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Review

Knowing the tumour microenvironment to optimise immunotherapy

Conoscere il microambiente tumorale per ottimizzare l'immunoterapia

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SUMMARY

Effective immunotherapy requires thorough knowledge of the tumour microenvironment. Indeed, the interplay among the immune system, the tumour and treatment is conditioned by the composition of the tumour microenvironment. In addition, it must be taken into account that homeostasis of the tumour microenvironment is highly dynamic and changes rapidly in function of many factors, such as inflammation, hypoxia, tumour volume, all of which change over time, and the effect of treatments. All these elements interact with each other and with conditions related to the tumour (i.e. mutational load, rate of clonal and subclonal mutations) and to host (life style, diet, obesity, age). All these factors as well as their interplay, affect the response to immunotherapy. The target of this short review is to summarise some of the major aspects that impact the homeostasis of the tumour microenvironment and how its structure can drive treatment choice.

KEY WORDS: Immunotherapy • Tumour microenvironment • Treatment targets

RIASSUNTO

Una immunoterapia efficace richiede una profonda conoscenza del microambiente tumorale. Infatti l'interazione fra il sistema immune, il tumore e il trattamento è condizionata dalla composizione del microambiente. Inoltre si deve tenere in considerazione che l'equilibrio del microambiente è altamente dinamico e cambia rapidamente in funzione di molti fattori, come il livello di infiammazione, l'ipossia, il volume tumorale, tutti fattori che cambiano nel tempo, e gli effetti dei trattamenti. Tutti questi fattori interagiscono gli uni con gli altri e con condizioni correlate al tumore, (il carico mutazionale o la percentuale di mutazioni clonali o subclonali) o all'ospite (stili di vita, dieta, obesità, età). Tutti questi fattori, così come la loro interazione, influiscono sulla risposta all'immunoterapia. L'obiettivo di questa breve review è di riassumere alcuni dei principali aspetti che interferiscono con l'omeostasi del microambiente tumorale e come la sua struttura può guidare la scelta terapeutica.

PAROLE CHIAVE: *Immunoterapia • Microambiente tumorale • Bersagli della terapia*

Introduction

The main target of immunotherapy is the immune system. The effects against cancer are the consequence of immune system repolarisation from a tumour supportive phenotype towards a tumour suppressive one.

The increasing number of solid and haematologic tumours that benefit from the same immunotherapy agent supports the central role of the immune system [1]. However, the extent of the benefit changes widely among different tumours [2]. In addition, the same immune cells, such as T regulatory (Treg) cells or Tumour Associated Macrophages (TAM), may show different prognostic values according to the tumour site [3 4], attesting that other factors, apart from the cancer itself, influence homeostasis between the host and disease.

These aspects suggest that additional factors intervene in the simplistic view of a match involving two players: the immune system and the tumour.

First of all, the plasticity of both immune system and tumour impacts the way they interact with each other. Immune system changes according to age (e.g.: immune aging) [5], life styles (e.g.: diet, obesity) [6], presence of chronic infections (e.g.: CMV, HIV, HPV) and factors related to geographic origin (e.g.: microbioma, HLA polymorphisms) [6 7].

In turn, cancer tissue is well known for its instability. Tumour instability acts on the way it faces the immune system, for instance leading to different mutational load and mutational heterogeneity within the same tumour types [8 9]. Moreover, specific mutations interfere with the immune system in different ways: mutation of the transforming growth factor beta (TGFβ) receptor II (TGFβRII) gene

generating a non-functioning protein, causes the accumulation of TGFβ in the microenvironment, leading to the inhibition of the immune response and, eventually, to the immune exclusion [10]. Another example is the frequently observed disruption of the WNT–β-catenin axis, driving up-regulation of β-catenin that prevents the activation of the immune system through inhibition of recruitment of *Baft3* dendritic cells (DC) [11].

Secondly, the feature of both the immune system and the tumour characterise the field in which they interplay: the tumour microenvironment (TME).

It is clear that the TME reflects the plasticity of both the immune system and the tumour, although other aspects influence its plasticity: the pre-existing immune structure of the organ in which the cancer develops, or the specific anatomical aspects acquired by the tumour during its life, such as the degree of hypoxia and necrosis [12].

Therefore, the TME represents the crossroad of many different and frequently opposite signals that control the relationship between the host and the tumour.

