Non-progressing cancer patients have persistent B cell responses expressing shared antibody paratopes that target public tumor antigens

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ABSTRACT

There is significant debate regarding whether B cells and their antibodies contribute to effective anti-cancer immune responses. Here we show that patients with metastatic but non-progressing melanoma, lung adenocarcinoma, or renal cell carcinoma exhibited increased levels of blood plasmablasts. We used a cell-barcoding technology to sequence their plasmablast antibody repertoires, revealing clonal families of affinity matured B cells that exhibit progressive class switching and persistence over time. Anti-CTLA4 and other treatments were associated with further increases in somatic hypermutation and clonal family size. Recombinant antibodies from clonal families bound non-autologous tumor tissue and cell lines, and families possessing immunoglobulin paratope sequence motifs shared across patients exhibited increased rates of binding. We identified antibodies that caused regression of, and durable immunity toward, heterologous syngeneic tumors in mice. Our findings demonstrate convergent functional anti-tumor antibody responses targeting public tumor antigens, and provide an approach to identify antibodies with diagnostic or therapeutic utility.

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1. Introduction

The relatively recent success of the checkpoint inhibitor (CPI) class of immunotherapeutic drugs has cemented a key role for active T cells in anti-tumor immune responses and positive clinical outcomes in cancer treatment. The role of B cells and their antibodies in anti-tumor immune responses, however, is less clear. In mice, whereas genetic B-cell deficiency enhances anti-tumor T-cell activity and tumor clearance [1], acute B-cell depletion has the opposite effect in most cases [2]. In several types of human cancer, there are correlations between improved prognosis [3,4] and the degree of B-cell infiltration, the expression of certain B-cell genes, and the antibody isotype. It has been proposed that B cells might aid in tumor control by producing antibodies that target tumor antigens and thereby induce tumor cell lysis [5] or prime anti-tumor T cell responses [6], or by producing antibodies that block factors critical for development of a supportive tumor microenvironment [7]. Indeed, tumor-reactive antibodies are detectable in the blood of cancer patients [8], and tumor-infiltrating B cells have been shown to produce tumor-reactive antibodies [9]. Moreover, administration of tumor-reactive antibodies has demonstrated tumor regression in several mouse models [2]. These findings provide a rationale to characterize the B cell response in metastatic cancer patients who have an effective response to therapy.

To better understand the B cell response in such patients, we analyzed plasmablasts circulating in the patients’ blood. Plasma blasts are generated in germinal centres during an adaptive immune response through the activation, affinity maturation, and differentiation of antigen-specific naive and memory B cells [10,11]. Once in the bloodstream, plasmablasts circulate transiently before migrating to inflamed...
tissues or secondary lymphoid organs. Their transient appearance, reporting on the recent output of affinity maturation, coupled with their accessibility in blood, makes them an ideal cell population to analyze for the presence of and information about active B cell immune responses [12–15].

Here, we show that plasmablast levels are elevated in individuals with non-progressing metastatic cancer indicating that these patients have ongoing, active B cell responses. Analysis of these plasmablast antibodies reveals clonal families of B cells that persist over time and show evidence of progressive affinity maturation and class switching. Using paratope clustering we identified convergent antibody groups elicited in the plasmablast populations across cancer patients. Both patient-specific and convergent group antibodies bind public tumor antigens at a surprisingly high rate, and antibodies from convergent groups showed higher rates of binding. Finally, we demonstrate that recombinant anti-tumor antibodies derived from plasmablast clonal families induce tumor regression, and for certain antibodies durable anti-tumor immunity, in a heterologous, syngeneic, mouse cancer model.

2. Results

2.1. Metastatic cancer non-progressors have a persistent B cell response

We examined the levels of circulating plasmablasts in individuals with melanoma (n = 19), lung adenocarcinoma (also referred to herein as non-small cell lung carcinoma (NSCLC); n = 3), or renal cell carcinoma (RCC; n = 3) that was metastatic but had not progressed for at least one year with either CPI or other treatment, whom we termed “non-progressors” (Supplementary Table 1). We isolated peripheral blood mononuclear cells (PBMCs) from these individuals and used flow cytometry to quantify the number of plasmablasts, defined as CD20low/−CD19+CD38highCD27+CD3+CD14+ IgA− IgM− cells (Fig. 1a).

