FLOW CYTOMETRIC EVALUATION OF PERIPHERAL BLOOD BIOMARKERS FOR SOLID TUMOURS IMMUNOTHERAPY GUIDING: A REVIEW

AVALIAÇÃO DE BIOMARCADORES NO SANGUE PERIFÉRICO POR CITOMETRIA DE FLUXO COMO MEIO DE ORIENTAÇÃO PARA A IMUNOTERAPIA EM TUMORES SÓLIDOS: REVISÃO DA LITERATURA

ANA CATARINA TRIGO[1,2,4], PATRÍCIA MAIA[1,3,4], INÊS GODINHO[1], CATARINA A. RODRIGUES[4,5], MARIA EMÍLIA SOUSA[1], ANA MARTA PIRES[1], CARLA AZEVEDO[1], LÚCIO LARA SANTOS[4,6,7,8], CARLOS PALMEIRA[1,4,6,8], GABRIELA MARTINS[1,4,8]

[1] Immunology Department, Portuguese Institute of Oncology of Porto, 4200-162 Porto, Portugal
[2] Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, 4050-013 Porto, Portugal
[3] Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
[4] Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, 4200-162 Porto, Portugal
[5] Faculty of Medicine of the University of Porto (FMUP), 4200-319 Porto, Portugal
[6] Health Science Faculty, University of Fernando Pessoa, 4249-004 Porto, Portugal
[7] Department of Surgical Oncology, Portuguese Institute of Oncology of Porto, 4200-162 Porto, Portugal
[8] Porto Comprehensive Cancer Centre (P.ccc), 4200-162 Porto, Portugal

ABSTRACT

Cancer treatment is an area that is constantly being updated, namely with the discovery of immunotherapy as a clinically effective therapeutic modality for various types of cancer. Among these innovative therapies, immune checkpoint inhibitors (ICI) have proven to provide significant and long-lasting responses. However, this response does not occur for all patients, i.e., some patients do not benefit from this therapy. Due to this heterogeneity in immunotherapy response, there is an urgent need to identify and establish biomarkers that will allow the identification of patients who will respond to the therapy, while sparing the non-responders from adverse effects. In addition, the use these biomarkers in monitoring the response during treatment seems promising. Given the recognized role of the immune system in the anti-tumour response, these cells have been intensively studied as potential biomarkers. Their study in peripheral blood (PB) has been of great interest and importance, given its easy accessibility and less invasive nature. The detailed and integral evaluation of peripheral immunity requires a multiparametric methodology such as flow cytometry (FC), applying the simultaneous analysis of lineage markers together with maturation, activation and functional state markers. In this narrative literature review, we intend to describe the "state of the art" on the FC study of PB immune cell populations as potential biomarkers for ICI therapy in solid tumours. The results found are presented for each of the major populations and their subsets, namely T lymphocytes, myeloid-derived suppressor cells (MDSCs), neutrophils, eosinophils, dendritic cells (CD), natural killer cells (NK), monocyte subsets, and B cells.

Keywords: *Cancer immunotherapy; Immune checkpoint inhibitors; Peripheral blood biomarkers; Peripheral immunoscore; Flow Cytometry.*

RESUMO

O tratamento do cancro é uma área em intensa e permanente atualização, nomeadamente com a descoberta da imunoterapia como modalidade terapêutica clinicamente eficaz para diversos tipos de cancro. Entre estas terapias inovadoras, as terapias com inibidores de checkpoints imunológicos (ICI) têm demonstrado apresentar respostas significativas e duradouras. No entanto, esta resposta não ocorre para todos os doentes, ou seja, alguns pacientes não beneficiam desta terapia. Devido a esta heterogeneidade na resposta à imunoterapia, persiste a necessidade urgente de identificar e estabelecer biomarcadores que permitam a identificação dos doentes que irão responder à terapia, poupando os que não respondem aos efeitos adversos. Para além deste aspeto, parece promissor o impacto na clínica da utilização destes biomarcadores na monitorização da resposta durante o tratamento. Dado o reconhecido papel do sistema imunológico na resposta anti-tumoral, estas células têm sido intensamente estudadas como potenciais biomarcadores. Com este objetivo, o sangue periférico (SP), tem revelado grande interesse e importância, dada a sua fácil acessibilidade e natureza menos invasiva. A avaliação detalhada e integral da imunidade periférica, exige uma metodologia multiparamétrica como a citometria de fluxo, recorrendo à utilização de marcadores de linhagem simultaneamente com marcadores de maturação, ativação e estados funcionais. Com esta revisão bibliográfica narrativa, pretendeu-se descrever o "estado da arte" sobre o estudo por citometria de fluxo das populações celulares do sistema imunológico no SP, como potenciais biomarcadores para a terapia com ICI em tumores sólidos. Os resultados encontrados são apresentados para cada uma das populações principais e suas subpopulações, nomeadamente linfócitos T, células supressoras derivadas da linhagem mieloide (MDSCs), neutrófilos, eosinófilos, células dendríticas (DC), células *natural killer* (NK), monócitos, e linfócitos B.

Palvras-chave: *Imunoterapia do cancro; Inibidores checkpoint imunológicos; Biomarcadores do sangue periférico; Imunoscore periférico; Citometria de fluxo.*

ABBREVIATIONS

CRC – colorectal cancer;
CTLA-4 – cytotoxic T-lymphocyte-associated protein 4;
DC – dendritic cells;
dNLR – dynamics of neutrophil-lymphocyte ratio;
FC – flow cytometry;
G-CSF – granulocyte colony-stimulating factor;
gMDSC – granulocytic myeloid-derived suppressor cells;
ICI – immune checkpoint inhibitors;
IFN-γ – interferon-gamma;
mDC – myeloid dendritic cells;
MDSC – myeloid-derived suppressor cells;
MHC – major histocompatibility complex;
M-MDSC – monocytic myeloid-derived suppressor cells;
NK – natural killer;
NLR – neutrophil-to-lymphocyte ratio;
NO^- – nitric oxide;
NSCLC – non-small cell lung cancer:
OS – overall survival;
PB – peripheral blood;
PD-1 – programmed cell death protein 1;
pDC – plasmacytoid dendritic cells;
PFS – progression-free survival;
PMN-MDSC – polymorphonuclear myeloid-derived suppressor cells;
ROS – reactive Species of Oxygen;
TCM – central memory T cells;
TEM – effector memory T cells;
TEMRA – terminal effector memory T cells;
TME – tumor microenvironment;
TNF-α – tumor necrosis factor-α;
TNM – tumor-node-metastasis;
T_{reg} – regulatory T cells.

