Article title: Role of GM-CSF signaling in cell-based tumor immunization

Short title: Role of endogenous GM-CSF in tumor immunity

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Abstract

GM-CSF is a potent adjuvant in cancer vaccination; however specific role of endogenous GM-CSF remains unknown. To address this question, we performed cell-based vaccination in two tumor models. First, we vaccinated C57BL/6 mice lacking either GM-CSF, IL-5 or beta-common chain (βc), a receptor subunit essential for GM-CSF and IL-5 signaling, with melanoma cells engineered to produce GM-CSF. Tumor vaccination was effective in both GM-CSF⁻/⁻ and IL-5⁻/⁻ mice showing that protective immunization is independent of both endogenous cytokines. However, all βc⁻/⁻ animals developed tumor. Loss of tumor immunity in βc⁻/⁻ mice does not reflect global impairment in cell-mediated immunity, as contact hypersensitivity reaction to haptens is unaltered. The importance of tumor cell-derived GM-CSF was highlighted by recruitment of dendritic cells at the vaccination site in wild-type, GM-CSF⁻/⁻ and IL-5⁻/⁻ but not in βc⁻/⁻ mice. In the second model, vaccination with unmodified RENCA cells showed similar results with efficient immunization in BALB/c wild-type and GM-CSF⁻/⁻ while all βc⁻/⁻ animals died. Altogether, our results strongly suggest that while endogenous GM-CSF and IL-5 are not required to induce tumor immunity, signaling through βc receptor is critically needed for efficient cancer vaccination in both genetically modified GM-CSF-secreting tumor cells and a spontaneously immunogenic models.
**Introduction**

Recent insights into the cellular and molecular mechanisms underlying the host anti-tumor response have led to the development of several strategies for enhancing anti-tumor immunity\(^1\text{-}^3\). Regardless of the antigenic sources (naked DNA, peptide, protein, antigen loaded dendritic cells, whole cells), Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF) has been shown to increase the immune response both in animal models and clinical trials\(^4\text{-}^6\). It is now widely used as an adjuvant in immunotherapy protocols. We and others have shown that vaccination with irradiated tumor cells engineered to secrete GM-CSF stimulates the generation of potent, specific, and long-lasting anti-tumor immunity in multiple murine tumor models\(^7\text{-}^{10}\). Moreover, this vaccination scheme consistently induces dense CD4+ and CD8+ T lymphocyte and plasma cell infiltrates, in metastatic lesions of patients with advanced melanoma. These inflammatory reactions result in extensive tumor necrosis, fibrosis, and edema\(^11\). In addition to melanoma, clinical trials using GM-CSF-secreting tumors cells have been reported in patients with several tumor types including non-small cell lung carcinoma\(^12\text{-}^{13}\), pancreatic\(^14\), prostate\(^15\) and renal cell carcinoma\(^16\). Despite the data from animal models and phase I clinical trials, the critical role of GM-CSF is not well characterized and several reports have raised concern about potential detrimental effect of this cytokine\(^17\). Indeed high doses of GM-CSF may prevent optimal immunization due to the expansion of myeloid-derived suppressor cells\(^18\). This has been further supported by the findings of Filipazzi and colleagues, who have identified the presence of myeloid suppressor cells in melanoma patients treated with subcutaneous administration of recombinant GM-CSF\(^19\). Moreover, GM-CSF induces the expression of milk fat globule EGF-8 in antigen presenting cells, which plays a critical role in the maintenance of FoxP3+ Tregs\(^20\). A deeper understanding of the functions of GM-CSF should help guide the use of this cytokine in immunotherapy.
The increased immunogenicity of GM-CSF-secreting tumor cells may be related to the ability to recruit and mature DC. While the critical role of DC in priming antigen-specific responses is well established, several studies have identified specific DC characteristics that are critical in the induction of a potent anti-tumor vaccination activity. For example, although both GM-CSF and Flt3-ligand induce the marked expansion of DC, we have shown that GM-CSF-secreting tumor cells promoted higher levels of protective immunity than vaccination with FLT3-L-secreting tumor cells. The superior efficacy of GM-CSF-secreting vaccines is in part associated with the higher expression of B7-1 (indicative of a better maturation) and CD1d (that evokes the involvement of NKT cells) on DC. We have also shown that tumor protection induced by GM-CSF-secreting tumor cells vaccine was abrogated in CD1d deficient mice whereas vaccinated wild-type (WT) mice mount protective tumor immunity. The abrogation of tumor protection in CD1d deficient mice is associated with impaired T cell cytokine response to tumor cells including GM-CSF, IL-5, IL-10, and IL-13, whereas T cell INF-γ secretion and tumor specific cytotoxicity remained unchanged.

