Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been and is still widely used as an adjuvant in clinical trials of vaccination with autologous tumor cells, peptides and/or dendritic cells in a variety of human neoplasms. This cytokine was administered either as product of gene-transduced tumor cells or as recombinant protein together with the vaccine given subcutaneously or intradermally. Results of these trials were heterogeneous in terms of induction of vaccine-specific immune response and of clinical response. Though in some of these studies GM-CSF appeared to help in generating an immune response, in others no effect or even a suppressive effect was reported. Here, we review the literature dealing with the immune adjuvant activity of GM-CSF both in animal models and clinical trials. As a consequence of such analysis, we conclude that GM-CSF may increase the vaccine-induced immune response when administered repeatedly at relatively low doses (range 40–80 μg for 1–5 days) whereas an opposite effect was often reported at dosages of 100–500 μg. The potential mechanisms of the GM-CSF-mediated immune suppression are discussed at the light of studies describing the activation and expansion of myeloid suppressor cells by endogenous tumor-derived or exogenous GM-CSF.

Key words: adjuvant, GM-CSF, immune stimulation, immune suppression

introduction

Different cytokines and chemokines have been used as immune adjuvants to facilitate antigen recognition and T cell expansion in vaccination studies both in animal models and in humans. One of the most frequently used cytokine is GM-CSF which has been administered both as cytokine released by gene-transduced tumor cells or even normal bystander cells, and as recombinant protein (given either systemically or locally) to animals or patients receiving differently formulated vaccines. However, GM-CSF, along with other cytokines, can be directly released by un-manipulated human tumor cells of melanoma [1], prostate [2], ovarian [3] and lung cancer [4]. Moreover, such an endogenous production of GM-CSF has been linked to the presence of CD34+ myeloid suppressor cells (MSC) infiltrating GM-CSF-producing head and neck carcinomas [5]; these MSC can release TGF-β which in turn inhibits T cell functions [6].

Thus, GM-CSF might display opposite effects on the immune system of tumor-bearing individuals causing either an augmentation or impairment of anti-tumor immune reactions. It is then of paramount importance to establish under which conditions such a cytokine can help in increasing the strength of the immune response in order to optimize its use as vaccine adjuvant.

biology of GM-CSF

GM-CSF, together with G-CSF, M-CSF, IL-3 and IL-5, belongs to the family of hematopoietic cytokines [see 7]. GM-CSF is secreted as a single chain glycoprotein containing 128 amino acids with a conserved disulfide bond by a variety of cell types (monocytes, endothelial cells, activated T-cells, fibroblasts, mitogen-stimulated B-cells, and LPS-stimulated macrophages). GM-CSF functions are mediated by binding to a high-affinity receptor composed by a GM-CSF-specific α chain and, for human cells, by a β chain which is shared with the IL-3 and IL-5 receptors. The receptor α chain is expressed as monomers on the plasma membrane of un-stimulated cells and it is responsible for the specific binding of the cytokine. After ligand binding, the β subunit of the receptor is recruited to the α chain/cytokine complex and interacts with the α-bound cytokine to activate signal transduction and functional responses. In addition, the receptor α chain exists as soluble external molecule (sGM-CSFRα) that is generated by an alternative splicing of α gene that lack the cytoplasmatic part. This soluble receptor is able to compete for cytokine binding with its transmembrane counterpart but it does not participate in agonistic signaling.
The GM-CSF receptor is expressed by CD34+ progenitor cells, by all myeloid lineages and by vascular endothelial cells. GM-CSF can promote myeloid differentiation and it has been initially discovered as a factor able to generate both granulocytes and macrophage colonies from bone-marrow precursor cells. However GM-CSF also mediates crucial functions in host response to external stimuli, in inflammation and in the anti-tumor immune response. These pivotal roles mainly derived from the ability of GM-CSF to affect properties and functional status of immature and mature myeloid cells such as granulocytes, dendritic cells (DCs), macrophages and eosinophils.

