Review

Immune Cytolytic Activity and Strategies for Therapeutic Treatment

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Posted Date: 31 January 2024

doi: 10.20944/preprints202401.2227.v1

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Review

Immune Cytolytic Activity and Strategies for Therapeutic Treatment

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Abstract: Intratumoral immune cytolytic activity (CYT), measured as the geometric mean of the expression of Granzyme-A (GZMA) and Perforin-1 (PRF1), has emerged as a critical factor in cancer immunotherapy, with significant implications for patient prognosis and treatment outcomes. Here, we review how different immune checkpoint pathways, the composition of Tumor Microenvironment (TME), antigen presentation and metabolic pathways, all regulate CYT. We also discuss the various methods through which CYT can be assessed. The detection and analysis of tumor-infiltrating lymphocytes using flow cytometry or immunohistochemistry provide important information on the immune cell populations within the TME. Gene expression profiling and spatial analysis techniques, such as multiplex immunofluorescence and imaging mass cytometry, allow researchers to study CYT in the context of the TME. We further report the significant clinical implications that CYT has, as its increased levels are associated with positive clinical outcomes and a favorable prognosis. Moreover, CYT can be used as a prognostic biomarker and aid in patient stratification. Measuring CYT through different methods offers promising paths for improving treatment responses. Overall, we highlight that the understanding and modulation of CYT levels is critical for improving cancer immunotherapy. Research into CYT and the factors that influence it has the potential to transform cancer treatment and improve patient outcomes.

Keywords: cytolytic activity (CYT); tumor-infiltrating lymphocytes (TILs); gene expression profiling; spatial analysis techniques; immune checkpoint pathways; tumor microenvironment (TME); antigen presentation; metabolic pathways

1. Introduction

1.1. Background on Cancer and the Immune System

Cancer is the second leading cause of death, worldwide and both the incidence of cancer and cancer-related mortality rates are constantly increasing. It is estimated that until 2060, cancer will be the main cause of death [1].

The immune system is a defence mechanism against infected antigens and self-antigens via a suitable balance between inhibition and activation of immune responses [2]. It also actively participates in cancer prevention, progression and therapy [3]. Cytotoxic T-cells (CTLs) via their T-cell Receptors (TCRs) recognize antigens derived from cancer cells and bound to Major Histocompatibility Complex (MHC) molecules on the surface of Antigen Presenting Cells (APCs) [4]. Moreover, the immune system creates immunological memory through the adaptive immune responses for a future more rapidly and effective response against the same antigens [5,6].

Tumors can evade the immune response, using various mechanisms, including the inhibition of the immune system, inducing T-cells exhaustion and restricting antigen recognition [7]. Evasion of cancer cells from the immune system can be defined as one of the cancer hallmarks [8].

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Furthermore, cancer cells proliferate and grow faster than the immune system can manage, resulting in the escape of an attack from immune cells. To achieve this, cancer cells create in their surrounding the TME which affects the efficacy of immune cell activation [9].

The TME plays a crucial role in cancer progression and cell migration [10]. It is composed of cancer cells, blood vessels, Extracellular Matrix (ECM), stromal cells and immune cells, including Natural Killer (NK) cells, B-cells, T-cells, Neutrophils, Myeloid-Derived Suppressor Cells (MDMCs) and Tumor-Associated Macrophages (TAMs) [11]. The interplay between the cellular and extracellular components of TME contributes to early diagnosis of cancer [12].

Cancer immunotherapy has enabled never-before-seen success rates in durable tumor control and enhanced survival benefit in patients with advanced cancers. There are two different forms of immunotherapy, the Immune Checkpoint Inhibitors (ICIs) and Adoptive Cellular Therapy (ACT) [13]. ICIs using specific monoclonal antibodies (mabs), including anti-programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) and/or anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) to treat several cancers. Specifically, anti-PD-1 and/or anti-CTLA-4 have increased the overall survival of patients with melanoma [14].

There are currently eight (8) approved ICIs to treat 18 different types of cancers, comprising tumors with deficient mismatch repair system/microsatellite instability—high (dMMR/MSI-H), lung cancer, renal cell carcinoma (RCC), ovarian cancer, gastric cancer (GC) and Hodgkin's lymphoma [15–17]**.** Nevertheless, many cancers create resistance to ICI therapies and do not respond to them.

1.2. The Role and Significance of Immune Cytolytic Activity in Tumor Control

CTLs and NK cells can target and kill target cells, using various mechanisms. One of the major mechanisms that CTLs kill cancer cells, is the granzyme/perforin pathway [18]. More specifically, upon recognition of the targeted cells, CTLs and NK cells release granzymes and perforins to enter and lead them to apoptosis [19].

Perforins are responsible to open pores in the membrane of the targeted cancer cells, facilitating the entry of the granzymes into them, which eventually eliminate cancer cells in an apoptotic manner (Figure 1) [20,21]. CYT is a new index of immune activation within a tumor and it is calculated by the expression levels of Granzyme-A (GZMA) and Perforin-1 (PRF1) mRNA expression levels [20,22].

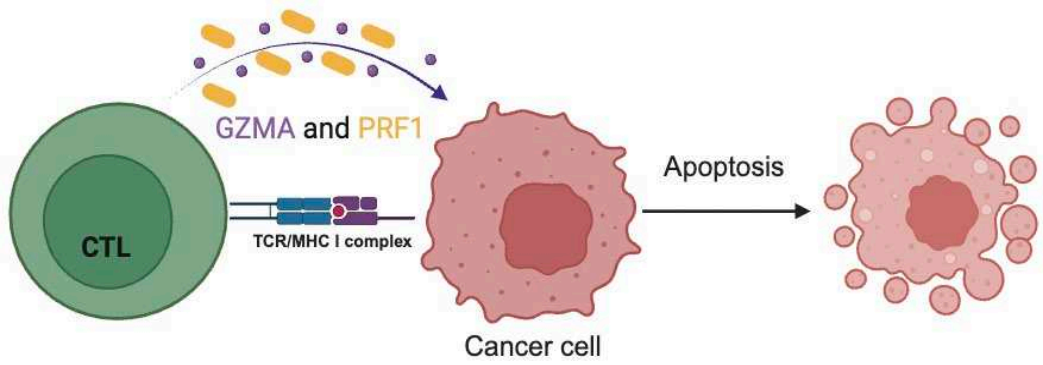

Figure 1. CTLs release GZMA and PRF1, leading cancer cells to apoptosis.

