhibitory Fc receptor (FcR) balance expressed on antibody-responsive cells.

Depending on antigen type and BCR affinity, activated B cells differentiate into effectors either extra-follicularly or within follicular germinal centres (GCs) during induction of primary B cell responses. B cells activated by T-independent antigens are first to respond (Berkowska et al. 2011), becoming extra-follicular plasmablasts which provide a wave of circulating IgM (Lee et al. 2011). SLO-traversing B cells with moderate affinity for T-dependent antigens are next to respond: CD40-only contact with CD4+ T cells causes these B cells, too, to become extra-follicular plasmablasts, which provide the next wave of circulating IgM (Kurosaki et al. 2015). Thus, extra-follicular plasmablasts—which have not undergone affinity maturation—provide early, transient, modest-affinity circulating IgM (Ahmed and Gray 1996; Kunkel and Butcher 2003), either from SLO/barrier tissues, or after infiltrating the inflammatory site (Kunkel and Butcher 2003).

Finally, SLO-traversing B cells with high affinity for T-dependent antigens respond most slowly, because they first undergo affinity maturation and class switching within GCs. Antigen is taken up by B cells after the APC-B cell synapse (Batista et al. 2001) for display to cognate CD4+ T cells: full synapsing (Batista and Neuberger 1998) provides T cell help that facilitates B cell transition into follicular plasmablasts. These B cells differentiate into GC centrocytes and undergo clonal expansion, affinity maturation, and class switching from IgM to IgA or IgG (MacLennan 1994; Haynes et al. 2012; Pennock et al. 2013). At the plasmablast stage, they become capable of antibody secretion and cells with sub-par affinity can undergo another round of affinity maturation (Vanderleyden et al. 2014; Roth et al. 2014; Kometani and Kurosaki 2015), while sufficient-affinity cells transiently provide antibodies from within SLO or at local tissue sites (Kometani and Kurosaki 2015; Halliley et al. 2015). Thus, follicular GC-derived plasmablasts—which have undergone affinity maturation and isotype switching—provide a delayed peak of circulating high-affinity IgG days to weeks post-antigen exposure (Haynes et al. 2012).

B cells likely utilise some or all of the above mechanisms in responding to tumour antigens. Because B cell responses represent only one facet of the complex and integrated immune response, the antibody response may reflect aspects of the broader immune response, including concurrent anti-tumoural CD8+ T cell responses. Since antibodies and T cells do exhibit shared antigen specificity as required for provision of mutual help by cognate B and T cells, measuring antibody responses—especially in patients exhibiting a clinical benefit, and especially in whom antibody specificity is shared with cytotoxic CD8+ T cells—may assist in identification of a subset of candidate cancer vaccine antigens. Although B and T cells may target shared antigens, this occurs via topographically distinct epitopes, although these can be partially or completely overlapping (Harris et al. 1996). Since most antibodies act in an aqueous environment, B cell-targeted epitopes are typically superficial/free and hydrophilic, as well as usually being discontinuous (non-adjacent amino acid residues brought together by protein folding) (Sharon et al. 2014). The MHC-presented peptide repertoire, on the other hand, is determined by various factors, including antigen availability and degradability, protease activity, chaperone and peptide editor/exchange catalyst availability and activity, HLA polymorphism (and consequent peptide affinity), protein intermediate states, and thermodynamic stability of the peptide-MHC complex (Wierczorek et al. 2017). Since measurable antibodies sharing peptide antigenic targets with CD8+ T cells may activate cognate antigen-specific CD8+ T cells, such antibodies may represent attractive therapeutic candidates.

**B cells in the tumour microenvironment**

Study of the TME enables characterisation of factors derived from leukocytes, other local cell types, and tumour cells, and this can provide critical prognostic insights and even guide therapeutic intervention (Da Gama Duarte et al. 2018b). The TME can be readily investigated, for example, via histologic analysis of archival resected tumour tissue (more recent fluorescence-based techniques enable simultaneous detection of multiple immune cell sub-populations).