**GM-CSF in the immune response to tumor antigens in animal models**

**evidence for stimulatory versus suppressive effects**

GM-CSF gene transduced tumor cells were described as the most potent, as compared to other cytokine gene-transduced counterparts, in generating a long-lived, systemic anti-tumor immune response in mice [8]. The mechanism underlying this augmentation of immunogenicity may lie in the local recruitment and maturation of DCs [9] which is likely to result in the improvement of tumor antigen presentation to lymph node T lymphocytes [10] in addition to the activation of macrophages, granulocytes and NKT cells [9, 11].

However, GM-CSF has also been shown to generate Gr1+CD11b+ MSC that in turn can activate CD4+CD25+ T regulatory lymphocytes thereby blocking the immune response leading either to potential clinical benefit in case of autoimmune reactions [12] or to down-regulation of immune defense in case of anti-tumor reactions [13]. How can this apparent paradox be explained?

As suggested by Reali and co-workers [14], in the mouse system the local administration of GM-CSF even under different forms usually increases the immune response. However, when its administration results in sustained systemic levels, immune suppression may ensue through the production of Gr1+CD11b+ MSC [15]. This latter aspect has been underestimated while it may have an impact on clinical studies of vaccination in humans and explain some results of previous trials using GM-CSF as adjuvant.

It is reasonable to assume that a minimal dose should exist for GM-CSF to prime anti-tumor immune response and a quantity of 36 μg/10⁶ tumor cells/24 h has been indicated as a threshold in mice [8]. Another threshold exists as upper level; in fact, while 300 μg/10⁶ tumor cells/24 h is still effective, 1500 μg of GM-CSF not only loses efficacy but induces the appearance of MSC early after vaccination [15]. Thus the cumulative serum concentration, that is uniform in mice but highly variable in humans, determines bone marrow mobilization of immature myeloid cells. These cells are Gr1+ and CD11b+ in mice but of uncertain phenotype in human (see below) and inhibit T cell function through the concomitant production of arginase 1 and a shift from nitric oxide (NO) to urea/ornithine production [see 13]. The expansion of these MSC generally occurs along with tumor progression and is proportional to tumor size. Thus, it is conceivable that tumor produced cytokines, other than GM-CSF, capable of bone marrow mobilization, like VEGF [16], can reduce the amount of GM-CSF necessary to activate this immune suppression mechanism.

**GM-CSF as immunologic adjuvant in human cancer vaccines**

**evidence for its adjuvant effect and underlying mechanisms**

After murine tumors, also autologous or allogeneic [17] human tumor cells transduced with the GM-CSF gene (GVAX vaccine, CellGenesys) have been tested as vaccine in a variety of neoplasms [17, 18].

Actually a series of publications suggested that GM-CSF gene transduced and, therefore, GM-CSF-releasing human tumor cells-based vaccines can induce an anti-tumor immune response in renal cancer [19], melanoma [20, 21], prostate cancer [22], lung and pancreatic cancer [23, 24]. However, with the exception of the renal cancer trial [19], no direct comparison has been made in these studies between GM-CSF and other cytokine gene-transduced tumor cells or mock–transduced tumor cells for the ability to elicit a vaccine-specific immune response. Nor was a dose response determined in terms of ability to elicit an immune response to the vaccine. Moreover, the frequency of patients mounting a systemic T cell response against tumor-associated antigens (e.g. gp100, Melan-A/MART1, PSA) was not higher than that generally obtained in other vaccination trials in which GM-CSF was not included [25]. Since a major limitation of this approach relies in establishment of autologous tumor cell lines, additional phase II trials were carried out in prostate cancer patients using two already available allogeneic cancer prostate lines transduced with the GM-CSF gene. Encouraging results were obtained in terms of PSA velocity and extension of time to progression. Thus two phase III trials are currently ongoing (VITAL-1 and –2) sponsored by Cell Genesys in metastatic patients comparing GVAX® with Taxotere® (VITAL-1) or GVAX®+Taxotere® vs. Taxotere® alone (http://www.cellgenesys.com). In a phase II study the allogeneic GVAX® was administered also to pancreatic cancer patients after surgery and chemotherapy with some promising results in terms of survival after 2 years of observation [26].