Tackling the TME with therapeutic interventions that are able to change the equilibrium in favour of the host is a challenge of the near future.

The tumour microenvironment

A neoplastic mass is made up of tumour cells along with a large number of non-tumour cells and stroma, which represent the majority of tumour volume. All these components, including tumour cells, communicate continuously with each other through cell to cell contact and a complex network of cytokines, proteins and chemokines, whose balance push the match in favour of the immune system or the tumour, driving the action of the former and the reaction of the latter. Hence, any change in the TME may reflect changes of the balance between immune system and tumour. Many factors affect the homeostasis of the TME.

TME changes according to tumour volume

The TME changes according to tumour volume. For instance, NKG2D is an important activator receptor of all natural killer (NK) cells and most CD8+, CD4+, natural killer T (NKT) and γδT cells. MIC-A and MIC-B are two surface proteins similar to HLA and are expressed by cells under conditions of stress. They represent the NKG2D ligands (NKG2D-L). The binding of the receptor with MIC-A or MIC-B triggers the activation of immune cells and leads to an immune response.

Their up-regulation should be associated with a favourable outcome. Surprisingly, in human tumours, up-regulation of MIC-A/B plays a conflicting prognostic role.

To explain this paradox, it must be considered that the binding of NKG2D-L to the receptor induces not only cell activation, but also endocytosis and degradation of NKG2D. This explains why the receptor is markedly reduced in many infiltrating and circulating T cells [13]. Unfortunately, NKG2D-L can be shed into the TME. Soluble ligand and membrane bound ligand play an opposite role in immune response against the tumour: while membrane bound ligand facilitates attack by immune effector cells, soluble ligand blinds the immune cells that become unable to lyse target cells. A specific protease, "A disintegrin and metalloproteases-9" (ADAM-9) is the major NKG2D ligand sheddases. The amount of soluble ligand in the TME is function of tumour *"age"* (i.e. tumour volume and stage) [14].

A second example is the change of tumour interstitial pressure related to tumour volume. Gutmann et al., as far back as 1992, observed that interstitial fluid pressure (IFP) in head and neck cancer changes according to tumour volume [15]. The increased pressure reduces O_2 diffusion, increases hypoxia and reduces pH.

These effects directly hamper not only immune response, but also favour the accumulation of TAM M2 (highly immunosuppressive) and induction of cytokines, such as VEGF, TGFβ and galectin 1, into the TME. All these cytokines are highly immunosuppressive. In particular, Galectin 1 is able to skew the immune balance toward Th2 response, hindering Th1, Th17 and CD8+ cells, inhibiting activity of NK cells, polarising TAM toward the M2 phenotype, up-regulating Treg cells and inhibiting trans-endothelial migration of cytotoxic T lymphocytes (CTL) [16]. Therefore, a tumour at a more advanced stage expresses more efficient immune escape mechanisms.

TME changes according to the site of tumour origin

As reported above, some immune cells, such as Treg or TAM, have opposite prognostic role according to the site of tumour origin. However, site of origin drives other differences that are able to affect the TME. For instance, one is mutation of TGFβRII or its pathway. It may occur in up to 66% of head and neck cancers [17], but is present in only 27% of non- hypermutated colon cancers [18].

Plasticity of many immune cells favours dissimilarity among primary sites. Indeed, immune cells are genetically stable, but highly plastic. CD4+ T helper (Th) cells may be redirected from one lineage to another. Only terminally differentiated Th1 or Th2 cells cannot be switched to a different state, while Treg, Th17 and non-terminally differentiated Th1 and Th2 cells maintain their plasticity and can be reprogrammed [19]. Therefore, under the pressure of mutated homeostasis, Th1 can be converted in Treg or Treg can become Th17, and so on. Basically, the domi-

nant microenvironment drives the phenotype of immune cells. Also, TAM M1 or M2 polarisation depends on the TME: high levels of IFN-γ and TNF-α induce M1 polarisation (tumour suppressive), while IL-4, IL-10 and TGFβ drive M2 polarisation (tumour supporting) [20]. Many drugs have shown the capacity to reprogram the main regulatory immune cells, and much preliminary data have confirmed this finding in humans so far. For instance, toll-like receptor 9 agonists (αTLR9) reprogram TAM toward the M1 phenotype when administered intra-tumourally. In the clinic, the combination of αTLR9 with anti PD-1 has shown high activity and induction of the abscopal effect in non-injected lesions [21 22].

