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Case Report

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Supporting Cases for the Depletion Model of Immune Checkpoint Inhibitor Therapy in Cancer

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ABSTRACT

Immune checkpoint inhibitor therapy has been introduced into cancer clinics for over a decade with big hopes and hypes, yet the true mechanism behind this therapy is still unclear in many ways. In a previous article we have introduced a new working model for this therapy based on the partial depletion of PD1 positive T cells. This model, as we called it the depletion model, explains all clinical observations including the trigger effect and the hyper-progression associated with anti-PD1/PDL1 antibody use. One critical prediction from this model is that under repeated dosing of anti-PD1 antibody, any antitumor response must be mediated by PD1-negative T cells, because that all PD1-positive T cells are removed by the antibody. Unless this prediction can be confirmed, the depletion model will not be supported by evidence. In this report, using few real-world cases, we provide supporting evidence to support the various aspects of the depletion model.

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Introduction

Thanks to ICI therapy in the past decade, there is no doubt today that the magnitude of immune responses against cancer is real and powerful that if activated in a right way, it can eradicate almost any commonly seen tumor burdens existing in a clinical setting. Yet, despite the high hopes and hypes in the past few years, ICI therapy so far is mostly effective in various clinical trial settings, but not in real world use [1,2]. Why there is such a huge difference between these two settings is not clear. Our own experience pointed to a confusion on the mechanism and, as a result, the wrong application in about 40% cases, causing harm instead of benefit [3]. According to the established mechanism of ICI therapy [4], immune attack of tumor causes tumor cells to express PDL1, this in turn down regulates immune response, thus preventing immune destruction of tumor. ICI antibodies block the interaction between T cells expressing PD1, and tumor cells expressing PDL1, thus saving T cells from being inhibited by PDL1. Based on such a mechanism, we applied ICI therapy when we observed resistance from tumor to activated immune attack, but observed 40% of patients experiencing hyper-progression subsequently [3]. In addition, three perplexing clinical observations could not be explained by the blocking model. The first is the "trigger effect" observed in some patients who for various reasons only got the chance to use the therapy once. Their tumors responded to this single treatment persistently, some time over a year. Most durable responders also demonstrated continued tumor regression long after stop of therapy for two years [5-7,8]. The second is the hyper-progression associated with ICI therapy [9, 10]. The third is the autoimmunity associated with therapy [11]. We have since investigated the differences between responders and non-responders and have come up with an alternative working model for ICI therapy. Based on this new model which we call depletion model [3], the location of tumor-infiltrating T cells is critical in that PD1-positive T cells located in the stromal and interstitial space are bond by the antibody and are depleted by various mechanism including ADCP [12], while T cells deeply infiltrating tumor mass are spared due to lack of antibody access and/or lack of PD1 expression. This depletion causes quick drop of T cells and is followed by homeostasis-driven expansion of residual T cells. This is the reason behind non-antigen specific activation of antitumor T cells. This initial activation results in the expansion of those T cells that could deeply infiltrate tumor mass to result the most effective responses. This model, although could explain the three most perplexing observations the blocking model could not explain, still leaves some perplexing questions to be answered. The most challenging one is about the continued and durable responses in the presence of continued antibody doing in a clinical setting. If T cell location could provide the initial hide-out place for some T cells infiltrating deeply in a tumor mass, the subsequent expansion will require these T cells to migrate out of tumor mass and into draining lymph nodes for most effective expansion. This change of location will expose these T cells to antibody-mediated depletion unless these T cells do not express PD1. But clinical observations suggest that these T cells may as well express PD1 and susceptible to antibody-mediated depletion. In quite a few cases, we have seen the initial robust responses following the initial ICI antibody dosing turned into hyper-progression following subsequent dosing (described in detail below). On the other hand, there are those durable responders that maintain long-term responses in the presence of continued antibody dosing while the same time in the absence of continued antigen release. How to explain the differences in these two situations is a challenge, too.

In the following sections of this report, we cite four real world cases to illustrate few points that together form a bigger picture. The combined observations and reasonable deduction point to a future direction in which we may find out a way to activate the most effective antitumor response.

Case description

Case 1: Repeated anti-PD1 dosing results in depletion of antitumor T cells and hyper-progression

A 59-year-old male with a large swollen mass in the neck (Figure: 1-1). Was diagnosed with melanoma upon biopsy. PET-CT examination showed additional bone metastases (Figure: 1-2). The patient had an elevated black mole in the forehead, but resected biopsy did not show malignant cells, thus the primary location of this melanoma was unknown. Patient went to us for help with treatment plan. We first examined the biopsy sample to evaluate the mode of tumor replication and status of concomitant antitumor immunity. Analysis showed that the tumor structure is typical of melanoma with packed tumor cells and lack of interstitial space between tumor cells (Figure: 1-3, HE). Tumor replication was active in that 40-70% of tumor showed strong Ki-67 staining depending on area (Figure: 1-3, Ki67). There was a large number of dispersed T cells in the tumor mass (Figure 1-3, CD3). These T cells are of the CD8 subtype and some show activated status. These T cells seemed to have antitumor activity in that tumor replication was most active in the area where there were fewer T cells while in the area there were more T cells, tumor replication was much less active. Based on these observations we believed that this was a case of highly active tumor replication with a concomitant antitumor immunity. The levels of the antitumor immunity in this case are relatively strong compared to most tumors at the time of diagnoses, especially some of the CD8 T cells inside the tumor showed activated state and there was a clear antagonism between T cells and tumor replication. Based on our accumulated experience in evaluating hundreds of tumor samples in the past 7 years, this case would range to the top 30% when it comes to the strength of antitumor immunity at diagnosis. Furthermore, the pattern of T cell infiltration in this case is a “mixed” type, indicating that it is like to benefit from ICI therapy with antibody to PD1 based on the depletion model of ICI therapy [3]. On the other hand, our observation of T cell-mediated suppression of tumor replication indicated that there was no tumor expression of PDL1 due to immune attack, which usually enhances Ki-67 staining. This was confirmed by a commercial third-party assay on PDL1 expression that concluded no tumor expression of PDL1 (not shown). The reason why tumor cells under such strong immune attack did not express PDL1 is not clear. Inasmuch as PDL1 expression is stimulated by IFN-gamma [13,14], it could not be the lack of IFN-gamma release because we saw clear suppression of tumor replication, which is the hallmark of T cell-released IFN-gamma. There must be other factors that prevented tumor cells from expressing PDL1.

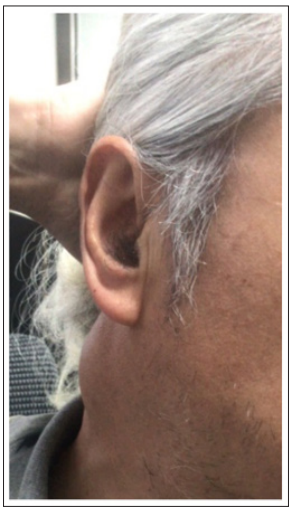

Figure 1-1: The large (8x6cm) neck swollen nodule at the time of diagnosis

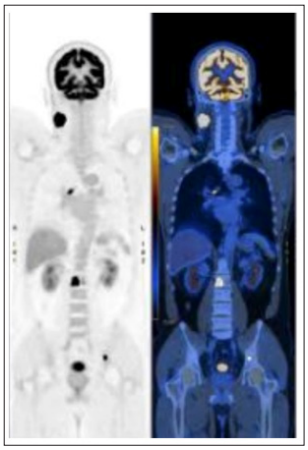

Figure 1-2: Pet-CT at The Time of Diagnosis Showing the Neck Nodule and A L4 Bone Metastasis

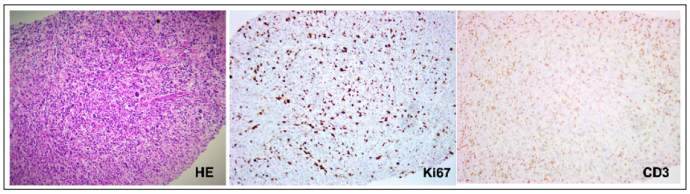

Figure 1-3: Biopsy tissue at diagnosis stained for Ki-67 and CD3, showing a compact structure of melanoma cells without interstitial space (left). Tumor replication is active with 40-70% tumor cells expressing Ki-67 (middle). There are large number of T cells mixed with tumor cells (right). Most T cells are of the CD8 subtype, some show sings of activation (concentrated CD3 membrane location).