In 2015, CYT levels were quantified and presented by Rooney et al. as a new index of local immune infiltrate , across 18 tumor types [23]. CYT index varies across different cancer types. Roufas et al. quantified the transcription levels of GZMA and PRF1 and acute myeloid leukemia, pleural mesothelioma, sarcoma, and stomach cancer are associated with high affinity in CYT. On the other hand, ovarian, liver, thyroid, esophageal, and prostate cancers, glioblastoma, glioma and adrenocortical carcinoma and uveal melanoma, shown the lowest CYT levels. Finally, head cancer, melanoma and neck cancer had considerably higher CYT levels, compared to the equivalent normal tissues [22]**.**

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In CRC, CYT is associated with different mutational events and high CYT tumors were related with increased Tumor Mutational Burden (TMB) [24–26] and upregulated the expression levels of immune checkpoint inhibitors, including PD-1, PD-L1/2, CTLA-4, Lymphocyte Activation Gene 3 (LAG-3) and Indoleamine 2, 3-Dioxygenase 1 (IDO-1) [22,24].

Moreover, high CYT levels were associated with high MSI status and better survival rates of patients with CRC, hepatocellular carcinoma (HCC) and triple-negative breast cancer (TNBC) [24,27–29].

CYT has a significant role in cancer control and progression and can be also used as a prognostic biomarker for reflecting immune status in hepatocellular carcinoma (HCC) [30], immunity and clinical outcome [25]. In addition, CYT-high skin cutaneous melanoma (SKCM) patients have better prognosis [31].

Although available therapies improved the prognosis for many patients with cancer, most patients still have a poor prognosis, or they develop resistance to them. Therefore, the mechanisms being responsible for the aberrations taking place within the TME need to be investigated further. Herein, we investigated the factors influencing CYT in cancer in order to develop better therapeutic approaches, identify new therapeutic targets or potential biomarkers and eventually help cancer patients manage better their disease, enhance their life span and increase their life span.

2. Results

2.1. Methods for Assesing CYT

2.1.1. Tumor-Infiltrating Lymphocytes

- Isolation and Characterization of TILs

TILs have the ability to recognize and kill autologous cancer cells, both in vitro and in vivo [32] and their isolation, especially in early stages of cancer is very important [33].

Isolation and characterizations of TILs have been evolved as TILs can be used for prognsis and therapeutic effects in tumors. Phenotypic analysis of TILs isolated from renal cell carcinoma (RCC), showed that mostly of TILs were composed of CD4+, CD8+ and CD56+ cells in different numbers [34]. As it is already mentioned, CYT is strictly related with the population and activity of CTLs. Therefore, CYT can be related also with the populations of TILs.

Moreover, isolation of TILs from tumor and non-tumor liver tissues of resected HCC patients was used to analyse the immune gene expression profiles and the results shown that the activation and proliferation of TILs at inflammatory immune microenvironment enhance the survival of HCC patients and the tumor progression [35].

Currently, immunotherapies using TILs have earned attention due to their efficacy and the failure of conventional immunotherapies [36]. Spatial characterization of TILs have been demonstrated as a key prognostic biomarker to predict treatment response in breast cancer [37].

- Analysis of TILs by flow cytometry or immunohistochemistry

TILs were expanded and analysed using different in vitro methodologies, including flow cytometry analysis and immunohistochemistry. In a study in 2017 TILs were surgically expanded from 19 patients with pancreatic adenocarcinoma in the presence of IL-2 and were characterized phenotypically by flow cytometry analysis. The results showed that the majority of TILs were composed of CD4+ T cells and CD8+ T cells [38].

In 1994, expanded TILs derived from NSCLC have been used in adoptive immunotherapy following surgery with promising results [32]. Flow cytometric immunophenotypic analysis of TILs immunophenotypes can predict both clinical effectiveness of immunotherapy [39] and early relapse in localized clear cell RCC [40].

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2.1.2. Gene Expression Profiling

- Identification of Cytolytic Markers

Upon activation, CTLs and NK cells mediate cytolysis of targeted cells using various mechanisms. One of this, is the granzyme-perforin pathway which is strictly associated with the CYT. Specifically, CTLs and NK cells upon their expose to infected/cancer cells, release cytolytic markers, such as granzymes and PRF1 to open pores, enter to the targeted cells and lead them to apoptosis [19,41].

GZMA and PRF1 are two key cytolytic effectors whose transcription levels are used for the quantification measurement of immune CYT. The expression of both GZMA and PRF1 is significantly affected upon CD8+ T cell activation [42].

- Measurement of CYT through RNA expression

Immune CYT is assessed on the expression levels of cytolytic effectors secreted from CD8+ T cells. It is measured using the mRNA expression levels GZMA and PRF1 [25,30,43] as well as using the RNA sequencing of the above-mentioned toxins [23].

2.1.3. Spatial Analysis Techniques

- Multiplex Immunofluorescence (mIF) and Imaging Mass Cytometry (IMC)

mIF and IMC are tissue imaging technologies used to detect multiple markers on a single tissue slide and their combination has been recently demonstrated as an accurate imaging method for single-cell segmentation [44].

mIF technique allows the simultaneous accurate detection of a variety of immune markers in different tissues types [45]. It has the unique ability to study the spatial interaction between immune cells and tumor cells without affecting the architectural features of the tumor [46]

IMC is based on the principles of flow cytometry analysis and Mass Spectrometry (MS) and it uses metal isotope-labelled antibodies that enables highly multiplexed imaging analysis of proteins, isotopes and markers within tumor tissues [47,48]. In 2021, it was used to characterize the TME of metastatic melanoma patients who received immunotherapy in order to find indicative biomarkers of treatment response with very promising results [49].

Among available spatial analysis techniques, both mIF and IMC are very significant in the field of immuno-oncology and they are used to study and understand better the TME in order to create more effective therapies and identify predictive biomarkers of response to immunotherapy [50].

- Assessment of CYT within the TME

Immune CYT and immune cells within the TME are strictly related. CTLs and NK cells secret granzymes and perforins such as GZMA and PRF1 to kill cancer cells. The population of the above-mentioned immune cell types within the TME is strictly associated with high levels of immune CYT and better survival [23,30,51]. On top of CD8+ T cells and NK cells, in GC the CYT score was associated with high infiltration of macrophages and low infiltration of Tregs [25].

Furthermore, CYT-high primary and metastatic skin melanoma tumors were enriched in CD8+ T cells, B cells and M1 macrophages, while CYT-low tumors were enriched in CD4+ T cells, monocytes and NK cells [52]. In glioblastoma, high CYT levels were related with higher infiltration of immunosuppressive cells including neutrophils and M2 macrophages and worst survival [43].