Plasmablasts constituted 0.094%–6.6% of the B cells in these metastatic cancer non-progressors, statistically higher levels than the 0.037%–0.46% seen in healthy individuals (Fig. 1b, P < 0.02, Wilcoxon rank-sum), but comparable to the levels seen following influenza vaccination [13] or in patients with rheumatoid arthritis who are mounting a chronic immune response against self-antigens (Fig. 1b, P = 0.010%–1.62%).

Plasmablast levels in the blood of metastatic cancer non-progressors who had received T cell-targeted CPIs against CTLA-4 (ipilimumab) or PD-1 (nivolumab or pembrolizumab), where an increased immune response against tumor was a reasonable rationale for lack of progression, were also elevated relative to healthy controls, with anti-CTLA4 CPI treatment associated with significantly higher blood plasmablast levels (Fig. 1c, P < 0.002, Wilcoxon rank-sum).

Because plasmablasts enter the bloodstream during active, adaptive immune responses [16], these results suggest that persistent B cell responses exist in a significant fraction of patients whose advanced cancers stop progressing after treatment.

2.2. Cancer non-progressors have persistent B cell responses indicative of ongoing antigen-stimulation

To understand more about the nature of the persistent B cell responses in these patients, we generated bias- and error-corrected sequences of the paired heavy- and light-chain antibody genes [15,17] expressed by individual plasmablasts (n = 25,775) isolated from patients with lung adenocarcinoma (n = 2), renal cell carcinoma (n = 3), and melanoma (n = 18). We sequenced 15 of these non-progressors at a depth of >500 plasmablasts, including three at a depth of >3500 plasmablasts (Supplementary Table 2). Cancer patient plasmablast antibodies showed hallmarks of somatic hypermutation (SHM, Fig. 1d), clonal expansion (Fig. 1e), and IgG class-switching (Fig. 1f) that are indicative of a canonical active humoral immune response similar to that seen in both rheumatoid arthritis [15] and long-term HIV-infected patients [18].

2.3. Persistent B cell responses in non-progressors display high levels of clonality and mutation

We then analyzed these antibody sequences to characterize the immunoglobulin lineages and clonality of the responses in more detail. Plasmablasts were considered possible members of the same clonal family if they originated in the same donor, expressed the same heavy and light chain V genes and had heavy and light chain complement determining region 3 (CDR3) regions of the same length. Assignment of antibody sequences to the same lineage was further restricted to clones that had a high degree of similarity between all 6 CDR amino acid sequences (CDR-H1/H2/H3/L1/L2/L3, see methods) which produced 15,236 clonal lineages among the 25,775 plasmablast IgG sequences from 23 patients with non-progressing metastatic cancer. Approximately 10% percent of these putative lineages, comprising 35% of the plasmablasts in the dataset, had 2 or more IgG clonal members with similar, natively-paired heavy and light chain CDR amino acid sequences, but that differ due to SHM (clonal variation indicative of affinity maturation). Of the lineages observed that contain only one clonotype in the dataset, 10% of them are lineages expressed by 2 or more plasmablasts, evidence of cellular proliferation representing 17% of the total plasmablasts sequenced. Overall, 52% of blood plasmablasts (n = 13,427) belong to families having evidence of clonal proliferation (n = 3054 lineages) with about half showing evidence of clonal expansion (n = 1544).

Antibody sequences from non-progressor patient repertoires carried an average of 48 nucleotide substitutions in the combined heavy chain and light chain variable regions as compared to germline, and exhibit similar degrees of mutation to that observed in autoimmune disease (Supplementary Fig. 1). Among 25,775 plasmablast cells sequenced from 23 cancer patients, only 0.64% expressed unmutated (naïve) full length heavy and light chain variable region germline sequences; the remainder had evidence of SHM (1–166 nucleotide mutations from germline across entire heavy and light chain variable regions). A significant association was observed between lineage expansion and degree of somatic hypermutation (Supplementary Fig. 2; P < 0.0001). Thus, both expanded and low-frequency clonal families exhibit high rates of mutation, with a trend toward increased somatic hypermutation in the largest clonal families.

