Nanomedicine and cancer immunotherapy – targeting immunosuppressive cells

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Abstract
The search for pharmacological strategies to reach and impact on immunosuppressive cells is, currently, one of the most exciting areas in cancer immunology and clinical oncology. In this context, it is increasingly accepted that the success of these therapies will largely depend on the availability of appropriate drug delivery strategies. Considering the critical role that nanotechnology plays in the development of these novel therapies, the main goal of this article is to provide an overview of the potential of nanomedicines targeted to immunosuppressive cells for the treatment of cancer. We present, first, a brief description of classical cancer immunotherapies based on therapeutic vaccination and monoclonal antibodies, with a special focus on the use of nanotechnologies and the targeting of immunological checkpoints. Second, we provide a thoughtful analysis of the possibilities to target the immunosuppressive cells, namely tumour-associated macrophages, myeloid-derived suppressor cells, tumour-associated neutrophils and regulatory T cells, at the tissue level (i.e. tumour, spleen, blood, lymph) and, also, at the cellular level. Finally, we wrap the article with a disclosure of strategies used to impair the generation, kill or re-educate these immunosuppressive cells, thus providing an up-to-date picture of the choices available for therapeutic intervention.

Keywords
Biopolymers, cancer immunotherapy, drug targeting, nanomedicine, lymphatics, oncologicals, tumour associated macrophages, tumour microenvironment

Introduction
Scientific research in the fields of Nanomedicine and Cancer Immunotherapy has advanced tremendously in the last years. In particular, the use of nanotechnology has provided innovative solutions for improving the delivery of cytotoxic agents to the cancer cells. As a result, numerous nanomedicines have been evaluated in clinical studies and some of them are already commercially available. These include liposomes (Myocet®, Doxil®/Caelyx®, Doxisome®, DaunoXome®, Depocyt®, Lipo-Dox®, Marqibo®, Mepact®), albumin nanoparticles (Abraxane®), polymeric micelles (Genexol-PM®), and polymeric (Oncasar®, Zinostim stimulamer®) and monoclonal antibody (mAb)–drug conjugates (Ontak®, Mylotarg®, Zevalin®, Bexxar®) [1–5]. Additional clinical achievements on the delivery of therapies, with significance on cancer treatment, include the first cyclodextrin-based polymer nanoparticles (NPs) delivering siRNA [6], and the first actively-targeted poly(lactic-co-glycolic acid)-polyethylene-glicol (PLGA–PEG) NPs, BIND-014, for the delivery of docetaxel [7,8]. In spite of these and other relevant advances, most of these nano-oncologicals have been designed to reach and act on the tumoural cells, without taking into account the important role of the immune system in the genesis, progression, dissemination and resolution of cancer.

On the other hand, in the last years, it has become clear that there are specific “immune cells”, which, rather than defending our body against cancer antigens [9], have developed relevant pro-tumoural activities [10–14]. In fact, there is strong clinical evidence of the correlation between the presence of these immunosuppressive cells in the tumour, and in a minor extent in the spleen, with the tumour resistance to therapies and with a poor prognosis [15,16]. This evidence suggests that a therapeutic intervention on immunosuppressive cells aimed at their depletion, inhibition of their recruitment or re-stimulation of their cytotoxic function, may represent a promising strategy to fight cancer.

The concept of “cancer-related inflammation”, suggested by Mantovani and colleagues as the seventh hallmark of cancer in 2008–2009 [17–19], has led to a new dimension in the “Cancer Immunotherapy” field. This new conceptual dimension is gaining increasing importance, as noted by the fact that “Cancer Immunotherapy” was considered “the breakthrough of the year 2013” by the journal Science [20], and a full issue on this topic was published in April 2015 [21].
This new and expanded concept of immunotherapy is nowadays associated with the administration of a variety of molecules, such as antibodies, cytokines and vaccines, as well as with gene and cellular therapies [22–24], whose targets may be the cancer cells or the immunocompetent cells. While some of these approaches may represent a breakthrough in oncology, it has become evident that their efficiency will largely depend on the availability of appropriate delivery strategies. The main goal of this article is to provide an overview of the new delivery technology in this field. Starting with a brief description of the nanotechnologies intended for either active or passive immunotherapy (i.e. therapeutic vaccination or mAbs, respectively), this review will address the main issues related to the nature, targeting and therapeutic intervention of the immunosuppressive cells, namely tumour-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), tumour-associated neutrophils (TAN) and regulatory T (Treg) cells (Figure 1).

**Cancer immunotherapy approaches using nanotechnology**

**Approaches based on the administration of mAbs – the concept of immunological checkpoints**

The use of monoclonal antibodies (mAbs) to target cancer cells has been established as one of the most promising anticancer therapies. mAbs have been designed to target, among others, tyrosine kinase (TK) receptors, the epidermal growth factor receptor (EGFR), the insulin growth factor receptor-1 (IGFR-1) and vascular endothelial growth factors (VEGFs) [25–28]. Although more than 10 mAbs have been approved already (National Comprehensive Cancer Network: www.nccn.org) [29], there are still critical problems associated with their inadequate pharmacokinetics and limited accessibility to the cancer cells. Nanotechnology offers the opportunity to improve the mAbs stability in vivo as well as their intracellular accumulation, reducing resistance problems [30–32]. For example, pH-sensitive polycrylic-based nanostructures have been developed to deliver antibodies within the tumour cells [33–35]. Overall, the literature available to date indicates that there is significant room for enhancing the performance of mAb-based therapies [30,36,37].