Regardless of tumor PDL1 expression, our depletion model for selecting patients for ICI therapy predicted that this would be a beneficial case. We therefore recommended anti-PD1 treatment. Unlike the mainstream use of PD1 antibody, our use based on the depletion model depends on the trigger effect of the antibody, and does not require repeated dosing unless necessary. Because PD1-positive T cells would be depleted, and this depletion is likely variable among patients who may have expressed different alleles of their FC receptor gene that affect IgG1 binding by macrophage and T cell removal, we monitored the blood cell counts from the patient before and after administration of anti-PD1 (Keytruda,

200mg). Blood cell counts indicated that there was a 23% drop of lymphocytes one day following antibody dosing (no drop of other white blood cells seen at the same time). This is not a large drop among the patients monitored for ICI therapy, which is often more than 30% drop immediately following the antibody dosing (our unpublished results), indicating that T cell depletion may not be severe. Since T cell activation depends on homeostasis-driven recovery by residual T cells, small depletion would drive a small recovery and probably less T cell activation.

Two weeks following the treatment, we could witness a response on the neck tumor nodule. By 5weeks, this nodule had shrunk significantly to <20% of previous volume (Figure: 1-4). This response began to wean down by the 6th week and neck tumor relapsed slowly. Other physicians the patient and his family members had consulted all blamed this lack of continued response on lack of continued antibody dosing (once every three weeks) and lack of combined chemotherapy. Because that the first PD1 antibody treatment did not show any sign of temporary tumor progression, a phenomenon associated with temporary depletion of PD1-positive antitumor T cells according to our depletion model, we thought a subsequent repeat of the treatment two months later should be safe, but we were against repeated dosing every three weeks due to the possibility of over-depletion of antitumor T cells and loss of control on tumor progression entirely leading to hyper-progression. Despite our warning and explanation, patient went on with his family and took the advice of the other physicians. By 6 weeks following the resumed PD1 antibody, accelerated tumor progression become obvious in a daily basis in that the neck tumor quickly became hard and larger, previous single nodule had split into four protruding nodules occupying large area of the neck Figure: 1-6. The patient also experienced back and leg pains. Based on our previous warning, we realized that the patient had experienced depletion of antitumor immunity and a hyper-progression as a result. Yet, the treating physician insisted that this is caused by the development of drug-resistant clones of tumor variation, not a loss of antitumor immunity. They insisted on continued antibody dosing. To resolve this dispute on the cause of tumor relapse, we asked for another biopsy on the neck tumor and another PET-CT to see whether tumor progression was limited to the neck tumor or new metastases had established. As (Figure: 1-6) shows, this PET-CT showed a massive presence of new metastases in many locations of the body. The biopsy of the progressing tumor showed active proliferation tumor cells without T cells inside the tumor mass (Figure: 1-7). Together, these observations support the conclusion of a total loss of antitumor immunity in the entire body, a result only explainable by antibody-mediated T cell depletion.

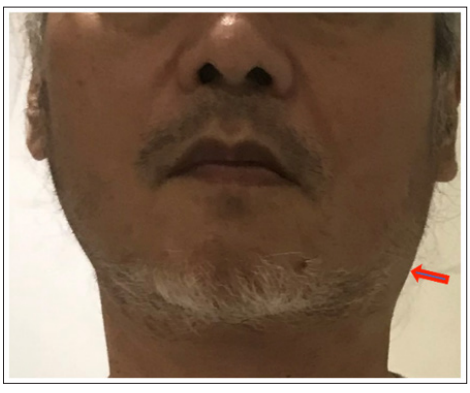

Figure 1-4: The neck nodule (a mirror imaging shown here) had shrunk to almost flat 5 weeks following first anti-PD1 therapy

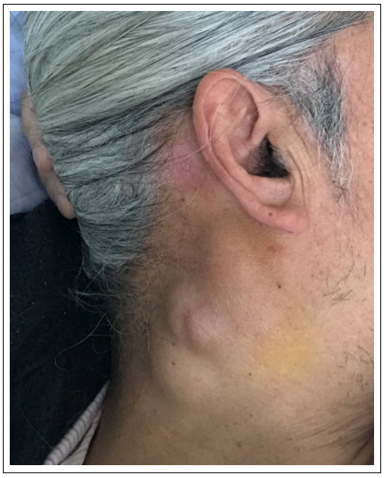

Figure 1-5: The neck nodule after hyper-progression following repeated PD1 antibody treatment

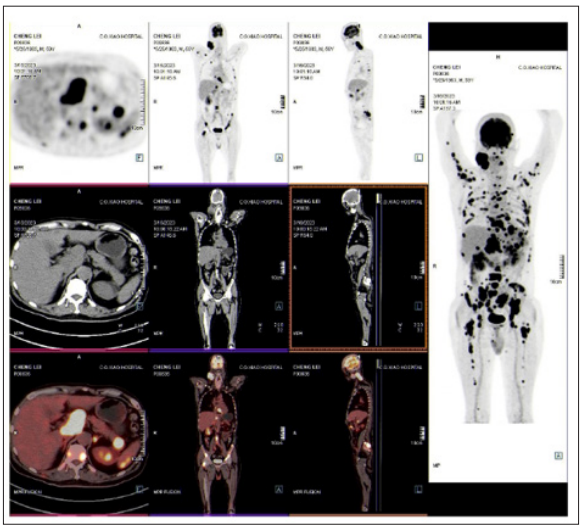

Figure 1-6: Second PET-CT showing massive newly established metastases in liver, lung, many bone and muscle locations

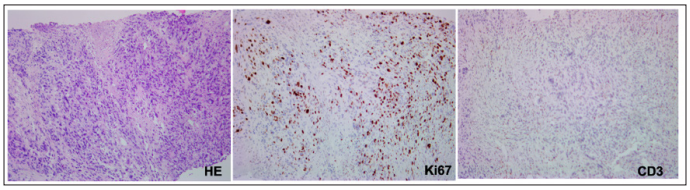

Figure 1-7: Biopsy tissue of the neck nodule following hyper-progression. The lack of T cell forms clear contrast to previous biopsy tissue at the time of diagnosis (Fig. 3).

Upon these findings, all previously involved physicians gave up on this patient. We explained to the patient and family members that the depletion of antitumor T cells was temporary as long as no more antibody was given. Immunity could recover eventually with time (2-3 months). In order to prevent more metastases from establishing, we suggested intermittent chemotherapy to suppress freshly established metastases. Yet our advice of chemotherapy was not carried out due to lack of cooperation by area hospitals. During this waiting time, around 9 weeks following the last antibody dosing, the patient started to experience regular 39°C fever that lasted few hours every day lasted more than two weeks. Despite the high fever, patient felt mostly normal. This was clearly different from the commonly seen "cancer fever" that is associated

with terminal stage cancer patients. With this fever, we noticed the partial softening of the neck tumor, indicating the return of antitumor immunity. In order to confirm this, we recommended another biopsy of the neck tumor. The biopsy indeed confirmed the return of T cells inside the tumor (Figure: 1-8).

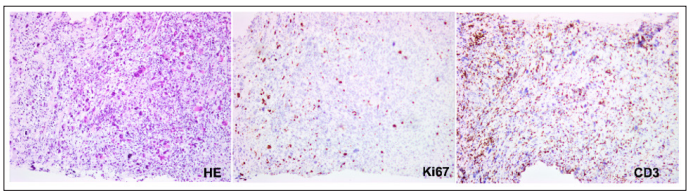

Figure 1-8: Biopsy tissue of the neck nodule 10 weeks following last PD1 antibody dosing. Compared to the last biopsy taking at the peak of hyper-progression (Fig. 7), large number of T cells returned to tumor mass. Tumor replication was clearly suppressed by these T cells, too.

Based on this observation, we suggested two options: 1) return to PD1 antibody treatment one more time, but only once at a time; 2) Use chemotherapy to activate antitumor immunity. Patient and his physicians did not accept the idea of using ICI therapy again due to the previous bad experience, so they opted to try one course of chemotherapy. The response from that chemotherapy was so dramatic, that not only the neck tumor shrunk quickly, a large degree of depigmentation appeared around the neck tumor following its regression also. At the same time, CT and MRI exams showed regression of many previously established metastases (not shown). With this massive tumor regression, the patient entered a state of rapid body weight loss accompanied by severe malaise resembling cancer cachexia. We believed that this was caused by a heightened immune response against the large tumor burden, and it should be suppressed partially to save the patient's life. Despite our advice on using immune suppressive measurements (for example, corticosteroids), the patient and his physicians did not intervene accordingly. He died soon after.