As immune CYT and immune cells are closely associated within the TME, it is considered that immune CYT can be assessment within the TME.

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2.2. Factor Influencing CYT

2.2.1. Tumor Intrinsic Factors

- Tumor Mutational Burden

The TMB is defined as the total number of genetic mutations in the DNA of cancer cells [53]. It is very significant index for doctors in the selection of the most effective treatment for each cancer patient.

Calculation of TMB is done by using different methods. The TMB was first measured from Whole-Exome Sequencing (WES), included only non-somatic mutations by the Next-Generation Sequencing (NGS) technology [54]. Both WES and NGS are used to measure the TMB [55].

Accumulating evidence suggests that TMB is a key predictive biomarker in cancer therapy. From the majority of studies it is clear that a high-TMB is related with clinical efficacy of ICI therapy in multiple cancer types [53,56]. It is worth mentioning that, between of all available immunotherapies and 27 different tumor types or subtypes, high-TMB is associated with better response to anti-PD-1 [57].

In 2015, a high TMB was strongly associated with better response of NSCLC patients to anti-PD-1 immunotherapy and longer Progression-Free Survival (PFS) [58]. Moreover, NSCLC patients with high TMB score, who received nivolumab (anti-PD-L1) had longer PFS and higher Objective Response Rate (ORR), than the patients who received chemotherapy [59]. On top of anti-PD-L1 alone, combination immunotherapy of nivolumab and ipilimumab in high-TMB patients with advanced NSCLC, showed a longer PFS than chemotherapy treatment [60].

The TMB is also linked with other emerging biomarkers. Specifically, MSI-H/dMMR tumors have been identified to exhibit high TMB and they also associated with better response to ICI therapy [61]. Besides of MSI-H, high TMB correlated with increased CYT and downregulated of various of immune checkpoint inhibitors in colon cancer [24].

More and more studies support that the TMB is a promising predictive biomarker, and its evaluation plays a key role in immuno-oncology. In addition, TMB could be very valuable in treatment selection for ICI therapy [55].

- Antigen Presentation and MHC Expression

APCs constitute an heterogenous group of immune cells that are responsible to process and present antigens from recognition to T cells through MHC [62]. APCs can be divided into professional, with haematopoietic origin and include B lymphocytes, macrophages and DCs and nonprofessional APCs that are not bone marrow derived and include hepatocytes and fibroblasts [63].

MHC proteins are key components of adaptive immunity and categorized in two different classes [64]. Both classes of proteins present antigens on the surface of APCs immune system and specifically to T cells [65].

MHC class I present antigens from virally infected cells to CD8+ T lymphocytes to kill them [66], however many human viruses develop proteins that interfere in the presenting procedure and enhance virally infected cells to escape the detection and destruction from immune system [67].

As opposed to MHC class I, MHC class II molecules are responsible to present antigens to CD4(+) T lymphocytes [68]. Moreover, the ability of MHC class I to present to APCs derived-peptides from cells, allow to CD8+ T cells to recognize and kill cells, e.g. cancer cells that synthesized abnormal proteins. It has been reported that loss of MHC class I antigen presentation leads to evasion of cancer cells from immune system [69]. It has been observed that some human cancers, including gastric cancer and melanoma are due to loss of MHC I expression [70,71].

Moreover, CD4+ T lymphocytes have a pivotal role in preventing tumor growth and targeting of the MHC class II antigen presentation pathway can be used to develop efficient vaccines to activate the immune system and enhance the immune responses against cancer [72]. There are also some types of tumors, such as solid tumors do not express MHC class II molecules, and the participation of CD4+ T lymphocytes depends exclusively on infiltrating APCs [73].

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Both MHC I and II pathways are taking into consideration in order to improve immunotherapy.

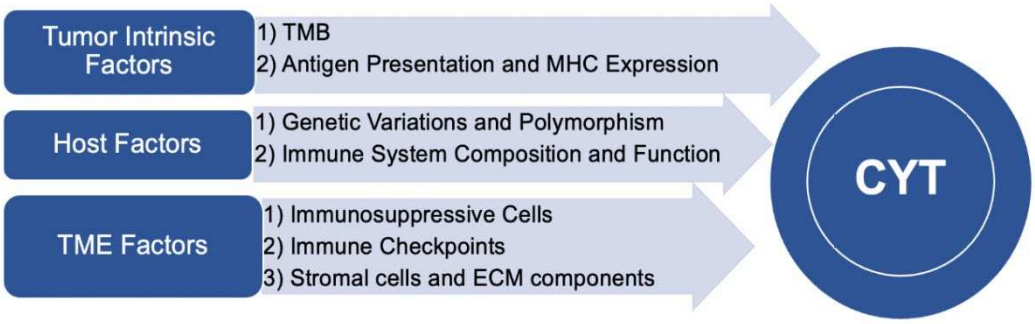

Figure 2. Factors Influencing CYT.

2.2.2. Tumor Microenvironment Factors

- Immunosuppressive cells

The TME has a pivotal role both in cancer progression [74] and affect the response of cancer patients to therapies [75]. It is composed by both cellular and extracellular components, including the category of immunosuppressive cells [11].

Regulatory T cells (Tregs) and Myeloid-Derived Suppressor Cells (MDSCs) are critical cells related with immune resistance [76], and they enhance the escape of cancer cells from the immune system [77]. Immunosuppressive cells have significant clinical value and targeting of them can enhance the effectiveness of immunotherapies and converse the immune resistance [76].

MDSCs are immature progenitor cells that suppress the immune responses and categorized into 2 subcategories depending on the mechanism of their action: the Monocytic (M-) MDSCs and the Granulocytic (G-) MDSCs [78]. Moreover, a subpopulation of T cells, the Tregs promote tumor progression [79] by suppressing both the proliferation of T-cells and the production of cytokines, to maintain immune homeostasis and self-tolerance [80].

More studies suggest that the elimination of immunosuppressive cells within the TME can inhibit the tumor progression. Specifically, inactivation or decrease in the number of MDSCs enhance the anti-tumor immunity and reverse the status of TME from immunosuppressive to immune-activate [81,82]. In addition, ICI therapies using anti-PD-1/anti-PD-L1 and or anti-CTLA-4 mabs decrease the number of Tregs by increasing to the number of CD8+ T cells [83]. Chemotherapy can eliminate the number of Tregs and MDCSs in the TME [84].