Within this frame, the idea of targeting the “immune checkpoint blockade” using mAbs to regulate the T-cell response, is one particularly appealing. Molecules that play a key role in checkpoint regulation include: the T-lymphocyte antigen 4 (CTLA-4), the programmed death-1 (PD-1) protein, the T-cell immunoglobulin and mucin domain containing protein 3 (Tim-3) and the lymphocyte activation gene-3 (LAG-3). The higher expression of these markers on tumour cells, intratumoural lymphocytes or Treg cells results in hyporesponsiveness of antigen-presenting cells (APCs) and inhibition of T cells function (immunotolerance) [38], thus facilitating the progression of cancer. Several checkpoint blockade mAbs have been designed to recognize these molecules, receptors or ligands, and hinder their interaction. For example, the anti-CTLA-4 mAb, ipilimumab, received FDA approval in 2011 for the treatment of metastatic melanoma [39,40]. Similarly, pembrolizumab, a mAb targeting PD1, has been recently approved by the FDA for patients previously treated with ipilimumab [41]. Despite these advances, the use of nanotechnology for targeting the immunological checkpoints has been hardly explored. However, given the nature of these pharmacological strategies, there is no doubt that nanotechnology will play a significant role in the delivery of mAb.

Beyond their role as therapeutic entities, mAbs have been proposed as a tool to target chemotherapeutic drugs. We will not expand in this particular approach as it has already been covered in many review articles [42–44].

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**Figure 1. Classification of cancer immunotherapeutics.**
Approaches based on the stimulation of the immune system

Therapeutic vaccination

Specific active immunotherapy, commonly known as therapeutic vaccination, consists on the stimulation of the patient’s immune system with the purpose of fighting the disease (i.e. cancer). The aim of this therapeutic strategy is to target and stimulate the antigen-presenting cells (APCs), i.e. macrophages and immature dendritic cells, which initiate the mechanisms of humoral and cellular immunity, and generate, as a result, a T-cell response [45]. Therapeutic vaccines, specifically designed to treat existing cancers, use attenuated whole cancer cells, tumour lysates, or purified tumour antigens, with or without adjuvants, to stimulate the patient’s immune system. Numerous nanostructures, including liposomes, micelles and polymeric NPs, have been developed to optimize the delivery of cancer antigens [46,47]. Some liposomal formulations, such as Emepemut-S (Stimuvax™), MAGE-3 and PRAME, indicated for the treatment of non-small cell lung cancer, are already in the advanced phases of clinical trials [48]. Interestingly, polymeric nanostructures, composed of PLGA or Chitosan (CS), which had been previously used to prepare micro- and nano-vaccines for infectious diseases [49–52], have been more recently applied to improve the delivery of cancer nano-vaccines [53,54]. For example, PLGA NPs encapsulating antigenic anti-tumour peptides (i.e. MART-1 and MHC class Ia, Ib and class II-restricted peptides) have shown effective anti-tumoural responses mediated by enhanced T-cell responses [55–58]. Similar results have been obtained using CS NPs decorated with the antigen K-ras mutant peptide for pancreatic cancer vaccination [59]. More importantly, there is already clinical evidence of the value of these polymeric carriers in anti-cancer vaccination (http://clinicaltrials.gov/ct2/show/NCT00005023).

Cytokines, interferons and other immunostimulants

Non-specific active immunotherapy refers to the stimulation of the immune system using immunostimulant molecules, to inhibit the progression of tumours in early stages, and to induce protective immunity. Several agents, such as monophosphoryl lipid A (MPLA) and imiquimod, which trigger the activation of Toll-like receptors (TLR) on APCs, have been approved by the FDA for the treatment of cancer [60]. Cytokines and interferons (IFNs) are also proteins secreted by cells of the immune system, which are known to activate anti-tumour immunity. Some of these molecules, such as IL-2 [61,62] or Type I IFNs [63], have already been applied in the clinic and several nanotechnologies have been adapted for their delivery alone or in combination with antigens. For example, Sánchez et al. reported the use of biodegradable PLGA/poxolaxmer micro- and nanoparticles for the controlled delivery of active IFN-alpha for up to 96 days [64]. In another example, PLGA NPs were reported to co-encapsulate two antigenic peptides (hgp100 and p180–188) and an immunostimulant MPLA. This nanovaccine was found to delay the growth of a melanoma tumour in a mouse model [65].

Overall, the results achieved in the field of vaccination and immunostimulation using nanotechnology underline the potential to enhance anti-cancer responses using appropriate combinations of antigens and immunomodulators.

Approaches based on the modulation of immunosuppressive cells

Over the last decade it has been shown that specific populations of “Immunosuppressive Cells”, including tumour-associated macrophages (TAM) [10], myeloid-derived suppressor cells (MDSC) [11], tumour-associated neutrophils (TAN) [12], and regulatory T cells (Treg) [13] have a key role in supporting tumour growth, invasion and metastasis [14,17,66]. Furthermore, these immunosuppressive cells are known to support the survival and proliferation of tumoural cells, promote tumour angiogenesis and suppress adaptive immune responses [10].

In contrast to the well-established therapeutic approaches presented above, those based on the modulation of immunosuppressive cells are still emerging and the use of nanotechnology to support these approaches has been hardly considered. In view of the promising impact of nanotechnology in this particular area, on the subsequent sections, we will present an overview of the knowledge concerning the nature and function of these cells, their localization, and strategies to target them and influence their activity.