A different approach was used by Small and co-workers [27] who vaccinated metastatic, hormone refractory prostate cancer patients with autologous DCs loaded with a recombinant fusion protein containing prostatic acid phosphatase (PAP) and GM-CSF (Provenge®). In this phase II study, encouraging results were achieved both in terms of PSA reduction and TTP. A double, placebo controlled (2:1) phase III trial of vaccination in 127 hormone resistant prostate cancer patients with Provenge® was therefore started whose early results were recently reported [28]. Patients with Gleason score of <7 receiving the vaccine experienced a better TTP (16.1 versus 9.1 weeks); this group of subjects also developed a stronger T cell response to PAP compared to those with GPS>8. More importantly, survival rate at 3 years was 33% in the treatment group compared with 11% of the control and OS was 25.9 versus 22.0 months in favor of vaccinated group. Despite these interesting results, these trials
could not investigate the role of GM-CSF and, therefore, it is not clear whether the cytokine is indispensable for the attainment of the clinical improvement described above. The GM-CSF protein was also injected at the site of vaccination to avoid the cumbersome procedure of gene transduction and to better define the dose of the administered cytokine (Table 1). In pre-treated but disease-free B cell lymphoma patients vaccinated with individual Ig idiotype, conjugated to the KLH carrier, and GM-CSF, this approach resulted in strong and frequent T cell response (19 out of 20 patients tested) and clinical responses [29]. However, in this trial no direct comparison was made to assess the role of GM-CSF, though an indirect comparison with a previous study [30] of a similar (Id-KLH) vaccine admixed with a different adjuvant suggests an advantage for GM-CSF in terms of both immune and clinical response (Table 1). Less convincing was the effect of GM-CSF administered with peptides in melanoma and other solid tumors owing to the small sample of patient populations [31–34], to differences in other vaccine components (e.g. addition of DCs) [35] and to lack of direct comparison within the same vaccine given with or without GM-CSF as adjuvant [32, 36].

The majority of anti-cancer vaccination trials in which GM-CSF was used as adjuvant given repeatedly s.c. at the site of vaccination at the dose of 100, 250 or 500 µg failed to show an increase frequency of immune and clinical response in patients bearing different types of tumors and receiving vaccines based either on autologous tumor cells [37], peptides [38] or recombinant antigens [39] (see Table 1). Remarkably, a randomized study of 133 cancer patients with trivalent influenza vaccine with or without GM-CSF administered s.c. at the dose of 250 µg also failed to show an increased immune response in the arm receiving GM-CSF [40].

Looking at the dosage used, it appears that trials performed with low doses of GM-CSF (40–80 µg given either i.d. or s.c. for 4–7 days within the site of vaccination) show an increased stimulation of the immune response [32–34, 36, 41] whereas when a higher dose was administered (100–500 µg/day as single dose or repeatedly) no additional stimulation of the immune response was obtained [37, 39, 40]. A possible exception is a study [35] reporting an adjuvanticity of 250 µg of GM-CSF in peptide vaccinated melanoma patients, a result that might be attributed to the single instead of repeated administration of the cytokine for each vaccination and/or to the counteracting effect of the subsequent injections of IL-2. In a different study by the same authors [42], vaccination with GM-CSF administered at the dose of 110 µg/m²/day for 3 days without subsequent IL-2, elicited peptide-specific T lymphocyte response to at least one of the 12 peptides injected in 67% of tested patients, again suggesting efficacy of the adjuvant combination GM-CSF + Montanide ISA-51 though a tetanus toxoid CD4 T helper epitope was also administered, rendering the interpretation of the specific role of GM-CSF rather difficult. A recent study in breast cancer patients vaccinated with HER-2/neu protein administered monthly for 6 months with 100 µg of GM-CSF i.d., also corroborates the idea that an effective adjuvant activity

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*As assessed by DTH assay with tumor unrelated recall antigen.
of the cytokine occurs since 24 of 27 patients developed a vaccine-specific T cell response [43]. It is conceivable that such a dose and timing of cytokine administration may fail to cause a systemic concentration high enough to activate MSC.