**Tumour-infiltrating B cells**

The presence of TME-infiltrating leukocytes has long been shown to predict a favourable clinical outcome in melanoma (Clark et al. 1989; Tefany et al. 1991; Mihm et al. 1996; Clemente et al. 1996). While innate and adaptive T cell
anti-tumour activities are well studied, knowledge regarding those of B cells lags behind (Zhang et al. 2014; Tsou et al. 2016). Although tumour lymphocytic infiltrates usually consist predominantly of T cells, B cells are also a component, and in some types of carcinoma even outnumber T cells (Spaner and Bahlo 2011). B cells infiltrate several cutaneous malignancies, including melanoma (Egbuniwe et al. 2015), and appear to have key functions within the cutaneous melanoma microenvironment (Karagiannis et al. 2013). Additionally, systemic B cell responses (e.g. alterations in circulating IgG levels) also occur during human cutaneous melanoma (Egbuniwe et al. 2015). However, despite such observations, B cell mechanistic roles in anti-tumour surveillance and -immunity remain relatively obscure (Zhang et al. 2014).

Like other leukocytes, B cells act on tumour cells both directly [via cell–cell contact (e.g. antigen presentation and co-stimulation)] and indirectly [via antibody production (including effects of antigen–antibody complexes), cytokine secretion, and effects on other cell types] (Martin and Chan 2006). Global B cell gene expression signatures and the presence of tumour-infiltrating B cells have been associated with a favourable prognosis in several cancer types (Schmidt et al. 2008; Mahmoud et al. 2012; Lohr et al. 2013; Iglesia et al. 2014). In melanoma, the high expression of a B cell signature was predictive of improved overall survival, based on mRNA sequencing data from The Cancer Genome Atlas (TCGA) database (n = 329) (Iglesia et al. 2016). When investigating the clonal diversity of these B cell infiltrates via BCR repertoire sequencing, BCR diversity was also associated with survival (Iglesia et al. 2016). More recently, an individual B cell score identified from transcriptomes of a cohort of treatment-naïve primary cutaneous melanomas (n = 703) was found to be strongly predictive of a favourable melanoma-specific survival (Nsengimana et al. 2018). These findings were validated using a primarily metastatic melanoma dataset from TCGA database (n = 472), when considering overall survival (Nsengimana et al. 2018). However, B cells are capable of engaging in both pro- and anti-tumour responses (He et al. 2014), influenced by the dynamic sub-population balance (Tan 2001; Fremd et al. 2013; Tsou et al. 2016). Despite this, B cell infiltrates are commonly identified using the pan-B cell marker CD20, rather than subset-specific markers (Flynn et al. 2017). Based on CD20 expression alone, melanoma metastasis B cell proportions range from 15 to 25% of the lymphocytic infiltrate (Hussein et al. 2006; Erdag et al. 2012). Particularly relevant to antibody production, the B cell infiltrate includes CD138+ plasma cells (Erdag et al. 2012; Bosisio et al. 2016). Based on lessons learnt from immunophenotyping circulating B cells, more inclusive B cell subset markers can be used when interrogating the TME. These may enable the identification of naïve B cells (CD19+, CD20+, CD27−), transitional B cells (CD19+, CD20+, CD24hi, CD38hi), follicular B cells (CD19+, CD20+, CD21+, CD24+, CD27−, CD38low), memory B cells (CD19+, CD20+, CD27+, CD38−), plasmablasts (CD19+, CD20−, CD27hi, CD38high, CD138+), and plasma cells (CD19low, CD20−, CD27hi, CD38high, CD138+) (Maecker et al. 2012).