Previous mouse studies exploiting gene-targeting techniques or neutralizing antibodies have established that both CD4+ and CD8+ T cells are required for efficient vaccination. Other investigations have revealed a central role for CD4+ T cells in the production of IFN-γ, IL-4 and the activation of eosinophils and macrophages to produce nitric oxide, and reactive oxygen species in GM-CSF-secreting tumor vaccination. Indeed, multiple effector mechanisms, including tumor-induced cytotoxicity, Th1 and Th2 cytokine production, high titer IgG antibodies to surface and intracellular tumor determinants and the selective destruction of the tumor vasculature have been attributed to the GM-CSF-secreting tumor cell vaccines. Studies of adoptive T cell therapy provide an alternative approach to identify specific effector functions associated with tumor protection. Although both Th1 and Th2 cells can mediate tumor...
destruction, in many model systems tumor-induced T cell production of GM-CSF and IFN-γ are tightly correlated with anti-tumor efficacy. In fact, GM-CSF secretion and cytotoxic activity of tumor activated T cells are closely linked with the ability of ex-vivo expanded tumor-infiltrating lymphocytes to mediate clinical responses in patients with metastatic melanoma. Together, these studies reveal important roles for GM-CSF in both the priming and effector phases of anti-tumor responses.

Surprisingly, the role of endogenous GM-CSF in tumor immunity has not been addressed. GM-CSF is known as a potent hematopoietic growth factor for granulocytes and macrophages expansion and it also induces dendritic cells recruitment and maturation. Most of GM-CSF activities are redundant with other hematopoietic growth factors such as IL-3, M-CSF, G-CSF and FLT3-L; furthermore, analysis of mice lacking GM-CSF did not reveal major hematopoietic defect. GM-CSF deficient mice showed modestly reduced numbers of DC populations in hematopoietic organs and tissue. Study of GM-CSF deficient mice revealed abnormal alveolar macrophage function with decreased surfactant clearance, leading to alveolar proteinosis. Spontaneous tumors were not described but increase in mortality to several pathogens, mainly encapsulated organisms, has been reported.

In an effort to further clarify the requirement for endogenous GM-CSF in anti-tumor immunity, we used two distinct anti-tumor vaccination models in two different mouse strains. In the first model, we compared the immunization induced by subcutaneous injection of irradiated B16 melanoma tumor cells genetically engineered to secrete GM-CSF in C57BL/6 mice deficient in GM-CSF (GM-CSF−/−), IL-5 (IL-5−/−) or βc (βc−/−) and WT littermates. The βc is a receptor subunit common for GM-CSF, IL-5 and IL-3. Both GM-CSF and IL-5 signaling are abolished in βc deficient mice, while IL-3 activity is maintained because of an additional β subunit, specific for IL-3 (β-IL-3) 34. In order to further strengthen the importance of βc signaling in tumor
immunization we have also tested vaccination in the spontaneously immunogenic renal adenocarcinoma RENCA model in BALB/c strain. Vaccination with irradiated, unmodified RENCA tumor cells was performed in GM-CSF−/−, IL-3−/−, GM-CSF&IL-3−/−, βc−/− in BALB/c background as well as WT littermates.
Methods

Animals. Mice deficient in GM-CSF, βc, IL-3 and both IL-3 and GM-CSF were backcrossed at least nine generations onto the C57BL/6 and BALB/c strains. IL-5 deficient mice were generated in a pure C57BL/6 background. Animals studied were at least ten weeks of age and all experiments were performed in accordance with local animal care regulations. The experimental protocols were accepted and approved by the Office Vétérinaire Cantonal, the regulatory body for animal experimentation at the Geneva University Hospital.

Antibodies. All monoclonal antibodies (mAb) and isotype controls were purchased from BD Pharmingen (San Diego, California) unless stated: phycoerythrin (PE)-conjugated anti-CD11c (hamster IgG, clone HL3) and isotype control (hamster IgG, clone A19-3); FITC-labeled MoAb including anti-Gr-1 (rat IgG2b, clone RB6-8C5) and anti-CD11b (rat IgG2b, clone M1/70); allophycocyanin (APC)-labeled anti-CD8 (rat IgG2a, clone 53-6-7); Alexa-Fluor®488 conjugated anti-F4/80 (rat IgG2a, clone R35-95 and rat IgG2b, clone A95-1).

Cell lines. B16-F10 melanoma cells (syngeneic to C57BL/6 mice) and RENCA cells (syngeneic to BALB/c) were cultured in DMEM with 10% FCS and penicillin/streptomycin (complete medium). B16-F10 cells secreting GM-CSF (B16-GM), FLT3-L (B16-FL) or IL-3 (B16-IL-3) cells were generated by retroviral mediated gene transfer, as previously described. GM-CSF secretion was approximately 150 ng/10^6 cells/24 hours, as determined by ELISA (BD Pharmingen, as indicated by the manufacturer). All the cell lines were confirmed to be mycoplasma free (Mycoplasma Detection Kit Enzyme immunoassay, Roche Laboratories, Germany).

