Role of the cytokine GM-CSF in cell-based anti-tumor immunity: learning from murine models to engineer new therapeutic strategies

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Learning from murine models to engineer new therapeutic strategies

Thesis submitted to the Medical School of the University of Geneva

for the degree of Privat-Docent
by

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Geneva

2012

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1) Summary

Cell-based cancer immunotherapy relies on tumor cells to provide the necessary antigenic material to antigen presenting cells during the priming phase of the immune response. The specific anti-tumor immune response is triggered by antigens although their specificity and numbers are not characterized. As all tumor are unique, autologous tumor cells has the best chance to provide a wide antigenic repertoire with the relevant antigens. In the past 20 years, antigen-based immunotherapy, immunizing with defined antigens, has focused most of the interest in the field of tumor immunology. Disappointing clinical results of antigen-based therapy and improvement in cell engineering techniques has brought cell-based immunotherapy back on track.

The work presented in this thesis shows the development of genetically modified tumor cells to produce stong immunomodulatory proteins and potential clinical applications.
Improvement in gene transfer technology has allowed the engineering of cytokine-secreting tumor cells. Prophylactic vaccination with irradiated tumor cells secreting various cytokines has been evaluated. Among more than 30 cytokines tested in a melanoma murine model, granulocyte-macrophage colony-stimulating factor (GM-CSF) was the most potent at inducing long lasting, tumor specific protective immunity. GM-CSF is a well known hematopoietic growth factor for many cell lines such as granulocytes, macrophages, dendritic cells, erythrocytes among others. It is secreted by lymphocytes and recombinant GM-CSF has been used for stimulating granulocytes recovery after chemotherapy.
Many research groups have tested GM-CSF-secreting tumor cells in many tumor types. Improvement in tumor immunity has been described in kidney, bladder, prostate, lung, breast, ovarian, colon, pancreas, liver or head and neck cancer as well as lymphoma, leukemia, neuroblastoma, melanoma, glioblastoma and sarcoma. Immunization with GM-CSF cancer cells has also demonstrated it capacity to increase tumor specific immunity after T-depleted, allogeneic bone marrow transplantation without stimulating graft versus host disease.

Comparative analysis of GM-CSF and FLT3-L (a strong dendritic cell growth factor) showed that GM-CSF was more potent than FLT3-L in tumor vaccination experiment by recruiting specific dendritic cell sub-population at the vaccination site.
Analysis of mice lacking GM-CSF (GM-CSF-/-), IL-3(IL-3-/-) both cytokines (GM-CSF/IL-3-/-), IL-5 (IL-5-/-) or the receptor for GM-CSF and IL-5 signalling (βc-/-) showed interesting data.
The generations of both IL-3-/- and GM-CSF/IL-3-/-mice are described in detail in the annexed documents.
The major findings observed was the absence of major hematopoeitc defect in mice lacking either GM-CSF, IL-3, both cytokines as well as a conserved anti-tumor immunity with GM-CSF-secreting tumors.
In marked contrast, 100% of mice lacking GM-CSF and IL-5 signalling develop tumor despite vaccination. This loss of immunization was observed in two mice strains and in both poorly immunogenic and immunogenic tumors (B16 melanoma in C57Bl/6 strain and Renca in Balb/c strain respectively). Good tumor protection of IL-5 mice after GM-CSF based vaccination confirmed the hypothesis that GM-CSF signalling is required for efficient tumor vaccination in the two models tested.
As Renca cells do secrete GM-CSF, we hypothesized that spontaneous cytokine secretion is leading to spontaneous immunogenicity. A set of experiments confirmed this hypothesis. The critical role of dendritic cell in GM-CSF signalling is also demonstrated with protective immunity obtained in βc-/- mice when immunized with wild-type dendritic cells. Altogether the data presented described the critical role of GM-CSF in cell-based immunization models.