A high number of intra- or peri-tumoural CD20+ B cells in primary melanoma samples provided melanoma patients with a survival advantage (Ladányi et al. 2011). Furthermore, such infiltrates were less prominent in primary melanomas that led to visceral metastases when compared to those not developing metastases or with lymph node metastases (Ladányi et al. 2011). Although intra-tumoural B cells were predominately observed in a dispersed pattern, follicle-like B cell aggregates occurred in 26% of patients (Ladányi et al. 2011). Garg et al. corroborated these findings, and further reported that primary melanomas containing many CD20+ B cells subsequently metastasised less frequently (Garg et al. 2016). Even in melanoma metastases, higher densities of CD20+ B cells were associated with increased overall survival (Erdag et al. 2012). Consistently, high CD20 mRNA expression was associated with a more favourable overall survival in melanoma, using TCGA database (n = 384) (Saul et al. 2016). However, Martinez-Rodriguez et al. reported that abundant tumour-infiltrating CD20+ B cells (> 15%) in primary cutaneous melanoma correlated with tumour recurrence and lymph node metastasis, as well as a shorter disease-free interval and overall survival (Martinez-Rodriguez et al. 2014). Other than differences in location (intra- or peri-tumoural) and B cell abundance, such contradictory findings may be attributed to the use of the CD20 pan-B cell marker, which is present on all B cell subsets prior to plasma cell differentiation. As such, the presence of heterogeneous B cell sub-populations among the detected CD20+ cells, including naïve, memory, and regulatory B cells (CD20low), may be exerting differential influences within the TME. Multiple immune and tumour factors may contribute to disrupting homeostasis and promoting cancer immune escape and tumour growth (Da Gama Duarte et al. 2018b). Further studies are required to fully comprehend how specific functionally distinct B cell subsets may confer a prognostic benefit or disadvantage.

Over 30 years ago, Weissmann et al. pioneered investigation into the presence of plasma cells within primary cutaneous melanoma infiltrates, discovering that this was a useful predictor of lymph node metastasis (Weissmann et al. 1984). Soon after, another study found that plasma cell infiltration (abundant plasma cells organised into nodules or patches, rather than dispersed or absent plasma cells) correlated with a poor prognosis (Mascaro et al. 1987). Recently, it has been found that dispersed CD138+
plasma cell infiltrates also correlated with favourable clinical outcomes in primary melanoma, while clusters were associated with a significantly worse prognosis (Bosisio et al. 2016).

A large meta-analysis of gene expression signatures demonstrated that a plasma cell signature was a significant predictor of survival in solid tumours in general (Gentles et al. 2015). Indeed, when investigating melanoma metastases, Erdag et al. reported that patients with higher densities of CD138⁺ plasma cells exhibited increased overall survival (Erdag et al. 2012). Although tumour-infiltrating plasma cells can actively produce anti-tumour antibodies (Punt et al. 1994), cross-talk with tumours can skew the humoral response to adopt regulatory or pro-tumoural functions (Saul et al. 2016). Namely IgG subclass distribution (65–80% IgG1, 2–4% IgG4) has been shown to be disrupted in melanoma, with a loss of IgG1 predominance (Saul et al. 2016). An immunosuppressive role has been reported for IgG4, the least predominant IgG subclass, with evidence of IgG4 antibodies impairing antibody—and thus anti-tumour—responses in melanoma (Karagiannis et al. 2013). Indeed, the melanoma microenvironment tends to favour type 2 helper T cell (Th2)-type inflammation (which promotes B cell IgG4 production), and elevated circulating IgG4 levels are generally associated with unfavourable outcomes, perhaps in part via interfering with IgG1’s tumoricidal effects (Egbuniwe et al. 2015). In agreement, elevated circulating IgG4 was found to be predictive of melanoma progression and poor clinical outcomes (Karagiannis et al. 2015). Only detailed antibody characterisation (specific isotype, subclass, and modification profile) can determine the specific role and associated prognostic value of TME antibodies. Furthermore, antibodies and immune complexes can recruit pro-tumourigenic myeloid cells to the TME, and can induce FcR- and complement-mediated chronic inflammation (He et al. 2014). Pro-tumoural immunity is likely partially attributable to suppressive cytokine-mediated signalling by Breg (IL-10 and TGFβ) and regulatory cell recruitment (Linnebacher and Maletzki 2012).