INTRODUCTION

Over the last few years, immunotherapy has become one of the backbones in cancer treatment, alongside with chemotherapy, radiation therapy and surgery. It is the only cancer therapy that makes use

of the host's immune system to target tumour cells[1]. The development of this novel therapy has shown some promising results, and has recently been nominated by American Society of Clinical Oncology as the major breakthrough in cancer[2]. These new immunotherapeutic strategies, namely immune checkpoint inhibitors (ICI), have been proven to be effective in distinct types of cancer, including non-small cell lung cancer (NSCLC), melanoma and squamous cell carcinoma, head and neck cancer, urothelial and renal carcinomas, hepatocellular carcinoma, amongst others[2-4]. ICI, described as monoclonal antibodies, allow the immune system to target the tumour by blocking some pathways that inhibit immune cells, thus stimulating their responses. Two of the currently approved ICI target the following molecules: programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[3, 5].

Immunotherapy has had a tremendous impact on the treatment of some tumours, whether it is used as a first-line or second-line of treatment. Several reports have shown that, with the usage of ICI, there is an increase in patient's overall survival (OS), as well as a higher durability of the clinical benefits that offer patients a better quality of life[4, 6]. However, the emergence of these drugs has led to a greater need for the identification and development of biomarkers associated with the immune system, in order to define the prognosis and to monitor immunological responses comparing the immune profiles before, during, and after the treatment. Besides, they would be helpful to determine the best patient and tumour specific immunological therapy[3, 7]. The concept of immunoscore has been a relevant tool to predict prognosis and response to therapy. It was initially described in colorectal cancer (CRC) patients, with the main purpose of complementing the already globally recognized standard for classification of the extent of spread of cancer, the tumour-node-metastasis (TNM) system, increasing its prognostic value. Indeed, the immunoscore system allows the quantification, through an immunohistochemical assay based on digital pathology, of *in situ* T cells ($CD3^+$, $CD8^+$ and $CD45\text{-}RO^+$), taking in consideration several immunological features, such as density, functional orientation and localization of the cells in the tumour [8, 9]. However, the immunoscore system displays some downsides, since it is a quantitative method that doesn't provide information regarding survival, function and metabolic processes of T cells, as well as the interactions that they may keep with other substances around the tumour[8]. In contrast, the identification of biomarkers in the peripheral blood (PB), namely T cell, myeloid-derived suppressor cells (MDSC), dendritic cells (DC) and natural killer (NK) cells subsets, monocytes, B cells, among others, allows for a global view of the host's immunological profile, in addition to the changes resulting from immunotherapy, thus defining the patient's immunological fingerprint, a sort of peripheral immunoscore[2, 6]. A PB sample is easily obtained and processed through non-invasive methods, usually in a considerable volume, and therefore immune cell's monitoring could be performed through a series of PB samples over the course of the treatment. Indeed, a PB biomarker presents several advantages in comparison to other biomarkers that derive from other types of samples, such as tumour biopsies, which are of more difficult access, consequently becoming an invasive process with possible inherent risks[4, 7, 10]. Nevertheless, when using a PB sample, the detailed information regarding tumour-immune cell's interaction may be limited and not representative[11]. Although a disadvantage, the previously referred advantages overcome it, and the discovery of PB biomarkers remains an area that has been extensively explored. Hence, the peripheral patient's peripheral immunoscore allows for a detailed information of the immunological state of the patient, and is based on the phenotypic characterization of the immune cells by reliable techniques, such as FC, which is a valuable technique due to its multiparametric and standardized features[12, 13].

The changes in the immunological profile of the patients may not only be associated with tumour progression, but also to subjacent immunotherapy responses[14]. Thereby, the establishment of reliable PB biomarkers is a challenge in cancer immunotherapy, as it will not only optimize the selection of patients who would clinically benefit from treatment, as well as sparing others from unnecessary treatment, and will also enable the monitoring of the immunotherapy response[15]. In this review, we will describe the latest advances published in the literature regarding PB immune cell populations as potential biomarkers for the prognosis and response monitoring to immunotherapy in solid tumour patients, focusing specifically on immunophenotyping by flow cytometry (FC).

BIOMARKERS

T cells

Immunotherapy, specifically ICI therapy, works by inhibiting immune checkpoint molecules, which are inhibitory receptors expressed on the surface of T cells, as well as tumour cells, which mediate the interactions between these cells[16].

Several subsets of T cells have been described, each one of them with diverse functions that may either promote or inhibit antitumor immune responses. Cytotoxic T cells, which represent most of $CD8^+$ T cells, recognize tumour-specific antigens through class I Major Histocompatibility Complex (MHC) molecules. On the other hand, $CD4^+$ T cells, mainly the T helper cell subset, , besides aiding in the activation of cytotoxic T cells and B cells, they contribute to regulatory mechanisms via secretion of diverse cytokines. Amongst the $CD4^+$ cells, the regulatory T cells (T_{reg}) have essentially an immunosuppressive function. In this way, they are indispensable for preventing autoimmunity, but also suppress effective tumour immunity. Lastly, effector memory (TEM) and central memory (TCM) T cells are memory T cells that have been exposed to specific antigens, and consequently will trigger a faster and stronger immune response when encountering the same antigen.

Since T cells have a major role in immune defence mechanisms against cancer, they have been intensively investigated as PB potential biomarkers regarding immunotherapy in solid tumours[17].