Translating the positive animal model into clinical application for cancer patients is a major issue. Detailed analysis of clinical trials based on autologous GM-CSF-secreting tumor cells in melanoma, lung, kidney cancers reveal the technical hurdles limiting further development. Despite interesting immunological findings and clinical results, personalized gene therapy of cancer cells proved highly variable, unpredictable and not reproducible. Investigators then tested allogeneic cancer cell line producing GM-CSF. With more than 800 patients treated with this approach disappointing results could be explained by early destruction of allogeneic cells leading to limited GM-CSF secretion. Lack of shared potent tumor-associated antigens between allogeneic vaccine and the patient's cells cannot be rule out.

Clinically meaningful cell based immunotherapy should fulfil the following criteria:
Wide antigenic repertoire / local GM-CSF release / sustained, stable and standardized GM-CSF secretion for several days / GMP production / limited toxicity / no need of customized gene therapy or skewed immune response toward viral vector

Novel immunization schemes for clinical applications are currently being developed and are described in detailed. As a team from John Hopkins has chosen a bystander approach with a MHC class I and II negative allogeneic cell line, our group has designed an innovative immunization strategy combining autologous tumor cells and encapsulated allogeneic, GM-CSF secreting cells.

The preliminary data showed good GM-CSF biodelivery of encapsulated cells as well as in-vivo biological activity. The proof of concept experiments in tumor immunization murine models are described.
Finally the first in human, Phase I, clinical trial combining irradiated autologous tumor cells and novel clinical grade capsules is presented.
This innovative clinical trial is based on data gathered over the last 15 years on GM-CSF secreting tumor models and also integrates the success and failures of reported clinical trials in the field of cancer immunotherapy.

2) Introduction

This work aims at explaining the tortuous path leading to innovative cell-based anti-tumor immunization. Learning from discoveries in basic sciences and analysis of clinical research in this field over the last 30 years is a very interesting and fruitful endeavour. Passive and active specific immunotherapies are often mentioned in the scientific and medical literature. Passive immunotherapy does not educate the immune system to mount lasting, specific response against a defined target and will therefore not be discussed further. This work will focus on active specific anti-tumor immunotherapy (ASI) namely the mounting of a specific, long-lasting response from the host immune system against cancer cells. As the field of active specific immunotherapy against cancer has been moving for more than 100 years it has segregated with time in two main paths: Cell-based and antigen-based immunotherapies. The similarities and differences between these two types of ASI will be discussed in greater detail. Among the cell-based therapies this current work will focus on cancer cells as sources of tumor associated antigen and not addressed cell-based therapies using other cells type combined with defined antigen (such as dendritic cells exposed to antigen).

a) Evidence for anti-tumor immunity

Experimental and isolated clinical observations showing spontaneous regression of growing tumors have been known for decades. For more than 50 years, increased knowledge in the basis of the immune system as well as observations of many experimental models and clinical situations has led to the conclusion that immune mechanism plays a role in cancer growth. The role of immune mediated mechanisms to explain tumor regression has been well documented in several tumor types.
Correlation between better prognosis (slower tumor progression, better survival) and endogenous host response such as T cell infiltration/recruitment within tumor deposit has been analyzed and reported for many common tumor types such as primary cutaneous melanoma (Clark, Elder et al. 1989), (Clemente, Mihm et al. 1996),(Mihm, Clemente et al. 1996) regional lymphnode metastasis of melanoma (Mihm, Clemente et al. 1996), colon carcinoma (Naito, Saito et al. 1998; Pages, Berger et al. 2005), renal cell carcinoma (Nakano, Sato et al. 2001), ovarian carcinoma (Zhang, Conejo-Garcia et al. 2003), head and neck cancers (Reichert, Scheuer et al. 2001), breast carcinoma (Marrogi, Munshi et al. 1997), lung carcinoma (Al-Shibli, Donnem et al. 2008) (Hiraoka, Miyamoto et al. 2006) and invasive bladder carcinoma (Sharma, Shen et al. 2007) as reviewed by Pages and al. (Pages, Galon et al. 2010).