2.4. B cell responses of non-progressors display clonal persistence across time and convergence across patients

To observe persistence of clonal families over time in these treatment responses, plasmablast antibody repertoires were generated for serial PBMC samples that were available from five metastatic cancer long-term non-progressors. Across timepoints, almost six hundred persistent lineages were detected: 189 in lung cancer (2 patients), 139 in melanoma (1 patient), and 265 in renal cell carcinoma (2 patients). Persistent clones were observed in patients treated with CPI and other treatments (Figs. 2a,b and Supplementary Figs. 3–4). CPI treatment with anti-CTLA4 or anti-PD1 resulted in significant expansion of clonal families (Fig. 2c and data not shown) and increased somatic hypermutation (Fig. 2d and data not shown). Persistent lineages were enriched for IgG2 (42% IgG2, 25% IgG1, 2% IgG3, 1%, IgG4, 30% multi-isotype lineages).

Further analysis of the plasmablast antibody heavy and light chain VJ genes revealed increased usage of certain heavy and light chain V gene combinations (Supplementary Fig. 5). Notably, our analysis of heavy and light chain sequences revealed shared sequence features across metastatic cancer non-progressors (Figs. 2e,f). An analysis of plasmablast antibody paratopes, which over-weights CDR amino acids at sequence positions likely to mediate antigen contact, identified sequences of high similarity across cancer patients. The method identified 301 convergence groups, containing a total of 1786 plasmablast antibody sequences, that contain representatives from at least two cancer subjects (Supplementary Fig. 6).
2.5. Antibodies generated in the persistent B cell responses of non-progressors bind public tumor antigens

To test whether plasmablast antibodies expressed by cancer non-progressors bind tumor tissue, we took advantage of the error-corrected nature of our sequences to express the complete natively paired variable regions of plasmablast antibodies as recombinant immunoglobulins for functional analysis. Because clonal expansion pinpoints B cells that have been selected during an adaptive immune response, we typically selected antibodies from clonal families, routinely selecting the plasmablast with the most “central” antibody sequence of each lineage. For some patients, we also selected antibody lineages only observed in one plasmablast, reasoning that this can represent a low frequency lineage.

Fig. 1. Sequences from the plasmablast antibody repertoires in cancer non-progressors exhibit affinity maturation, clonal proliferation, lineage evolution, and class-switching typical of acute immune responses. (a–c) Elevations in blood plasmablasts in individuals with metastatic cancer who are non-progressors. Flow cytometry was performed on PBMCs stained with fluorescently-conjugated antibodies specific for CD20, CD19, CD38 and CD27 to identify the CD20<sup>low</sup>-CD19<sup>+</sup>-CD38<sup>high</sup>-CD27<sup>+</sup>-CD3<sup>−</sup>-CD14<sup>−</sup>-IgA<sup>−</sup>-IgM<sup>−</sup> plasmablasts. Representative plots of the plasmablast stains of PBMCs derived from patients in health, with cancer non-progression for >1 year in response to non-checkpoint inhibitor therapies, ~3 months following anti-CTLA4 treatment, or ~3 months following anti-PD1 treatment (a). Graph of plasmablast levels in healthy individuals (n = 12), cancer non-progressors (n = 25), and in humans with the autoimmune disease rheumatoid arthritis (RA, n = 37) (b). Graph of plasmablast levels in cancer patients treated with anti-CTLA4 within 3 months, anti-PD1 within 3 months, non-checkpoint inhibitor therapies (Other Therapy), or who were previously treated with anti-CTLA4, anti-PD1, or IL2 and taken off immuno-modulatory therapy (Anti-CTLA4/PD1 > 3 mo) (c). (d–f) Characteristics of humoral immunity seen in plasmablast IgG sequences. Degree of identity between IgH germline gene sequences and heavy chain sequences in cancer patients’ plasmablasts, post-vaccination influenza plasmablasts, total CD27<sup>−</sup>-IgG and total CD27<sup>+</sup>-IgG from healthy controls (d). Clonal expansion (count of clones) observed in 500 randomly selected lineages per individual in total CD27<sup>−</sup>-IgM (naive), total CD27<sup>+</sup>-IgM, and total CD27<sup>+</sup>-IgG from healthy controls compared to cancer patients’ and post-vaccination influenza plasmablasts (e). Representative affinity maturation lineages exhibiting IgG class-switching (f) (Green: IgG3, Orange: IgG1, Red: IgG2, Purple: IgG4).
While patient-specific neoantigens have been demonstrated to play a key role in anti-tumor immune responses [19–23], the availability of antibodies from plasmablasts of non-progressors presented an opportunity to investigate if these B cell responses might also be directed to “public” antigens, i.e., those tumor-associated antigens and epitopes present in more than one person. To this end, we performed
immunohistochemistry (IHC) with these chosen recombinant antibodies on a diverse set of tumor tissues obtained from different patients. Surprisingly, patient antibodies bound to non-autologous tumor tissues at a high rate: 54% (228/425) of recombinantly expressed antibodies bound non-autologous tumor tissue samples from two or more other patients. Tumors recognized were often of the same type as that present in the patient from whom the plasmablasts were derived (Fig. 3a); however, in certain cases, they also bound to other, distinct tumor types (Fig. 3a and Supplementary Fig. 7). Such antibodies rarely bound to normal tissues at appreciable levels (Fig. 3a and Supplementary Fig. 7).