Myeloid derived suppressor cells (tumour supporting) can be induced to maturation toward DCs or TAMs (M1) by many agents, such as retinoic acid [23] or some chemotherapy agents such as gemcitabine [24].

Finally, Tregs can be selectively depleted using, for instance, low dose cyclophosphamide [25], or can be reprogrammed towards the Th1 phenotype targeting CCR8 or OX40 that can both avoid expansion of Tregs and the shift from Th1 to Treg [26 27].

TME changes due to cancer treatment

All anti-cancer treatments induce TME changes.

Many drugs interfere with the TME in different ways depending on their structure and/or mechanism of action. Chemotherapy can modulate immune cells depending on the drug and scheduling. Ghiringhelli et al. demonstrated that low dose cyclophosphamide selectively kills Treg cells, but not CD8+ cells or other CD3 lineages [25]. This selective effect might be due to the increased expression of pro-apoptotic molecules induced by the transcriptional factor Foxp3 that is mainly expressed by Treg. Foxp3 might contribute to the higher sensitivity to low-dose cyclophosphamide (reviewed in Sistigu et al. [28]). In addition to cyclophosphamide, many other drugs affect immune system. Bracci et al. reviewed this topic a few years ago [29]. Moreover, some chemotherapy agents are able to induce immunogenic cell death [30], a particular cell death leading to a potential increase of tumour immunogenicity that can induce strong changes in the TME and favour activity of the immune system.

Targeted therapies may alter TME as a consequence of their main activity. Cetuximab and bevacizumab serve as examples.

Cetuximab is a monoclonal antibody (mAb) targeting the EGFR expressed on the cell membrane and induces arrest of cell proliferation and migration. In addition, cetuximab is able to trigger antibody dependent cell cytotoxicity (ADCC) [31]. Activation of NK cells through the binding of Fc fragment of cetuximab to FcγRIII *(*CD16) induces release of cytotoxic granules by natural killer (NK) cells and release of pro-inflammatory cytokines, such as IFNγ and TNFα, which deeply impact the TME [32]. Furthermore, the link between the Fc fragment with FcγRI *(*CD 64) on DCs, leads to the priming of specific CD8+ clones targeting cells with high EGFR expression [33]. Indeed, the immune system can be activated not only by the presence of "non-self" antigens, but also by an excess of "self" antigens, such as the overexpression of EGFR on tumour cells.

Bevacizumab is a mAb directed against vascular endothelial growth factor (VEGF). Its activity results in remodeling of the vasculature and reactivation of the endothelial cells that favours trafficking and homing of T effector cells and oxygenation of hypoxic (immunosuppressive) areas. However, this effect is largely dose-dependent, since high dose bevacizumab, such as those routinely used for the treatment of most human cancers*,* induces the pruning of the microvasculature, reduces the homing of CTL and worsens hypoxia [34].

Immunotherapy directly interferes with the TME. Indeed, blocking the PD-1 – PD-L1 axis induces a number of major changes leading to the restoration of immune activity [35].

The immune checkpoint inhibitors may facilitate the homing of T effector cells preventing their contact with PD-L1 expressed on the endothelial cells or may hinder Treg cells.

Radiotherapy induces a number of immune effects both activating and immunosuppressive, such as up-regulation of MHC-I or up-regulation of chemokines recruiting effector cells, and of TGFβ or IL-10. These effects depend on total dose, dose per fraction and scheduling and require more investigation in humans.

TME drives resistance

Resistance to immunotherapy is largely due to the structure of the TME. Hedge et al. identified three different TMEs [36]. The "*inflamed*" tumours are characterised by infiltration of immune cells. These immune cells are inefficient because they are kept in check by immunosuppressive mechanisms. Inflamed tumours, such as many head and neck cancers, have a high chance to respond to immune checkpoint inhibitors. Immune cells localised at the margins of the tumour nests characterise the "*excluded*" tumours; this phenotype shows reduced response to ICIs. Finally, the "*desert*" tumours are characterised by lack of immune cells, both within the tumour and at its margins. These tumours usually do not respond to ICIs.