In few studies where patients receiving vaccine plus GM-CSF were compared with patients vaccinated without GM-CSF, the cytokine appears even to inhibit the vaccine-induced immune response. In fact, an inhibitory effect of GM-CSF was reported on the immune response to a recombinant (ALVAC-CEA) vaccine in patients with CEA+ metastatic carcinoma receiving 250 µg/day for 5 days of the cytokine s.c. [41], and in melanoma patients vaccinated with peptides since GM-CSF was shown to decrease the induction of a specific T-cell response when given s.c. at the dose of 100 or 500 µg for 6 days [44]. A further example of the potential suppressive action of GM-CSF, as assessed by DTH, and on the clinical response assessed as reduction of circulating PSA, is that described by Simmons and co-workers [45] using PSMA peptide-pulsed DCs alone or with 75 µg/m²/day GM-CSF s.c. for 7 days in a group of 44 metastatic prostate cancer patients as compared with 51 patients who received the vaccine without GM-CSF.

GM-CSF was also administered systemically as a therapeutic agent either as monotherapy or in combination with other agents. In a phase II, open labeled study [46], stage III and IV melanoma patients were treated with long-term, chronic, intermittent GM-CSF after surgical resection. GM-CSF was given s.c. in 28-day cycle at the dose of 125 µg/m² daily for 14 days; treatment continued for one year. These authors obtained an OS and DFS that were considered significantly prolonged as compared with matched historical controls with a MST of 37.5 versus 12.2 months. The authors hypothesized that GM-CSF might work through activation of anti-tumor killer macrophages but no immunobiological data were presented to substantiate such an idea. An important comparative phase II study is being conducted by Kirkwood and co-workers in 120 stage IV melanoma patients who receive three melanoma peptides (MART-1, gp1000, tyrosinase) alone, peptides plus GM-CSF 250 µg daily for 14 of the 28 days cycle each month, or peptides plus IFNα2b 10 MIU/m² TIW or peptides plus both cytokines. T cell specific immune response was documented in 26/71 (37%) evaluable patients that was not influenced by GM-CSF or IFNα2b treatments [47].

The emerging picture, therefore, suggests that while low doses of the cytokine administered locally can help the immune reactions to the vaccine, the highest doses (100–500 µg) of GM-CSF administered in the vaccination studies and causing systemic effects, not only fail to stimulate but may even depress the immune response as compared to the vaccine alone. Moreover, GM-CSF given systemically at doses and schedules considered potentially therapeutic (i.e. 250–500 µg/day for 14 of the 28 day cycle) does not provide evidence of adjuvanticity [47]. It is of note that even these negative trials are heterogeneous in terms of type of vaccines, tumor histotype, and schedules and doses of GM-CSF, making comparison and conclusions uncertain.