The nature and function of immunosuppressive cells

TAM are important regulators of tumourigenesis that may represent up to 50% of the tumour mass [14,67–69]. TAM derive from circulating lymphocyte antigen 6C (Ly6C+) monocytes originated in the bone marrow, and also from reservoirs in the spleen [70]. Upon reaching the tumours, these monocyte-derived macrophages receive, from tumour cells, local cytokine and inflammatory signals that influence their phenotype and functions [71]. This plasticity is a key hallmark of monocyte-macrophage lineage and explains its capacity to acquire distinct functions and phenotypes in response to distinct microenvironmental signals [72]. At the extremes of their functional and phenotypic continuum, macrophages range from M1 (anti-tumoural) to M2 (pro-tumoural) polarization states. M1 cells are involved in efficient antigen presentation and pathogen killing, secreting high amounts of proinflammatory cytokines and promoting Th1 cell responses. In contrast, M2 macrophages show high phagocytic activity, poor antigen presentation properties, contribute to the dampening of inflammation, promote wound healing, angiogenesis, tissue remodelling and support tumour progression [73]. In advanced solid tumours, TAM are polarized towards M2-like macrophages by the secretion of cytokines (IL-4, IL-13) from polarized Th2 cells, metabolic signals (lactic acid and hypoxia) or byproducts of tumour cells themselves (e.g. TGF-β, CSF1, CCL2). These TAM promote suppression of innate and adaptive immune responses via different pathways, including the production of immunosuppressive cytokines (e.g. IL-10 and TGF-β), prostanoid (PGE2), indoleamine 2,3-dioxygenase (IDO) metabolites and expression of PDL1 (B7H1). Furthermore, TAM are a major source of proteases, such as cysteine...
cathepsins, which support tumour progression and therapeutic resistance in multiple cancer types [74].

Based on this knowledge, it seems reasonable to expect that the development of new strategies to manipulate the M1/ M2 balance in tumours will allow us to effectively impact the resolution of the disease. The goal would be to target and re-educate TAM from their immunosuppressive M2-like phenotype into a tumour-suppressing M1 phenotype. Alternative approaches might consist on killing the TAM, inhibiting their function or blocking their recruitment to the tumour (see the “Therapeutic interventions on TAM” section).

MDSC are a heterogeneous population of activated immature myeloid cells that are characterised by a morphological mixture of granulocytic and monocytic but lack the expression of cell-surface markers associated with fully differentiated monocytes, macrophages or dendritic cells [75]. In tumour-bearing mice, MDSC accumulate within primary and metastatic tumours, in the bone marrow, spleen and peripheral blood. MDSC are defined in mice by the characteristic co-expression of myeloid lineage differentiation antigens Gr-1 and CD11b [76]. The equivalent MDSCs in humans are defined by the CD14-CD11b+CD33+CD15+ phenotype or cells that express the CD33 marker but lack the expression of the major histocompatibility complex (MHC) class II molecule HLA-DR [77]. MDSC mediate their tumour-induced immunosuppression through several mechanisms involving the inhibition of T-lymphocytes, but also through their action on other cells such as Treg [77]. Overall, the accumulation of MDSC has been associated with the progression of human cancers, and MDSC elimination was shown to improve anti-tumour immune responses [77]. As in the case of TAM, therapeutic strategies targeting MDSC include: the prevention of their generation, their elimination, the inhibition of their immunosuppressive functions or even their conversion into mature APCs (see the “Therapeutic interventions on MDSC” section).

TAN have received little interest as targets for immuno-therapeutic drugs due to their short life span. However, nowadays, it is known that certain cytokines, such as IL-1, or microenvironment conditions, such as hypoxia, can prolong neutrophils survival [78]. In addition, it is known that TAN can differentiate between N1 (anti-tumoural and N2 (protumoural) type neutrophils [79]. TAN are commonly found in several tumours, including kidney, breast, colon and lung [80], and a correlation between TAN infiltration and poor outcome has been described in renal cell carcinoma, bronchoalveolar cell carcinoma, hepatocarcinoma and breast cancer [12,81]. TAN contribute to tumour growth by promoting angiogenesis, cell proliferation and metastasis [12]. As was the case with macrophages, the depletion of TAN, impairment of their recruitment to the tumour, and, their re-education into anti-tumoural (N1) neutrophils will have a significant impact in the treatment of cancer.

FOXP3+CD25+CD4+ regulatory T (Treg) cells, crucial for the maintenance of immunological self-tolerance, are found at high frequencies in tumour tissues of various types of cancers, such as breast, lung, liver, pancreatic and gastrointestinal cancers and malignant melanoma [82]. Treg can be considered as the best characterized immunosuppressive cells, consisting in the a population of CD4+ T cells with high-level expression of the IL-2 receptor α chain (CD25) and the transcription factor forhead box P3 (Foxp3) [83]. Treg cells are recruited into tumours by chemokines (i.e. CCL22) produced by tumour cells or TAM. Once in the tumour, Treg cells are activated and expanded, presumably, via the recognition of tumour-associated antigens released from dying tumour cells (i.e. surviving) [84]. It is worth noting that, Treg cells are better than effector T cells at recognizing these antigens, because of their sensitive TCR repertoires [85]. Indeed, Treg cells are able to suppress the activation and expansion of tumour-antigen-specific effector T cells in the tumour. For example, the immune response to the HLA-DP-restricted NY-ESO-1 peptide antigen in cancer patients was hampered by the presence of Treg cells. Furthermore, the depletion of Treg cells has been shown to be able to activate and expand effector T cells, thus mediating potent anti-tumour immune responses [86,87].

Taking into account the most recent discoveries concerning the important role of the immunosuppressive cells in the origin, progression and resolution of tumours, it is expected that a significant number of therapies targeting these immunosuppressive cells will be designed in the immediate future. Indeed, this paradigmatic change in oncology has already started and it is envisaged that major improvements in cancer therapy may be associated to the adequate engineering of nanocarriers. Some strategies for the targeting and impacting on the immunosuppressive cells have already been endeavoured, as described in the following sections.