Immune evasion constitutes one of the hallmarks of cancer progression [81]. Immunosuppressive cells inhibit antitumor immune responses and enhance tumor immune escape, resulting to tumor cell extravasation and metastasis [77]. Additionally, in order to avoid immune cells, cancer cells enlist Tregs to upregulate tumor antigen expression, active inhibitory immune checkpoint molecules and create an immunosuppressive TME [85,86]. Immunosuppressive cells are critical in cancer progression and metastasis.

- Immune checkpoints

Immune Checkpoint Proteins (ICPs) are expressed on the surface of cancer cells to inhibit T cell-mediated immune responses and they are using to treat many different types of cancer [87].

ICI immunotherapy is used to bind to ICP, active T cells and allow them to kill cancer cells. It is currently one of the most novel and promising cancer therapies [88].

There are different immune checkpoint inhibitors that used to treat a range of cancer types. The most well-studied ICIs consists of anti-CTLA-4, anti-PD-1 and anti-PD-L1 [88,89].

In 2011, Ipilimumab, an anti-CTLA-4 ICI, was successfully approved and introduced a promising new form for cancer therapy [90]. Anti-PD-1 (nivolumab, pembrolizumab and cemiplimab) and anti-PD-L1 (atezolizumab avekumab and durvalumab) were then received the

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Food and Drugs Administration's (FDA) approval and they are using to treat about 15 different types of tumor [91] (Figure 3).

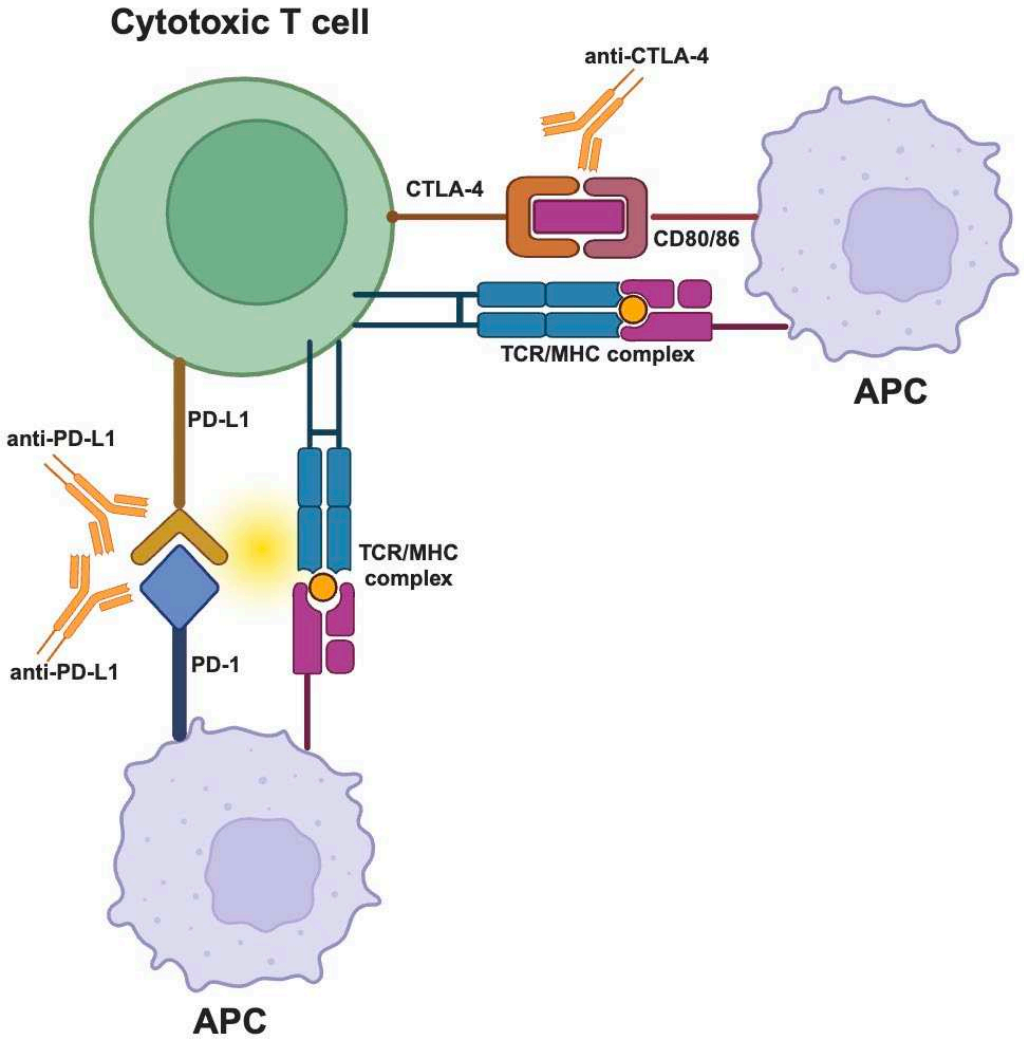

Figure 3. Immune checkpoint Inhibitors.

Moreover, other studies have been focused to study for other antibodies against other immune checkpoint proteins, including V-domain immunoglobulin-containing suppressor of T-cell activation (VISTA), lymphocyte activation gene-3 (LAG-3), B7-homolog 3 (B7-H3) and T cell immunoglobulin, and mucin domain 3 (TIM-3), [89].

ICI therapy is a vital and very promising form of therapy to treat cancer [92], however only a small number of cancer patients respond to this form of immunotherapy. Most of the ICPs are expressed on different type of immune cells and their expression varies between a cell type [88]. In addition, it was acknowledged that the TMB, MSI-H, dMMR, neoantigen expression and certain gene mutations are predictive biomarkers for the efficacy of ICI therapies to cancer patients [56,93]

ICPs influence CYT and high CYT increases the expression of immune checkpoint inhibitors and for this, CYT-high tumors are better candidates to respond to ICI therapies [26,27,94].

ICI therapies targeting CTLA-4 and/or PD-1/PD-L1 improved outcomes and survival for many patients with different types of cancer. Despite the challenges, additional inhibitory pathways and immune checkpoint proteins must be explored in order to apply the ICI therapies to more cancer patients and improve the responses of cancer patients to it.

- Stromal Cells and Extracellular Matrix Components

Stromal cells have fundamental roles in health and disease and they are responsible for the building and infrastructure of organs [95]. Both the presence of stromal cells in TME and their

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interplay with cancer cells are crucial for the cancer initiation. More studies increasingly show that stromal cells and their products, actively participate and promote tumorigenesis [96].