We have recently conducted a phase II trial of vaccination of stage IV melanoma patients with autologous tumor-derived heat shock proteins gp96 (HSPPC-96), GM-CSF (75 µg/day for 3 days given s.c. at the site of vaccination starting the day before vaccine) and low dose of IFN-α administered twice/week after vaccination [48]. Both the frequency of the T cell-mediated immune response to melanoma antigens assessed by ELISPOT and that of clinical response, were found to be lower in comparison with those obtained in a clinically similar group of patients vaccinated with heat HSPPC-96 without addition of cytokines [49]. We have analyzed the PBMC of these patients before and after vaccination and found that GM-CSF increased the frequency of immature CD14+ HLA-DR-negative, TGFβ-producing myeloid cells in their PBMC; such an increase was associated with lack of anti-melanoma T cell response (Rivoltini et al., unpublished data). Since our dose was low (75 µg/day x3 days) i.e. a dose that should not cause significant systemic effects, other factors possibly exist that can tip the balance in favor of inhibition rather than stimulation of the vaccine induced T cell reactivity likely through MSC. As already pointed out, one such factor could be the ongoing and constitutive release of GM-CSF or other hematopoietic factors by melanoma cells which, by providing additive effects may reduce the threshold of external GM-CSF necessary for the activation of MSC.

**Immunosuppressive effects of GM-CSF and Its Underlying Mechanisms**

Tumor cells, through the release of myeloid growth factors such as GM-CSF, and/or tumor-activated NKT cells in an IL-13-dependent fashion [50], are believed to be responsible for MSC expansion in tumor-bearing hosts, as depicted in Figure 1.

In contrast to that observed in mouse tumor models, MSC phenotypic and functional features remain elusive in humans. Although the existence and expansion of MSC in cancer patients is clearly demonstrated, data regarding their phenotype and, most importantly, their mechanism of action are still inconclusive and tumor histotype-related. In fact, patients with head and neck carcinoma have increased numbers of CD34+ myeloid precursor cells in their peripheral blood and in tumor lesions, exerting inhibitory activity on different immune responses [6]; the percentage of these cells correlates with the ability of autologous tumor cells to release GM-CSF, which is in turn influenced by a poor prognosis [51]. On the other hand, patients with breast and lung carcinoma have increased frequency of Lineage<sup>neg</sup> (i.e. MSC staining negative for CD3, CD19, CD57 and CD14), HLA-DR negative cells, representing myeloid cells at different maturation stage [52]. These immature cells actively suppress antigen-specific T cells responses through NO-independent mechanisms likely mediated by the release of oxygen reactive species, and requiring cell-to-cell contact. In renal cancer patients, however, MSC appear to be mostly represented by CD11b<sup>+</sup>CD14<sup>+</sup> myeloid cells expressing the granulocyte marker CD15 [53], thus highly resembling the CD11b<sup>+</sup>Gr1<sup>+</sup> subset of tumor-bearing mice. These cells, which are barely detectable in healthy donors, suppress lymphocyte proliferation by an arginase 1-dependent mechanism. MSC could also represent myeloid DC precursors whose differentiation arrest would impair the generation of fully functional DC and, through the production of NO, might inhibit T cell functions [54, 55].
The usage of GM-CSF as adjuvant, either injected locally at low doses at the vaccine site or released by genetically modified tumor cells, should be hardly the direct cause of generation of MSC population whereas high doses, by inducing bone marrow mobilization of myeloid cells, could result in the appearance of MSC both in circulation and in tumor tissue. However, it is conceivable that even low doses of GM-CSF when administered in cancer patients bearing abnormalities in the myeloid compartment, may results in a further expansion and/or activation of MSC, with important and detrimental implications on the effector functions of anti-tumor T cell responses (Figure 1).

conclusions

Though the heterogeneity of the different clinical studies of vaccination that made use of GM-CSF as adjuvant does not allow drawing strong conclusions, it appears that the widespread use of this cytokine as adjuvant is not justified by the present data particularly at doses higher than 80 μg administered for 1–5 days s.c. or i.d. In fact, it is entirely possible that tumor growth itself, by directly releasing the cytokine or by generating chronic inflammatory reactions, can activate MSC endowed with the ability to interfere with spontaneous or vaccine induced immune responses. In such a case, administration of GM-CSF to these patients can actually worsen their clinical conditions through the activation of MSC which can be found in the blood, lymph nodes and/or in tumor tissues. Therefore, caution should be exercised in the use of GM-CSF as adjuvant in vaccination trials.

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