T_{reg} cells

Several authors have reported that higher baseline frequencies of circulating $CD4^+CD25^+FoxP3^+$ T_{reg} cells are associated with improved OS, in patients who were subjected to ipilimumab, a monoclonal antibody against CTLA-4[12, 18, 19]. T_{reg} cells represent direct target cells of ipilimumab due to their constitutive CTLA-4-expression. Therefore, a high baseline frequency might render patients more susceptible to anti-CTLA-4 antibodies[12]. However, there have been conflicting results when it comes to this cellular subpopulation over the course of the treatment. Some authors report that an increase in the frequency of T_{reg} cells throughout the treatment is associated with improved clinical benefit[20, 21], whereas other authors describe the exact opposite, this is, patients who show a decrease in the frequency of circulating T_{reg} cells at the time of the treatment are the ones who will respond to therapy[22, 23].

Included in the first group of authors, Tarhini et al.[20] compared changes in the percentage of circulating T_{reg} cells at baseline and 6 weeks after neoadjuvant treatment with ipilimumab in 27 regionally advanced melanoma patients. They found that there was a significant increase in the percentage of circulating $CD4^+CD25^{hi+}Foxp3^+$ cells after treatment, and this increase was found to be associated with improved progression-free survival (PFS). They also found that the significant increase in circulating T_{reg} cells was accompanied by a higher proportion of circulating non-T_{reg} $CD4^+$ T cell population. Since this increase in circulating

T_{reg} cells was associated with a better PFS, the authors raise questions about the functional status of these T_{reg} cells. Simeone et al. [21] also noticed that, in advanced melanoma ipilimumab treated patients, the number of $Foxp3^+$ T_{reg} cells in the periphery increased in patients with disease control and decreased in patients without disease control. In line with these results, Martens and colleagues[24] also found that $CD4^+CD25^+Foxp3^+$ T_{reg} cells were increased at delayed time-points, in comparison with baseline frequencies, in melanoma patients treated with ipilimumab. However, the changes in the frequencies of T_{reg} cells were not significantly associated with OS, thus the authors note that these changes cannot be qualified as potential biomarker.

As opposed to these findings, other authors have reported decreasing frequencies in responsive patients[23, 25]. In fact, a study lead by Hotson and colleagues[22] revealed that patients who did not respond to ipilimumab treatment tended to have a greater T_{reg} frequency in PB, when compared to those who responded to therapy. Retsek et al.[23] also found that an increase in circulating T_{reg} cells, with their suppressive activity confirmed ex vivo, was significantly associated with a decrease in PFS in patients treated with ipilimumab.

As previously referred, there is no consensus in the literature and the prognostic role of T_{reg} cells for patients treated with ICI therapy remains controversial. These contradictory results may be related to differences in the cellular populations studied in the various works, regarding their maturation and functional state. According to Lepone and collaborators[26], while the percentage of T_{reg} cells was similar between cancer patients and healthy donors, cancer patients had higher levels of a T_{reg} subset that expressed CTLA-4, which is a suppressive marker for these regulatory cells. Others like Retsek et al.[23], argue that the association of an increase in circulating Treg following CTLA-4 blockade with an increase in PFS may relate more to a concomitant and greater increase in the overall T cell population, of which Treg are a small fraction, than in the functionality of Treg. The impact of ICI on Th may outweigh its effect on Treg.

Therefore, new studies should include not only the evaluation of Treg subpopulations but also the evaluation of their suppressive activity, in order to assess the potential usefulness of these cells as a biomarker for the ICI therapeutic response.

CD4+ and CD8+ T cells

Regarding the non-T_{reg} $CD4^+$ and the $CD8^+$ cells, the findings in the literature are more consensual, in which a higher expansion of $CD4^+$ and $CD8^+$ cells is related with positive clinical outcomes[17, 24, 27, 28]. Martens et al. [24] found that melanoma patients subjected to ipilimumab therapy had higher frequencies of $CD4^+$ and $CD8^+$ cells expressing Ki67, a marker for diving cells, over the course of the treatment, that were associated with improved OS. Wang and colleagues[29] had also observed increasing frequencies of $CD4^+Ki67^+$ and $CD8^+$ $Ki67^+$ cells in patients subjected to ipilimumab treatment, however they were not correlated with clinical outcome. A study lead by Felix et al.[27] reported a significant increase in $CD4^+$ and $CD8^+$ cells 3 weeks after the first dose of ipilimumab, as well as an increase in HLA-DR activated T cells. The authors also identified naïve and memory T cell subsets, namely TCM, TEM and terminal effector memory T cells (TEMRA) based on their expression of CD45-RA and CCR7, and detected an early effect on $CD4^+$ and $CD8^+$ TCM and TEM, with an increase in both percentages and absolute counts, and a delayed increase in $CD8^+$ TEMRA, suggesting that the major dynamic changes between naïve and memory subsets operate within 3 weeks after the first dose of ipilimumab. They observed that only the patients with a significant expansion of $CD4^+$ and $CD8^+$ cells over the course of the treatment had higher rate of disease control. This data is supported by previous clinical studies employing ipilimumab[19, 30]. Wistuba-Hamprecht et al.[28] also

studied the role of TEM and TEMRA subsets as potential biomarkers to monitor ICI therapy in the PB, and found that the abundance of $CD8^+$ TEM cells was positively, and the $CD8^+$ TEMRA cells negatively associated with OS.

In addition to these functional cell subsets, Lepone et al.[26] highlights the possibility of identifying, by FC, surface molecules in these cells, which are targets for therapies such as CTLA-4, PD-1, and PD-L1, as well as other potential targets for developing immunotherapies.

Even though different authors report similar results regarding these subsets of T cells, confirmation from well-designed, controlled clinical trials is required before these findings can be applied to clinical practice.