In pancreatic cancer (PC), neoplastic and cancer cells create a specific environment which enhance the malignant properties of cancer cells [97]. Multipotent Stromal Cells (MSCs) a subpopulation of stromal cells is present in multiple tissues and have the ability to differentiate in different lineages, encompassing osteoblasts, chondrocytes and adipocytes. In cancer, MSCs can migrate from primary tumors to metastatic organs, contributing to the progression of carcinogenesis. Moreover, cancer cells enhance the migration of MSCs and their crosstalk is crucial in tumor development and can be targeted for therapeutic approaches [98].

Another form of stromal cells that are present in TME are the cancer associated fibroblasts (CAFs). They promote tumor growth, invasion, angiogenesis and metastasis [99]. Moreover, CAFs can be activated by TAMs and enhance the development of malignant tumors [100]. In breast cancer, CAFs were associated with therapeutic resistance [101].

The crosstalk of immune cells and stromal cells is crucial in both cancer progression and anticancer activity. In addition, immune cells and stromal cells conduce to immune suppression within the TME and induce ani-tumor immunity [102].

Extracellular Matrix (ECM) is a three-dimensional highly dynamic structural network that has a pivotal role in normal development as well as in disease development and progression [103]. It is composed of different macromolecules, including collagens, glycoproteins, elastin, proteoglycans, fibronectin, etc., which join each other as well as cell-surface receptors, creating a scaffold for cells and present in all tissues and organs [104]. The interaction between ECM components is crucial in different cellular processes and functions, including proliferation, differentiation, migration and survival [105].

2.2.3. Host Factors

- Genetic Variations and Polymorphisms

Genetic sequences called polymorphisms and/or variants are different DNA sequences among individuals compared to the reference genome with different functional significance. DNA polymorphisms occur in ≥ 1% of the population and the majority of them do not have impact on protein or gene function.

However, some polymorphisms affect the function of proteins and/or genes, called mutations with significant value on cancer progression. There are four types of DNA polymorphisms: The Single Nucleotide Polymorphism (SNP), the Restriction Fragment Length Polymorphism (RFLP), the Simple Sequence Repeat (SSR) and the Variable Number or Tandem Repeats (VNTR) [106].

The SNPs are the most common polymorphism and it is estimated that they occur every 1000 base pairs usually found in protein-coding regions [107]. SNPs are also used as a tool to identify determinant of different diseases [108]. In addition, SSRs are the most frequently used in mapping studies due to they are highly polymorphic, very informatic and consistent [109].

The role of polymorphisms in cancer has been evaluated and it is found that gene polymorphisms are implicated in different stages of tumor development and cancer progression. Specifically, polymorphisms affect tumor growth, invasion, metastasis and respond to cancer therapy [110].

- Immune System Composition and Function

The immune system is very important in cancer management. Its failure to recognize and kill cancer cells contributes to cell migration and cancer progression [10,92].

It is composed of different types of cells, cytokines, proteins and soluble bioactive molecules. Each of them has different role to recognize and defend against ''foreign'' antigens or proteins [5]. NK cells and phagocytes, including macrophages, neutrophils and monocytes are the key players in cell-mediated innate immune responses. NK cells act using the MHC I complex proteins and phagocytes facilitate immune protection either by swallowing cells that express non-self-antigens or by using lysosomal enzymes [111]. Innate immune responses also include other cells such as

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basophils and eosinophils which using different inflammatory mediators to attract more immune cells to the inflammation/injured site [112].

The adaptive immunity is antigen-dependent and antigen-specific [113] and it is comprised of lymphocytes and APCs. Lymphocytes are categorized into two major types: the B cells that mature in the bone marrow and the T cells that mature in the thymus and their function is to recognize specific antigen that present on the surface of APCs [112].

Other immune cells that participate to the cellular innate immunity are the granulocytes, consists of neutrophils, eosinophils and basophils and they are responsible to release inflammatory mediators and attract more immune cells at inflammation and/or infection sites [5].

CYT index is strictly associated with the composition of immune system. Specifically, CD8+ T cells release perforin and granzymes to kill targeted cells [114]. Therefore, since CYT is based on the expression of granzymes and perforin, the composition of immune system and the percentage CD8+ T cells can affect the index of CYT.

The immune system is a defence mechanism against infected and/or self-antigens [2] and its role is very important both for prognosis and treatment of cancer. Unfortunately, cancer cells evolve different mechanisms to escape effective immunosurveillance. They produce immunosuppressive cytokines and prostaglandins to inhibit the division of NK cells as well as the proliferation and function of T helper and CTLs. Moreover, antigen-processing of mutant proteins through MHC complexes by immune-resistant cancer cells, reducing antigenicity and reinforce the destruction of malignant cells from immune system [115].

The interplay between the immune system and the cancer pathogenesis enhances understanding of immune activation and response against cancer. Immune-oncology is a fiend that is rapidly constantly evolved and knowledge of immune composition especially in the TME will significantly contribute to create more effective therapies [5].

2.3. Clinical Implications and Prognostic Significance

2.3.1. Association between CYT and Patient Outcomes

CYT is closely related with patient prognosis and outcomes and the expression of both GZMA and PRF1 genes synergistically, or alone can be affect the overall survival of cancer patients. In Adrenocortical Carcinoma (ACC), skin melanoma and bladder cancer, high CYT levels associated with better patient outcome and survival. Roufas et. al. reported that high CYT skin melanomas, activate immune-related genes and increase the levels of CD8+ T cells, NK cells, B cells, M1 macrophages and DCs [22].

Increase of T-cells and M1 macrophages within CRC and HCC patients in combination with high CYT, correlating with better survival, longer OS and Disease-Specific Survival (DSS) [27,29].

In endometrial carcinoma (EC), and high-grade serous carcinoma (HGSC), high expression levels of both GZMA and PRF1 or alone, associated with higher overall survival (OS) rates. Conversely, low CYT levels related to worst clinical outcome [26,116]. In addition, high CYT lung adenocarcinoma had significantly longer PFS and OS [117]. Unlike ACC, skin melanoma, bladder cancer, EC, HGSC and lung adenocarcinoma, in glioblastoma (GBM) high CYT associated with poor prognosis and worse survival [43,118].

In a study of 7533 breast cancer patients, TNBC group had the highest CYT levels and better survival, compared to the estrogen receptor (ER)-positive and Human Epidermal Growth Factor Receptor 2 (HER2)-negative subgroups [28]. On the other hand, breast tumors with high mutant allele tumor heterogeneity (MATH) score are related with low CYT levels, less immune responses and worse survival [119].