The graft versus leukaemia effect observed in allogeneic bone marrow recipients is another example of the strong anti-tumor effect triggered by antigen within the leukemic cells (Bleakley and Riddell 2004)

Clinical observations of patients taking immunosuppressive medications (for either allogeneic organ transplant or inflammatory diseases) revealed an increase risk of cancers, especially tumor driven by oncogenic viruses such as lymphoma, basal cell carcinoma, oral and anal squamous carcinoma and Kaposi sarcoma (Birkeland, Storm et al. 1995; Rama and Grinyo 2010) (Vajdic and van Leeuwen 2009) but also many other tumor types (Grulich, van Leeuwen et al. 2007)
The HIV epidemic provided further evidence for the risk of cancer in immunocompromized hosts. Similarly to transplanted patients taking immunosuppressive medications, incidence of cancers is dramatically increased in this population. Incidence of anal squamous cell

carcinoma is increased 14 to 81 times in this selected group of patients(Sunesen, Norgaard et al. 2010).
Indeed, the risk of cancer in the HIV infected population is related to the level of immunosuppression, the more severe the immunosuppression, higher the risk of cancer such as lymphoma (Engels, Pfeiffer et al. 2010). The role of the immune system in modulating the cancer growth is also well illustrated in immunocompromized patients suffering from post transplant lymphoproliferative disorders (PTLD) (Gottschalk, Rooney et al. 2005). A decrease in immunosuppressive drugs leads to lymphoma regression in most cases in a large serie of cardiac transplant recipients (Aull, Buell et al. 2004). Similar effect has been observed when switching from calcineurin inhibitors to mTOR inhibitors in kidney transplanted patients (Cullis, D'Souza et al. 2006).

Experiments in animal models have confirmed these finding. In addition to the very strong clinical data observed in immunocomporomised patients, scientific experiments in immunocompromized animal models clearly demonstrated a correlation between cancer growth and immunosuppression
Indeed, animal lacking genes key for immune functions such as RAG1-2, INF gamma, Trail, develop spontaneous tumors and chemically induced tumors (Dunn, Koebel et al. 2006).

The detailed explanation for the role of the immune system for decreasing the risk of cancer growth is beyond the scope of this work but refers to both immunosurveillance and abnormal cell destruction(Zitvogel, Tesniere et al. 2006; Stagg, Johnstone et al. 2007).

b) Rationale for anti-tumor immunotherapy

The strong evidence of modulation of cancer growth by the immune system led to a major effort to go one step further. As the immune system modulates tumor growth, can this effect be oriented in order to prevent or treat cancer? More than 100 years ago, long before any knowledge on the basis of the immune system, clinical experiments were performed based on empirical observations. The clinical experiments by Cooley, injecting adjuvants such as live streptococcus pyogenes and Serratia marcesens bacterial extract into more than 1000 patients has been very well documented in the medical literature (Nauts, Fowler et al. 1953; Dranoff 2004). Tumor control was rarely observed for patient suffering from carcinoma but response rate for soft tissue sarcoma was reported to be as high as 30% to repeated injections of bacterial extracts (Cooley's toxin) either directly into the tumor or intravenously.

The lack of standardisation and the lack of good tools to measure the activation of the immune system prevented any analytical understanding of the observed phenomenon. Nevertheless, administration of adjuvant within tumor deposit has remained a valid treatment as bladder instillation of the adjuvant Bacille-Calmette-Guerin (BCG) is recommended for localized bladder cancer. Strong scientific and clinical evidences support this immunotherapy.
Genetic and molecular description of the key elements of the immune systems (Major Histocompatibility Complex system, innate immune response, T Cell Receptor, Immunoglobulin specificity, Co-stimulation molecules and identification of tumor associated antigens) over the last 40 years paved the road to more specific targeted therapies.
In the same period, vaccination against infectious agent achieved tremendous progress with the identification of potent immunogens such as proteins or extracts from bacteria and viruses. Learning from the success of efficient prevention of infectious diseases with specific proteins, the search of similar targets against cancer became a legitimate goal.