Myeloid-derived suppressor cells (MDSCs)

MDSCs are described as a heterogeneous population of immature cells with immunosuppressive properties, at the systemic level, as well as the tumour level[13, 31]. This immunoregulating population is described in the literature as being important in homeostasis, controlling inflammation. Nonetheless, in an atypical situation, specifically in chronic situations, such as cancer, the levels of MDSCs are increased. In a situation like this, the tumour microenvironment (TME) will favour its expansion, thus favouring cancer progression. MDSCs provide immunological suppression in the TME, since they inhibit the function of T cells, by secreting molecules, such as ARG1, IL-10 and TGF- β[13, 32]. Due to their immunosuppressive capacity, the presence of this cell population has been shown to hamper the efficacy of anti-tumour therapies, specifically immunotherapies, and has been described in patients with various solid tumours, including NSCLC, pancreatic cancer, breast cancer, and head and neck squamous cell carcinoma. Therefore, the constant research on MDSCs as a PB biomarker is relevant, as the monitoring of clinical outcome will contribute to better selection of patients who will potentially benefit from the therapy[13, 33].

In humans, MDSCs can differentiate into two main subpopulations: monocytic cells (M-MDSCs), and granulocytic or polymorphonuclear cells (gMDSCs or PMN-MDSCs), each with a distinct phenotype, $CD11b^+$ $CD14^+$ $HLA\text{-}DR^{low}$, and $CD11b^+$ $CD15^+$ $LOX\text{-}1^+$ $CD14^-$, respectively[10, 31, 34], and different suppression mechanisms[14]. On account of their heterogeneity, the determination of a combination panel of surface markers for MDSCs is not totally established in the literature, and there have been some different approaches. Nevertheless, in general, authors have been using a model outlined by Gabrilovich and colleagues[33, 34] to identify MDSCs by FC, taking into consideration its phenotypic characteristics.

Various studies published in the literature reveal the role of MDSCs in immunotherapy, in particular ICI therapy. In general, there is consensus in the literature that a lower baseline frequency of MDSCs, as well as a decrease in their frequency over the course of the treatment, is associated with better clinical outcome[13, 31, 35-37]. According to Poschke et al.[38], an increase in MDSCs is related to a higher activity of melanoma. However, with the application of an anti-CTLA-4 therapy, the peripheral frequency of these cells decreased, especially PMN-MDSCs, thus resulting in improved OS. In line with these results, Meyer et al.[35] analysed the role of $CD14^+$ $HLA\text{-}DR^-$ MDSCs, also in melanoma patients, and observed lower frequencies of these cells in patients who responded to ipilimumab, versus patients who did not respond to therapy. However, they refer that with this therapy, T cells produce higher amounts of interferon-gamma (IFN-γ), resulting in a higher recruitment of MDSCs. Hence, they would expect higher frequencies of MDSCs in patients treated with ipilimumab. Nonetheless, it is well established that patients who present a lower increase have better clinical benefits. In a study conducted by Sade-Felman et al.[36], the authors mention that,

due to the immunosuppressive characteristics of MDSCs, when increased in the PB, they may lead to alteration of T cell function, and consequently, to a limited response to the established immunotherapy. Accordingly, they observed several particularities in advanced-stage melanoma patients. First, they found that decreased frequencies of circulating MDSCs before the first cycle of ipilimumab were associated with prolonged OS and with clinical benefit, whereas higher frequencies were associated with lower OS and with minimal or no clinical benefit at all. Moreover, they noted that advanced stage melanoma patients who did not respond to therapy presented inflammatory properties characteristic of this type of tumour, namely a higher production of nitric oxide (NO^-) and reactive species of oxygen (ROS), which are directly related to higher frequencies of MDSCs. As reported by Coaña et al.[37], there is a considerable reduction of MDSCs after ipilimumab treatment, that is rapidly observed after the first dose. They also specify that MDSCs exhibit suppression mechanisms, like the expression of ARG1, that negatively influence the function of T cells. In a study lead by Martens et al.[24], they found that, when submitted to ipilimumab therapy, the patients presented a decrease of circulating MDSCs, given that the therapy limits the inhibitory function of these cells. Also Limagne et al.[39] found an association between M-MDSCs and anti-PD-1 therapy, in which the administration of nivolumab provokes a decrease in the frequency of these cells in metastatic NSCLC patients that obtained clinical benefit from the therapy.

Given the analysed data in different studies, it is reasonable to affirm that, independent of the type of cancer or type of treatment, the frequency of MDSCs in PB should be low so that the patient could clinically benefit from the therapy[10]. A higher frequency of these inhibitory cells after therapy may indicate an unfavourable response. Thus, the enrolment of these cells as a predictive biomarker would be clinically relevant.

Neutrophils

Inflammation plays a key role in the development of the tumour and is associated with the prognosis of solid tumours. On one hand, it is a critical component in different stages of cancer development, such as initiation, proliferation, and invasion. On the other hand, it also affects the patient's immune response to the tumour[40].

Neutrophils are a key component of the inflammatory response, as they target tumour cells directly, but also the TME, through the release of cytokines, chemokines, and tumour growth factors, that will initiate or promote tumour developing. Besides, the tumour cells can release granulocyte colony-stimulating factor (G-CSF), thus increasing the number of neutrophils. There is, therefore, a mutual relationship between tumour cells and neutrophils. Given the roles of neutrophils and lymphocytes in tumour growth, changes in neutrophil-lymphocyte ratio (NLR) can reflect the body's anti-tumour status. An elevated NLR indicates a chronic inflammation status, which could be used to reflect the immune status of patients[4, 40]. Several studies have reported that high pre-treatment NLR in PB are significantly associated with worse OS and PFS[4, 40, 41]. An increase in NLR indicates an increase in the number of neutrophils and/or a decrease in the number of lymphocytes and, consequently, a decrease in the anti-tumour capacity of the immune system. Thus, this is associated with a poorer response of cancer patients to immunotherapy. Accordingly, a decrease in NLR might lead to an improved response of the immune system against the tumours, and might indicate a good response to immunotherapy[40]. Actually, Möller et al.[41] has reported that patients with partial/complete clinical response to ICI therapy are characterized by the reduction of neutrophils and an increase of lymphocytes, resulting in a declining NLR. Li et al.[40] has had similar results, in which patients with higher baseline NLR had shorter PFS. Moreover, the authors calculated the dynamics

of NLR (dNLR) after 6 weeks from baseline and showed that an increase in dNLR after 6 weeks from baseline was associated with poorer PFS.