Tumor models. For tumorogenicity experiments, C57BL/6 and BALB/c mice were injected subcutaneously (s.c.) in the interscapular region with 1x10^5 live B16 and 2x10^6 live RENCA
cells, respectively. Animals were sacrificed when tumors reached 10 mm in diameter or became ulcerated. For vaccination experiments, C57BL/6 mice were injected s.c. in the abdomen with 1x10^6 irradiated (3500 rads) B16-GM cells and challenged seven days later with 5x10^5 live B16 cells injected s.c. in the upper back; BALB/c mice were injected s.c in the abdomen with 1x10^6 irradiated (3500 rads) RENCA cells and challenged seven days later with 5 x10^6 live RENCA cells injected s.c. in the upper back. Mice were followed for three months after tumor challenge. In all experiments, confirmation of genotype was performed by PCR analysis using somatic DNA (data not shown).

**Cytokine assays.** Tumor-induced cytokine production was measured as previously described 10. Briefly, splenocytes (10^6 cells) were harvested 7 days after vaccination with irradiated B16-GM cells, depleted of erythrocytes, and cultured with irradiated (10000 rads) B16 cells (2x10^4) in 2 ml of complete medium supplemented with 10 units/ml of IL-2. Supernatants were harvested after 5 days and assayed for GM-CSF, IL-5, IL-10, IL-13 and INF-γ by ELISA using the appropriate monoclonal antibodies (Endogen, Woburn, MA and PharMingen, San Diego, CA).

**GM-CSF ELISA.** The GM-CSF secretion from RENCA cells was determined by ELISA Kit (OptEIA™ from BD Biosciences Pharmingen), as indicated by the manufacturer. Briefly, the RENCA cells were irradiated at 3500 rads and seeded at a density of 10^6 cells per well. The GM-CSF release in the supernatant was measured after 24h.

**Cytokine measurement using cytometric bead assay (CBA).** Irradiated B16-F10, B16-GM, B16-FL and B16-IL-3 cells were seeded in triplicates at a density of 10^6 cells per well during 24 hours. Evaluation of cytokine secretion was done using Th1/Th2 and Inflammatory cytometric bead array kits (BD PharMingen) by flow cytometry according to the manufacturer's instructions and analyzed by BD CBA system (BD PharMingen). Standard curves were determined for each cytokine from a range of 20–5000 pg/ml. The following cytokines were measured: IL-2, IL-4,
IL-5, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), IFN-γ, tumor necrosis factor-α (TNF-α) and IL-12p70.

**Contact hypersensitivity.** WT and βc−/− mice from both C57BL/6 and BALB/c strains were sensitized epicutaneously on day 0 with 70 μl of 4% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, Sigma) in acetone/olive oil (4/1). Mice were challenged five days later on the right ear with 20 μl of 0.5% oxazolone in acetone/olive oil and with carrier acetone/olive alone on the left ear. The ear thickness was measured on both ears with a micrometer at 24 hours post challenge. Results are presented as the increased thickness of the hapten-treated ear minus the non-specific swelling (carrier treated ear). Data represent mean values of five mice per group. Similar results were obtained in three independent experiments.

**Histopathology.** Vaccination sites were removed at day five and processed for pathological examination. Tissues were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin, cut to 5 micron thickness, and stained with hematoxylin and eosin (H&E).

**Flow cytometric analysis of cells at the vaccination site.** Mice were immunized subcutaneously with 10⁶ irradiated tumor cells (B16 or B16-GM). Four days later, mice were sacrificed, the site of vaccination was dissected, and cells were extracted as described previously 39. Briefly, tissue was minced with scalpel and enzymatically digested with PBS containing 2 mg/ml of collagenase I (Sigma) and 2 mg/ml of hyaluronidase I (Sigma) at 37°C during 30 min. After mechanical dissociation using a syringe piston, the cells were filtered through a cell strainer (Falcon). The filtered suspension was centrifuged and cells were incubated with the indicated antibodies and analyzed on a FACSCalibure cytometer (Becton Dickinson, Mountain View, California). Living cells were identified by means of the nonpermeant DNA dye 7-amino-actinomycin D (Sigma). Data were analyzed with WINMDI software written by J. Trotter.
(Scripps, La Jolla, California) and Cell-Quest software (Becton Dickinson). FACS analysis of the vaccination site was performed in at least 6 mice in each background.

**Statistics.** Tumor vaccination and contact hypersensitivity experiments were repeated at least 3 times with a minimum of 4 mice per groups and gave similar results. The two-tailed Student’s t-test was used to evaluate $p$ values between experimental groups. A $p$ value $<$0.05 was considered as statistically significant.
Results

Comparison of tumor immunity between WT, GM-CSF<sup>−/−</sup>, IL-5<sup>−/−</sup> and βc<sup>−/−</sup> mice. Since GM-CSF production by T cells involved in vaccination and adoptive therapy is closely associated with tumor protection, we first evaluated the ability of GM-CSF deficient mice to generate anti-tumor immunity. In these experiments, adult female C57BL/6 GM-CSF<sup>−/−</sup> animals and littermate controls were vaccinated with 10<sup>6</sup> irradiated B16-GM and challenged one week later with 5x10<sup>5</sup> live B16 cells. Consistent with previous reports, vaccinated WT mice efficiently rejected tumor challenge (long term protection of 75% of animals), whereas non-immunized WT animals uniformly developed progressive tumors (Fig. 1A). Interestingly, vaccinated GM-CSF<sup>−/−</sup> mice showed tumor protection that was equivalent to immunized WT controls, with 75% of animals rejecting tumor challenge (Fig. 1A).