Conversely, tumour-infiltrating B cells may promote anti-tumour immunity via antigen processing and presentation, stimulatory cytokine-mediated signalling, formation of tertiary lymphoid structures (TLSs), antigen-driven clonal expansion, antibody production, and class switching (Nielsen and Nelson 2012; Yuen et al. 2016; Chiaruttini et al. 2017), and their effects on other leukocyte types (especially cytotoxic leukocytes). Specifically, antibody production can potentiate anti-tumour immunity via antibody-dependent cytotoxicity, complement-mediated tumour cell destruction, or opsonisation (Nelson 2010).

**Tertiary lymphoid structures**

Driven by chronic inflammation, tumour-infiltrating B cells (peri- or intra-tumoural) often organise into follicle-like aggregates resembling GCs (Ladányi et al. 2011), known as ectopic or TLSs. TLSs are also evident in various chronic inflammations, infections, and autoimmunity settings (Drayton et al. 2006; Carragher et al. 2008), and—like SLO—exhibit a B cell zone containing follicular dendritic cells, a T cell zone with mature dendritic cells, and high endothelial venules (HEVs) (Dieu-Nosjean et al. 2014). HEVs provide immune cells with a gateway to enter the TME, thereby facilitating immune cell recruitment and mobilisation (Dieu-Nosjean et al. 2016), and contribute to the generation of both effector and memory T and B cells (Germain et al. 2015). Intra-tumoural TLSs are thought to orchestrate both local and systemic anti-tumour responses, and are largely associated with a favourable clinical prognosis (Sautès-Fridman et al. 2016). With regards to B cells specifically, the majority of studies across several solid tumours investigating the presence of CD20⁺ B cell aggregates in TLSs demonstrated a correlation with improved prognosis (Sautès-Fridman et al. 2016). It has even been suggested that the presence or composition of TLSs may facilitate patient stratification approaches with regards to suitability for immunotherapy (Hiraoka et al. 2016).

TLSs have been called local antibody producing factories, where B cells are capable of undergoing dynamic tumour antigen-driven development into plasma cells that secrete large amounts of high-affinity antibodies, which may differ from those secreted into circulation from distant sites (Teillaud and Dieu-Nosjean 2017). It has even been suggested that tumour escape mechanisms may trigger in situ production of tumour-specific antibodies (Teillaud and Dieu-Nosjean 2017). As indicated above, whether these antibodies contribute to anti- or pro-tumour responses depends on the class of antibodies produced (and their glycosylation patterns), with IgG1 and IgG3 being favourable and IgG2, IgG4 and IgA unfavourable (Teillaud and Dieu-Nosjean 2017). As such, antibody responses are diverse and polyfunctional, and, accordingly, can have differential prognostic implications (Germain et al. 2015).

Regulatory cell types, such as regulatory T cells and myeloid-derived suppressor cells also occur in TLSs, and high frequencies may indicate an increased risk of relapse and a poor clinical prognosis (Colbeck et al. 2017). As such, although most evidence is supportive of an anti-tumour role, in the absence of adequate stimuli (or as modified by tumour products) TLSs may also foster immunosuppression or tumour growth. As such, the specific location of TLSs in the TME and their cellular composition is essential in determining clinical significance, a parameter which differs
across cancers and may be a contributor of contradictory findings (Colbeck et al. 2017).

Melanoma histologic analysis reveals functional TLSs with evidence of antigen-driven B cell responses (first reported in cutaneous metastases), while incomplete TLSs in primary lesions display poorly organised B cell aggregates (Cipponi et al. 2012). Furthermore, analysis of the immunoglobulin repertoire supports a local antigen-driven B cell response, with evidence of within-TLS clonal amplification, isotype switching, and somatic hypermutation (Cipponi et al. 2012). Despite Cipponi et al. reporting the absence of B cell follicles in primary melanoma, such follicles were observed in a subsequent study employing a larger patient cohort, although at lower levels than in cutaneous metastases (Ladányi et al. 2014). It is likely that TLS-derived B cell responses may occur in both early- (after induction by chronic inflammation) and late-stage disease. These structures have also been identified in metastatic melanoma using a unique chemokine gene expression signature enriched for immune- and inflammation-related genes, and were associated with improved patient outcomes (Messina et al. 2012).