The mechanisms responsible of these diverse TME architectures are already known [37], and consequently the necessary approaches to counteract the resistance resulting

from them are known, at least in theory. Briefly, the immune resistance of inflamed tumours can be overcome by ICIs. The excluded tumours may benefit from drugs able to facilitate trafficking and homing of lymphocytes into the tumour nests, while immune desert tumours may take advantage by treatments that are able to improve the immunogenicity of cancer cells [38].

Tumour histotype does not necessarily correspond to one of these different TME but, rather, can coexist in any tumour type, probably with different ratios [36]. In addition, there is evidence that in human metastatic cancers, metastases may express any TME, regardless of the characteristics of the originating tumour and other metastatic sites [39]. Taken together, these observations can explain why the same immune checkpoint inhibitor reaches different activity in diverse tumour histotypes and within the same tumour.

Taking advantage of TME characteristics to achieve the best response

The knowledge of TME characteristics can allow for identification of the best treatment for each situation. For example, our group demonstrated in colon cancer patients treated with cetuximab and presenting with high basal ADCC activity, a significantly better overall survival compared to those treated with the same drug but expressing low basal ADCC [40]. We also analysed a series of patients treated with cetuximab and radiotherapy for locally advanced head and neck cancer not suitable for chemoradiation. In this population, high basal ADCC activity correlated with significantly better survival ($p = 0.033$) compared to low ADCC. On the contrary, ADCC did not correlate with better outcome in a control group treated with chemoradiation [41].

In addition, considering only patients with over expression of EGFR (+++) in which there is the highest probability of binding cetuximab and EGFR, the difference between high and low basal ADCC was stronger ($p = 0.024$) and patients in the group with high ADCC have 100% overall survival, compared to 49% in the low ADCC group at a maximum follow-up of 44 months [41].

It has also been observed that high mutational load predicts response to immunotherapy, while low mutational burden predicts response to chemotherapy. Indeed, in a randomised phase III study, Carbone et al. observed that tumours expressing high mutational load have a greater chance to achieve objective response and long benefit with nivolumab rather than with chemotherapy. On the contrary, tumours with low mutational burden correlate with an opposite attitude [42]. Interestingly, Riaz et al. observed that the mutational burden decreases during successful treatment with ICIs in patients with melanoma [43]. If this observation is extended to other tumours, it will pave the way to beneficial treatment with chemotherapy after prior immunotherapy. Actually, reports showing unexpected responses to single agent chemotherapy after immunotherapy already exist, at least, in lung cancer [44 45] and in head and neck cancer [46] and a similar observation was also reported at the 2018 ASCO meeting [47].

Selected promising agents targeting TME in clinical development in head and neck cancer

Anti PD-(L)1

PD-1 is a receptor expressed by immune cells following their activation and physiologically its role consists in limiting the immune response to avoid serious damage to the host tissues. Its ligand, PD-L1, is expressed in tumour cells and in stromal cells with regulatory functions, such as TAM and endothelial cells. Targeting PD-1 with mAb changes the TME from a Th2 phenotype (immunosuppressive) to Th1 phenotype (immunostimulatory) in a consistent proportion of lymphocyte infiltrated tumours. Treatment with anti PD-1 mAbs in patients with relapsed-metastatic head and neck cancer after failure of chemotherapy leads to a small but reproducible rate of long-term survivors [48 49].

Very recently, the KeyNote 048 study, comparing the anti PD-1 mAb pembrolizumab alone to the "extreme" regimen (cisplatin, fluorouracil and cetuximab) in patients never treated for recurrent disease, was presented at the 2018 ESMO meeting. Pembrolizumab showed a large and significant improvement in overall survival compared to extreme, with a strong reduction in adverse events, at least in patients with high expression of PD-L1 [50]. Many other randomised trials are in progress with agents targeting the PD-1/PD-L1 axis in relapsed/metastatic disease and in combination with radiotherapy with cetuximab and/or chemotherapy in locally advanced disease and results are awaited soon.

Toll-like receptor agonists

The Toll-like receptors (TLRs) are able to trigger the immune response when they recognise danger signals (alarmin, danger-associated molecular patterns – DAMPS – or pathogen-associated molecular patterns – PAMPS -).

SD101 is αTLR9 oligodeoxynucleotide. SD 101 induces a rapid IFN type I production, which, in turn, induces activation of NK, promotes CD8+ homing into the tumour and initiates an immune response while blocking immune suppression.

SD 101 was injected directly into tumour lesions of 22 patients with relapsed metastatic squamous cell carcinomas of the head and neck.