In a parallel set of experiments, we tested the ability of our recombinant antibodies to bind immortalized tumor cell lines using immunofluorescence (IF) or whole cell ELISA (Fig. 3b). Testing against a panel of commercially available cell lines showed a high frequency of reactivity, with 47%, 43%, and 55% of expressed antibodies from lung cancer, renal cell carcinoma, and melanoma patients, respectively, binding at 10 μg/ml. A subset were tested across a range of concentrations for whole cell binding and had EC50 values as low as 3 nM. Some antibodies that bound to human tumor cell lines were also found to potently bind one or more mouse tumor cell lines: 5% bound mouse lung cancer cell line, 10% bound mouse melanoma cell line. The results were confirmed by whole cell ELISA (Fig. 3b).

**Fig. 3.** Clonal family antibodies from cancer non-progressors bind public tumor antigens. (a) Immunostaining of tissue arrays containing human lung adenocarcinoma, renal cell carcinoma, melanoma, and corresponding normal tissues with recombinant clonal family antibodies AB791, AB381, or AB339. Recombinant antibodies representative of a heavy and light chain native pair for each of the selected families were cloned, expressed and used to stain tissue sections. Recombinant antibodies were tested for reactivity to fresh frozen sections generated from tumors matched to the tumor-type of the source patient as well as from tumors from individuals with non-related tumor types. Tumor 1, Tumor 2: non-autologous tumor sections as indicated below each antibody name. Other Tumor: AB791, melanoma; AB381 and AB339, lung adenocarcinoma (NSCLC). Scale bars, 40 um.

(b) Immunoreactivity of human and syngeneic mouse tumor cell lines. Upper row: Immunostaining of tumor cell lines by Control antibody, AB860, or AB680 on line A549; and immunostaining with AB956 on line KLN205; and immunostaining with AB670 on cell line SK-Mel-3. Recombinant antibodies were tested for reactivity against a panel of human cancer cell lines and mouse syngeneic tumor cell lines. EC50 = 14 nM, EC50 = 26 nM, EC50 = 150 nM, EC50 = 5.5 nM. Bottom row: Analysis of binding to A549 cells determined by whole cell ELISA. A549 monolayers were fixed, permeabilized, blocked and incubated with indicated primary antibodies. Binding was quantitated using an anti-human-HRP conjugate followed by luminescence detection.
KLN205; 5% bound mouse melanoma line, B16; and 14% bound mouse breast cancer line EMT6. To identify the targets of these antibodies, we employed commercially available antigen arrays and demonstrated binding to proteins previously hypothesized to play a role in or serve as biomarkers for disease, as well as some previously not connected to cancer (Fig. 4a and data not shown). Antibodies from the same lineage, but that differ in sequence due to SHM, were found to bind the same target albeit with varying potencies (Fig. 4b,c).