Although not investigated in the context of TLSs, a high HEV density often accompanies increased lymphocytic infiltration and enhanced tumour regression in primary melanomas (Martinet et al. 2012; Avram et al. 2013). A recent study also in primary melanoma confirmed these findings, including the presence of TLSs, but found no correlation to patient prognosis (Sebestyén et al. 2018). TLSs have also been observed in desmoplastic melanoma, a subtype characterised by a high mutational burden and frequent lymphocytic infiltrates (Stowman et al. 2018).

**Circulating B cells and antibodies**

An ideal biomarker should be measurable in readily accessible peripheral fluids and be highly sensitive and specific for melanoma to achieve diagnostic, prognostic, and therapeutic monitoring utility in a clinical setting. B cell differentiation and the production of tumour-specific antibodies are dynamic processes influenced by changing tumour antigen profiles and microenvironmental as well as systemic signals. Circulating antibody characteristics and dynamics can thus provide insight into bi-directional engagement between the immune system and the tumour(s). As mentioned, melanomas are highly heterogeneous and unstable, experiencing changes in tumour antigen expression profiles—including CT and melanoma differentiation antigens—over time (Elder et al. 1989; Svobodová et al. 2011). This poses a challenge to effector leukocytes, including B cells, which must continually develop new antigen-specificities. The ability to simultaneously assay a significant proportion of the systemic tumour antigen-specific antibody repertoire would thus provide a unique snapshot of the tumour at a given time point, while circumventing the influence of inter- and intra-tumour heterogeneity that affects tissue-based techniques, thereby offering an attractive route to the discovery of novel blood-based biomarkers. Furthermore, unlike tumour antigens (which are rapidly degraded upon entry into circulation), specific antibody titres are relatively stable (Belousov et al. 2008).

Circulating antibodies can be measured using several techniques that differ in terms of assay complexity and necessary specificity. The simplest of these is the enzyme-linked immunosorbent assay (ELISA), which offers limited sensitivity and multiplexing capacity. High-throughput protein microarrays overcome these limitations (Duarte and Blackburn 2017), while generating ELISA-concordant data (Gnjatic et al. 2009; Beeton-Kempen et al. 2014). Microarrays containing only peptide antigens (rather than polysaccharide antigens) reflect T-dependent rather than T-independent responses. Indeed, T-dependent responses probably constitute the majority or at least a significant proportion of the response phase to established melanoma, especially in light of the strongly observed immunogenicity of certain melanoma peptide antigens.

**Diagnostic and prognostic biomarkers**

Circulating tumour antigen-specific antibodies are produced in response to most cancers, and often precede clinically apparent disease by months to years (Tan 2001), with the signature often being more pronounced at earlier time-points (Ladd et al. 2013), which probably reflects increasing tumour and immune response heterogeneity over time, especially after invasion and metastasis. This highlights the applicability of tumour antigen-specific antibodies as diagnostic or prognostic biomarkers (Caron et al. 2007). However, detection by protein microarray depends on prior identification of antibody-targeted tumour antigens, a limiting factor for antibody profiling coverage (Tan et al. 2009).