Recently, in Breast Cancer (BC), was reported that overexpression of GZMA alone, increased the infiltration of DCs and CD8 + T cells and related with better OS, DSS and Progress Free Interval (PFI) [120], while in 2019, high expression levels of GZMA, were significantly correlated with better survival in HCC [121]. On top of that, high CYT levels within TNBC were significantly associated with high inflammatory scores, better DSS and OS [122]. On the contrary, late recurrence BC

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patients have lower CYT index compared with survivors and low CYT levels in late recurrence patients were associated with worse Disease free survival (DFS) [123].

CYT was also evaluated between tumor and tumor-free tongue patients with Squamous Cell Carcinoma of the Oral Tongue (SCCOT) and it is remarkable that, tumor-free tongue patients had higher CYT levels with better survival, contralateral to the tumor patients that CYT is not predictive for their survival [51].

2.3.2. Predictive Value of CYT for Immunotherapy Response

In recent years, cancer immunotherapy has enabled never-before seen success rates in durable tumor control and enhanced survival benefits in patients with advanced cancers. Importantly, ICI therapies using monoclonal antibodies (mabs), including anti-PD-1 to treat cancer patient has spawned overwhelming enthusiasm for immunotherapy in the disease [124,125].

CYT is identified as a new immunotherapy biomarker that reflects the antitumor immune responses and activity of CD8+ cytotoxic T lymphocytes and macrophages [23] and it can be used to predict the respond of the patient to ICI therapies. It is indicative that the expression levels of both GZMA and PRF1 can predict favourable survival of cancer patients following ICI immunotherapy [126].

High CYT levels within CRC, EC and Non-small cell lung cancer (NSCLC) were associated with high expression of immune checkpoint proteins, including PD-L1 and CTLA-4 and may be respond better to ICI therapies [26,27,94]. It is also reported that, CYT-high GC tumors respond better respond to anti-PD-1 therapy [25] and CRC patients with high CYT levels showed a more sensitivity to ICI therapies, compared to CYT-low CRC patients [24]

CYT-high skin melanomas patients who receive anti-CTLA-4 and/or anti-PD-1 therapy, have better clinical results due to higher immunophenoscore, compare to the patients with low CYT levels [52]. Metastatic melanoma patients with high GZMA expression levels, exhibited significantly higher expression levels of CTLA-4, PD-L1 and PD-L2 and have better respond to anti PD-L1 (nivolumab) treatment, resulting to better clinical benefit and long-term survival [127,128].

In addition of nivolumab, in melanoma patients that treated with anti-CTLA4 antibody (ipilimumab), intratumoral immune CYT increases the infiltration of CD8+ T cells and the expression of MHC class I [129].

In cutaneous melanoma, high GZMA expression levels lead to immune activation and infiltration of CD8+ T cells, making the patients more sensitive to anti-PD-L1 immunotherapy [130]. Furthermore, CYT-high Prostate Cancer (PCa) patients are more sensitive to ICIs, due to the fact that, high GZMA and PRF1 levels, increase the number of CD8+ T cells and the expression of immune checkpoints inhibitors, including PD-L1 compared with the low CYT patients [131].

Although, the different forms of immunotherapy have achieved success in treating a lot of cancer types, only a small number of patients respond to these therapies and some patients create resistance to them. Therefore, the evaluation of CYT as a prognostic factor in treatment selection and immunotherapy response will help cancer patients to receive the most appropriate therapy for their cancer, with the best results.

2.3.3. Integration of CYT Assessment into Clinical Practice

- Potential Biomarkers for CYT Evaluation

CYT is a biomarker of antitumor immunity, and it is calculated based on the expression levels of GZMA and PRF1, released from CTLs through granzyme/perforin pathway [23]. CYT is also significantly related with the expression of other different biomarkers.

The TME plays a crucial role in cancer progression and its composition may predict patient's prognosis. CYT depends on the proportion of immune cells within the TME which can be used as a biomarker for CYT evaluation. Notably, it has been recorded that the presence of T cells within the TME relates with favourable prognosis [132], and CYT is positively correlated with the proportion of tumor-infiltrating CD8+ T cells and M1 macrophages.

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Additionally, CYT-high levels are strongly related with high infiltration of the above mentioned immune cells [22,29,129–131,133]. Further of GZMA and PRF1, GZMB has been also to be present in high amounts of CD8+ T cells [134,135].

CYT is positively associated with the status of MSI, the mutational burden and the rate of tumor-mutated peptides, called neoepitopes. Rooney et al, shown that neoepitopes can handle CYT in a number of tumors [23]. CYT is also correlated with increased mutational burden and neoepitope load in GC and colon tumors [24,25].

Moreover, MSI-high colon and colorectal tumors are related with increased levels of both CYT and mutations [24] and dMMR/MSI-high patients with colorectal adenocarcinoma respond better to anti-PD-1 immunotherapy [136]. In contrast, in Pancreatic ductal adenocarcinoma (PDA) increase of CYT levels does not correlated with increase in the mutational burden or neoepitope load [20].

Checkpoint inhibitors, including CTLA-4 and PD-L1 are important indicators for both CYT-low and CYT-high cancer patients. Most studies supports that high levels of PD-L1 and CTLA-4 are exhibited in CYT-high cancer patients [26,27,94] and these patients are more sensitive to ICI mab therapies [24].

- Role of CYT in Patient Stratification and Treatment Selection

In past decades, standard treatment option for cancer therapy includes immunotherapy, chemotherapy, radiotherapy, surgical removal, and/or targeted therapy, depending on the tumor's stage [137]. CYT plays a pivotal role in patient stratification and its levels has significant value to choose the suitable treatment for each patient with different types of advanced cancer.

As it is already mentioned, CYT-high and CYT-low patients have different respond to therapies and high CYT levels increase the activation and infiltration of CD8+ T cells [22,129–131,133].

CYT is significantly related with the amount of active CTLs that can be used in the treatment selection. In vitro studies of Paclitaxel (TAX), Doxorubicin (DOX) and Cisplatin (CIS) showed that all of the above mentioned drugs enhance the cell-mediated killing by CTLs [138–140]. It has been also reported that, low non-cytotoxic doses of 5-Fluorouracil (5-FU), Taxotere, Cisplatin and Irinotecan (CPT-11), and CIS induce antitumor responses of CD8+ T cells which they are connected with high CYT levels in colon cancer [141]. On the contrary, CYT-low Androgen Receptor (AR) and ER-positive BC were associated with low infiltration and CD8+ T cells, less overall anticancer immunity and survival [142].