Eosinophils

Over the past decade, eosinophilic accumulation in cancer has been observed, not only at the tumour site, but also in the PB of patients with different types of solid tumours. This accumulation is often linked to a better survival of cancer patients, as well as response to ICI therapy[42]. Indeed, Martens and colleagues[24] show that an accumulation of eosinophils in the PB of melanoma patients being treated with ICI therapy is associated with improved OS. Gebhardt et al.[43] also found that an increase in the frequency of circulating eosinophils over the course of the treatment was associated with an improved clinical response. Similarly, Simon et al.[42] found an increase of peripheral eosinophil frequency after the first administration of ICI therapy in responders, which was not observed in 50% of non-responders. The authors also measured the expression of CD69 on eosinophils, since it is a well-known activation marker for eosinophils, and observed a high expression in some of the responders after the first dose of the treatment, although this trend was not significant. However, two patients with over 90% of $CD69^+$ circulating eosinophils with stage III tumours did not develop visceral metastasis within 3 years after the start of the treatment, illustrating the value of peripheral eosinophils as a putative biomarker.

Even though an increase in TME and circulating eosinophils has been associated with better prognosis in most cancer cases studied, the mechanisms of beneficial effects of eosinophils in tumour-bearing hosts remains unclear[42]. In the B16 melanoma mouse model, it has been showed that tumour-infiltrating eosinophils can guide T cells into the tumour, which resulted in tumour eradication and improved survival[44]. Nevertheless, this is an area that requires further investigation in order to develop a reliable biomarker.

Dendritic cells (DC)

DC represent about 1% of total circulating mononuclear cells, and are defined as antigen-presenting cells, with a high expression of MHC class II (HLA-DR), and a lack of expression for other leukocyte markers, such as CD3, CD14 or CD19. Based on their lineage origin, blood DC can be divided into two major subsets, plasmacytoid DC (pDC), and myeloid DC (mDC), each with distinct functions. mDC regulate pro-inflammatory responses by inducing T_H1 and cytotoxic T lymphocytes responses upon a bacterial and viral infection, whereas pDC are the major producers of type I interferons[45]. Möller and colleagues[41] studied DC's potential as putative biomarkers for ICI therapy. They showed that cancer patients with low baseline percentages of pDC, $CD1c^+$ mDC, and $CD141^+$ mDC had the lowest PFS. When comparing patients undergoing ICI therapy as first-line therapy versus second-line therapy, the authors found that, in both settings, a clinical response was associated with an increase of DC levels, and that patients with a PFS $\leq$ 1 month had the lowest frequencies of total DC. Over the course of the treatment, therapy responders were also the ones with the highest percentages of mDC, and patients with tumour progression had a decrease in total DC number. This study shows the promising value of DC as potential predictive biomarkers for ICI therapy.

Natural killer (NK) cells

NK cells are lymphocytes of the immune system with cytotoxic properties and a rapid and potent response to tumour cells and intracellular pathogens infected cells[46]. In PB, they are characterized by the surface phenotype $CD3^-$ $CD56^+$. However, they can be subdivided into two populations whose CD56 expression is distinct, namely $CD56^{dim}$ and

$CD56^{bright}$. The main difference between these two subpopulations remains on their functions, since they reveal high levels of cytotoxicity, or immunoregulatory properties, respectively[47]. NK cells contribute to an improved immunological surveillance, as they exhibit several stimulatory or inhibitory receptors on their surface. Besides, they secrete cytokines, such as IFN-γ and tumour necrosis factor-α (TNF-α), that will favour anti-tumoral immunity[46].

Thereby, the use of NK cells as a biomarker may be a favourable tool to identify patients who will benefit from therapy, especially anti-PD-1 therapy[48]. Taking into account a study lead by Tang and colleagues[49], they found that high frequencies of NK cells identified by FC ($CD3^-CD16^+CD56^+$) in the PB of melanoma, urothelial and renal cell carcinoma patients, at the beginning of anti-PD-1 therapy, are related to a better clinical outcome. Accordingly, in a study conducted by Youn et al.[50], anti-PD-1 therapy has induced an efficient anti-tumoral response by NK cells. They observed that, in nivolumab responding NSCLC patients, there was a significant increase of NK cells after the first cycle of treatment, when compared to non-responding patients.

Given the importance of NK cells in anti-tumoral immunity, specifically due to their cytotoxic functions, it is accepted that higher frequencies of these cells before, during and after the treatment, are associated with better clinical outcomes. Although the results seem promising, there remains a need for further studies regarding NK cells as a biomarker for ICI therapy response.

Monocytes

In whole blood, monocytes are classified according to their CD14 and CD16 expression profiles. $CD14^+$ $CD16^-$ monocytes correspond to classical monocytes, with the main function of phagocytosis after their recruitment to tissues following inflammation or damage. Intermediate monocytes have the phenotype $CD14^+$ $CD16^-$, which are a pro-inflammatory subset, also with phagocytosis capability. Lastly, non-classical monocytes are characterized as $CD14^-$ $CD16^+$, with surveillance functions[10, 51].

There are only a few indications of changes in blood monocytes associated with clinical outcomes after ICI therapy, and with different results depending on the monocytes subset and type of ICI. A study in melanoma patients treated with ipilimumab (an anti-CTLA-4 antibody) uncovered that responders displayed higher baseline percentages of non-classical monocytes than non-responders[10]. Other authors found different associations with anti-PD-1 therapy studies. Cloughesy et al. [52] found that a decrease in $CD11c^+$ $CD14^+$ $CD16^+$ $HLA\text{-}DR^{hi}$ intermediate monocytes was associated with a significant increase in OS in glioblastoma patients who received neo-adjuvant anti-PD-1 therapy before surgery and Krieg et al.[53] confirmed by flow cytometry that a frequency of $CD14^+$ $CD16^-$ $CD33^{hi}$ $HLA\text{-}DR^{hi}$ classical monocytes higher than 19.38% before therapy was associated with a better treatment outcome. These results suggest that the presence of activated classical monocytes may be a prerequisite for a successful response during anti-PD-1 immunotherapy.