Tumor-associated antigen

It is now widely accepted by the scientific community that tumors do have specific antigen. Such antigen may results from mutated proteins, over-expressed proteins, aberrantly glycosylated proteins or reactivation of protein express in other organ or during development. The concept of tumor antigen, or protein express on tumor cells and not on normal cells raised extraordinary expectations in the field of clinical oncology.

Many different types of tumor antigen have been described. In experimental immunization models the most potent antigen are 'Tumor-rejection antigen'. Tumor rejection antigen are strongly immunogenic and lead to a protective immune response upon tumor challenge.

Since the first reported experiments on immunity to transplantable chicken tumors by Rous and Murphy (Rous and Murphy 1914) and the description by the team of T. Boon of the first cancer antigen in human (van der Bruggen, Traversari et al. 1991; Traversari, van der Bruggen et al. 1992) (MAGE-1 for **m**elanoma **a**nti**ge**n) the definition of tumor antigen has been refined. Indeed several families of tumor associated antigen have been described.

-Tumor specific antigen: altered protein found only on tumor cells (altered immunoglobulin in lymphoma, fusion protein resulting from translocation)
-Tissue associated antigen: proteins found only in specific organs (Melan-A or gp100 on melanoma and melanocytes, PSA on normal prostate cells and prostate carcinoma)
-Onco-foetal antigen: proteins expressed during development and by tumor cells: **C**arcino**E**mbryonic **A**ntigen, MAGE, surviving (Andersen, Svane et al. 2007)
-Tumor-testis antigen: antigen express only in testis and on cancer cells (NY-ESO 1)
-Viral associated antigen: viral protein express by cancer cells previously infected (EBV, HPV, HBV, HHV8, etc)

Characterization and production of known tumor associated antigen allowed testing in experimental animal models in order to demonstrate the proof of principle that antigen specific anti-tumor immune response indeed induce tumor protection and or tumor regression.

Experimental model of Ag-based immunization.

There are strong experimental data in many different murine models such as melanoma, sarcoma and breast cancers showing induced specific immunotherapy upon vaccination with specific antigens. This field was pioneered by L. Old at the Sloan–Kettering Institute in New-York. Using tumor specific rejection antigen, protective immunity has been observed in several animal models of chemically induced sarcoma. Chemically or virally induced changes in cancer cells are recognized as strong antigen, inducing a potent immune response. (Srivastava, DeLeo et al. 1986).

In melanoma and neuroblastoma as reported as early as 1972 by Hellstrom and Hellstrom (Hellstrom and Hellstrom 1972) but also in colon cancer model with immunization againt CEA peptide (Bei, Kantor et al. 1994), or breast cancer model with vaccine against Her2-neu (Disis, Schiffman et al. 2000).

c) 20 years of clinical research in Antigen-based cancer immunotherapy

ASI using defined tumor associated antigen in clinical research has benefited greatly from both the identification of TAA and the progress observed in immunization of several infectious diseases using defined bacterial or viral antigens.

The discoveries of CD8 T cells specific immune response in addition to major improvement in monitoring tools such as cytotoxic T lymphocytes assays, elispot, cell sorting for antigen specific CD8 cells led to the design of numerous clinical trials in many tumor types with many TAA. Looking back for more than 30 years in clinical research addressing the issue of TAA-

based cancer immunotherapy clinical is quite interesting. The clinical trials performed over the last 30 years are very difficult to compare as most are small phase I trial of selected patients. The differences in the immunization pattern are numerous:

- Cancer types are variable: most trials are performed in melanoma patients, colon and breast cancer patients with matching HLA profile (most trials are targeting HAL A2 patients)
- Many different TAA: many melanoma associated antigen have been tested as well as CEA, PSA, Her2-neu, mutated EGFR, viral protein such as HPV E6,E7. Some immunization include more than one antigen.
- The formulation of a given TAA is variable: protein, peptide, RNA, naked DNA, enclosed into a viral plasmid,
- The routes of immunization are diverse: intra-tumoral, intradermal, sub-cutaneous, intra-nodal, i-p
- The schedules of treatment are variable: single or repeated injections
- The uses of adjuvant are variable: no adjuvant, systemic adjuvant before or during immunization such as IL-2, INF gamma, GM-CSF, local application such as Toll like receptor agonist such as imiquimod or CPG, QS21, KLH, BCG or mixed with the antigenic material (fused protein or mixed plasmid)

Most clinical trials are small-size Phase I or non-randomized Phase II studies. Few Phase III trials have ever been performed and/or published to date with very limited success.
With more than 100 clinical trials published and 25 years of effort, no compound has proven its efficacy and none has been registered for clinical applications by regulating health authorities.

The deception has matched the high expectation in the fight against cancer contrary to the field of infectious diseases. In deed successful immunization programs against a very wide range of bacterial, viral or parasitic pathogens are obvious and ongoing.
The reasons for the lack of efficacy in oncology despite more than 20 years of clinical trials are numerous. **First, prevention against infectious agents with foreign antigen is much more efficient than treating ongoing cancer with self proteins.** Experimental animal models may have been misleading or overoptimistic as most tumor in rodent are chemical or virally induced and inoculated within days before or after the treatment is performed. In addition many experimental models have demonstrated a specific T /B cell response against defined antigens in a xenogenic models, far from the clinical setting of patients with ongoing exposure to autologous (self) antigenic material. Experimental and Clinical situations are quite distinct, most cancer patients have a tumor growing for months or years with TAA not potent enough to initiate and maintain a meaningful immune response. True tumor-regression antigen may be responsible for spontaneous cancer regression but trials testing known antigen have not yet been able to induce major clinical benefit in patients with advanced disease. Many additional mechanisms can explain the lack of efficacy observed in clinical trials. Some are listed below:

- Weak immunogenicity of the known human antigens
- Lack of potent adjuvants
- Loss of antigen on tumor surface, immunoediting
- Loss of expression of MHC class I on tumor cells
- Lack of necessary co-stimulatory molecules on antigen presenting cells
- Strong inhibitory signalling in and surrounding tumors such as TGF beta
- Clinical trials performed in very sick patients with massive tumor infiltration are unlikely to show clinical benefit as such therapy should be performed in minimal residual disease in good performance status patients.

The gap between infectious disease and cancer is well demonstrated with the immunization against several Human Papilloma Virus (HPV) serotypes. HPV is a well known viral organism causing infections (warts and oro-genital ulcers) but also an oncogenic virus (mainly

serotypes 16 and 18), responsible for cervical, anal and oral squamous cell carcinoma. Although preventive immunization with HPV proteins shows very positive results with induction of a strong immunity, preventing acute infection and late onset of cervical cancer in female subjects (Lehtinen, Paavonen et al. 2012), therapeutic immunization in female patients with ongoing HPV induced cervical cancer failed to improve clinical outcome (Lehtinen, Paavonen et al. 2012).

Some adjuvants are used alone in close contact with tumor cells. The best example of clinical application of non-specific adjuvant therapy is the local bladder instillation of Bacille Calmette Guerin (BCG) for the treatment of small superficial bladder carcinoma. This treatment is performed without adding TAAs and has demonstrated its efficacy in randomized trial (Herr, Schwalb et al. 1995). Topical application of TLR7 agonist imiquimod is approved for the treatment of basal cell carcinoma (Geisse, Caro et al. 2004) and has shown very promising result in vulvar intraepithelial neoplasia as published in 2008 in the New England Journal of Medicine (van Seters, van Beurden et al. 2008). Some trials have compared the efficacy of several adjuvant in melanoma patients such as Incomplete Freund's adjuvant (IFA), GM-CSF and QS-21, showing less potency in IFA containing vaccine combined with tyrosinase peptide (Schaed, Klimek et al. 2002).