Studies have shown that while βc mediates most GM-CSF signaling, some evidence suggests that the α chain alone may transduce signals<sup>40</sup>. As melanoma cells express the α chain in the absence of βc, part of the vaccination activity of GM-CSF-secreting melanoma cells may involve autocrine effects<sup>41</sup>. In order to learn more about the pathways stimulated by GM-CSF-secreting tumor cells, we have evaluated the ability of βc<sup>−/−</sup> mice to generate tumor immunity. Although the tumorigenicity of B16 cells in WT, GM-CSF<sup>−/−</sup>, βc<sup>−/−</sup> and GM-CSF & IL-3 double knock-out mice appeared the same (Fig. 1C), vaccination with irradiated B16-GM cells fail to induce any tumor protection in βc<sup>−/−</sup> mice (Fig. 1A). All mutant animals rapidly developed growing tumors at the challenge site, with kinetics comparable to unvaccinated WT animals (Fig. 1A). Since βc is also implicated in IL-5 signaling<sup>35,42</sup>, we therefore evaluated the role of endogenous IL-5 to generate tumor immunity. IL-5<sup>−/−</sup> mice vaccinated with irradiated B16-GM cells showed a 75% survival rate, in comparison to an 80% survival rate in WT mice (Fig. 1B). Tumor immunity obtained
after efficient vaccination is sustained in WT, GM-CSF−/− and IL-5−/− mice. All the protected animals rejected secondary tumor challenge performed on day 60 (data not shown). Similar experiments were performed in the BALB/c background, using the RENCA tumor cells. BALB/c WT and GM-CSF−/− mice, immunized with 10^6 irradiated RENCA cells, were protected in a comparable manner from subsequent 5x10^6 live RENCA cells challenge (Fig 2A). Similarly to the result obtained in the C57BL/6 strain, the loss of protective immunity is also observed in BALB/c βc−/− mice that were vaccinated with irradiated RENCA cells. While all vaccinated βc−/− animals succumb to tumor challenge, all vaccinated WT and GM-CSF−/− mice showed protective anti-tumor response (Fig 2A). We then evaluated the tumorogenicity of RENCA cells in different knock-out mice and found out that this remains identical between WT, GM-CSF−/−, βc−/− and GM-CSF & IL-3−/− double knock-out mice (Fig 2B).

As vaccination with RENCA cells induce tumor immunity in WT and GM-CSF−/− but failed in βc−/− mice, we hypothesized that GM-CSF protein should be present during the priming phase of vaccination. We, therefore, assessed the possibility of GM-CSF secretion, necessary for the induction of anti-tumor response, from RENCA tumor cells. Supernatant of irradiated RENCA cells was analyzed by ELISA and murine GM-CSF secretion was confirmed at a low but detectable level (Fig 2C). In contrast, unmodified B16-F10 melanoma cells do not release any GM-CSF (Fig. 2C). We can, therefore, postulate that the murine GM-CSF present in the supernatant of RENCA cells might be responsible for the induction of protective tumor immunization.

Tumor immunity in IL-3−/− and GM-CSF&IL-3−/− mice. We have previously described that BALB/c IL-3 deficient mice showed no impairment in tumor vaccination using the RENCA model 36. Here we present additional data showing similar results in mice lacking both GM-CSF
and IL-3. Similarly to WT littermates, GM-CSF&IL-3+/− mice have no defect in tumor immunization and are protected from subsequent tumor challenge (Fig. 2A).

βc−/− mice immunization with different cytokine-secreting tumor cells. In the next set of experiments, we aimed to study the ability of other cytokines to immunize βc−/− mice. We, therefore, immunized WT and βc−/− mice either with irradiated B16-IL-3, B16-FL or B16-GM. In WT animals, immunization with B16-IL-3, B16-FL induces 40% and 25% (respectively) of survival upon tumor challenge as compared to 75% tumor protection following B16-GM vaccination (Fig. 3A). This is consistent with the previously published data where GM-CSF remains the most potent cytokine in the induction of tumor protection7. On the contrary, none of the above-mentioned cytokine-producing cells was able to protect βc−/− mice, with all mice showing progressive tumor growth upon challenge (Fig. 3A).