In many patients with different types of cancer, conventional therapies do not provide satisfactory clinical results. Therefore, in recent years, the combinational therapy of cancer immunotherapy and chemotherapy is gaining credibility due to its high efficacy, and it is used to treat patients with advanced types of cancer. CYT can be used to predict the response of cancer patients to ICI therapies. Specifically, CYT-high cancer patients were exhibited significantly higher levels of immune checkpoint inhibitors, including PD-L1 and CTLA-4 and respond better to ICI therapies with mabs [25–27,52,94,127,128,130,131].

Furthermore, anti-CTLA-4 mab therapy alone and/or with anti-PD-1 therapy has been offered important therapeutic benefits to cancer patients [143], and it enhances the immune anti-tumor activity due to increase the number of tumor-infiltrating CD8+ and CD4+ T cells. Moreover, anti-CTLA-4 therapy combined with CIS chemotherapy was significantly more effective, increases the expression of perforins and Granzyme B and improved the survival of patients, compared to the anti-CTLA-4 and CIS alone therapies in murine mesothelioma [144].

Even though, there is an improvement in the prognosis for many patients depending on the stage of cancer, most cancer patients still have a poor prognosis, or they develop resistance to immunotherapy. CYT as a relatively new index, and its index can be used as a key tool in cancer - patient treatment plan.

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2.4. Modulation of CYT

2.4.1. Current and Emerging Immunotherapy Strategies

- Immune Checkpoint Inhibitors

ICIs have made tremendous progress and they approved and widely used in cancer immunotherapy [145]. In addition, ICIs targeting CTLA-4 and/or PD-1/PD-L1 have shown unparalleled clinical efficacy in multiple types of cancer [146].

Ipilimumab (anti-CTLA-4 antibody) constitutes the first checkpoint inhibitor that approved for cancer treatment and it showed improvements in the OS of metastatic melanoma patients [147,148]. Moreover, the use of pidilizumab (anti-PD-1 antibody) in metastatic melanoma patients that 51% of them, they previously treated with ipilimumab showed 1-year better OS [149].

- Adoptive Cell Therapies

ACTs refer to a type of personalized cancer immunotherapy in which lymphocytes are expanded and grown ex-vivo and re-infused into the cancer patients [150,151] and they give a possibly dynamic general treatment for cancer [152].

ACTs with TILs or gene-modified T cells expressing TCRs or chimeric antigen receptors (CARs) are alternative type of therapies that trigger the immune system in order to recognize and kill cancer cells and they have shown promising results in many cancer types [153].

- Vaccines and Oncolytic Viruses

OVs are created to target and kill cancer cells directly, without harming the normal cell [154] and constitute a promising category of cancer therapy. They enhance anticancer immune responses either by the production of tumor associated antigens or inflammatory signals [155]. OVs also modulate the TME by increase the maturation of tumor-specific T cells within it dye to the high number innate immune cells including of DCs [156].

OVs can be used as immunotherapeutic anti-cancer vaccines to enhance tumor-specific T -responses and mediate the killing of unharmed cancer cells [157]. Moreover, it has been shown through both in-vitro, in-vivo and pre-clinical studies that the combination of OVs with anti-CTLA-4 and/or anti-PD-1 ICIs has high oncolytic efficacy [154,158].

2.4.2. Combination Approaches to Enhance CYT

- Dual Immune Checkpoint Blockade

Despite that ICB therapies have received heightened interest in strategies, the efficacy of them alone is restricted by low response. Dual ICB constitutes one of the most promising form of immunotherapy for cancer treatment that enhance the antitumor activity of Cytotoxic T cells and it is already used to treat solid tumors, including melanoma and RCC as well as advanced HCC with great efficacy and promising results [159].

Different combinatorial therapies with mabs are used to treat cancer both in preclinical models and clinical trials. Combination of anti-CTLA-4 and anti-PD-1 mabs enhance antitumor activity by increased the infiltration of CD8+ T cells and decreased the number of Tregs. As a results of this, the TME converts from immunosuppressive to inflammatory with better clinical results [83,160]. Moreover, in NSCLC patients, immunotherapy using nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) mabs showed survival benefit [161].

On the contrary, in head and neck squamous cell carcinoma, combinatorial immunotherapy of PD-1/CTLA4 mabs causes decrease in the activation of CD8+ T cells and increase the activation of Tregs [162]. In addition, phase II clinical trials of concurrent treatment with the above-mentioned mabs showed higher response rate and PFS, compared to the monotherapies in melanoma patients [163,164].

Dual ICB is a powerful therapy, and it can be considered almost certain that in the near future the use of this type of immunotherapy will increase with greater success rates and in more types of cancer.

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- Modulation of the TME

TME involves in different biological processes that implicated with the initiation and treatment and it constitutes an important tool for both prevention and treatment of cancer [165,166]. Modulation of the TME is crucial in different stages of cancer including of primary tumor growth, development of metastasis and immune evasion and it plays a leading role in strategies for cancer treatment [74].

The TME is modulated from cancer cells by the production due to their high consumption of glucose. This metabolic modulation of the TME increases to ICPs and infiltration of TILs [167]. Moreover, the treatment of cancer through immune modulation of the TME makes headway and studies focuses to understand more about the TME components and their interactions between themselves in order to use it as a prognostic tool. TME is already a prognostic tool for enhancing cancer immunotherapy and it used to predict its efficacy [168].

2.4.3. Challenges and Future Directions in CYT Modulation

As previously mentioned, CYT is a measure of immune activation based on the expression of GZMA and PRF1 and it varies across different cancer types [23]. Moreover, the expression of GZMA was either lowly expressed or absent, while PRF1 was not detected from 20 different tumor types [22]. CYT is also associated with the TMB events as well as with different mutational signatures and it deregulates the expression of immune checkpoint inhibitors [24].

It is remarkable that, CYT-high skin melanoma tumors express immune-related genes and vice versa, and it is suggesting that CYT-high skin melanoma patients are more sensitive to ICI therapies due to their higher immunophenoscore [52].

CYT constitutes a significant prognostic biomarker for cancer treatment, and it can be used to predict the respond of cancer patients to ICI immunotherapies, as well as measure the immune activation within the tumor. Therefore, its use is expected to solve the problem that occurs with many cancer patients who are not sensitive, or they show resistance to this type of immunotherapy.

As CYT described first from Rooney et al., in 2015 and it is relatively new index, more research is needed in future to investigate how this important prognostic/predictive tool can be used in cancer strategies to treat different tumor types.

