Blocking inflammation to improve immunotherapy of advanced cancer

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Abstract
The ability to induce functional reprogramming of regulatory T (Treg) cells in the tumor microenvironment is an extremely important therapeutic opportunity. However, when discussing such an approach, the opposing effect that the activation of the Treg cell compartments may have in inducing the immune inflammatory response and its link with the efficacy of immunotherapy should be considered. In fact, Treg reprogramming has a dual effect: immediate, with mechanisms that activate immunosurveillance, and late, mediated by the macrophage activation that yields an inflammatory status that is deleterious for the antineoplastic efficiency of the immune system response. Persistence of the inflammatory response is associated with specific changes of oxidative and glycolytic metabolic pathways that interfere with conventional T-cell activation and function and may be one of the reasons for the failure of immunotherapy in advanced cancer patients. Therefore, in addition to modulating Treg cell action, the combined use of drugs able to block chronic inflammation mediated mainly by macrophages, to counteract the oxidative stress, and to positively regulate the metabolic derangements, could improve the effectiveness of modern immunotherapy. In conclusion, reprogramming of Treg cells may be an appropriate strategy for treating early stages of neoplastic diseases, whereas other immunosuppressive mechanisms should be the target of a combined immunotherapy approach in more advanced phases of cancer.

Keywords: immunotherapy; inflammation; macrophages; regulatory T cells; tumor immunology.

Introduction
The crucial contribution of regulatory T (Treg) cells to cancer immunosuppression, and their role in immunotherapy, has been extensively reviewed.1 Treg cells represent a T-cell lineage assigned to suppressive activities and characterized by the expression of the transcription factor Foxp3. Foxp3 is crucial in stabilizing the Treg cells by inducing a gene expression profile specific for their suppressive functions.2,3 Although, Treg cells present a lineage stability,4 under certain circumstances, such as inflammatory perturbations of microenvironment, they can switch their fate and phenotype by changing their gene expression program and convert into effector T cells (Treg reprogramming), which may be characterized by loss of Foxp3 expression and production of pro-inflammatory cytokines and interferon-γ (IFN-γ).5

In a recent review series in Immunology, Gallimore et al.1 indicated that the induction of selective recruitment and modulation of different Treg cell molecular profiles and functions in the tumor microenvironment was an extremely important therapeutic opportunity.

However, Gallimore et al.1 in their Editorial also highlighted the inadequate clinical responses to immunotherapies observed in poorly defined subsets of patients, and hence the need to provide a basis for patient stratification.
that is useful for identifying effective combination immunotherapy protocols. Starting from the observations of Gallimore et al., we believe that, when determining an immunotherapy protocol based on Treg reprogramming, the opposing effect of this strategy in inducing an inflammatory response, and its link with the efficacy of immunotherapy, should be considered. This review aims to discuss how Treg reprogramming may not be sufficient for cancer immunotherapy, but that other suppressive and counter-regulatory mechanisms underlie the lack of effectiveness of such therapy, especially in the advanced stages of neoplastic disease.

To date, Treg reprogramming can be obtained by several methods. In vitro and in vivo preclinical experiments in murine models have achieved Treg reprogramming by targeting the interleukin-2 (IL-2) receptor, the glucocorticoid-induced tumor necrosis factor receptor (GITR), and the neuropilin-1 receptor.

In particular, Rech et al. found that treatment of Treg cells isolated from healthy human donors with daclizumab, a monoclonal antibody against the CD25 subunit of IL-2 receptor, decreased expression of CD25 and Foxp3 on Treg cells and increased their secretion of IFN-γ (consistently with Treg reprogramming). The same authors in a further clinical trial found that daclizumab, administered in association with a cancer vaccine in patients with metastatic breast cancer, obtained a decrease in Treg cells associated with a robust response of CD8 and CD4 T cells with autoimmunity avoidance.

Also, an anti-GITR antibody (TRX518) in a phase I clinical trial in patients with advanced cancer confirmed its ability to reduce intratumoral and circulating Treg cells. The exposure to pro-inflammatory cytokines, mainly IL-6, is also able to induce Treg reprogramming with down-regulation of Foxp3 and loss of Treg cell suppressive activity, as demonstrated in both in vitro and in vivo studies in murine tumor-bearing models.