2.6. Antibodies from clonal families with shared paratope sequences exhibit increased rates of binding to public tumor antigens

We found evidence that the convergence we observed across patients by CDR3 clustering and paratope analyses (Fig. 2e,f) was functionally relevant. Although antibodies from lineages that showed convergence across cancer patients bound to tumor cell lines at a higher rate than antibodies from lineages observed only in one patient, 58% (42/72) versus 48% (283/594), the difference was not significant ($P > 0.1$). However, when the analysis was restricted to only those convergent lineages seen in cancer patients and excluded those convergent lineages also detected in RA patients (Fig. 2f), the difference was significant: 69% (22/32) of cancer-specific, convergent lineages bound to tumor cell lines versus 48% (283/594) of lineages that were seen only in one cancer patient ($P = 0.028$).

Together, these IHC and IF data demonstrate that a sizable portion of the persistent B cell responses within and across cancer non-progressors are generating antibodies that are not restricted to private or “neo” antigens; rather, they bind “public” tumor antigens, i.e., those tumor-associated antigens present in more than one person and, in certain cases, conserved across species.

2.7. Antibodies from persistent B cell response of non-progressors reduce tumor burden, modify the tumor microenvironment, and induce durable anti-tumor immunity in vivo

To examine whether the presence of these antibodies in the persistent B cell response of non-progressors might have functional ramifications in patients, we selected several human tumor-reactive antibodies that also bound the EMT6 mouse tumor cell line for testing in the EMT6 syngeneic tumor model. In this model, treatment of mice with escalating doses of CPIs that target CTLA-4 or PD-1 can eliminate tumor in a dose-dependent manner [24], and this model is known to have tumors with relatively low levels of immune infiltrate (a “cold” tumor) [25]. To facilitate these analyses, the variable regions of several non-progressor

![Fig. 4. Identification of clonal family antibody target antigens using protein arrays. (a) Recombinant clonal family antibodies were used to probe antigen arrays containing approximately 19,000 human proteins. Representative images of reactive antigen features are presented. Antigen reactivity was confirmed, and binding potency was determined, by ELISA using a range of antibody concentrations. Examples of the identified candidate antigens are presented. (b) Analysis and rank-ordering of the binding potency of recombinant antibodies representative of clonal sub-clusters within a clonal family. Recombinant antibodies representing the phylogenetically-identified sub-clusters were expressed, and their binding potencies to target antigen determined by ELISA using a range of antibody concentrations. (c) Heavy and light chain CDR3 amino acid alignments from the clonal family presented in (b).](image-url)
antibodies were grafted without modification onto the mouse IgG2a heavy chain and mouse kappa or lambda constant regions, to permit engagement with the murine host immune system and reduce development of anti-drug antibodies. As several of these antibodies had measurable ADCC activity on tumor cells in vitro (Supplementary Fig. 8 and data not shown), they were prioritized for analysis in vivo.

In an initial screen of 20 non-progressor antibodies, two displayed strong effects in vivo. AB213 was a member of a persistent (10 months) clonal family identified in a stage IV lung adenocarcinoma non-progressor who had been treated with anti-PD1 therapy. AB213 bound to human lung (data not shown) and breast tumor tissues as well as mouse mammary carcinoma.
EMT6 cells cultured in vitro and EMT6 tumor tissue (Fig. 5a). When syngeneic Balb/c mice harboring subcutaneous grafts of EMT6 tumor cells were treated with the mouse IgG2a version of AB213 (AB213m) we observed significant growth suppression and regression of EMT6 tumors ($P < 0.01$, Fig. 5b and Supplementary Fig. 9a). Treatment of EMT6 tumor-bearing mice with a combination of AB213m and either a PD-1 inhibitor (Fig. 5b,c) or an OX40 agonist (Supplementary Fig. 9b) also had profound effects, with at least additivity and possible synergy at lower doses of AB213m (data not shown). Mice demonstrating complete tumor regression following initial therapy with a combination of anti-PD1 + AB213m (75%; 15/20) or anti-PD1 alone (25%; 5/20) were subsequently re-challenged with an EMT6 cell inoculum into the contralateral flank and received no further therapy (Fig. 5d). Mice that had responded to the initial combination of anti-PD1 + AB213m showed better durable survival following re-challenge (55%; 11/20) than those that had initially received only anti-PD1 therapy (20%; 4/20).