Previously, a significant anti-tyrosinase IgG autoantibody presence was noted in both melanoma and vitiligo patients (Fishman et al. 1997). Anti-tyrosinase autoantibodies were also detected in healthy individuals, albeit at significantly lower levels (Fishman et al. 1997). To assess the correlation between autoimmunity (spontaneous autoantibody production) and melanoma survival, Maire et al. screened advanced melanoma patients for the presence of anti-thyroid and anti-nuclear autoantibodies (Maire et al. 2013). Indeed, evidence of autoantibodies predicted improved survival, independent of prior immunotherapy status (Maire et al. 2013). Recent evidence further reported that vitiligo was to some degree protective against skin cancers, including melanoma (Rodrigues 2017).
Hoon et al. first detected circulating IgG antibodies targeting MAGE-1 in stage III/IV melanoma patients (Hoon et al. 1995). Soon after, these findings were extended in a cohort of stage III melanoma patients \((n = 23)\) who exhibited IgG antibodies targeting tyrosinase, tyrosinase-related protein 1 (TRP-1) and 2 (TRP-2), and gp100, when compared to healthy individuals (Huang et al. 1998). Jäger et al. investigated whether a melanoma patient with elevated IgG NY-ESO-1-specific antibody titres exhibited a matching effector T cell response, and unsurprisingly verified that B and T cell responses can occur simultaneously (Jäger et al. 1998). Next, Stockert et al. screened melanoma patient \((n = 127)\) serum for IgG antibodies targeting a subset of tumour antigens, successfully detecting anti-NY-ESO-1, -MAGE-1, -MAGE-3, and -SSX2 antibodies that were not present in healthy individuals \((n = 70)\) (Stockert et al. 1998). Furthermore, in an additional melanoma patient cohort \((n = 62)\), the presence of tumour-specific antibodies indeed indicated expression of corresponding antigens by the tumour, with all anti-NY-ESO-1+ antibody patients possessing matching NY-ESO-1+ tumours and NY-ESO-1− tumour patients exhibiting no such antibody responses (Stockert et al. 1998). A more recent study also detected significant anti-NY-ESO-1 antibodies in only half of a metastatic melanoma cohort \((n = 11)\) with NY-ESO-1+ tumours refractory to all standard treatments (Robbins et al. 2011), implying that the expression of even highly immunogenic antigens may not always lead to a matched circulating antibody response. Furthermore, tumours expressing poorly immunogenic tumour antigens may be less likely to induce antigen-driven B cell and antibody responses. Nonetheless, the potential diagnostic utility of circulating tumour antigen-specific antibodies is clear and is enhanced now by the availability of novel, highly reproducible technologies with improved sensitivity and multiplexing capacity for antibody quantitation/isotyping, such as protein microarrays.

Investigating the evolution of NY-ESO-1-specific antibody titres in patients \((n = 10)\) with NY-ESO-1+ tumours relative to tumour antigen expression revealed that antibody titre varied with the burden of NY-ESO-1+ tumours (including increasing antibody titres accompanying disease progression, and decreases accompanying partial regression or curative surgical resection) (Jäger et al. 1999). This indicates that persistent antigen contributes to the maintenance of the corresponding antibody response and implies that monitoring antibody repertoire changes may thus indicate disease regression, progression, or recurrence (provided that memory was successfully induced). In addition, a cohort of primary \((n = 66)\) and metastatic \((n = 13)\) uveal melanoma patients was recently found to exhibit anti-gp100, -MART-1, and -tyrosinase antibodies during both disease stages examined, whereas anti-NY-ESO-1 antibodies were less frequent in this melanoma subtype (and only detected in metastatic disease) (Triozzi et al. 2015). Regarding additional B cell-relevant circulating prognostic biomarkers, an elevated circulating plasmablast \((CD19^{+}CD38^{high})\) frequency was observed in non-progressing metastatic melanoma, demonstrating persistent and functional anti-tumour B cell responses (DeFalco et al. 2017). When considering circulating antibodies, Litvak et al. assessed the prognostic capacity of 90 kDa tumour-associated antigen (TA90) antibodies in prognostic feature-matched patients with primary melanoma \((1–2 \text{ mm and tumour-negative regional lymph nodes})\) who either did (group 1) or did not (group 2) experience recurrence within at least 7 years (Litvak et al. 2004). Interestingly, although there was no difference in IgG TA90 antibody titre, elevated levels of IgM TA90 antibodies correlated with prolonged survival, whereas their absence was associated with metastatic disease in both groups (Litvak et al. 2004). A more recent study assessed antibodies against a panel of 29 tumour-associated antigens using a bead-based immunoassay in stage I–IV melanoma patients \((n = 365)\) (Zörnig et al. 2015). However, this study reported associations between IgG antibodies against specific antigens and poor clinical outcomes, with decreased overall survival and/or progression-free survival (Zörnig et al. 2015). As such, in addition to the antibody class produced, the antigen specificity itself may also have differential prognostic implications.