**Targeting immunosuppressive cells**

**Tissular targeting: tumour, spleen, lymph**

In cancer patients, the “harmful” immunosuppressive cells accumulate predominantly in the spleen and in the tumour; whereas the blood and the lymph are the mainline fluids through which these cells (i.e. TAM) and their precursors (i.e. monocytes) are transported. Consequently, to rationally design nanotherapies specifically directed to these cells, we need to understand the anatomical and physiological barriers they must surmount to reach these compartments. Traditionally, the nanotechnological approaches to improve the delivery of anti-cancer drugs to the tumoural tissue have been classified into passive and active mechanisms of targeting. The passive targeting of nanoscale structures to the tumoural tissue can be achieved through the manipulation of their physicochemical properties, such as size, shape, charge and stability [2,88,89]. This preferential accumulation of NPs in the tumour, coined as the enhanced penetration and retention (EPR) effect, was attributed to their prolonged blood circulation time and their subsequent transport across the fenestrations in the imperfect tumour blood vessels. This phenomenon, being influenced by a number of biological processes, among them, angiogenesis, vascular permeability, hemodynamic regulation, and lymphangiogenesis [90], has inspired the use of VEGF inhibitors [91,92], hypertensive molecules (i.e. angiotensin II) or vasodilators (i.e. nitric oxide or prostaglandins) [93], to improve the tumour accumulation of NPs, through the manipulation of the blood vasculature.

Once the NPs are extravasated into the tumour, the main challenge for the delivery of the therapeutic molecule(s) to the
immunosuppressive cells, is the nature of this abnormal tissue. Tumours are complex tissues, comprising not only malignant cancer cells but also stromal cells, such as endothelial cells, fibroblasts and immune cells among many others [14,17,19,94]. This heterogeneous population of cells plus the extracellular matrix they produce constitute the tumour microenvironment (TME) [95]. The immunosuppressive cells have the ability to regulate the TME through their engagement in complex bidirectional interactions with tumour cells, cancer stem cells, fibroblasts, mesenchymal stem cells, endothelial cells, and T, B, and NK cells [14,17,19,94]. This intricate network influences the response to therapy [95,96]. Within this context, NPs offer an interesting potential given the avidity of macrophages towards them [97,98].

The drug-loaded long-circulating nanocarriers accumulate not only in the tumour tissue but also in highly irrigated organs, preferentially liver and spleen. Paradoxically, the preferential accumulation of NPs in the spleen, which was initially viewed as a potential limitation for the use of NPs in medicine, is now considered an opportunity for targeting the splenocytes. Indeed, the spleen known as a key organ for the origin and accumulation of immunosuppressive cells in cancer patients is a key targeting compartment for cancer immunotherapeutics. Several splenic immune cell populations (i.e. T and B cells, dendritic cells and macrophages) remain in the spleen until they are activated. In addition, immune cells are recruited to the spleen in response to a diseased state (i.e. cancer), and, then, mobilized from the spleen to other tissues (i.e. the tumour). Actually, a constant supply of immunosuppressive cells from the spleen towards the tumour has been described [99]. All these data suggest that the therapeutic targeting of the spleen is important for controlling tumour-induced myelopoiesis and immune suppression.

The delivery of therapeutics to the spleen allows to target the monocytes, precursors of the immunosuppressive cells, before they reach the tumour. For example, upon intravenous administration, siRNA-CCR2 NPs were shown to reach their target receptor on inflammatory Ly6C hi monocytes in the administration, siRNA-CCR2 NPs were shown to reach their before they reach the tumour. For example, upon intravenous targeting of the spleen is important for controlling tumour-described [99]. All these data suggest that the therapeutic targeting of tumoural cells and macrophages in tumours [106–110] (for more information see the “Cellular targeting: how countermands [103].

The characteristics mentioned above make the lymphatic system a compartment of preferential interest for the delivery of anti-cancer–immunomodulatory–nanomedicines. While several strategies have been attempted to reduce lymphangiogenesis, for example the use of PEI-alginate NPs containing VEGFR-3 siRNA [104], other lymphotargeting strategies have been designed to reach metastatic and immunocompetent cells. Our team, as leader of the LYMPHOTARG European Consortium, has found that lipid nanocapsules surrounded by a polymeric shell accumulate significantly in the lymphatics [105]. Other strategies relied on the use of targeting ligands. For example, the use of the peptide, LyP-1 led to an increased affinity for tumour lymphatics and specific targeting of tumoural cells and macrophages in tumours [106–110] (for more information see the “Cellular targeting: how to reach the immunosuppressive cells?” section).

Overall, in addition to the possibility of using targeting ligands, the accumulation of nanocarriers in the tumour, spleen and lymphatics is dependent on the modality of administration (iv versus sc) and also on their physicochemical properties, i.e. size, shape, surface charge and stability. Following iv injection, small size nanocarriers may be removed from the blood by renal clearance (<5 nm) or rapid liver uptake (10–20 nm), whereas the larger ones (>200 nm) are filtered in the sinusoidal spleen or are recognized and cleared by the mononuclear phagocytic system (MPS) [111]. Those with a size between 20 and 200 nm may remain in the circulation for an extended period of time. A recent study showed that different-sized particles (30, 50, 70 and 100 nm) with similar blood circulation profiles distributed comparably in tumours when the tumours were hyperpermeable (i.e. murine colon adenocarcinoma), however, only NPs smaller than 70 nm accumulated efficiently in poorly permeable tumours (i.e. human pancreatic adenocarcinoma) [112]. It has also been shown that the shape of the NPs influences their interaction with the MPS. For example, elongated polymer micelles, designated as filomicelles, remained in circulation 10 times longer than their spherical counterparts [113]. The charge of the NPs may also influence
their recognition by the MPS and their overall plasma circulation profile and intratumoural distribution [111]. The positive charges commonly favour interactions of the nanostructures with the tumour blood vessels and limit their diffusion in the tumour [114,115]. Finally, the development of strategies to enhance the in vivo stability of drug-loaded NPs remains a challenge for an efficient tumour-targeting strategy [116].