B cells

B cells function in the humoral immunity component of the adaptive immune system by secreting antibodies. Additionally, they can be potent antigen-presenting cells and secrete cytokines[54].

Das et al.[55] found that anti-CTLA-4 and anti-PD-1 combination therapy increased $CD21^{lo}$ B-cell subsets and plasmablasts, suggesting a germinal center activation, which was not observed in monotherapy, either with anti-CTLA-4 or anti-PD-1 therapy. Although these changes are not related to survival, they seem to be related to immune-related adverse events.

The role of peripheral B cells as a biomarker for ICI therapy is not established in the literature, and

remains a need to further investigate their putative contribute in the prediction and monitoring of immunotherapy.

CONCLUSION

ICI therapies are rapidly evolving and revolutionizing the treatment of a variety of malignancies. However, only a limited number of patients benefit from this therapy, and this inability to predict treatment efficacy and patient clinical response to the treatment is a major issue of cancer immunotherapy. It is therefore urgent to find predictive biomarkers that allow to select patients who will obtain clinical benefits, this way minimizing unnecessary exposure to immune-related toxicities in non-responders, and reducing the financial burden of healthcare systems, due to the expensive nature of these therapies.

A potential predictive biomarker should gather a series of relevant features. In order to be applied to the clinical setting, a predictive biomarker should be cost-effective, accurately predict clinical response, and be easily accessed through standardized methods. A PB sample has the potential to gather these features. Therefore, the demand for PB biomarkers for ICI has been the subject of intense research due to their safety and less invasive nature. In this research, FC is one of the most valued techniques in the interpretation of immunological changes arising from immunotherapy, as it provides a comprehensive view of peripheral immunity. Its multiparametric character allows the identification of differences among immune cell subsets, expressing molecules associated with different maturation, activation, and functional states.

In this review, we covered several potential PB biomarkers for ICI, such as T cell, MDSCs, neutrophils, eosinophils, DC, NK cells, monocyte subsets and B cells. Different types of experimental study designs have been conducted, and several promising results have been described (**Table 1**), demonstrating the potential of these biomarkers in predicting and monitoring the efficacy of ICI therapy. Nonetheless, these results should be validated in well-designed, controlled clinical trials, with defined patient populations and endpoints, in order to be implemented into routine clinical practice.

The overall evaluation of these biomarkers may allow the establishment of peripheral immunological profiles, like peripheral "immunoscores", which would help clinicians in the application of immunological therapies, either in patients selection or in the monitoring of the response to therapy.

REVIEW METHODOLOGY

In this literature review, a search was performed to identify studies focusing on the role of flow cytometric evaluation of peripheral blood immune cell populations as potential biomarkers for immunotherapy, in solid tumours. A recent and up-to-date literature search was performed in three different data-bases, namely PubMed, Scopus, and Web of Science, and studies were identified using the following terms: "peripheral blood biomarkers", "peripheral immune populations", "biomarkers for immunotherapy", "peripheral immunoscore", "cancer immunotherapy", and "flow cytometry". The connector words AND and OR were used to combine search sets. Articles that were published before 2010, written in languages other than English, and not performed in humans were excluded. Besides, only studies that performed analysis of potential biomarkers on peripheral blood by flow cytometry, in patients submitted to immunotherapy, were considered.

AUTHOR CONTRIBUTIONS

ACT, PM and CP article design and writing; IG, GM and LLS critical review; CR, MES, AMP and CA literature review. CP and GM approved the final version of the article.

Table 1 – **Potential peripheral blood immune cell biomarkers of response to ICI therapy.**

Biomarker	Clinical significance	References
T_{reg}	Higher baseline frequencies associated with improved OS	[12, 18, 19]
	Increase throughout the treatment associated with prolonged PFS	[20]
	Higher frequency over the course of the treatment in patients with disease control	[21]
	Increase throughout the treatment not significantly associated with OS	[24]
	Greater T_{reg} response in patients who did not respond to therapy	[22]
	Higher frequency during the treatment associated with a decrease in PFS	[23]
$CD4^+$ and $CD8^+$ T cells	Increase in $CD4^+$ $Ki67^+$ and $CD8^+$ $Ki67^+$ levels associated with improved OS	[24]
	Increase in $CD4^+$ $Ki67^+$ and $CD8^+$ $Ki67^+$ levels not correlated with clinical outcome	[29]
	Higher expansion of $CD4^+$ and $CD8^+$ T cells during the treatment associated with higher rates of disease control	[19, 27, 30]
	$CD8^+$ TEM cells positively and $CD8^+$ TEMRA negatively associated with OS	[28]
MDSCs	Lower baseline frequencies associated with better clinical outcome	[13, 31, 35-37]
	Decrease in the frequency of MDSCs associated with better OS	[38, 39]
	Reduction of MDSCs after the first dose of the treatment	[24, 37, 39]
Neutrophils	Higher pre-treatment NLR associated with worse OS and PFS	[4, 40, 41]
	Response to ICI treatment characterized by a decrease in NLR	[41]
	Increase in dNLR associated with poorer PFS	[40]
Eosinophils	Increase over the course of the treatment associated with improved OS	[24, 43]
	Increase after the first dose of treatment in responding patients	[42]
DCs	Low baseline percentages of pDC and $CD1c^+$ mDC and $CD141^+$ mDC associated with lower PFS	[41]
	Increase during the treatment associated with better clinical response	
NK	High baseline frequencies associated with better clinical outcome	[49]
	Increase after the first dose observed in responding patients	[50]
Monocytes	Responders displayed higher baseline percentage of non-classical monocytes with anti-CTLA-4 therapy	[10]
	Decrease frequency of intermediate monocytes associated with increase OS	[52]
	Higher frequency of classical monocytes was higher in better outcome patients after anti-PD1 therapy	[53]
B cells	Increase $CD21^{lo}$ B-cell subsets and plasmablasts not associated with survival but with immune-related adverse events	[55]

Abbreviations: CTLA-4 – cytotoxic T-lymphocyte-associated protein 4; DC – dendritic cell; dNLR – dynamics of neutrophil-lymphocyte ratio; ICI – immune checkpoint inhibitors; mDC – myeloid dendritic cells; MDSC – myeloid-derived suppressor cells; NK – natural killer cell; NLR – neutrophil-to-lymphocyte ratio; OS – overall survival; PD-1 – programmed cell death protein 1; pDC – plasmacytoid dendritic cells; PFS – progression-free survival; TEM – effector memory T cells; TEMRA – terminal effector memory T cells; T_{reg} – regulatory T cells.