**Therapeutic efficacy biomarkers**

Data indicate that tumours likely to benefit from immunotherapy can be defined as having a pre-existing robust general antibody response (Yuan et al. 2016). Immunotherapy may also reactivate suppressed immunity to trigger antibody production, and monitoring changes in the antibody repertoire may thus reflect treatment efficacy. However, robust pre-analytic, analytic, and clinical validation of prospective immunotherapy efficacy biomarkers is required to prove their clinical predictive or monitoring utility (Dobbin et al. 2016; Masucci et al. 2016).

An early study investigating the presence of tumour-specific antibodies in a cohort of stage III/IV melanoma patients treated with a melanoma cell vaccine found that more than half of patients developed IgG-specific MAGE-1 antibodies (Hoon et al. 1995). This study was expanded to stage III melanoma patients \((n = 23)\) receiving a polyvalent melanoma cell vaccine which induced IgG targeting TRP-2 and gp100 (Huang et al. 1998). Clinical efficacy was assessed in stage II melanoma patients treated with a polyvalent vaccine plus BCG (although circulating antibodies were only assessed against the tumour-associated antigen TA90): elevated IgM TA90 antibody titres correlated with improved overall survival (Hsueh et al. 1998; DiFronzo et al. 2002), while elevated IgG TA90 antibody titres correlated with decreased survival (Hsueh et al. 1998).
Stage IV melanoma patients vaccinated with NY-ESO-1 developed matched antigen-specific cellular CD8+ and CD4+ T cell and antibody responses (Jäger et al. 2000b), with evidence that the CD8+ T cell response preceded the antibody response (Jäger et al. 2000a). Similar findings were reported in patients vaccinated with NY-ESO-1 and ISCOMATRIX (NY-ESO-1+ tumours and minimal residual disease) (Davis et al. 2004), with NY-ESO-1 and CpG in Montanide (stage II/III/IV) (Valmori et al. 2007), and with vaccinia/fowlpox NY-ESO-1 (stage III/IV) (Jäger et al. 2006). A multiplexed approach employing a protein microarray containing 100 different CT antigens also demonstrated induction of anti-NY-ESO-1 antibodies, among antibodies against several other tumour antigens, after vaccination of stage IV melanoma patients (NY-ESO-1+ tumours) with NY-ESO-1 and ISCOMATRIX (Beeton-Kempen et al. 2014).

In stage III/IV melanoma patients undergoing CTLA-4 blockade, pre-existing or induced NY-ESO-1 antibodies exclusively identified patients experiencing clinical benefit (Yuan et al. 2008). However, a subsequent study reported no correlation before or after treatment (Goff et al. 2009). Yuan et al. later reiterated their initial findings in a larger cohort of advanced metastatic melanoma patients, with NY-ESO-1 seropositive patients being more likely to exhibit clinical improvement after CTLA-4 blockade (Yuan et al. 2011). Nonetheless, a subsequent study of stage III/IV melanoma patients investigating circulating antibodies against five distinct tumour antigens showed no association between the post-CTLA-4 blockade induction of tumour-specific antibodies and clinical benefit (Weber et al. 2012). Currently, no studies have investigated whether there is a correlation between circulating B cells and antibodies and PD-1 blockade efficacy. Both CTLA-4 and PD-1 blockade can directly or indirectly regulate B cell activity and antibody production (Yuen et al. 2016) through immune de-repression. As expected, immunotherapy can induce antibody production in melanoma patients, but anti-tumour activity of antibodies can also be down-regulated by immune regulatory pathways.