In conclusion, strategies aimed at targeting the different body compartments (tumour, blood, lymphatics and spleen) must be considered in order to reach the immunosuppressive cells. A description of the state of the art of the molecular targeting approaches for each specific population of immunosuppressive cells is provided in the next section.

**Cellular targeting: how to reach the immunosuppressive cells?**

The tumour passive targeting strategy mentioned in the previous section has often resulted in an irrelevant enhancement of the therapeutic effect [117]. A broad spectrum of “active targeting” strategies has been designed, involving the decoration of NPs with specific ligands, such as antibodies, peptides, aptamers, saccharides, folic acid (FA) and other small molecules [7,8,106–108,118–129]. The identification of molecules exclusively present in TAM, MDSC or TAN maybe an unrealistic objective due to the nature and plasticity of these type of cells. However, the indiscrimination among these cell populations might be seen as an advantage in the design of specific therapies. For example, both MDSC and inflammatory monocytes are identified as Ly6C highCCR2+ precursors and have a synergistic impact on the treatment of cancer.

Most of the work aimed at identifying specific targeting strategies has been oriented to TAM (Table 1). Many biomarkers are overexpressed on the surface of TAM (M2-like macrophages) when compared to M1-like or even resting “non-activated” macrophages. These biomarkers include the mannose receptor (MR or CD206) [131–133], the haemoglobin scavenger receptor CD163 [134], the galactose-type lectin (Mgl) receptor [135], the p32 receptor, neutrophil-1 and -2 (NRP1 and NRP2) receptors, triggering receptor expressed on myeloid cells-1 (TREM-1), the folate receptor beta [136–138] and the legumain receptor [139–141]. The pharmacological and delivery strategies making use of these targeting receptors is disclosed in detail in the next paragraphs.

Sugars, such as mannose and galactose have been used as ligands for the targeting of nanomedicines to TAM. The mannose receptor (MR or CD206) [131–133] is expressed on the surface of macrophages and dendritic cells for the recognition of both, endogenous and pathogen-associated ligands [142]. Interestingly, the level of expression of this receptor is higher in M2 macrophages than in M1 macrophages, thus making it particularly suitable for the targeting of TAM [143]. A number of mannosylated nanocarriers aimed at reaching TAM, including liposomes [144] and PLGA NPs [145], have been described so far. In order to favour the uptake of the mannosylated PLGA NPs by the TAM, the NPs were shielded with PEG moieties, which were released upon degradation of the linker at the acidic pH of the TME (~pH 6.8) [145]. Such a carrier has been used for the delivery of doxorubicin (DOX) [146] and siRNA into TAM [147]. In another experiment, galactosylated cationic dextran nanocomplexes were engineered to deliver oligonucleotides to the Mgl receptor expressed by TAM [135]. These NPs were coated with a pH-sensitive PEG-histidine-modified alginate (PHA), so as to trigger their delivery specifically in the acidic TME. This nanomedicine, designed to inhibit IL-10 signalling, was able to switch the M2 protumoural phenotype of TAM to proinflammatory macrophages (M1) and simultaneously activate macrophages and dendritic cells via TLR9. The result was a significant suppression of the tumour growth in an hepatoma murine model.

An array of peptides and proteins has also been used to target TAM specifically. For example, LyP-1 peptides, known for their affinity for the p32 receptors overexpressed in TAMs [108,109,148] have been used for the design of TAM targeted NPs [108]. In particular, LyP-1-conjugated PEGylated liposomes loaded with DOX were shown to reach TAM in metastatic lymph nodes causing the inhibition of lymphatic tumour metastasis [108]. The truncated form of LyP-1 (tLyP-1) targets NR1 and NR2 receptors, which are overexpressed in human TAM [68]. In addition, tLyP-1 is known to promote the penetration through the tumour tissue, property that makes this peptide particularly attractive for the decoration of NPs. Another peptide, known as GF9, has also been used as a ligand to target lipoprotein-based NPs to TREM-1 [149]. Similarly, the protein “Legumain” and the Ly6C antibody were proposed for the targeting of liposomes and silicon NPs, respectively, intended to re-educate TAMs [141,150,151] (this issue is discussed in detail in the “Strategies to kill TAM” section). Finally, the targeting of TAM to the folate receptor was also explored using folate-conjugated liposomes loaded with zoleodronic acid [137,138]. Unfortunately, the results obtained upon administration of this formulation to KB (human nasopharyngeal) and colon 26 (mouse colon adenocarcinoma) tumour-bearing mice were negative.

In addition to the approaches to target TAM, some authors have dedicated efforts to discriminate other cell populations. In particular, Fridlender et al. [152] conducted a transcriptomic analysis of TAM, naïve bone marrow neutrophils (NN) and granulocytic MDSC and concluded that these two cell populations differ markedly in their genetic profile. Other authors have described the molecular characterization of Treg cells [83] and identified mAbs as well as other molecules aimed to reach specific receptors (CD4, CD25, CTLA-4, GITR, OX40 and folate receptor-4) [153,154]. Unfortunately, the efficacy of these molecules to target Treg cells was not satisfactory. On the other hand, the specific “cellular targeting” of MDSC, TAN or Treg cells has hardly been explored. From our knowledge, the only example attempting to target MDSC was based on the use of RNA aptamers with specific affinity for the murine or human IL-4 receptor α (IL-4Rα/CD124) [155]. The targeted aptamers showed a preferential binding to M-MDSC in the spleen, and to M-MDSC and TAM in murine tumours. However, the selectivity of this approach is limited because the IL-4...
**Table 1. Cellular targeting: nanocarriers designed to reach TAMs.**

| Strategy/nanomedicine | Receptor | Observations | Reference |
|-----------------------|----------|--------------|-----------|
| AzEAMA, 2-azidoethyl methacrylate; BMA, butyl methacrylate; DMAEMA, 2-(dimethylamino)ethyl methacrylate; FITC, fluorescein isothiocyanate; PAA, 2-propylacrylic acid; PEG, polyethylene glycol; PLGA, poly(lactic-co-glycolic acid). |