To determine whether the introduction of the different transgenes into B16-F10 could induce the secretion of other types of cytokine that might interfere with their respective properties, we analyzed the supernatants of cells for the presence of IL-2, IL-4, IL-5, IL-6, IL-10, MCP-1, INF-γ, TNF-α and IL12p70 using CBA system. 10^6 irradiated B16 WT, B16-FL, B16-IL3 and B16-GM cells were cultured during 24 hours; their supernatant was collected and analyzed with the Inflammation and Th1/Th2 kit of CBA system for the presence of the indicated cytokines (Fig. 3B). We have not detected any of the above cytokines in the supernatant of different B16-F10 transformed cells; all the tested values were under the detection limit.

Characterization of effector function following GM-CSF-based tumor vaccination in different knock-out mice. Vaccination with irradiated B16-GM cells stimulated comparable anti-tumor effector mechanisms in GM-CSF−/− mice as in WT controls. The tumor-induced production of IFN-γ, IL-5, IL-10, and IL-13 from splenocytes in GM-CSF−/− mice was similar to control animals, although the mutant animals were unable to secrete GM-CSF (Fig. 4A). In order
to delineate the basis for the loss of anti-tumor immunity in βc-/- mice, we characterized the generation of anti-tumor effector mechanisms. In contrast to WT or GM-CSF-/- animals, βc-/- mice showed reduced production of IFN-γ, GM-CSF, IL-5, IL-10, and IL-13 (Fig. 4B). Furthermore, consistent with the results of tumor immunity (Fig. 1B), the development of anti-tumor effector response in immunized IL-5-/- mice was comparable to WT animals (Fig. 4C). In particular, the production of IFN-γ, GM-CSF, IL-10, and IL-13 was unimpaired, although the mutant animals were unable to secrete IL-5 (Fig. 4C).

**Histopathological analysis of the immunization site in different knock-out mice vaccinated with B16-GM.** In order to understand the lack of vaccination efficacy in βc-/- mice, we performed histopathological analyses of the site of tumor antigen capture five days after B16-GM immunization. This enabled us to investigate potential defects in the early phase of the response. In WT (Fig. 5A) and GM-CSF/- (Fig. 5B) animals, B16-GM cells elicited a robust cellular infiltrate and inflammation at the site of vaccination. Central necrosis of the vaccination site is also observed. Histopathologic analysis of the vaccination sites in IL-5-/- animals was similar to WT mice (Fig. 5C) except for reduced eosinophils. In contrast, the vaccination site in βc-/- mice showed minimal infiltrates and no inflammation (Fig. 5D). Interestingly, this reaction was comparable to the response evoked in WT mice by vaccination with irradiated unmodified B16 cells (Fig. 5E).

**βc-/- mice immunized with GM-CSF-secreting tumor cells failed to recruit myeloid DC at the site of vaccination.** It has been shown that GM-CSF-secreting tumor cells induce the generation of potent anti-tumor immunity by increasing the local recruitment and maturation of myeloid derived DC 10. We therefore evaluated the capacity of immunized GM-CSF-/-, IL-5-/- and βc-/- mice to recruit DC at the vaccination site.
WT mice vaccinated with irradiated B16-GM tumor cells have significantly increased numbers of CD11c+CD11b+DC (36% ±3.4; S.E.M of 6 independent experiments) as compared to WT animals vaccinated with irradiated unmodified B16 tumor cells (3.4% ±1.9; S.E.M of 5 independent experiments) (Fig. 6A). Furthermore, all recruited CD11b+CD11c+DC were CD8α negative (data not shown).

Similarly to WT animals, DC recruitment was increased following B16-GM immunization in GM-CSF−/− (43% ±3.3; S.E.M of 6 independent experiments) (Fig. 6B) and IL-5−/− (39% ±3.9; S.E.M of 6 independent experiments) mice (Fig. 6C). GM-CSF−/− (5%± 0.5; S.E.M of 6 independent experiments) (Fig 6B) and IL-5−/− mice (3% ±0.6; S.E.M. of 5 independent experiments) (Fig. 6C) vaccinated with unmodified B16 tumor cells showed a low percentage of DC recruitment that was similar to WT mice. In contrast, βc−/− mice fail to recruit DC following B16-GM vaccination (4% ±0.9; S.E.M. of 3 independent experiments) (Fig. 6D). This recruitment was similar to the vaccination of βc−/− mice with B16 WT tumor cells (4.25% ±0.6; S.E.M. of 3 independent experiments) (Fig. 6D). Together, these observations suggest that the loss of anti-tumor immunity in βc−/− mice is not due to the lack of IL-5 signaling and establish a requirement for βc signaling in the early phase of GM-CSF-based vaccines.

Contact hypersensitivity reaction in βc−/− mice. To address whether βc−/− mice demonstrate a general impairment in cell-mediated immunity, we assessed the ability of these mice to generate contact hypersensitivity (CHS) in both mouse strains. This reaction is a form of delayed-type hypersensitivity in which hapten-protein conjugates are presented by cutaneous DC, following their migration to regional lymph nodes, to hapten-specific CD4 and CD8 positive T lymphocytes. Upon secondary hapten challenge, sensitized T cells initiate a local inflammatory response. As shown in Fig. 7, βc−/− mice mounted contact hypersensitivity reactions that were equivalent to WT controls in both C57BL/6 and BALB/c background.