What was the reason behind the big swing in responses following ICI therapy from one extreme to the other? Our analyses based on the depletion model point to the initial activation of antitumor T cells following one single administration of anti-PD1 antibody. Continued dosing of the same antibody caused the near complete depletion of the activated T cells and hyper-progression. Subsequent return of antitumor T cells after stopping giving more antibody resulted in spontaneous tumor control. But we did not expect the dramatic sustained antitumor response following a single course of chemotherapy with paclitaxel that eventually caused the death of the patient. Depigmentation of melanocytes following melanoma immunotherapy has been described before. It is usually associated with self-sustained antitumor responses that often resulted in cancer eradication [15]. Apparently, this type of sustained response is not usually associated with chemotherapy, less to say a single course of chemotherapy. The true reason for this sustained response seen in this case comes not from the selection of chemotherapy drug, but the activation of returned antitumor T cells. Since we have seen the best responses following ICI to be mediated by PD1-negative T cells (see later section on case 3), we went back to check the PD1 expression status of T cell in the first and the third biopsy samples (since the second biopsy did not contain T cells). As Figure: 1-9 illustrated, in the sample of the first biopsy taken at the time of diagnosis, nearly all T cells inside the tumor mass expressed PD1 marker. In clear contrast, in the third biopsy taken at time of spontaneous tumor control 9 weeks following cessation of repeated anti-PD1 antibody, there was large number of T cells in the tumor, but less than half of these T cells expressed PD1.

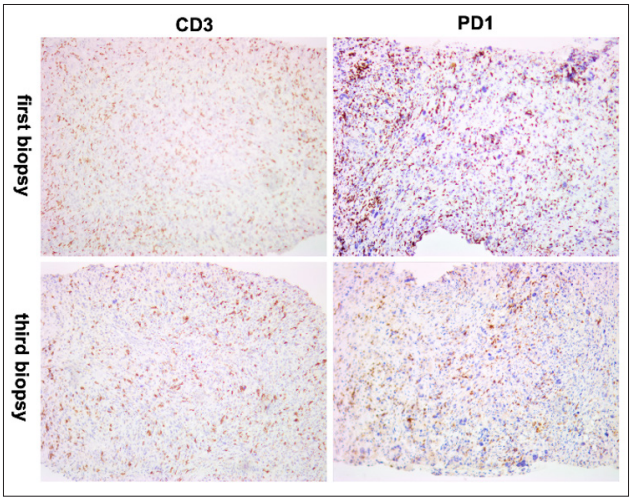

Figure 1-9: PD1 expression ratio in T cells infiltrating tumors in the first and third biopsy samples. As shown, nearly all T cells in the first biopsy taken at the time of diagnosis expressed PD1, whereas in the third biopsy taken at a time when spontaneous tumor control retuned following anti-PD 1 antibody induced hyper-progression, less than half of T cells inside the tumor expressed PD1.

We could not conclude that these PD1-negative T cells would remain PD1-nagative after chemotherapy during sustained antitumor response, but based on our observation from other case (Case 3), we believe so. It is not even clear whether the sustained antitumor response following chemotherapy was activated by the single course of chemotherapy, it could as well be the continuation and expansion of the spontaneous T cell recovery process already observed before chemotherapy. In that case, we would be witnessing a selective process of PD1 antibody for PD1-negative T cells to expand only. Had the treatment with anti-PD1 antibody not stopped upon observing hyper-progression, we may actually see the subsequent tumor regression after the PD1-nagative T cells caught up eventually. In as much as some of the most durable responses following ICI therapy are carried out under continued antibody administration, this would be a reasonable explanation.

Case 2: PDL1 expression by tumor is not a safe indication to avoid hyper-progression following ICI therapy

In the above case, the status of PDL1 expression was negative both by our evaluation and by a third-party immunohistochemistry analysis. This is not the reason anti-PD1 should not be used in that case, because that ICI treatment was given and was highly effective following the first dosing. On the other hand, mainstream guideline for selection of ICI therapy candidates often uses the status of tumor expression of PDL1. A correlation between the expression levels of PDL1 by tumor cells and responses to ICI therapy has been established by clinical data [16]. Although many studies have since demonstrated that patients with PDL1-negative status may benefit from ICI therapy as well, but high expression of PDL1 by tumor is generally a better indicator of better responses [17]. In light of the finding that tumor expression is stimulated by IFN-gamma released by T cells [13, 14], this high expression of PDL1 by tumor cells at least indicates the nearby location of antitumor T cells and the ability to release IFN-gamma, a hallmark for the preferred Th1 antitumor response. Even by our depletion model, this nearby location of T cells to tumor often points to a mixed T cell infiltration within the tumor mass, an indicator of potential benefit following ICI therapy. But the status of tumor expression of PDL1 is not a guaranty that depletion of antitumor T cells by ICI antibodies would not take place. The protective factors for

depletion are 1) T cell location inside tumor mass; and 2) lack of PD1 expression on T cells, but not that whether T cells stimulated tumor cells to express PDL1. The following case is an illustration for this point.

A 52-year-old man was diagnosed with lung cancer following symptoms of persistent coughing and chest pain. A PET-CT exam showed a 4CM primary tumor in the left lung and multiple metastases all over the body (Figure: 2-1), securing a stage IV designation. Analysis on driver gene mutation and any potential use of targeted therapy did not yield any hope. The patient who was a physician by training and who had familiarized himself with current treatment guidelines on stage IV lung cancer went to us for assessment of prognosis and treatment plan suggestions. We asked to evaluate the status of his concomitant antitumor immunity by looking into the biopsy sample for the mode of tumor replication and the presence of antitumor immunity. The analysis with his biopsy samples showed (Figure: 2-2) a low-differentiated adeno carcinoma (Figure: 2-2, HE) with few autonomously replicating tumor cells (Figure: 2-2, Ki67) that with enlarged nucleus and stained heavily with Ki-67 expression, a sign of extremely active in recruiting local inflammation (the reason for heightened symptoms). There were large number of T cells present in the biopsy sample (Figure: 2-2, CD3). The distribution of T cells was mainly in the interstitial space surrounding small patch of tumor mass, but some clearly infiltrated inside the tumor mass to form a mixed pattern of infiltration with tumor cells. Most of these T cells are of the CD8 subtype and did not show activated status. Together, these observations put this case into a category of relatively strong concomitant antitumor immunity with a widely metastasized tumor distribution. By the TNM staging, this is a Stage IVb, very late-stage cancer with the worst prognosis, whereas by our compiled staging system incorporating the status of antitumor immunity, this case is not desperate as it seems and if antitumor immunity can be activated to eradicate most metastases, the case could be salvageable with a good long-term prognosis. Based on this assessment, we suggested to activate antitumor immunity with ICI therapy using one single treatment of anti-PD1 antibody. It should be pointed out that the selection of ICI therapy was also supported by a third-party analysis on tumor PDL1 expression that showed >90% tumor cells expressed PDL1 (Figure: 2-3). However, based on our observation of his biopsy samples, we made it clear to the patient that anti-PD1 therapy could only be given once at a time.

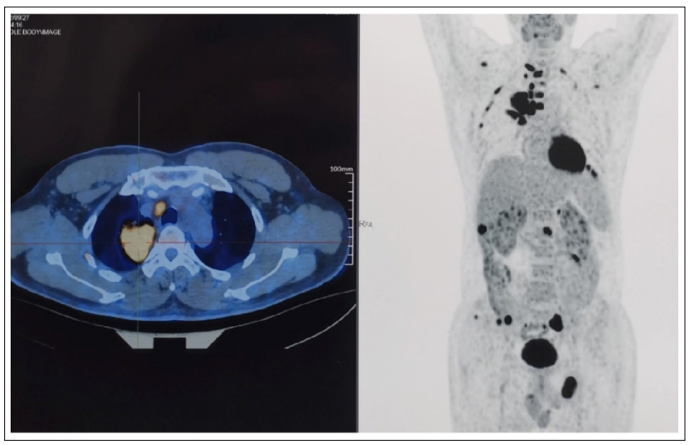

Figure 2-1: PET-CT showing the primary tumor in the lung (left) and the multiple metastases (right) all over the body including lung and nearby lymph nodes, peritoneal metastases, liver, bone and muscles.