Biomarkers predicting immune-related adverse events (irAEs)

A common side effect of immunotherapy is onset of high-grade irAEs that mimic classic autoimmunity, since such therapies generally reverse immunosuppressive effects (including circumventing some tolerance mechanisms) (Chen and Mellman 2017). Perhaps expectedly, vitiligo and other dermatologic immune-related events are common adverse effects of immunotherapy in patients with cutaneous melanoma (Michot et al. 2016). Interestingly, a systemic review of a large number of melanoma patients undergoing immunotherapy reported that those who developed vitiligo exhibited significantly improved progression-free and overall survival (Teulings et al. 2015). Manifestation of irAEs may in fact thus also reflect the induction of robust organ-specific (‘organotypic’) anti-tumour immunity.

Recent evidence identified B cells as potential irAE-predictive biomarkers in the context of immune checkpoint (CTLA-4 and/or PD-1) blockade: early reduction in total peripheral B cell frequency and enrichment of differented CD21lowPD-1+ memory B cells and plasmablasts preceded high-grade irAEs (Das et al. 2018; Liudahl and Coussens 2018). Since plasmablasts are immature plasma cells, such changes may impact antibody production. Circulating autoantibodies have been suggested as potential predictors of irAEs in an organ-specific manner (Michot et al. 2016). Indeed, using the Immunome protein array, we have shown that profound increases in the autoantibody repertoire preceded high-grade irAEs in a small cohort of melanoma patients treated with intralesional BCG followed by CTLA-4 blockade (Da Gama Duarte et al. 2018a). A subsequent study, utilising the HuProt Human Proteome array, recently published similar findings in a cohort of melanoma patients undergoing checkpoint blockade monotherapy or combination therapy: baseline predictive autoantibody profiles could readily distinguish patients who did or did not go on to experience severe irAEs (Gowen et al. 2018). However, these early studies are limited by low sample sizes, and require validation in larger cohorts.

Challenges and perspectives

Induction and modulation of primary and secondary B cell responses by tumours and their influence on tumour cells are complex and dynamic processes, meaning that no broad consistent conclusions can yet be drawn regarding whether B cell activities in an oncology context are beneficial or detrimental. Differential anti-/pro-tumour effects of leukocytes (including B cells and antibodies) are likely due to whether they are active early versus later during tumourigenesis and progression, to ratios of B cell sub-populations and their phenotypes, and to ratios of antibody functional subtypes (including pro-inflammatory vs. suppressive B cells/antibodies).

The tumour and its products likely significantly influence B cell phenotype and function. Tumour cell mechanisms mediating immune evasion and escape are numerous, including selection of non-immunogenic clones for outgrowth, antigen masking, and significant modulation of the micro- and systemic environment in ways that promote tumour progression. Despite such immunosuppression, the tumour still represents a chronic inflammatory stimulus, giving rise to a situation in which opposing inflammatory and resolving biological programmes are simultaneously (and abnormally) active. Thus, immunosuppressive tumour activities extend
to induction of regulatory cell types in the TME: the chronic inflammation characterising established malignancy can skew leukocytes away from pro-inflammatory and towards regulatory types.

As a result of the incredible heterogeneity of established tumours, as well as their ability to adapt to and survive within their environment, it may appear at first glance that a focus on immune responses during advanced cancer would not be helpful in designing broadly applicable and durable therapeutic modalities. However, an understanding of immuno-oncology of advanced cancers has provided some of the most broadly applicable and clinically effective anti-cancer therapies yet, including immune checkpoint blockade. Overall, immune-oncology is critical to melanoma pathology and therapeutics. Recent developments and a switch in focus from treating the tumour to treating the immune response to it strongly support a role for the immune system in both the prevention and progression of melanoma. The measurement of circulating tumour-specific antibodies, particularly, represents a promising avenue in the search for new, validated melanoma biomarkers. In addition, antibody responses may reflect concurrent CD8+ T cell responses and help identify novel therapeutic antigen or antibody candidates.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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