REFERENCES

1. Burugu, S., A.R. Dancsok, and T.O. Nielsen, *Emerging targets in cancer immunotherapy.* Semin Cancer Biol, 2018. **52**(Pt 2): p. 39-52.
2. Spencer, K.R., et al., *Biomarkers for Immunotherapy: Current Developments and Challenges.* Am Soc Clin Oncol Educ Book, 2016. **35**: p. e493-503.
3. Voutsadakis, I.A., *Prediction of Immune checkpoint inhibitors benefit from routinely measurable peripheral blood parameters.* Chin Clin Oncol, 2020. **9**(2): p. 19.
4. Li, S., et al., *Emerging Blood-Based Biomarkers for Predicting Response to Checkpoint Immunotherapy in Non-Small-Cell Lung Cancer.* Front Immunol, 2020. **11**: p. 603157.
5. Rotte, A., J.Y. Jin, and V. Lemaire, *Mechanistic overview of immune checkpoints to support the rational design of their combinations in cancer immunotherapy.* Ann Oncol, 2018. **29**(1): p. 71-83.
6. Zhang, M., et al., *Monitoring checkpoint inhibitors: predictive biomarkers in immunotherapy.* Front Med, 2019. **13**(1): p. 32-44.
7. Quandt, D., et al., *Implementing liquid biopsies into clinical decision making for cancer immunotherapy.* Oncotarget, 2017. **8**(29): p. 48507-48520.
8. Guo, L., et al., *Colorectal Cancer Immune Infiltrates: Significance in Patient Prognosis and Immunotherapeutic Efficacy.* Front Immunol, 2020. **11**: p. 1052.
9. Galon, J., et al., *Immunoscore and Immunoprofiling in cancer: an update from the melanoma and immunotherapy bridge 2015.* J Transl Med, 2016. **14**: p. 273.
10. Hernandez, C., et al., *Systemic Blood Immune Cell Populations as Biomarkers for the Outcome of Immune Checkpoint Inhibitor Therapies.* Int J Mol Sci, 2020. **21**(7).
11. Schnell, A., et al., *The Peripheral and Intratumoral Immune Cell Landscape in Cancer Patients: A Proxy for Tumor Biology and a Tool for Outcome Prediction.* Biomedicines, 2018. **6**(1).
12. Martens, A., et al., *Baseline Peripheral Blood Biomarkers Associated with Clinical Outcome of Advanced Melanoma Patients Treated with Ipilimumab.* Clin Cancer Res, 2016. **22**(12): p. 2908-18.
13. George, A.P., et al., *The Discovery of Biomarkers in Cancer Immunotherapy.* Comput Struct Biotechnol J, 2019. **17**: p. 484-497.
14. Whiteside, T.L., *Immune responses to cancer: are they potential biomarkers of prognosis?* Front Oncol, 2013. **3**: p. 107.
15. Riemann, D., et al., *Blood immune cell biomarkers in lung cancer.* Clin Exp Immunol, 2019. **195**(2): p. 179-189.
16. Pardoll, D.M., *The blockade of immune checkpoints in cancer immunotherapy.* Nat Rev Cancer, 2012. **12**(4): p. 252-64.
17. Nishino, M., et al., *Monitoring immune-checkpoint blockade: response evaluation and biomarker development.* Nat Rev Clin Oncol, 2017. **14**(11): p. 655-668.
18. Jochems, C., et al., *A combination trial of vaccine plus ipilimumab in metastatic castration-resistant prostate cancer patients: immune correlates.* Cancer Immunol Immunother, 2014. **63**(4): p. 407-18.
19. Delyon, J., et al., *Experience in daily practice with ipilimumab for the treatment of patients with metastatic melanoma: an early increase in lymphocyte and eosinophil counts is associated with improved survival.* Ann Oncol, 2013. **24**(6): p. 1697-703.
20. Tarhini, A.A., et al., *Immune monitoring of the circulation and the tumor microenvironment in patients with regionally advanced melanoma receiving neoadjuvant ipilimumab.* PLoS One, 2014. **9**(2): p. e87705.
21. Simeone, E., et al., *Immunological and biological changes during ipilimumab treatment and their potential correlation with clinical response and survival in patients with advanced melanoma.* Cancer Immunol Immunother, 2014. **63**(7): p. 675-83.
22. Hotson, D., et al., *CTLA-4 Defines Distinct T Cell Signaling Populations in Healthy Donors and Metastatic Melanoma Patients*. 2012. 760-760.
23. Retseck, J., et al., *Phenotypic and functional testing of circulating regulatory T cells in advanced melanoma patients treated with neoadjuvant ipilimumab.* J Immunother Cancer, 2016. **4**: p. 38.
24. Martens, A., et al., *Increases in Absolute Lymphocytes and Circulating CD4+ and CD8+ T Cells Are Associated with Positive Clinical Outcome of Melanoma Patients Treated with Ipilimumab.* Clin Cancer Res, 2016. **22**(19): p. 4848-4858.
25. Pico de Coaña, Y., et al., *Ipilimumab treatment decreases circulating Tregs and GrMDSC while enhancing CD4+ T cell activation.* Journal of Translational Medicine, 2015. **13**(Suppl 1): p. O7-O7.
26. Lepone, L.M., et al., *Analyses of 123 Peripheral Human Immune Cell Subsets: Defining Differences with Age and between Healthy Donors and Cancer Patients Not Detected in Analysis of Standard Immune Cell Types.* J Circ Biomark, 2016. **5**: p. 5.
27. Felix, J., et al., *Ipilimumab reshapes T cell memory subsets in melanoma patients with clinical response.* Oncoimmunology, 2016. **5**(7): p. 1136045.
28. Wistuba-Hamprecht, K., et al., *Peripheral CD8 effector-memory type 1 T-cells correlate with outcome in ipilimumab-treated stage IV melanoma patients.* Eur J Cancer, 2017. **73**: p. 61-70.
29. Wang, W., et al., *Biomarkers on melanoma patient T cells associated with ipilimumab treatment.* J Transl Med, 2012. **10**: p. 146.
30. Ku, G.Y., et al., *Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting: lymphocyte count after 2 doses correlates with survival.* Cancer, 2010. **116**(7): p. 1767-75.