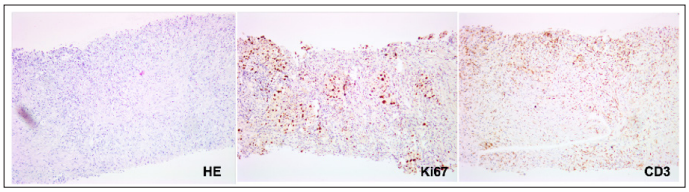

Figure 2-2: Biopsy tissue at diagnosis stained for Ki-67 and CD3, showing a low differentiated adeno carcinoma of the lung (left). Tumor replication was active as indicated by enlarged Ki-67 stained tumor cell (middle). There are large number of T cells mixed with tumor cells (right). Most T cells are of the CD8 subtype, few showed activated status.

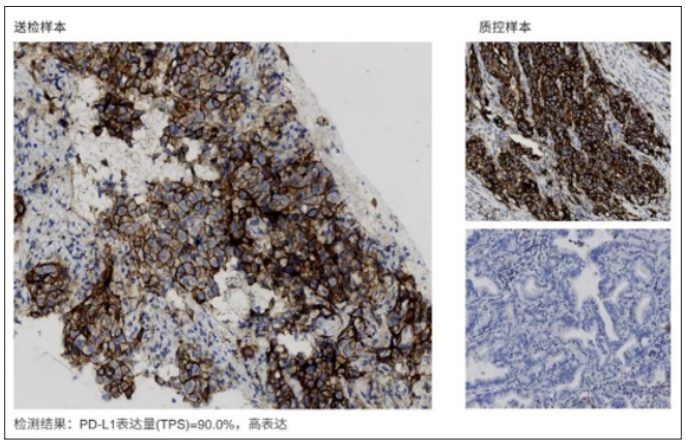

Figure 2-3: Tumor expression of PDL1 as tested by a third-party commercial laboratory. The positive (top) and negative (bottom) controls are shown in the right panels whereas the tested sample with high PDL1 expression on >90% of tumor cells is shown on the left.

Figure: 2-4 is the change of sensitive tumor markers before and at various times after the first dosing of anti-PD1 antibody. All three sensitive markers showed a temporary rebound 2 weeks after the administration of antibody, a phenomenon often seen with ICI therapy. This is explained by the depletion model as the short-term effect when those interstitial infiltrating T cells were removed by the antibody. Since these T cells were responsible for controlling tumor progression, their removal would result in tumor rebound. Subsequently all tumor markers dropped quickly and continuously for the next 12 weeks at which time a rebound of only marker CEA was seen. The sustained response following a single anti-PD1 antibody treatment was expected based on the depletion model we have described before [3], but the rebound of CEA without the other two markers rebounding was unexpected and pointed to an escape event rather than general decaying of antitumor immunity activated by ICI therapy. In order to confirm this, we asked for a second PET-CT exam. (Figure: 2-5) shows the comparison between the two PET-CT results. There were dramatic differences in tumor burdens between these two tests, illustrating a dramatic antitumor response activated by a single dose of anti-PD1 antibody. This dramatic and durable response supports the trigger-effect as explained by the depletion model [3]. Further, we also found the reason for CEA rebound as there was one newly established bone metastasis (Figure: 2-6) among all previously identified nodule regressing. This is a clear demonstration that the ongoing antitumor immunity, regardless the strength, could not recognize this nodule. Since the other two tumor markers (Cyfra21-1 and NSE) did not rebound, replication of this nodule was not represented by these two markers, thus was likely a new variant in replication and an immune escape as well.

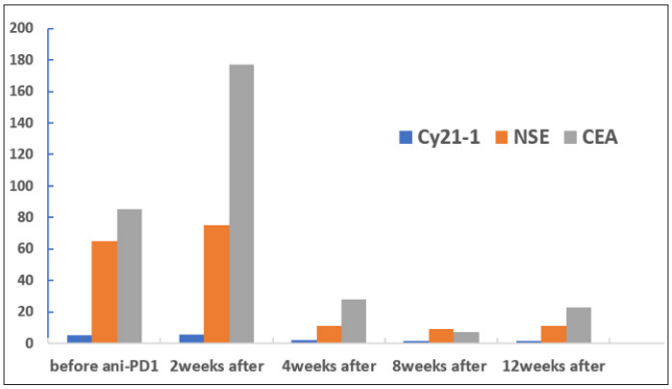

Figure 2-4: Change of sensitive tumor markers (Cyfra21-1, NSE and CEA) before and after initial anti-PD1 treatment.

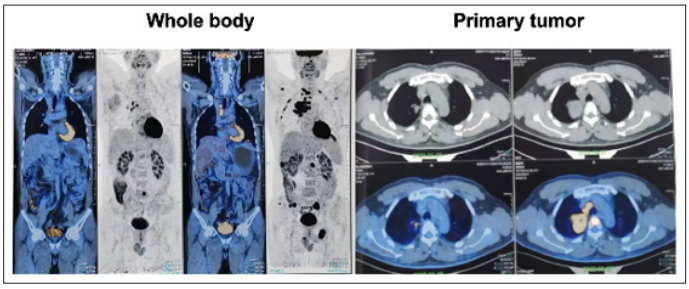

Figure 2-5: Comparison between PET-CT images before and 13 weeks after ICI therapy with a single dose of anti-PD 1 antibody. The left side shows the whole body image comparison. The initial PET images are on two the right-hand side panels, while the after treatment PET images are next to the left. The regression of primary tumor is presented on the right side as labeled. Again, the before treatment images are on the two right-side panels and the after images are on the left.

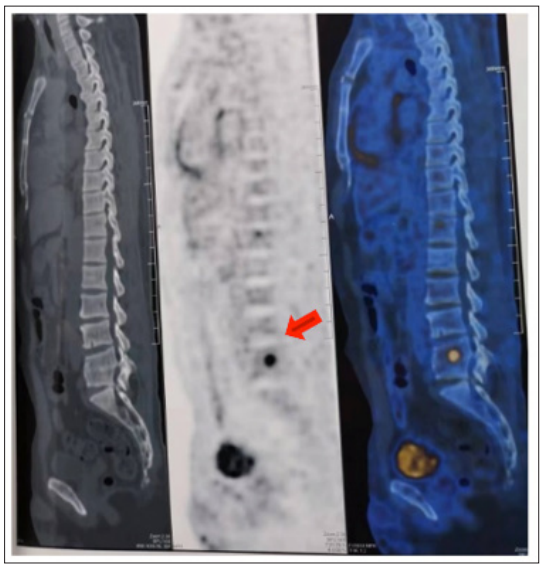

Figure 2-6: The new bone metastasis as indicated by the 2nd PET-CT despite massive tumor regression following ICI therapy as shown in Figure 2-5.

In light of the overall tumor regression with one escape metastasis, we suggested a radiation treatment of this bone metastasis while leaving the rest tumors to continue regressing. But other physicians the patient and his family consulted insisted on giving more antit-PD1 antibody. While we explained the reason why ICI therapy has trigger effect and that the three-month response pattern from the initial anti-PD1 antibody supported this view, and that T cell infiltration pattern in this tumor may not withstand repeated dosing of ICI antibody, the patient chose to do radiation treatment on the newly established bone metastasis while the same time taking repeated dosing of anti-PD1 antibody. Two months later after radiation therapy and two consecutive anti-PD1 antibody treatment, tumor markers showed rapid rebound, indicating a loss of tumor control. Patient went back to us for explanation and suggestion. We asked for a third PET-CT to see the changes of tumor burden. As Figure: 2-7 shows, there was clear relapse of some of the previously regressing tumors including the primary tumor by the time of the third PET-CT exam. In addition, there were also numbers of newly established metastasis. The single bone metastasis identified by the second PET-CT (Figure: 2-6), which was treated by radiation showed reduced metabolism. Together with rapidly rebounding tumor markers, these observations indicate that T cells that were responsible for suppressing tumor was removed by repeated anti-PD1 antibody, thus we saw the rapid regrew of the primary tumor and the appearance of new metastases. In contrast, since the single bone metastasis identified by the second PET-CT was an immune escape, T cell depletion would not affect its growth. Indeed, this metastasis was suppressed by radiation treatment and showed reduced metabolic activity.

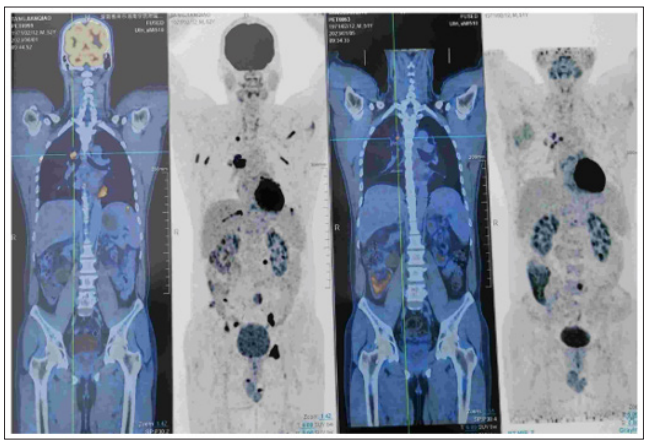

Figure 2-7: Comparison between the third (left-hand side two panels) to the second (right-hand side two panels) PET-CT images, showing rebound of previously regressing tumors and appearance of new metastasis.