Discussion

Many studies of tumor vaccination and adoptive T cell therapy indicate important roles of GM-CSF in both the priming and the effector phases of anti-tumor responses. GM-CSF is thought to be one of the most potent adjuvants and is used in many tumor immunization schemes including DNA, peptide, tumor cell or dendritic cell-based vaccination. Despite numerous demonstrations of strong immunostimulatory effects in animal models and clinical trials, some reports have raised concerns regarding the detrimental effect of this cytokine when used at high concentration levels. In animal studies, GM-CSF has proven active when delivered or produced at the vaccination site. The adjuvant or anti-tumor immunization effect describes to be maximal when prolonged and sustained release can be achieved at the inoculation site. Indeed, sustained local release of GM-CSF at the vaccination site by GM-CSF-secreting cells proved to be efficient in the induction of tumor immunity in many animal models when using cells engineered to release 90-300ng/10^6 cells/24hrs. In contrast, local release of high dose GM-CSF at the vaccination site or injected intra-peritoneally, has been shown to block the immune response leading to the down-regulation of immune defense in anti-tumor reactions. Additionally, high doses of GM-CSF injected sub-cutaneously have shown to be detrimental, resulting in the expansion of myeloid suppressor cells.

The role of endogenous GM-CSF for generating and maintaining crucial hematopoietic cell types involved in immune responses has not been addressed fully. The design of this study aims at a better understanding of the selective role of endogenous GM-CSF and GM-CSF produced locally at the vaccination site by the implanted tumor cells and does address the role of other cytokines and chemokines.

Our experiments were undertaken in an effort to learn more about the requirements for GM-CSF signaling in anti-tumor immunity. The results showed that host-derived GM-CSF is dispensable
for both the priming and the effector phases of GM-CSF-based tumor cell vaccines in two different anti-tumor immunization models using either genetically modified GM-CSF-secreting B16 tumor cell line in C57BL/6 or the spontaneously immunogenic RENCA tumor cell line in BALB/c. In the B16-GM model, the local release of exogenous GM-CSF by irradiated genetically modified tumor cells at the vaccination site is sufficient to trigger an efficient immune response in WT and GM-CSF−/− mice. These findings further imply that endogenous GM-CSF is not required for the development and survival of the various cell populations involved in GM-CSF-based anti-tumor immunization in at least two distinct tumor models.

In contrast, our experiments in βc deficient mice demonstrated that GM-CSF signaling is crucial during the priming phase of vaccination. The tumor protection elicited by GM-CSF-secreting B16 cells and the unmodified RENCA cells were both completely abrogated in βc−/− mice. This was associated with a failure to develop granulocyte, macrophage, DC and lymphocyte infiltrates as a consequence of vaccination. The β-subunit of the GM-CSF receptor is identical for IL-3 and IL-5 receptors. The α subunits of GM-CSF, IL-3 and IL-5 receptors are distinct and the α/β heterodimer forms a high affinity receptor for the respective cytokines. In mice, a second βc for IL-3 has been identified (βc-IL-3), which binds to the IL-3α subunit with low affinity, and forms a high affinity receptor to transmit the proliferation signal 34. Mice lacking the βc subunit are therefore lacking both GM-CSF and IL-5 signaling but have adequate IL-3 signaling. In addition, we have previously shown that IL-3 deficient mice do not have any defect in tumor vaccination in the RENCA tumor model 36.

Our results formally established that α chain signaling is not sufficient for the generation of anti-tumor immunity in these models and that specific functions mediated through the βc subunit receptor are required. Previous studies have demonstrated that the GM-CSF α chain receptor alone is insufficient to mediate in vitro survival of hematopoietic cells 45. Future experiments
involving the adoptive transfer of defined cell populations from WT animals should help further elucidate the cellular requirements for effective priming.

Since $\beta_c$ is involved in both IL-5 and GM-CSF signaling, one explanation for the loss of tumor immunity was impaired IL-5 function. However, our results argue strongly against this possibility, by showing that the vaccine responses were not diminished in IL-5$^{-/-}$ mice. Furthermore, our results fail to confirm a previous report suggesting a significant role for IL-5 in this system$^9$. Although the basis for the discrepancy between the two studies is currently unclear, age-related B cell defects in IL-5$^{-/-}$ mice or the amount of GM-CSF produced at the vaccine site may contribute to differences in immunization$^{38,46}$.