31. Peranzoni, E., et al., *Myeloid Cells as Clinical Biomarkers for Immune Checkpoint Blockade.* Front Immunol, 2020. **11**: p. 1590.
32. Kotsakis, A., et al., *Myeloid-derived suppressor cell measurements in fresh and cryopreserved blood samples.* J Immunol Methods, 2012. **381**(1-2): p. 14-22.
33. Apodaca, M.C., et al., *Characterization of a whole blood assay for quantifying myeloid-derived suppressor cells.* J Immunother Cancer, 2019. **7**(1): p. 230.
34. Bronte, V., et al., *Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards.* Nat Commun, 2016. **7**: p. 12150.
35. Meyer, C., et al., *Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab.* Cancer Immunol Immunother, 2014. **63**(3): p. 247-57.
36. Sade-Feldman, M., et al., *Clinical Significance of Circulating CD33+CD11b+HLA-DR- Myeloid Cells in Patients with Stage IV Melanoma Treated with Ipilimumab.* Clin Cancer Res, 2016. **22**(23): p. 5661-5672.
37. Pico de Coana, Y., et al., *Ipilimumab treatment results in an early decrease in the frequency of circulating granulocytic myeloid-derived suppressor cells as well as their Arginase1 production.* Cancer Immunol Res, 2013. **1**(3): p. 158-62.
38. Poschke, I., et al., *Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign.* Cancer Res, 2010. **70**(11): p. 4335-45.
39. Limagne, E., et al., *Tim-3/galectin-9 pathway and mMDSC control primary and secondary resistances to PD-1 blockade in lung cancer patients.* Oncoimmunology, 2019. **8**(4): p. e1564505.
40. Li, Y., et al., *Pretreatment Neutrophil-to-Lymphocyte Ratio (NLR) May Predict the Outcomes of Advanced Non-small-cell Lung Cancer (NSCLC) Patients Treated With Immune Checkpoint Inhibitors (ICIs).* Front Oncol, 2020. **10**: p. 654.
41. Moller, M., et al., *Blood Immune Cell Biomarkers in Patient With Lung Cancer Undergoing Treatment With Checkpoint Blockade.* J Immunother, 2020. **43**(2): p. 57-66.
42. Simon, S.C.S., et al., *Eosinophil accumulation predicts response to melanoma treatment with immune checkpoint inhibitors.* Oncoimmunology, 2020. **9**(1): p. 1727116.
43. Gebhardt, C., et al., *Myeloid Cells and Related Chronic Inflammatory Factors as Novel Predictive Markers in Melanoma Treatment with Ipilimumab.* Clin Cancer Res, 2015. **21**(24): p. 5453-9.
44. Carretero, R., et al., *Eosinophils orchestrate cancer rejection by normalizing tumor vessels and enhancing infiltration of CD8(+) T cells.* Nat Immunol, 2015. **16**(6): p. 609-17.
45. Splunter, M.V., et al., *Plasmacytoid dendritic cell and myeloid dendritic cell function in ageing: A comparison between elderly and young adult women.* PLoS One, 2019. **14**(12): p. e0225825.
46. Paul, S. and G. Lal, *The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy.* Front Immunol, 2017. **8**: p. 1124.
47. Krijgsman, D., et al., *Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile.* Cancer Immunol Immunother, 2019. **68**(6): p. 1011-1024.
48. Cao, Y., et al., *Immune checkpoint molecules in natural killer cells as potential targets for cancer immunotherapy.* Signal Transduct Target Ther, 2020. **5**(1): p. 250.
49. Tang, B., et al., *Natural killer cells as a predictive biomarker for response to anti-PD-1 therapy in patients with advanced solid tumors.* Journal of Clinical Oncology, 2017. **35**: p. e21055-e21055.
50. Youn, J.I., et al., *Peripheral natural killer cells and myeloid-derived suppressor cells correlate with anti-PD-1 responses in non-small cell lung cancer.* Sci Rep, 2020. **10**(1): p. 9050.
51. Holl, E.K., et al., *Examining Peripheral and Tumor Cellular Immunome in Patients With Cancer.* Front Immunol, 2019. **10**: p. 1767.
52. Cloughesy, T.F., et al., *Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma.* Nat Med, 2019. **25**(3): p. 477-486.
53. Krieg, C., et al., *High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy.* Nat Med, 2018. **24**(2): p. 144-153.
54. Wennhold, K., A. Shimabukuro-Vornhagen, and M. von Bergwelt-Baildon, *B Cell-Based Cancer Immunotherapy.* Transfus Med Hemother, 2019. **46**(1): p. 36-46.
55. Das, R., et al., *Early B cell changes predict autoimmunity following combination immune checkpoint blockade.* J Clin Invest, 2018. **128**(2): p. 715-720.

Correspondência:
CARLOS PALMEIRA
e-mail: carlospalmeira@ipoporto.min-saude.pt

Data de recepção do artigo:
14-12-2020

Data de aceitação do artigo:
22-12-2020