This case was designated as potential high-responder to ICI therapy by extremely high tumor expression of PDL1 (Figure: 2-3). On the other hand, it was also recognized by the depletion model as potential beneficiary of ICI therapy by the structure of lowly differentiated tumor and presence of mixed T cell infiltration of tumor mass (Fig. 2-2). The actual response form ICI therapy was a dramatic antitumor effect as witnessed by the two PET-CT tests before and after the initial ICI therapy (Figure: 2-5). The subsequent dosing of anti-PD1 antibody was carried out three months later at a time when tumor control was still apparent except for one variant escape. It is difficult to blame a second dosing of ICI antibody for the subsequent reverse from dramatic response to hyper-progression, not even by the depletion model. What caused the dramatic reverse should be the third antibody dosing spaced three weeks away from the second. According to the depletion model, T cells not hiding inside solid tumor mass and present in the interstitial and stromal space are subjected to antibody binding and removal unless they do not express PD1. Following 2nd antibody dosing, T cells hiding deeply in the tumor

migrated out of the tumor mass for expansion, this was the time when they were most accessible by anti-PD1 antibody for removal. Thus, a repeated antibody dosing given at this time would result in massive removal of T cells responsible for tumor control, causing total loss of tumor control. The actual hyper-progression supported this speculation. This event, therefore, predicted that all of the antitumor T cells following initial anti-PD1 antibody still retained PD1 expression, therefor was susceptible for removal by anti-PD1 antibody. To test this prediction, we went back to look for PD1 expression in the biopsy sample shown in Figure 2-2. Figure 2-8 shows that at the time of diagnosis, all T cells infiltrating the tumor expressed PD1. One may assume from this observation that upon removal of interstitial T cells, T cells that came out of tumor mass for homeostatic expansion may retain their PD1 expression status. These T cells therefore were susceptible for antibody-mediated depletion. Had this case not taken subsequent anti-PD1 antibody, whether T cells activated by the initial ICI therapy treatment could sustain the antitumor response till complete tumor regression is an interesting question.

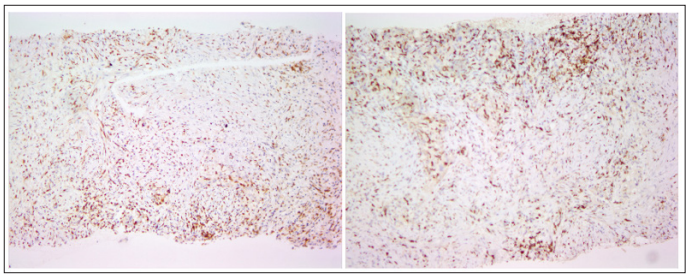

Figure 2-8: Biopsy tissue at diagnosis stained for CD3 and PD1, It shows that the density of CD3-positive T cells was similar to that of PD1-positive cells, indicating all T cells expressed PD1.

Case 3: PD1-nagative T cells may be responsible for durable, hyper-progression-resistant antitumor responses activated by ICI therapy

One critical prediction from the depletion model is that durable antitumor responses following repeated ICI therapy must be carried out by PD1-nagative T cells. Inasmuch as PD1 is known to be expressed by activated T cells [3], and the prerequisite for ICI therapy is expression of PD1 by antitumor T cells based on the blocking model [17], it is not known whether there are activated T cells that do not express PD1, less to say these T cells are responsible for the best antitumor response. It is rather contradictory that an antibody against PD1 on T cells activates a response by PD-1 negative T cells, but if the depletion model is correct, this must be true. To test this prediction, we looked surgical tumor samples for the PD1 expression by activated T cells in durable ICI therapy responders. The following is such a case.

A 60-year-old man went for hospital following persistent chest pain in 2016. Chest CT found a large (8cm) nodule near the hilum of left lung with multiple swollen lymph nodes in the mediastinum. Biopsy confirmed presence of a lowly differentiated adeno carcinoma. The hospital chose to carry out chemotherapy followed by radiation to the primary tumor. This combined treatment brought short-term tumor shrinkage but followed by tumor relapse and distant metastases to the shoulder, adrenal gland, brain and liver two months after radiation. Upon a biopsy of the shoulder mass confirmed it being a lung metastasis, the case was designated hopeless and family member went to us for help. We looked the biopsy sample from the shoulder metastasis to evaluate the presence of antitumor immunity. Figure: 3-1 shows the biopsy sample stained with HE, Ki-67 and CD3. As shown, this is a lowly differentiated tumor of mixed adeno and squamous carcinoma (HE), tumor replication was active with some patches of tumors reaching over 70% tumor cells expressing strong Ki-67 (Ki-67).

There were large number of T cells in the entire tumor area, some were mixed with tumor cells, others were in the interstitial space (CD3). These observations provided an explanation for the previous response to chemo and radiation treatments followed by tumor relapse and spread. First of all, this was a case of lowly differentiated tumor (a mixed type between adeno and squamous carcinoma). Tumor was highly malignant with active replicating activity. But this was also a case with concomitant antitumor immunity. Response to the initial chemotherapy was due to activation of antitumor immunity with chemotherapy. But the subsequent radiation destroyed primary tumor as well as most antitumor immunity present inside the tumor. This radiation-mediated suppression of antitumor immunity is a common presence in the clinic, often more than abscopal antitumor effects radiation therapy may activate (our unpublished observations). It was this suppression of immunity that resulted the relapse of primary tumor and the establish of the distant metastases. However, with the return of tumor burden, concomitant immunity returned to the tumor and this was what we saw in the biopsy sample (Figure 3-1). This re-establishment of concomitant antitumor immunity would prevent future establishment of metastasis and allow subsequent therapy to be supported by activation of antitumor immunity. By theory, if we could eradicate all metastasis, this case may be curable upon final removal of the primary tumor. With this outlook, we suggested new rounds of tumor reductive treatments.

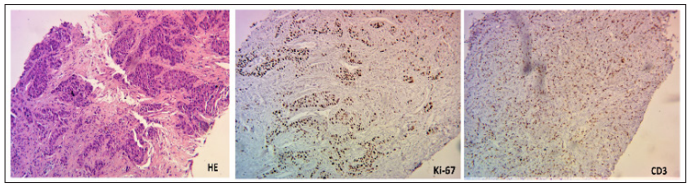

Figure 3-1: Biopsy of shoulder metastasis stained with HE, Ki-67 and CD. HE staining indicated that this was a lowly differentiated tumor of mixed adeno and squamous carcinoma from the lung. Tumor replication was active with more than 70% of some patches of tumor expressed Ki-67. Three were many T cells present in the area, some infiltrated the tumor mass to mix with tumor cells and the rest were spread in the interstitial space.

The brain metastasis was treated by a brief course of gamma-knife radiation. By monitoring sensitive tumor markers, we could evaluate responses to therapies. As Figure 3-2 shows, this treatment brought clear drop of CEA. The shoulder metastasis was then treated with local intervention chemotherapy. CEA continued to decrease following this treatment. Subsequent whole-body chemotherapy did not bring further response, and this was interpreted at the result of tumor resistance by expression of PDL1. Because at the time tumor expression of PDL1 was taken as an indicator for ICI therapy with anti-PD1 antibody, we therefore suggested so. We have also suggested local use of interleukin-12, an experimental drug that had significant antitumor activities in pre-clinical models [18,19]. This combination did bring down CEA deeply after a brief pause and continuously. At the time, we had not developed the depletion model for ICI therapy, therefore the antibody was given once every three weeks for totally three times. As can be seen from the tumor marker change, this repeated dosing of anti-PD1 antibody did not cause tumor relapse. During the continued response, the adrenal gland metastasis was treated by radiation frequency ablation (RFA). Finally, with continued tumor regression by tumor marker, we proposed to remove the remaining primary tumor and the surrounding mediastinum lymph nodes by

surgery, a critical step that may achieve clinical cure of this case. A PET-CT test was carried out for the purpose of evaluation. As Figure: 3-3 shows, there were three major high SUV area by the test: the faint signal from the primary tumor with a low SUV (about 5), a high SUV (about 9) signal of the mediastinum metastases and a very high SUV signal (about 13) on the shoulder metastasis. This signal distribution pattern suggesting active tumor metastases by the mediastinum and shoulder is a clear contradiction to the continued drop of tumor marker that had reached the "normal" range (Figure: 3-2) indicating there was almost no active tumor replication existing. Since our own experience had indicated that high PET-CT signal could be caused by immune response/ inflammation (our unpublished results), we took the high SUV signals from PET-CT as indication of immune response and went ahead with recommendation of surgery.