In combination with the anti PD-1 pembrolizumab, SD 101 induced reduction of tumour volume in injected and non-injected lesions (abscopal effect) in 6 patients (27%)

and stopped tumour progression in another 6 [51]. Further studies on SD 101 and other agonists of TLRs are in early clinical development.

STAT-3 inhibition

STAT-3 is a "double-edge sword" transcriptional factor that drives both pro-immune activities and suppressive immune activity. Its role depends on the level of activation: intermittent activation induces pro-immune activity, whilst continuous activation, such as in cancer, manages a number of immune suppressive activities including up-regulation of VEGF, TGF-β, IL-10 and down-regulation of HLA, IFN type I and II, CXCL10, CD80 and CD86.

AZD 9150 is an antisense oligonucleotide that is able to decrease STAT-3 expression in advanced clinical development in lymphoma and lung cancer [52].

Cohen recently reported preliminary results of AZD 9150 in combination with anti-PD-L1 in RM-HNC showing response rate higher than expected with the inhibition of the PD-1/PD-L1 axis and with no additional toxicity [53]. The approach looks highly promising.

Anti TGF-β

TGF-β is among the most immunosuppressive cytokines in cancer, whilst the physiological role of TGF-β is to preserve tissue homeostasis. Indeed, one of its main functions is to keep under control the cell proliferation. In cancer, TGF-β inhibits most effector cells, and contributes to maintaining an immunosuppressive TME as well as to drive epithelial-mesenchymal transition (EMT).

EMT is a phenotypical change of cancer cells that promotes invasion and metastatisation.

Increased level of TGF-β has been reported in the majority of HNC [54]. Therefore, it represents an interesting target of immunotherapy in this disease.

Preliminary results of a phase 1 study based on a fusion protein targeting both PD-L1 and TGF-β ("TGF-β trap") were presented during the 2018 ESMO meeting. With a very favourable toxic profile, TGF-β trap achieved a tumour burden reduction of 50 to 90% in 6 of 11 patients [55].

Anti NKG2A

HLA-E is a non-classical HLA class I molecule, which can be expressed in cancer cells. Around 80% of HNCs express HLA-E, which is the highest value among solid tumours together with renal cancer and melanoma. HLA-E binds to NKG2A, which is an inhibitory receptor expressed on NK cells and CD8+ cells, and induces a potent inhibitory signal. The prevention of the binding of HLA-E with NKG2A results in restoration of immune cytotoxicity, including ADCC. A monoclonal antibody (monalizumab) is currently under clinical investigation in combination with cetuximab in heavily pretreated RM-HNC. Preliminary results show responses in 27% of patients and this value is more than double of that expected with cetuximab alone. Moreover, overall survival of 10.3 months compares favourably to the extreme regimen (10.1 months in non-pretreated RM-HNC) and to the anti PD-1 monoclonal antibody pembrolizumab and nivolumab (around 8 months in similar patients) [56].

Conclusions

The key for successful treatment of cancer resides in the TME. The problem is its plasticity that leads to a continuous change over time and represents the result of an incredible number of crosstalks among host characteristics, cancer cells, immune cells and cancer therapies.

Therefore, the solution is to identify the characteristic of the TME in a specific patient at the time of treatment. Clearly, this is a very daunting challenge.

We already know many cards of the puzzle and can positively drive the outcome in many tumours, including some, such as metastatic melanoma, which were hopeless until a decade ago. This is largely due to the huge improvements in our ability to interfere with the TME thanks to the impressive development of immune oncology.

However, we need to further improve our knowledge focusing on the mechanisms driving TME plasticity. We have to enhance our skills to distinguish one specific clinical situation among many that we consider similar on the basis of histology, TNM, or stage.

Finally, it is also necessary to change the way used to design, conduct and analyse clinical trials.

In the tremendous heterogeneity of cancer, small phase II trials, designed to detect remarkable advantages in highly selected and strictly homogeneous patient populations, along with strong translational studies, might be more useful than classical large clinical trials at the present status of clinical research.

Conflict of interest statement

MCM received honoraria consultancies, speakers bureau from M.S.D., Merck Serono, Astra Zeneca and research fundings from Merck Serono, M.S.D. and for participation in conferences from B.M.S., Merck Serono and Novartis. AA, ND, OG. declare no conflict of interest.

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