Preclinical experiments have induced Treg reprogramming by genetic disruption or pharmacological inhibition of different transcription factors such as Eos, Helios, nuclear receptor 4A proteins, Enhancer of Zeste Homolog 2 histone methyltransferase. Targeting the specific metabolic profile of Treg cells can also be a modality to induce their reprogramming. In this context the deletion of two important regulators of oxidative phosphorylation (OXPHOS), peroxisome proliferator-activated receptor c co-activator 1a or sirtuin 3 impairs Treg cell functions both in vitro and in vivo. Also, the metabolic pathway mediated by phosphatase and tensin homolog (PTEN) expression and downstream suppression of phosphatidylinositol 3-kinase (PI3K) is critical for the maintenance of Treg cell suppressive function. Hence, reprogrammed Treg cells have been generated from suppression of PTEN expression in Foxp3 CD25 Treg cells in vitro. Indoleamine 2,3-dioxygenase (IDO) can also induce differentiation of naive T cells into Treg cells. Preliminary data in mice with melanoma showed that combined treatment with an IDO inhibitor and a tumor vaccine induced conversion of Treg cells into T helper type 17-like cells and increased conventional T (Tconv) effector cell activation and anti-tumor efficacy.

Beside Treg reprogramming, Treg cell depletion strategies have also been tested as anticancer therapy. In detail, monoclonal antibodies against CD25 have been used to kill Treg cells by antibody-dependent cell-mediated cytotoxicity and complement-mediated cytotoxicity in preclinical and clinical studies alone and in combination with dendritic cell vaccines. Additionally, a monoclonal antibody against the chemokine CCR4, which represents a main promoter of intratumoral Treg cell recruitment, has been tested in phase I/II clinical trials and achieved Treg cell depletion with well-tolerated antitumor activity, although long-term effects are unclear. In this context, Gyori et al. obtained Treg cell depletion in a preclinical in vivo model with a specific Treg deletion of PI3K. They found that isolated disruption of Treg activity had only a limited antitumoral effect and was accompanied by increased numbers of immunosuppressive CSF1R tumor-associated macrophages within tumor; in contrast, co-depletion of Foxp3 Treg cells and CSF1R tumor-associated macrophages increased the recruitment and activity of CD8 Tconv cells and achieved almost complete tumor rejection. Gyori et al. suggested that compensatory immunosuppressive mechanisms activated by Treg cell reprogramming and deletion may drive resistance to immunotherapy.

More recently, Pilato et al. described an attempt to improve immune checkpoint inhibitor therapy by reprogramming Treg cells towards the synthesis of IFN-γ. This cytokine, together with IL-2, is normally inhibited by Treg cells and is typically synthesized by Tconv cells to promote lymphocyte growth, proliferation and cytotoxicity. Interferon-γ also promotes expression of major histocompatibility complex class II (MHC II) on macrophages, thus increasing their antigen-presenting capacity. At the same time, IFN-γ induces MHC I expression on neoplastic cells, improving targeting by cytotoxic T cells.

The approach proposed by Pilato et al., involved blocking the immunosuppressive action of Treg cells. Briefly, they found that after disruption of the CARMA1–BCL10–MALT1 (CBM) signalosome in Treg cells, achieved by crossing Foxp3 Cre to CARMA1 flox/flox mice, tumor-infiltrating Treg cells produced IFN-γ and exhibited a pro-inflammatory profile. However, in doing so, this approach stimulated immune escape mechanisms. In fact, the increase of the levels of IFN-γ favored, as expected, the expression of programmed cell death protein 1 (PD-1) on effector T cells as well as synthesis of the PD-1 ligand (PD-L1) by
cancer cells and macrophages, so turning off effector Tconv cells and reactivating an immune escape mechanism. The same authors observed that the increase of IFN-γ associated with Treg reprogramming coincided with increased macrophage activation.