3. Conclusions

3.1. Recapitulation of Key Findings

In this review, we summarize the role of intratumoral immune CYT in cancer progression and its implication for cancer patient prognosis and treatment outcomes. CYT, as measured by the geometric mean of GZMA and PRF1, reflects a tumor's immune activation and plays a significant role in its control. Along with GZMA and PRF1, GZMB can also be used to calculate CYT levels. Different methods are used to evaluate CYT, including of isolation, characterization and analysis of TILs by flow cytometry, due to their ability to recognize and kill autologous cancer cells, and gene expression profiling. In addition, immune CYT can be calculated within the TME by mIF and IMC and the immune cells which composed the TME, constitute an indicator to assess the CYT.

CYT is influenced by different factors, such as the TMB, where cancer patients with high TMB (especially MSI-H/dMMR ones) show better response to ICI immunotherapies. Finally, CYT is strictly related by the antigen presentation and MHC I and II complexes, as well as by TME factors, including immunosuppressive cells, immune checkpoints, stomal cells and ECM components.

CYT is importantly related with the composition of immune system, mainly within the TME. It is important to refer that the interplay between the immune cells and cancer cells is crucial for primary growth of tumor and treatment. Most studies observed that an increase in the CYT index enhances the antitumor immune response by increasing the infiltration of CD8+ T cells and decreasing the number of immunosuppressive cells (Tregs and MDSCs). On the contrary, CYT-low tumors are characterised by higher number of immunosuppressive cells and inhibition of antitumor

 doi:10.20944/preprints202401.2227.v1

immune responses and immune checkpoint molecules, resulting in cancer progression, metastasis and immune evasion.

3.2. Potential Clinical Implications and Future Directions

Although, in recent years, a tremendous progress has been recorded in the field of cancer therapy, there is a big number of patients that still have poor prognosis and they develop resistance to therapies. Therefore, it is considered imperative to find out accurate prognostic and predictive biomarkers in order to create the most effective therapy for each cancer patient. As inferred from this review, CYT can constitute one of these biomarkers.

CYT is expected to take a central role in cancer treatment plans in the near future due to its high clinical value. Currently, it is an important prognostic biomarker for patient response in cancer therapies. In ACC, skin melanoma, bladder cancer, BC, HCC, EC, CRC, HGSC, TNBC, lung adenocarcinoma and SCCOT, high levels of CYT showed longer OS, PFS, PFI and DSS [15,22,26–29,51,116,117,122]. On the contrary, only CYT-high GBM patients were related with poor prognosis and worse survival [43,118].

Moreover, CYT is an accurate immunotherapy biomarker that it is strictly related with the expression of immune checkpoint inhibitors. Importantly, CYT-high tumors exhibit significantly higher levels of immune checkpoint inhibitors, too, whereas CYT-high patients are more sensitive to ICI immunotherapies, using mabs with higher respond rates. It is indicated that high expression levels of GZMA and PRF1 synergistically or alone in different types of cancer, including of CRC, EC, NSCLC, GC and cutaneous melanoma, increased the levels of CTLA-4 and/or PD-1/PD-L1, resulting to better clinical outcome and survival for CYT-high patients who received anti-CTLA-4 and/or anti-PD-1/PD-L1 immunotherapies (Figure 4).

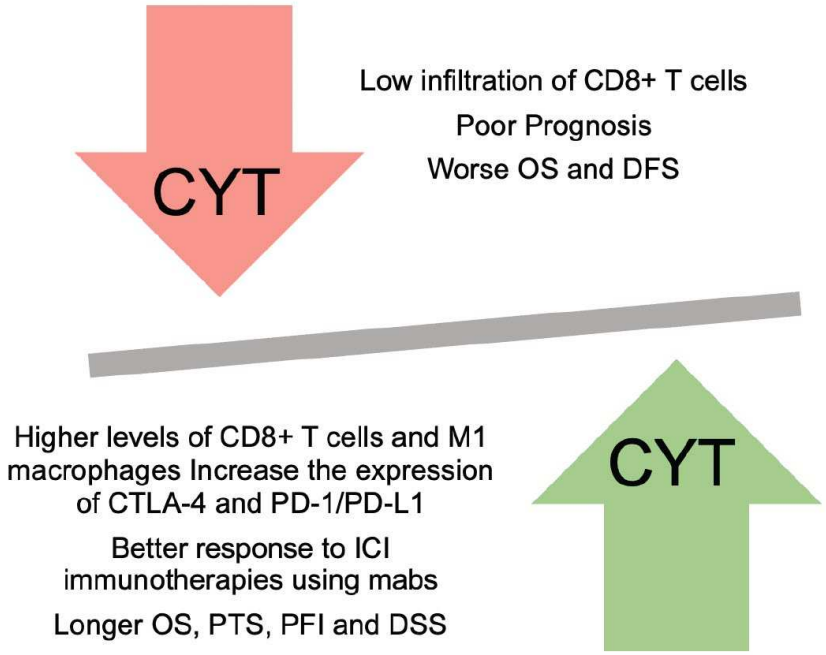

Figure 4. CYT-high and CYT-low levels affect infiltration of immune cells, patient's prognosis and survival.

Most studies showed that CYT-high patients are related with better clinical outcome and long-term survival in different types of cancer. In the near future, more research into CYT is needed, that will focus to recapitulate its importance in cancer immunotherapy, either with dual ICB or by targeting their metabolic pathways to promote an immune-stimulatory microenvironment.

3.3. Overall Significance of Understanding CYT in Cancer

Considering all of the above parts, current review highlights the significance of understanding the implication of intratumoral immune CYT in cancer, from primary growth to distant metastasis in order to use it for therapeutic purposes.

Intratumoral immune CYT arises as an indicator of immune activation based on the expression of granzymes and perforins and through research, it becomes a critical prognostic and predictive factor in cancer immunotherapy. Importantly, increased levels of CYT are related with high expression levels of immune checkpoint molecules like CTLA-4, PD-1 and PD-L1 and blocking of them by using mabs, enhances antitumor immunity. Finally, CYT-high patients are related with positive clinical outcomes and a favourable prognosis.

On top of that, TME composition directly affects the intratumoral immune CYT and the full known and understanding of immune cells and cancer cells within the TME can modulate in in order to improve CYT, create an immune-stimulatory microenvironment and enhance antitumor immune responses against cancer cells.

In summary, understanding and modulating CYT is crucial for improving cancer immunotherapy. Research into CYT and the factors that influence it eventually transform cancer treatment, improve patient outcomes, and increase their life span.

Author Contributions: All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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