**Therapeutic interventions on immunosuppressive cells**

**Therapeutic interventions on TAM**

**Strategies to impair the generation of TAM**

TAM are primarily derived from inflammatory monocytes that are recruited into the tumour [157]. Thus, it is feasible to impair the generation of TAM by targeting and impacting the monocytes, either in the blood or in the lymphoid organs (Figure 2). A relevant example of this strategy is the co-delivery of colony stimulating factor-1 receptor (CSF-1R) antagonists and common cytotoxic drugs, which resulted in a significant decrease of primary tumour progression and metastasis. The use of a mAb (RG7155), inhibiting CSF-1R, has been reported to reduce TAM and increase T cells in a murine model of colon carcinoma [158]. In the same study, patients treated with the same mAb showed satisfactory responses, with striking reductions of TAM in tumour tissues. Despite these positive results, the targeting of CSF-1R using NPs has not been explored yet.

The first nanotechnology-based approach aimed at inhibiting the recruitment of macrophages was disclosed in 2011 [100] (Table 2). Their approach used siRNA-loaded lipid NPs intended to inactivate the chemokine receptor CCR2, which is required for the monocyte recruitment to the tumour. Following intravenous administration these NPs accumulated in the spleen and bone marrow, where they delivered the associated siRNA into the Ly6C<sup>high</sup> monocytes, precursors of TAM. Importantly, the application of this approach on two different mouse tumour xenograft models resulted in lower accumulation of TAM and reduced tumour volume [100].

**Strategies to kill TAM**

Different strategies have been developed to kill TAM specifically (Figure 2). Importantly, at the end of 2013,
Figure 2. Strategies for the therapeutic intervention of immunosuppressive cells. Molecules which showed therapeutic effects and could be used in nanomedicines for killing, impairing the recruitment or re-educating the immunosuppressive cells into anti-tumoural cells.
Germano et al. observed for the first time the positive effect of the cytotoxic drug Trabectedin [159] on the depletion of TAM. They showed that this anti-tumour agent, registered for soft tissue sarcoma and ovarian cancer, induces selective apoptosis in human and mouse monocytes, but not in other leukocytes, such as granulocytes and lymphocytes. They further demonstrated the specific depletion of circulating and splenic Ly6Chigh monocytes and the reduction of TAM in four different mouse tumour models. The clinical relevance of this finding was confirmed by the reduction of monocytes and TAM in soft-tissue sarcoma patients treated with the drug [159]. Apart from this important discovery, the use of nanotechnological approaches for the targeting of cytotoxic drugs towards TAM has just started (Table 2). Niu et al. have recently reported the use of PLGA mannosylated NPs for the delivery of DOX to TAM [146]. These NPs were able to deplete TAM in a mouse model of human melanoma, thereby improving significantly the anti-tumour efficacy of DOX.

A different category of drugs, whose toxic effect against TAM has benefited from the nanotechnology are the bisphosphonates (Table 2). Overall, the results in different animal models using these drugs encapsulated in liposomes were a reduction on tumour growth, impaired angiogenesis and decreased metastasis [160–165]. In a recent study, the authors compared the effect of liposomes encapsulating two different bisphosphonates: zoledronate or clodronate [166], using an in vitro co-culture containing macrophages and breast cancer cells. The results showed that clodronate-liposomes were very toxic for TAM, whereas zoledronate-liposomes promoted the re-education of TAM. Other researchers analysed the synergistic effect of liposomal clodronate and an anti-VEGF antibody and the results were very positive in terms of reducing tumour blood vessels density and inhibiting tumour growth [165]. Finally, the bisphosphonate, alendronate was also administered in the form of a conjugate with glucosannan [167], a polysaccharide that was intended to target mannose receptors in TAM. The results obtained following intratumoural injection of this conjugate to sarcoma-bearing mice, evidenced the efficient depletion of TAM [167]. In conclusion, the impact that bisphosphonate liposomal formulations have showed on TAM was dependent on the type of bisphosphonate, the delivery carrier and the co-administration of synergistic drugs.

**Strategies to re-educate TAM**

The re-education of TAM consists in the re-programming of macrophages with M2-like protumour properties as M1-macrophages with active defensive activity, including anti-tumour functions (i.e. direct killing of tumour cells and eliciting of vascular damage and tissue destruction) [72,74]. Several pharmacological agents with the ability to re-switch the polarization of macrophages from M2-like immune-suppressive macrophages to M1-like cytotoxic effectors have been discovered in the last years [168] (Figure 2). IFN-γ is known to activate the M1 polarization of M2 macrophages [169], however, its clinical application has been hindered by a variety of side effects. More effective approaches include the CD40 antibody used in patients with advanced pancreatic cancer [170] or BLZ945 (a highly selective small molecule
inhibitor of CSF-1R) used in patients with glioblastoma multiforme [171]. Both strategies resulted in a clear enhancement of the patient’s survival [25,171].