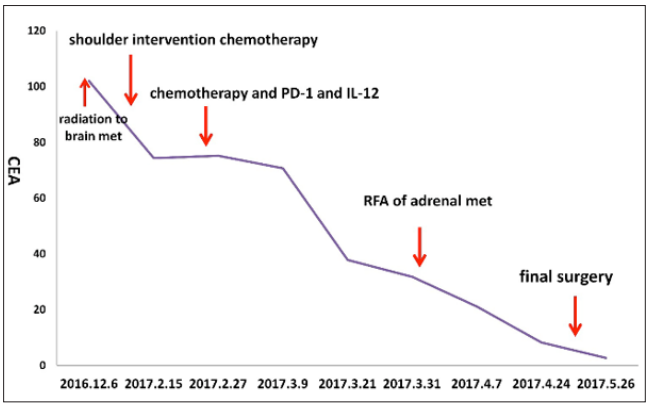

Figure 3-2: Change of tumor marker CEA with various treatments since TANLUN involvement in December 2016. Surgery date was 5/26/2017. Patient remains recurrence-free till now (>6 years after final surgery).

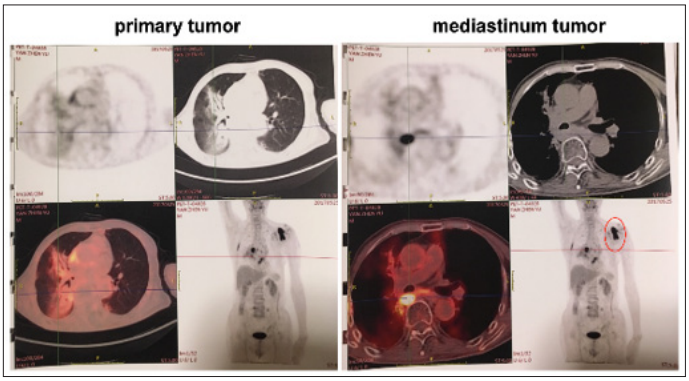

Figure 3-3: PET-CT imaging before final surgery. The primary tumor had much low metabolic activity compared to the mediastinum metastasis. The shoulder metastasis (indicated by the red circle in the lower right panel) had very high SUV signal.

The post-surgery analyses confirmed this speculation. Figure: 3-4 and Figure: 3-5 show surgical tumor samples from the primary and mediastinum metastasis, respectively, stained with HE, Ki-67 and CD3. As seen, tumor structure by HE staining was lowly-differentiated. There was clear difference between the two tissues as patches of tumor cells were obvious in the primary tumor section, whereas in the mediastinum section, tumor cells were less obvious. Tumor replication in the two samples was also different in that only sporadic replicating tumor cells were observed in the mediastinum tumor (Fig. 3-5, Ki-67), while more tumor cells expressing Ki-67 were seen in the primary tumor (Fig. 3-4, Ki-67). Inasmuch as the primary tumor contained more tumor cells and had more active tumor replication, this tumor appeared on the pre-surgery PET-CT with much less metabolic signals (Fig. 3-3, primary tumor) than that from the mediastinum metastasis (Figure: 3-3, mediastinum tumor). As we speculated, this difference in metabolic activity on PET was likely caused by differences in immune responses. Although, both samples contained large number of T cells, density-wise, more T cells were found in the mediastinum tumor. Furthermore, much higher ratio of T cells in this tumor demonstrated activated state (circular staining pattern) than in the primary tumor. These observations indicate that immune responses contributed to a larger portion of the observed PET signal in this case. Deduced from this fact, we believed that the shoulder metastasis, although not resected, would contain high immune activity that may eradicate the residual tumor eventually, a speculation that proved to be true by time. The patient remains recurrence-free till now, more than 6 years after the final surgery.

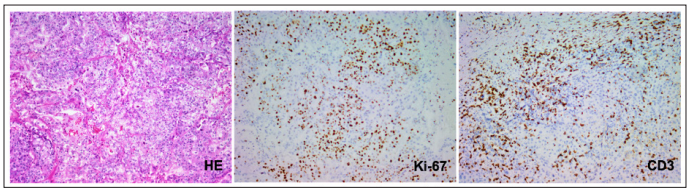

Figure 3-4: Surgical sample from primary tumor showing plenty residual tumor cells still presenting with active replication (Ki-67). There were large number of T cells (CD3) inside the tumor. This is the nodule that showed low metabolic (SUV) activity on PET-CT before surgery (Fig. 3-3).

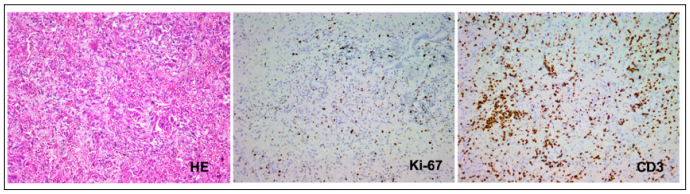

Figure 3-5: Surgical sample from the mediastinum showing residual tumor with low replication (Ki-67) and high antitumor immunity (CD3). The density of T cells in this tumor was much higher than that in the primary tumor (Fig. 3-4). This is the nodule that showed high metabolic (SUV) activity on PET-CT before surgery (Fig. 3-3).

Because the shoulder metastasis was not resected and was eradicated by immunity alone, this case has demonstrated durable antitumor response following treatment with ICI therapy. Although not given many times, anti-PD1 antibody was administrated three times in a row, a practice that may cause depletion of antitumor immunity and tumor relapse based on the above two cases. Yet, there was no such event in this case. By prediction, the T cells in this case must withstand depletion and thus had to be PD1-nagative. We therefore wanted to see the PD1 expression status of the activated T cells in the final surgical samples. As Figure: 3-6 shows, massive activated T cells were present in the surgical sample of the mediastinum tumor (CD3, 100x), and most of these T cells showed activated state (CD3, 400X). But when it comes to PD1 expression, nearly all of them were PD1-negative (PD1, 100X). Among the few PD1-positve T cells in the surgical sample most did not exhibit activated state (PD1, 400X). These observations indicate that strong antitumor response following repeated anti-PD1 antibody treatment are mediated by PD1-nagative T cells, and thus supports the depletion model for ICI therapy. It is interesting to mention that the residual tumor cells expressed high levels of PDL1 as well (not shown), indicating that the strong antitumor immune response mediated by the PD1-

negative T cells was also Th1 type that released IFN-gamma and stimulated tumor expression of PDL1. Nevertheless, these T cells were not interfered by tumor expression of PDL1, and carried out tumor eradication in a self-propelling manner until complete antigen clearance (tumor eradication), and deposited a strong protective immune memory that ensured clinical cure.

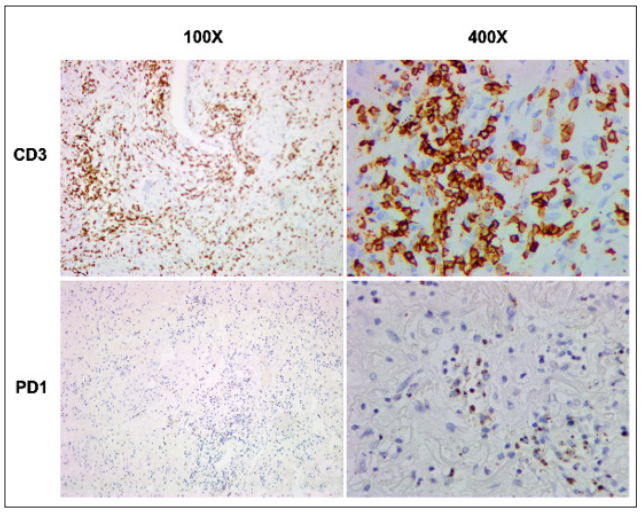

Figure 3-6: Expression of PD1 and T cells in the mediastinum tumor. Large number of T cells were present. Many of these T cells showed activated state (400X). Nearly all the T cells were PDI 1-positive T cells not showing activated state (400X).