Role of macrophage activation with Treg reprogramming

Moreno Ayala et al. recently discussed how reprogramming Treg cells has a dual effect: an immediate one with mechanisms that activate immunosurveillance, and a late one mediated by macrophage activation that yields an inflammatory status deleterious for the antineoplastic efficiency of the immune system response (Fig 1). Several in vivo and in vitro experimental models involving Treg reprogramming into IFN-γ-producing cells, have shown that loss of Treg cell activity is accompanied by pro-inflammatory polarization of peritoneal macrophages with associated release of pro-inflammatory and immunosuppressive cytokines. Persistent activation of macrophages by neoplastic cells does not favor sustained antitumor T-cell responses. This may be exacerbated by IFN-γ-associated with Treg reprogramming, which specifically polarizes tumor-associated macrophages into an M1 pro-inflammatory phenotype. Characterized by increased synthesis of IL-6, production of reactive oxygen species (ROS), specific changes of glucose metabolism and a capacity to modulate iron metabolism. Macrophages normally present an iron-sequestering phenotype that leads to intracellular iron accumulation and, consequently, low iron release and availability (functional-iron deficiency) for several vital cell processes, such as DNA and protein synthesis, enzyme activity, integrity of energy oxidative pathways and cell proliferation. These conditions result in progressive loss of T-cell function as disease advances. Indeed, Mascaux et al., by analyzing a data set of different morphological stages of the development of lung squamous cell carcinoma obtained from cancer patient biopsies, showed that the adaptive immune response is strongest at the earliest stages, whereas the most advanced stages are characterized by an increased expression of co-inhibitory molecules and suppressive cytokines, such as PD-L1, IL-10 and IL-6.

On the basis of these considerations, blocking chronic inflammation should be considered alongside Treg reprogramming.

Highlighting the role of energy/oxidative metabolic changes on lymphocyte function

In the tumor microenvironment, both the presence of cancer cells and the prevalence of activated M1 polarized macrophages, as demonstrated by us in advanced ovarian cancers, contribute to suppression of glucose uptake and impair oxidative energy metabolism in tumor-infiltrating Tconv cells. In particular, low iron availability consequent to functional iron deficiency significantly blunts oxidative phosphorylation and tricarboxylic acid cycle activities, where iron is fundamental for the maintenance of the mitochondrial membrane potential and adenosine triphosphate production. Exposure to excess lactate, as a consequence of the Warburg effect, also impairs Tconv cell activation, disrupts their motility, and reduces the cytolytic function of CD8+ cells. T-cell metabolism is also influenced by the direct action of cytokines associated with the chronic inflammatory response, mainly IL-6, a potent regulator of glucose uptake, trafficking and glycolysis. Notably, IL-6 can exert inhibitory effects on the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway so inhibiting primary cellular energetic and anabolic processes. Moreover, by acting at the systemic level, IL-6 induces specific changes of energy, protein, lipid and glucose metabolism such as insulin resistance, lipolysis and free-fatty acid mobilization. Additionally, IL-6 is one of the main mediators of cancer-related anemia and anorexia with consequent impairment of nutritional intake of energy substrates as well as micromolecules (e.g. glucose, iron, zinc), fundamental for optimal lymphocyte activity. Overall, the above events determine a condition of energy deficiency, which, in turn, represses T-cell receptor (TCR)-related signaling, IFN-γ production, cytotoxicity and motility of Tconv cells with detrimental effects on the immune antineoplastic response.

Among the changes induced by chronic inflammation and macrophage activation in the tumor microenvironment, oxidative stress plays a key role in negatively affecting the immune response. When ROS is produced excessively, as in chronic inflammation, exceeding the neutralization rate achieved by intracellular antioxidants (mainly glutathione), T cells experience oxidative stress whose persistence may alter their function and blunt effector T-cell responses. Prolonged oxidative stress can cause different types of DNA damage, triggering genomic instability and transcription errors, leading to failed biosynthetic pathways, compromised proliferation and cell death. In this regard, our previous works demonstrated that the in vitro addition of antioxidants, such as α-lipoic acid and N-acetyl cysteine, reversed the oxidoreductive state and restored the blastic response of lymphocytes isolated from patients with advanced stage cancer. Moreover, ROS may cause peroxidation of lipids in the cell membrane, as well as conformational and structural protein changes, with consequent protein dysfunction. High concentrations of ROS induce also specific alterations in TCR signaling, including...
conformational changes of TCR-ζ and the tyrosine kinase LCK, thereby reducing phosphorylation and calcium flux, with consequent suppression of antigen-mediated Tconv cell responses. The difficulty of remodeling lymphocytes with these functional defects could explain the failure of immunotherapy noted by Gallimore et al.1