Despite the promising results achieved with the therapies indicated above, a more effective and long lasting functional repolarization of TAM is still needed. To achieve this goal specific nanotechnological approaches have been explored (Table 2). For example, Huang et al. designed nanocarriers for the delivery of anti-IL-10 and anti-IL-10RA oligodeoxynucleotides (ODN) into TAM [135]. These nanocarriers were made of PEG-modified alginate meant to be released in the acidic microenvironment of the tumour, and galactosylated cationic dextran (gal-C-dextran), intended to associate ODN and to target a galactose-type lectin highly expressed in TAM. Their experiments using an allograft hepatoma murine model showed that, after intravenous administration, these nanosystems accumulated into TAM, thereby inducing a suppression of the protumour functions and stimulating the anti-tumour activities of TAM [135].

Another approach to re-educate TAM was based on the use of hydrazinocurcumin-loaded legumain-targeted liposomes. Following intravenous administration to a mice model of metastatic breast cancer, this formulation was able to decrease the tumour cell proliferation, migration and invasion. Mechanistic studies revealed that this activity was associated to the suppressed STAT3 activity and the reversion of M2-like polarization to an M1 phenotype [141]. Similarly, the association of imidazole, another inhibitor of STAT3 activation, to legumain-targeted liposomes [150] in combination with an HER-2-DNA vaccine, resulted in the protection from cancer recurrence through improved immune surveillance against a tumour-specific antigen.

**Therapeutic interventions on MDSC**

The reduction of the number of MDSC could be achieved through their specific elimination, but also by preventing their generation (Figure 2). The generation of MDSC can be prevented by manipulating the signalling pathways that are responsible for their expansion. The extracellular signalling molecules involved in this process include different cytokines, growth factors and transcription factors [11]. For example, STAT3 activation has a primary role in promoting MDSC proliferation and survival, hence its inhibition by peptides and chemical compounds may be exploited to interfere with cancer-induced MDSC accumulation [172]. Moreover, STAT3 signalling has been reported to control MDSC function by upregulating ARG1 expression [173]. Some authors have identified a way to manipulate this pathway in MDSC through the use of curcumin-loaded exosomes [174] (Table 3).

Interestingly, several studies have demonstrated that low doses of cytotoxic drugs are able to kill MDSC (Figure 2). Some of these chemotherapeutics are: sunitinib, sorafenib, bortezomib, gemcitabine, fludarabine, 5-fluorouracil and docetaxel [130,175,176]. Among them, gemcitabine and 5-fluorouracil have been reported to exert the strongest cytotoxic activity against MDSC, at doses that have a minimum or no effect on tumour growth [130,175]. It could be, then, expected that the loading of these drugs into adequate carriers could further enhance the efficiency of this strategy. Finally, the specific elimination of MDSC could also be achieved using IL-4Rz RNA aptamers, as described in the previous section (targeting MDSC) [155].

There is only preliminary knowledge on the possibility to re-educate or re-program MDSC, by abolishing their immunosuppressive functions or, even better, by converting them into mature APCs with anti-tumoural properties. For example, the all-trans-retinoic acid, a derivative of vitamin A primarily employed in the treatment of acute myeloid leukaemia, has been found to promote the in vitro differentiation of mouse splenic MDSC [177], or immature myeloid cells [178] into more mature cells devoid of immunosuppressive activity. Several other drugs, such as phosphodiesterase-5 (PDE5) inhibitors [179], nitroaspirin [180] or AT38 [181], have been used to reduce iNOS and ARG1 expression, resulting in the inhibition of MDSC immunosuppressive functions (Figure 2).

The first example of NPs inducing the re-education of MDSC in mice has been reported for a vaccine adjuvant, based on very small size proteoliposomes (VSSPs) [182] (Table 3). VSSPs were prepared by the combination of the outer membrane protein complex from Neisseria meningitides and were tested in clinical trials as such for immunotherapy.
and accumulation of CD11b+ myeloid cells, with consequent mice, VSSPs were found to induce the immunostimulation of tumour models [182]. Namely, following injection to healthy mice, VSSPs were found to induce the immunostimulation and accumulation of CD11b+ myeloid cells, with consequent splenomegaly. Nevertheless, these splenic myeloid cells did not exert any relevant immunosuppression on T cells. In addition, the injection of this adjuvant in tumour-bearing mice was able to modulate the phenotype of splenic MDSC towards APCs and strongly reduce their ability to impair cytotoxic T cell activation [182,186]. A similar example was provided by Thomas et al., who showed that poloxamer-stabilized poly (propylene sulphide) NPs (30 nm), loaded with either CpG or paclitaxel, were able to specifically target tumour-draining lymph nodes and reduce MDSC activity [187] (Table 3). Thus, overall, the delivery of CpG oligonucleotides and other immunostimulants through nanosystems could be considered in future studies as a strategy to interfere with MDSC differentiation and activity.

Therapeutic interventions on TAN

The current understanding of TAN biology and the development of therapeutic strategies for their manipulation is in its infancy (Figure 2). Tumoural cells recruit neutrophils through the release of chemokines, thus theoretically, the therapeutic targeting of these mediators would result in the impairment of neutrophils recruitment. Several strategies targeting the CXCL8/CXCR1-CXCR2 axis have been attempted. For example, using an IL-8 neutralizing antibody, it was found an effective inhibition of neutrophils influx and reduction in tumour angiogenesis and invasation in models of fibrosarcoma and prostate cancer [188]. Other strategies have been explored with the aim to block the protumoural functions of TAN (re-education). For example, the inhibition of TGF-β was also shown to be able to ‘‘re-educate’’ – N2 (protumoural neutrophils) towards an N1-like (anti-tumoural) phenotype [189]. In the same study, the monoclonal anti-ly6G antibody 1A8 was used for the systemic depletion of neutrophils in mice previously treated with the TGF-β inhibitor, SM16. This strategy resulted in a significantly reduced effect of SM16, confirming that neutrophils participate on the anti-tumour activity of TGF-β blockade. Importantly, in another study, it was found that TAN from early tumours are more cytotoxic toward tumour cells and produce higher levels of TNF-α, NO and H2O2, while in established tumours, these functions are down-regulated and TAN acquire a more protumoural phenotype. Thus, the depletion of neutrophils at late stages of the tumour development could result in the inhibition of the tumour growth [190]. In conclusion, there are possibilities for killing or re-educating TAN, and the development of nanotechnological approaches for the specific targeting of these cells is expected to help to reach this goal.