Case 4: PD1-nagative T cells are involved in durable and self-sustaining antitumor responses not activated by ICI therapy

The preferential expansion of PD1-negative T cells in the presence of anti-PD1 antibody is a natural consequence by the depletion model. Yet we found that even in other durable antitumor response not activated by ICI therapy, PD1-negative T cells may also play a major role. The following is such a case.

A 64-year-old women with persisted virginal bleeding and lower abdominal pain went to hospital. Test results suggested cervical cancer. Biopsy pathology confirmed presence of cervical squamous cancer. Tumor marker SCC was elevated. Besides primary cancer of >3cm, there were multiple pelvic metastases. Hospital selected the standard chemo and radiation plan for cervical cancer. Following the treatment, the primary tumor nearly disappeared, SCC dropped to below normal range and the patient was put on observation. Eight months later, SCC began to increase and subsequent PET-CT showed two prominent lung metastases (Figure: 4-1). A family member went to us for help. We noticed that there was no recurrence of the primary tumor and pelvic metastases, indicating the previous chemo and radiation treatments had completely eradicated these tumor burdens. But In light of the common effect of radiation-mediated immune suppression, we suspected that the lung metastases were the result of such suppression. If so, upon the establishment of new metastases, the previously suppressed immunity may return and form new balance with the tumor. This return of antitumor immunity is often accompanied by spontaneous stabilization or even drop of sensitive tumor marker since tumor replication is suppressed by returned immunity. Indeed, few weeks later, we caught a brief drop of SCC spontaneously (Figure: 4-2), which indicated the return of antitumor immunity. With the presence of concomitant immunity, we suggested chemotherapy to activate this immunity. One course of chemotherapy brought persisted drop of SCC (Figure: 4-2) for 5 weeks followed by rebound. Second course of chemotherapy did not bring SCC drop but a rapid increase (Figure: 4-2). With known presence of antitumor immunity, such tumor marker rebound reflects rebound of tumor replication with is often the result of tumor expression of PDL1 (our unpublished observation). This is because that chemotherapy activates antitumor immunity that releases IFN-gamma, which in turn stimulate tumor expression of PDL1. We had prepared to combine the use of IL-12 for further activation of antitumor immunity [18,20] before the second course of chemotherapy. Knowing that IL-12-modified T cells may be resistant to negative regulation [21], we suggested to give IL-12 injection in light of this SCC rebound. With three IL-12 injections, SCC continued to increase quickly, that it reached the levels even higher than that at the time of diagnosis with the primary tumor and all of the multiple pelvic metastases. CT imaging showed that the two lung metastases increased in size (about 3 cm) without other new metastasis, supporting that this rapid increase of SCC was the result of tumor expression of PDL1.

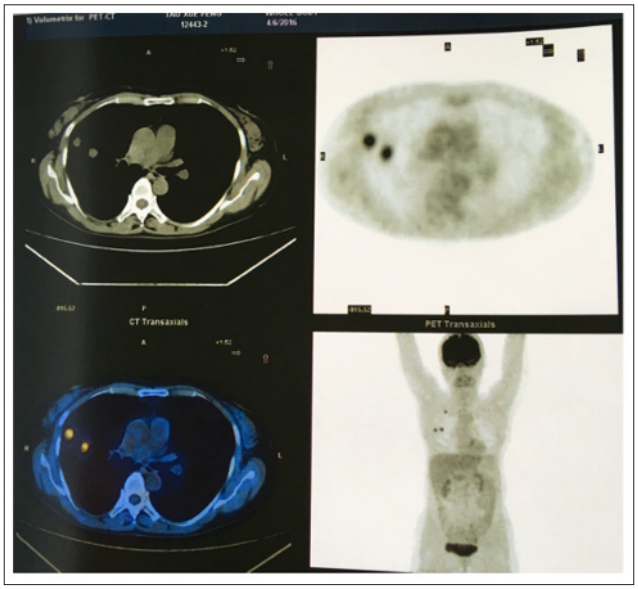

Figure 4-1: PET-CT imaging showing two lung metastases 10 months following chemo and radiation treatments of the pelvic primary tumor and metastases. Note the lack of pelvic recurrence.

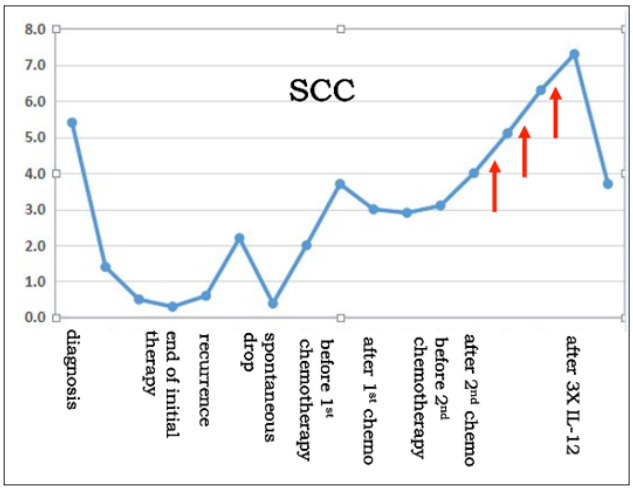

Figure 4-2: Change of tumor marker SCC from diagnosis to following IL-12 after chemotherapy. Note that with the initial combination treatment, SCC dropped to normal range. The recurrence was accompanied by SCC rebound. The return of antitumor immunity was marked by the spontaneous drop of SCC following recurrence.

Since we did not observe tumor response following IL-12 administration, an alternative treatment using anti-PD1 antibody was proposed. One day before anti-PD1 antibody treatment, we saw a steep drop of SCC (Figure: 4-2). This drop, taking place 4 weeks after 2nd chemotherapy and in the background of previous rapid increase, was rather a surprise, but was nevertheless, consistent with our predicted purpose of IL-12 administration. Seeing, this sudden response, we halted the ICI therapy and continued to follow the change of tumor marker. SCC continued to decrease steadily for the next month with shrinkage of the two lung metastases to about 1-2 cm in size. Not sure whether this antitumor response could eradicate the entire tumor burden and with the location of these two metastases easily accessible for surgery, we proposed to remove them surgically to secure a chance of clinical cure. The patient remains recurrence-free till now, more than 5 years after surgery.

The post-surgery analysis showed a dramatic antitumor response in the surgical samples. As Figure: 4-3 shows, the tumor has typical squamous cell structure (HE), supporting its source of cervical cancer. Tumor replication was suppressed in that most Ki-67-positive tumor cells showed faint staining only (Ki-67). As expected, massive presence of T cells (mostly CD8 subtype) was seen in the tumor (CD3). Majority of these T cell showed activated state. These observations indicated that the pre-surgery treatment with chemotherapy and IL-12 indeed activated a strong antitumor response. We had also stained the tumor section for PDL1 expression and the result showed that most remaining tumor cells, especially those that located near T cells expressed PDL1 (not shown). This observation confirmed our previous speculation that the rapid increase of SCC following 2nd chemotherapy and IL-12 was indeed caused by tumor expression of PDL1. Since the sudden drop of SCC after IL-12 was not due to loss of tumor expression of PDL1, it must be caused by an antitumor immune response that was PDL1-resistant. Upon recent realization that durable antitumor responses could be mediated by PD1-nagative T cells, we recently went back to stain the tumor section form this case with anti-PD1 antibody. As Figure: 4-4 shows, the same area that showed massive CD3 in the tumor section had much less PD1-positive T cells. The few that did express PD1 were not activated. This retrospective analysis thus confirmed that in this case where no ICI therapy was applied, a strong and durable antitumor response was also mediated by PD1-nagative T cells.

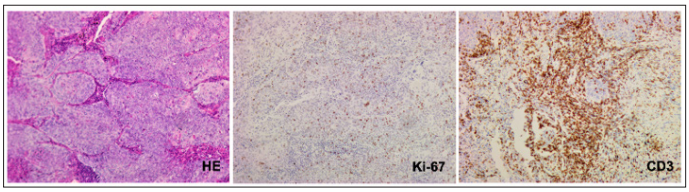

Figure 4-3: Surgical sample form one of the two lung metastases stained with HE, Ki-67 and CD3. Tumor structure and morphology resembles squamous carcinoma, supporting cervical cancer origin. Tumor replication seemed suppressed as many Ki-67-positive tumor cells had faint staining. There was a massive presence of T cells in the tumor, surrounding tumor structure. Most of these T cells were CD8 majority showed activated state.