Rationale for a combination immunotherapy approach

Considering the evidence discussed above, the basis for immunotherapy and immunomodulation should require clinicians to verify: (i) the presence of an immunogenic
Figure 2. A mechanism-based combined approach to improve the effectiveness of immunotherapy. In addition to Treg reprogramming/depletion and amplification of T-cell activation with immune-checkpoint inhibitors (anti-CTLA4, anti PD-1/PD-L1), the concomitant use of drugs targeted against the pathogenetic mechanisms responsible for Tconv cell functional impairment, mediated mainly by macrophage-induced chronic inflammation, could improve the effectiveness of modern immunotherapy. Such a combined approach should include drugs able to modulate the macrophage population and related chronic inflammation (such as medroxyprogesterone acetate, cyclophosphamide and COX-2 inhibitors), counteract the associated oxidative stress (i.e. antioxidants) and improve the derangements of energy (such as carnitine) and iron metabolism (i.e. lactoferrin). The drugs that are supported by high-quality experimental evidence are indicated in blue and those that are hypothetical are in grey. Abbreviations: IFN, interferon; IL, interleukin; ROS, reactive oxygen species; CTLA-4, cytotoxic T-lymphocyte antigen 4; MHC, major histocompatibility complex; Fe^{2+}, ferrous iron; Fe^{3+}, ferric iron; TCR, T-cell receptor; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; TCA, tricarboxylic acid cycle; FAO, fatty acid oxidation; PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; COX-2, cyclooxygenase-2; Treg, regulatory T.

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antigen, (ii) PD-1 expression in T cells, (iii) the integrity of effector T-cell functions, (iv) the absence of immunosuppressive events and cells (such as the presence of Treg cells, immunosuppressive macrophage populations, PD-L1 expression, or cytokines with immunosuppressive actions), and (v) the presence of a chronic inflammatory status associated with oxidative stress and consequent changes of cell energy metabolism.

The last point may be particularly important in explaining the inferior response to immunotherapy in some subsets of advanced stage cancer patients. This supports combining immunotherapy with drugs that modulate chronic inflammation, counteracting oxidative stress and correcting derangements of energy and iron metabolism (Fig 2). Some studies by our groups have already tested the efficacy of a combination treatment approach with immunotherapy (recombinant IL-2), anti-inflammatory agents (medroxyprogesterone acetate) and antioxidants in patients with advanced cancer.58,59 More recently, we demonstrated the effectiveness of combination therapy with the anti-PD-1 antibody, nivolumab, and cyclophosphamide, an immunomodulatory chemotherapy agent that down-regulates macrophage activity and inhibits pro-inflammatory cytokine synthesis,60 thereby synergistically enhancing the action of immunotherapy.61 Of note, low-dose cyclophosphamide can also induce Treg depletion by inhibiting their proliferation and intratumoral migration and to dampen their activity by decreasing the FOXP3 and GITR expression.62–66

Consistent with the above findings, a very recent review67 discussed cyclooxygenase-2 inhibitors among drugs under investigation in combination with anti-PD-1 antibody immunotherapy.

Among the drugs with a strong rationale to be included in the design of a combined immunotherapy approach, the potential role for lactoferrin should be emphasized. Lactoferrin has the ability to lessen inflammation associated with M1 macrophage polarization68 and to modulate positively the related changes of iron metabolism (iron trafficking and storage).69 This contributes to restoring Tconv cell responses.70 In addition, carinactine is a promising agent that may be useful for augmenting the effectiveness of modern immunotherapy with immune checkpoint inhibitors because of its ability to increase oxidative mitochondrial energy metabolism, exert antioxidant effects,71–73 and thus improve T-cell activation and functions.

In conclusion, we believe that the reprogramming of Treg cells is appropriate for specific stages of neoplastic disease, but that other suppressive mechanisms should be the target of a combined pharmacological approach in more advanced cancer phases. In this context, understanding the precise role of the immune response in specific subsets of patients in relation to the stage of disease74,75 and tumor types76 is a goal to be pursued vigorously.

Disclosures
The authors declare no competing interests.

Author contributions
AM and CM contributed to the conception or design of the work; the analysis and interpretation of data; drafted the work and approved the submitted version.

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