The compromised tumor protection in $\beta_c^{-/-}$ mice did not reflect a requirement for host-derived GM-CSF, as the efficiency of tumor vaccination in GM-CSF$^{-/-}$ mice was indistinguishable from WT controls. The generation of anti-tumor effectors in immunized GM-CSF$^{-/-}$ animals was also similar to WT littermates. While these findings show that host-derived GM-CSF is dispensable for GM-CSF-based tumor vaccination, they do not preclude the possibility that the coordinated activities of GM-CSF and other factors are required for optimal tumor protection. Indeed, our previous studies of mice deficient in both GM-CSF and IL-3 established overlapping roles for these cytokines in hematopoiesis and immunity$^{37}$. Furthermore the close interrelation between inflammation and cancer is well illustrated by the study of mice lacking both GM-CSF and IFN-$\gamma$. The inability to uptake apoptotic cells by GM-CSF$^{-/-}$ antigen presenting cells (APC) led to autoimmunity via decreased numbers of regulatory T cells (Treg), while double knock-out mice lacking both GM-CSF and IFN-$\gamma$ showed a marked increase in the incidence of both solid and hematological tumors$^{20,47}$.

Finally, tumor vaccination in $\beta_c^{-/-}$ mice was completely inefficient and this was associated with a marked decreased in IL-5, IL-10 and IL-13 production and lack of inflammatory cells influx at
the vaccination site (Fig. 4B and 5D). This is probably due to the lack of DC recruitment at the site of vaccination in βc−/− mice (Fig. 6D) that are mandatory in the initiation of effective anti-tumor responses. The critical role of βc signaling is further illustrated by the loss of protective immunity in the RENCA model. As production of GM-CSF by cancer cell lines has been reported for solid tumors including renal cell carcinoma, our hypothesis was that spontaneous release of GM-CSF by RENCA cells may trigger the priming phase. Indeed, analysis of supernatant from irradiated RENCA cells revealed a spontaneous production of GM-CSF. These results point to an unexpected role of GM-CSF signaling in immunogenicity. This is also illustrated by the inability of βc−/− mice to develop protective immunity when using B16 cells secreting other cytokines such as FLT3-L or IL-3. Our results parallel the recently published data revealing the critical role of βc signaling in lung inflammation and Th-2 responses.

Nevertheless βc−/− mice do not have generalized severe impairment in cell-mediated immunity as the contact hypersensitivity reaction is similar to WT control. The CHS data showed that at least some antigen-specific T cell responses do not rely upon βc function. These observations point to a specific defect crucial for cell-based anti-tumor immunization. Working hypotheses include the lack of recruitment and/or differentiation of a subclass of DC critically needed for the coordination of an efficient cell-based tumor immunization. In addition, a recent paper demonstrated a novel role for GM-CSF as being a potent driver of Th17 cells. It is therefore interesting to determine whether GM-CSF-based tumor rejection is mediated via Th17 effector responses and whether this response is abolished in βc−/− mice. Experiments analyzing the protective effect of other known potent adjuvants (cytokines and chemokines) in mice lacking GM-CSF signaling will be of great interest. Additional studies will help to better characterize the molecular defect responsible for the loss of anti-tumor immunity observed in βc−/− mice and may bring further understanding to improve cell-based anti-tumor immunization schemes.
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Authorship

Contribution: S.Z. performed research and wrote the paper, F.S. analyzed the data, P.L. performed the research, M.A-L. provided new reagent and analyzed the data, P.M. analyzed the data, M.K. provided critical reagents G.D. designed the research and analyzed the data, N.M. designed the research and wrote the paper.

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Figure references and Legends:

Fig. 1. GM-CSF-secreting tumor cell vaccination in WT, GM-CSF, IL-5 or βc knock-out C57BL/6 mice. (A) GM-CSF−/−, βc−/−, WT or (B) IL-5−/− and WT C57BL/6 mice were immunized s.c. on the abdomen with 1x10^6 irradiated, B16-GM cells (five per group). One week later, the vaccinated mice as well as the unvaccinated WT controls, were challenged s.c. on the back with 5x10^5 live B16 cells. The difference observed in survival time between B16-GM vaccinated βc−/− mice and unvaccinated WT controls is not statistically significant. This experiment is representative of three independent experiments. (C) Tumorogenicity of B16-F10 cells in different knock-out mice. Survival of WT and indicated knock-out C57BL/6 mice inoculated with 5x10^5 B16-F10 cells. This experiment is representative of three independent experiments.

Fig. 2. (A) Tumor protection of RENCA cells in WT and different knock-out BALB/c mice. WT and indicated knock-out BALB/c mice were vaccinated with 10^6 irradiated RENCA cells and challenged one week later with 5x10^6 RENCA tumor cells. The survival curve indicates percentage of mice that survived the challenge. The graphic represents data of four independent experiments (n = 4 mice/group). (B) Tumorogenicity of RENCA cells in different knock-out mice. WT and indicated knock-out BALB/c mice were inoculated on the back with 2 x10^6 of RENCA tumor cells. Mice were sacrificed when tumor size reached 15 mm in diameter or was ulcerated. This experiment is representative of three independent experiments. (C) Spontaneous
production of GM-CSF by RENCA cells. $10^6$ irradiated RENCA or B16-F10 cells were seeded in a 10 mm plate and GM-CSF release was detected by ELISA from their supernatant after 24 hours. The graph is representative of three independent experiments.