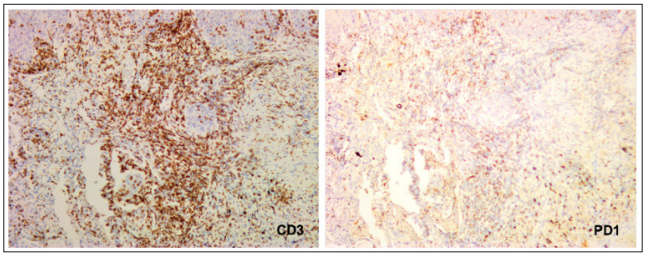

Figure 4-4: The same Lung metastasis in Fig.4-3 stained with CD3 and PD1. The same area of the slide was compared here. It is clear that among the massive T cells, very few expressed PD1. The ones that did express PD1 did not show activated state.

Discussion

PD1 is a molecule expressed on the surface of activated T cells [22]. But many studies also show that PD1 expression is a hallmark for exhausted T cells in tumor environment [23,24]. The overall analyses on PD1 expression tend to show that it is a negative regulator of T cell function. On the other hand, tumor cells express PDL1 by the stimulation of IFN-gamma [13,14]. Therefore, we have a situation where tumor-infiltrating T cells inside tumor mass are met with tumor cells expressing PDL1. The net effect is the survival of tumor with presence of antitumor T cells, a situation we call concomitant immunity. The antitumor immunity inside a growing tumor may be "exhausted" as many tend to believe, but also "functional" as very few realize. The functions of these tumor-infiltrating T cells include: 1) to restrict the growing (proliferating/replicating) rate of the tumor; and 2) to restrict the establishment of new metastasis. These functions are easily seen in animal models when T cells are removed, but hardly recognized in human cancer patients until recently when ICI therapy bring many hyper-progression cases. The essence of ICI therapy-induced hyper-progression is the depletion of antitumor T cells, those many thought to be exhausted cells that co-existing with growing tumor. By this measurement, we should not consider these co-existing T cells "exhausted" and "functionless", but recognize their important role as functional antitumor T cells.

Clearly these T cells do not eradicate their accompanying tumors, which is the reason many believe that they are functionless. It seems from the above cases, and many other cases we have experienced in the past few years since we begin assessing the status of antitumor immunity in individual cancer patients, that these T cells do not overcome their accompanying tumor burdens because they are usually not "fully" activated. First of all, their numbers are often low and consistently increasing with growing tumor burdens, but not more than the tumor cells they battle with.

Secondly, these T cells are not in an activated state. This is often detectable by looking at the staining pattern of these T cells, because activated T cells show focused circular signal instead of diffused distribution of signal though the entire cell (a good example is the shape and straining pattern of CD3-positive T cells in Fig. 3-6, 400X). In the above case 3 and case 4, we can see what kind of T cell pattern is associated with tumor regression by immune attack. At least a large number of T cells and activated state were present.

But just meeting these two conditions are still not enough to witness the persistent tumor regression in these two cases, as these two conditions have been met in many of our cases following activation of T cells by chemotherapy (our unpublished observation). The biggest difference between activated T cell response in a regular chemotherapy and the responses in Case 3and 4 seems to be the self-sustaining feature of the later. A self-sustained immune response does not require antigen release by repeated intervention (for example, tumor killing by chemotherapy drugs). Once activated, it searches for antigen and clear it while maintain activated until antigen clearance. This is common in immunity-mediated anti-viral response as no intervention by man was required and the result is always complete eradication of virus. But when it comes to antitumor immune response, the self-sustaining response is rarely seen. The reasons have been discussed but no consistent clue emerged.

Many studies have shown that the infiltrating T cells are in a state of exhaustion [23,24]. Subsequently, this was attributed to down regulation of T cell function by the expression of PDL1. This argument, in light of the self-sustaining antiviral response, does not seem to make sense because antiviral responses are Th1 type by nature, and should also stimulate expression of PDL1 by infected cells or surrounding uninfected endothelial cells. In addition, the immune responses in the above Case 3 and 4 also stimulated PDL1 expression by tumor cells. One explanation, of course, is that the T cells in these two cases did not express PD1, thus were not able to be interacting with PDL1 on tumor cells. In Case 3, PD1-nagative T cells may be selected by continued presence of anti-PD1 antibody. But there was no such selection in Case 4, thus PD1-negative T cells may be a group of T cells mediating self-sustaining immune response naturally. In contrast, antitumor T cells we see inside a tumor are more likely in a state of inactivation, although they do have antitumor activity. The question is how to activate a strong and self-sustaining antitumor response?

In the case of ICI therapy, this is sometimes achieved by selection of PD1-nagative T cells through depletion of PD1-posotitive T cells according to the depletion model. The depletion causes a state of temporary homeostatic disbalance of T cells and a subsequent expansion of any surviving T cells. When all antitumor T cells are PD1-positive and are depleted, there will be a short-term loss of tumor control, and a possible tumor outgrowth. But when some T cells, although PD1-positive, hide inside a tumor mass not accessible to antibody binding, they may be activated through homeostasis recovery. This activation results in T cell number expansion, and changes the activation status of the T cells. These T cells in turn infiltrate and attack tumor, resulting in antitumor response. In Case 1 and 2, this seemed to be the case following initial anti-PD1 antibody. But from the subsequent hyper-progression in these two cases, homeostatic activation perse does not seem to lead to PD1-nagative T cells. The question how PD1-negative T cells arise remains a mystery.

But there is always this possibility that there are some naturally occurring PD1-nagative T cells in a concomitant antitumor immunity, which will expand over PD1-positive T cells under presence of PDL1-mediated suppression. There is also this possible that any T cell activation may also lead to transition from PD1-posiitve to PD1-nagative T cells. In this aspect, if we know how to convert more T cells to PD1-nagative, the chance of better tumor control will certainly increase. In this regard, the use of IL-12 in Case 4 may be one measurement to achieve this goal. This is consistent with reported finding that IL-12 modification results in loss of PD1 expression [21]. IL-12 was used in both Case 3 and 4. It was clear that in Case 4, this treatment was effective to activate a response that was resistant to PDL1 interference, most likely through activation of a PD1-nagative T cell response. But its role in Case 3 was not clear. Our belief is that the activation of antitumor response to overcome the resistance was by the ICI therapy, because IL-12 was used after witnessing resumed tumor regression. If IL-12 further helped to push for a PD1-negative response, it does not change our conclusion that such a response was not disrupted by continued ICI therapy. IL-12 had been used in other cases with benefits (our unpublished results), but we rarely see dramatic antitumor responses as seen in Case 3 and 4. On the other hand, we have evidence to show that T cells activated by IL-12 in local setting such as tumor vaccine are often susceptible to depletion by anti-PD1 antibody, indicating that IL-12 modification perse does not guarantee a generation of PD1-negative T cells (our unpublished results).

If there was any remaining doubt, the combined findings from the above four cases settled the dispute between the mainstream blocking and our depletion model for the true working mechanism of ICI therapy. One ironic question following the settlement is this: If anti-PD1 antibody target PD1-positive antitumor T cells for its antitumor effect, what is the use of such antibody in a durable response mediated by PD1-nagative T cells? Current clinical practice for ICI therapy is continued antibody dosing in every three weeks. Some durable responders received dozens of doses of antibody in 1-2 years. Was this necessary? There is certainly no proof from ICI therapy developer that continued dosing of anti-PD1 antibody is necessary. It's continued dosing is a natural thinking based on the blocking model. On the other hand, based on the depletion model, ICI therapy has trigger effect that only requires a single dosing of antibody to generate T cell activation and antitumor response. This was demonstrated by Case 1 and 2 following the initial treatment. And these two cases also demonstrated that repeated antibody dosing may reverse a previously antitumor response into a hyper-progression.

We do not have an accurate account of how many such cases had taken places in the real-world clinic, but based on our own experiences, roughly 40% of ICI therapy-treated cases ended up with loss of tumor control. This high ratio of harm to benefit for ICI therapy may explain the low response ratio and lack of clear impact in real world use of ICI therapy [1, 2]. On the other hand, since we have recognized the depletion model in the past 15 months, we have established a record of >90% accuracy in selecting potential responders and avoiding all harmful use of ICI therapy. In a few cases where ICI therapy was used without our knowledge and generated harm, there was no exception that had we evaluated the case for selection of ICI therapy, we would not have recommended it. These clinical records indicate that the depletion model must be correct. If adapted by the mainstream medicine, many lives could be saved. After all, ICI therapy is a great development for cancer management, it is just that it is

like a double-edged sword that may benefit or harm its users. By understanding its true working mechanism, we should be able to save the benefit while prevent the harm.

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