Therapeutic interventions on Treg cells

Strategies to impact Treg cells include their depletion or their functional ‘‘re-education’’ (Figure 2). Anti-CD25 mAbs, specifically targeting the IL-2 receptor, have been demonstrated to augment anti-tumour immune responses through the depletion of Treg cells. However, the same receptor is also present in effector T cells resulting in their concomitant elimination [191,192]. Interestingly, a recent study combining anti-CD25 and anti-CTLA-4 antibodies has led to promising results in the treatment of prostate cancer with immunotherapy [193]. The application of anti-tumoural drugs, such as cyclophosphamide or fludarabine, in combination with vaccines has led to the reduction of the number of Treg cells and the enhancement of immune responses, improving the survival of renal cell carcinoma patients [194]. Other molecules, such as the glucocorticoid-induced TNF-receptor family (GITR) protein or OX40 [195], have been used to suppress the activity of Treg cells and activate effector T-cell function, thus being important candidates for the ‘‘re-education’’ of Treg cells. Finally, the clinical benefit of the immune-checkpoint blockade could be attributed, at least in part, to the depletion or re-education of Treg cells from tumour tissues [196]. Notably, the use of anti-CTLA-4 mAb (i.e. Ipilimumab) is known to promote the depletion of Treg, rather than activation of effector T cells, which results in augmented anti-tumour immunity [197]. Similarly, the blockade of PD-1 signalling was shown to cause both, decreased Treg cell ratios and augmented T effector cell function in a murine melanoma model [198]. Overall, we foresee that combination therapeutics for reducing Treg cells or attenuating their suppressive activity in tumour tissues together with therapeutics for the activation and expansion of tumour-specific effector T cells (i.e. by cancer vaccines) will significantly improve the outcome of patients with cancer.

Conclusions: the future of cancer nano-immunotherapy

The use of nanotechnology with the purpose of enhancing the immune response against cancer is receiving increasing attention. Here, we provide our view of the most relevant ‘‘cancer nano-immunotherapeutics’’ developed so far and the strategies designed to tackle them. Immunotherapeutic strategies, involving the use of mAbs or therapeutic vaccines, have provided important advances for the treatment of cancer through the targeting of cancer cells or APCs, respectively; however, the therapeutic intervention of immunosuppressive cells remains to be properly addressed. Although, the key role of tumour associated macrophages, myeloid derived suppressor cells, tumour associated neutrophils and Treg in the development of the tumour has been established, a better understanding of the phenotypic and functional properties of these cells is still required. In addition, an improved knowledge of the plasticity of these cells and their adaptation to the microenvironment could provide new ideas for the development of novel immunotherapies.

Among the immunosuppressive cells, TAM, representing in some cases up to 50% of the tumour mass, are known to play a major role in the development of the tumour and also in the response to therapy. Several studies have used nanotechnology aimed at targeting TAM and at the pursuit of therapeutic intervention strategies against TAM, and several researchers have presented promising pre-clinical results, which, hopefully, will be soon translated into clinical applications. However, the main problems for the effectiveness of this
approach are still the specificity of the tissue (i.e. the tumour instead of other tissues with high abundance of macrophages such as the liver) and cellular targeting (i.e. TAM instead of “healthy” tissue macrophages). Although, less information is available on the biology and therapeutic intervention of MDSC or TAM than of TAM, some strategies have also been presented on the former two types of cells. Interestingly, the targeting and manipulation of MDSC in the spleen could provide an important alternative to the tumour targeting in some types of cancer. However, further investigations are needed for the precise characterization of these cells in each tissue. The targeting of Treg cells may also be of foremost importance on the treatment of some cancers. Recent clinical investigations point out the targeting of the “immune checkpoint blockade” (i.e. PD-1), resulting in the unleashing of an appropriate T cell response, as a breakthrough in the treatment of cancer. In this regard, we would suggest the precise manipulation of the regulatory effector T cell ratio as the next step to be further explored.

Overall, the nanotechnological approaches explored so far have already led to an improvement in the concentration of the drug at the appropriate site for its action (i.e. TAM in tumours or MDSC in spleen). We expect that the development of nanostructures allowing a sustained release of the drug on the immunosuppressive cell will be of foremost importance for the effective and maintained re-education of these cells. Furthermore, we expect that the next step will be the engineering of novel nanostructures allowing the combination of several immunomodulatory molecules (i.e. antibodies, cytokines, other immunomodulatory molecules) and/or anti-tumoural drugs (i.e. cytotoxic drugs) into the same nanomedicine. Several recent clinical trials have evaluated the combination of immunotherapy with “targeted therapies”, showing encouraging results. It is highly expected that ongoing investigations in the field of cancer immunology will reveal soon new “molecular biomarkers” of immunosuppressive cells, which will allow for a rational molecular design. The development of novel nanotechnological approaches will be crucial to make the most of these discoveries.

In summary, we foresee that the combination of knowledge acquired in the fields of nanotechnology, immunology and cancer will lead to the development of novel nanomedicines with cancer immunotherapeutic properties, which might represent a break-through in the treatment of cancer.

Declaration of interest

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