**Fig. 3. Tumor protection in WT and $\beta c$ knock-out mice vaccinated with different cytokine-producing tumor cells.** (A) Survival of C57BL/6 mice vaccinated s.c. with irradiated cytokine-producing B16 cells. Mice were vaccinated with $10^6$ B16 cells secreting GM-CSF, FLT3-L or IL-3 and challenged one week later with $5 \times 10^5$ viable unmodified B16 cells. The graph is representative of three independent experiments. (B) Cytokine production by the modified B16 tumor cell lines used in the experiments. Cytokine-producing B16 cells were irradiated and seeded in triplicate at a density of $10^6$ cells per well. Supernatant of cells were collected after 24 hours and IL-2, IL-4, IL-5, IL-6, IL-10, IL12p70, MCP-1, INF-$\gamma$ and TNF-$\alpha$ were measured using Inflammation and Th1/Th2 CBA kits. The positive and negative controls represent the DMEM complete medium and the 1250 pg/ml of CBA standard curve, respectively. Each plot is representative of one single cell supernatant and illustrates one of the two independent experiments.

**Fig. 4. Comparison of tumor-induced cytokine profile in B16-GM vaccinated mice.** Quantification of cytokines release by ELISA in cell suspension supernatants from spleen of B16-GM immunized C57BL/6 mice (black bars) or knock-outs (A) GM-CSF$^{-/-}$ (B) $\beta c^{-/-}$ and (C) IL-5$^{-/-}$ mice (white bars), co cultured with irradiated B16 cells during five days, in the presence of IL-2. Values are in ng/ml. Error bars represent the standard deviation from triplicate samples of one single experiment. Similar results were obtained on three independent experiments.
Fig. 5. **Histopathological analysis of the vaccination site.** (A) WT, (B) GM-CSF$^{+/+}$, (C) IL-5$^{-/-}$ and (D) βc$^{-/-}$ mice were immunized s.c. on the abdomen with $10^6$ irradiated B16-GM cells. As control, WT mice were immunized with $10^6$ irradiated B16 cells (E). Five days later, tissue samples were collected and fixed in Formalin prior to paraffin embedding. Samples were then stained with hematoxylin and eosin. Magnification: x 25 and x 200.

Fig. 6. **Flow cytometric analysis of DC recruitment at the site of vaccination following B16 or B16-GM immunization.** C57BL/6 WT (A), GM-CSF$^{+/+}$ (B), IL-5$^{+/+}$ (C), and βc$^{-/-}$ (D) mice were immunized with irradiated, B16 or B16-GM ($10^6$ cells). Four days later, cells at the vaccination sites were isolated and subsequently analyzed by flow cytometry for the presence of CD11c, CD11b and CD8α positive DC. Results are percentages and are representative of one of at least three animals per group. The difference observed between B16 and B16-GM in each group was highly significant (**P ≤ 0.005) except for the βc$^{-/-}$ group (*P =0.45).

Fig. 7. **Contact hypersensitivity reactions in βc$^{-/-}$ and WT mice.** C57BL/6 and BALB/c mice were sensitized with oxazolone on the abdomen and foot pads on day zero. Five days later, mice were challenged on the right ear with 0.5% oxazolone in acetone/olive oil. The left ear was treated with the carrier (acetone/olive) alone. The ear thickness was measured with a micrometer at 24 hours post challenge. Results are presented as the increased thickness of the hapten treated ear minus the non-specific swelling (carrier treated ear). Data represent mean values of five mice per group. Similar results were obtained in three independent experiments.
Fig. 1

A

B

C

Days following tumor challenge

Days after tumor challenge

Days after tumor challenge
Fig. 3

A

Days after tumor challenge

Mice Survival %

B16-GM in WT
B16-FLT3-L in βc-/-
B16-IL-3 in βc-/-
B16-GM in WT
B16-FLT3-L in WT
No vaccination in WT

B

Inflammation

Unmodified B16  B16-GM  B16-FL  B16-IL-3  positive control  negative control

Th1/Th2

IL-4  IL-10  MCP-1  TNF-γ  IL-12p70  IL-2  IL-4  IL-5  IFN-γ  TNF-α

IL-2  IL-4  IL-5  IFN-γ  TNF-α
Fig. 4

**A**
B16-GM vaccination: Cytokine profile in GM-CSF-/-(white) and WT control (black) mice

**B**
B16-GM vaccination: Cytokine profile in βc-/-(white) and WT control (Black) mice

**C**
B16-GM vaccination: Cytokine profile in IL-5-/-(white) and WT control (Black) mice
Fig. 5
Increased thickness (μm)
Role of GM-CSF signaling in cell-based tumor immunization

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