AB419, derived from a metastatic melanoma patient, bound to human melanoma as well as mouse mammary carcinoma EMT6 cells cultured in vitro and EMT6 tumor tissue (Supplementary Fig. 10a). When syngeneic Balb/c mice harboring subcutaneous grafts of EMT6 tumor cells were treated with AB419m in combination with anti-4-1BB, we observed significant growth suppression and regression of EMT6 tumors ($P < 0.01$, Supplementary Fig. 10b).

IHC analysis of tumor tissue in a separate cohort of AB213m treated animals showed increased immune infiltrates, compared to vehicle (PBS) or anti-PD1 treated mice. Treatment with AB213m correlated with increased levels CD8$^+$ T cells, NK cells, and macrophages, concomitant with increased iNOS expression, a marker of M1 polarization (Supplementary Fig. 11). These results suggest that treatment with AB213m induces a coordinated anti-tumor immune response.

3. Discussion

Plasmablasts are products of the germinal center environment, and provide a window into active B cell responses [13]. We observed persistently elevated blood plasmablast levels in many patients with multiple types of metastatic cancer who had exhibited a lack of disease progression for at least one year. Notably, non-progressors who were not treated with CPI immunotherapy could also exhibit increased levels of blood plasmablasts. This suggests that either B cell responses develop de novo to some tumors or that conventional cancer therapies are inducing B cell responses, presumably by exposing the immune system to antigens liberated through tumoricidal action.

Our observations are consistent with previous findings that treatment of tumors with radiation [26], anti-tumor antibody drug conjugates [27], or tyrosine kinase inhibitors [28] can enhance anti-tumor immune responses. Our results suggest that a lack of disease progression over time induced by more conventional as well as immunoncology treatments may involve a persistent B cell response in many patients. Recent findings indicate that ectopic follicular structures are present in tumor tissue [29–31], raising the possibility that such follicular structures orchestrate the persistent plasmablast response we observe in cancer patients. However, it appears not all tumors are infiltrated with immune cells [30,31]. Comparing the B cell response in individuals with inflamed vs. non-inflamed tumors, and in individuals who progress vs. exhibit long-term non-progression, will be addressed in future studies.

Recently, much attention has focused on tumor neoantigens that arise from DNA damage and are targeted by checkpoint inhibitor-induced anti-tumor T-cell responses [19–23,32–35]. Here, we show that a surprising percentage of the clonal families generated in the persistent B cell responses of non-progressors target public antigens, and that those families with shared paratopes target public antigens at an even higher rate. This is consistent with the targeting of wild-type tumor antigens being associated with effective anti-tumor immune responses and favorable outcomes [5]. Changes common across neoplastic cells could lead not only to the mis-expression of otherwise unmutated antigens, but also to their mis-localization, mis-folding, or mis-modification, all of which could cause such antigens to be seen as “non-self” and immunogenic. As private, patient-specific tumor neoantigens (mutated antigens) are also likely to be immunogenic, some of the clonal families not reacting with public antigens would be expected to encode antibodies targeting neoantigens.

Our data are also consistent with observations in other patients having persistent B cell responses. While healthy humans have repertoires of antibodies with short CDR3 regions found in larger “clusters” [36], in our metastatic cancer non-progressors, as well as autoimmune disease patients [15] and patients with chronic HIV infection [18], reactive antibodies from ongoing B cell responses are frequently found in small clonal families and exhibit a significant level of somatic hypermutation. Likewise, we were able to identify with relative efficiency, from the ongoing active B cell response of cancer patients, antibodies that could direct tissue immune infiltration and destruction in vivo, similar to what has been observed for a subset of reactive antibodies found in autoimmune disease patients. Our findings provide a new paradigm for the discovery of anti-tumor antibodies for diagnostic and therapeutic purposes.

4. Methods

Methods and associated references are available in the online version of the paper.

Author contributions


Competing financial interests


Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2017.10.002.

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