More recently ipilimumab, a drug designed to inhibit the immune system dowregulator CTLA-4 has been tested in a three arm Phase III trial in advanced melanoma. Monotherapy with ipilimumab or in combination with gp100 vaccine resulted in similar improvement in progression free survival, statistically and clinically meaningful compared to treatment with gp100 alone. (Hodi, O'Day et al. 2010). These positive results lead to the registration of ipilimumab both in USA and Europe for metastatic melanoma progressing after first line therapy. Additional data combining ipilimumab with chemotherapy in first line metastatic melanoma recently showed improved overall survival compared to chemotherapy alone (Robert, Thomas et al. 2011).

d) Cell-based immunotherapy

Another way of inducing anti-tumor specific immunity is cell-based immunotherapy. It is in a sense a rediscovery of initial anti-tumor strategy developed by Cooley in the late 19th century and followed by others using adjuvant such as BCG or Corynebacterium parvum in the 20th century (Bartlett and Zbar 1972; Ribi, Granger et al. 1975; Dye, North et al. 1981). Discoveries in the field of biotechnology over the last 20 years allowed the testing of novel approaches in cell-based immunotherapy. One of the main reasons for going back to cell-based immunotherapy is the lack of success of antigen based immunotherapy as presented previously. The table below illustrates the major differences between single antigen versus cell based cancer immunotherapies.
Cell-based immunotherapy is an ASI as described before. In most situations the immunization is prepared from the patient's own tumor as the goal is to educate the immune system against one/several TAAs present on the cancer cells to be targeted. As autologous tumor cells harvesting may be difficult and required personalized therapy, strategies have been developed to evaluate allogeneic source of tumor cells, assuming immunogenic antigen are present on both the patient's tumor cells and the allogeneic cells used for immunization.

Differences between Antigen and Tumor cell-based Immunization

	Antigen-based Immunization	**Cell-based Immunization**
Principles	Target: defined antigens	Target: any antigen
Targets	1-10 antigens	Unlimited (tumor cells)
TAAs	Needs to be determined	No predefined TAAs
Sources	TAA in Proteins, peptides, DNA, viral vector	Non replicative tumor cells autologous vs allogeneic
Patients selection	Tumors need to harbour TAA	Need to harvest tumor cells
Patients selection	HLA restricted	No HLA restriction
Tumor type	Only tumor with known TAA	Any tumor type
Monitoring tools	Well validated techniques	Difficult as TAAs are not known
Adjuvants	Required for efficacy	Required for efficacy
Products manufacturing	Similar for all patients	Patient specific if autologous cells

In cell-based immunotherapy the quality and the quantity of targets are not defined. Despite this lack of knowledge about the targets the principle of tumour specificity remains as the goal is to induce an immune response against TAAs. Selecting tumor cells as the source of TAAs is a simple way to have a very **large repertoire of TAAs** for any given tumor cells. One of the driving hypothesis for selecting autologous tumor cells as the source of TAAs is that each tumor may have a distinct set of TAAs that requires specific immunization. In addition, the lack of proven clinical benefit in immunotherapy trials with currently described TAAs raises the question that truly strongly immunogenic TAAs have not yet been discovered. The statement made in 1997 by E. Jaffe and D. Pardoll:'..but until more common tumor antigens have been identified at the genetic level, and the prevalence and biorelevance of these antigens have been assessed, an individual's tumor is the only source of tumor antigens for vaccination at this time' (Jaffee and Pardoll 1997) may still be accurate today.

Experimental data in immunogenic models

Very strong data have been published over the years showing that inactivated tumor cells induce specific, long lasting anti-tumor immunity in many experimental animal models. The 1993 publication by Dranoff et al. set the basis for a better understanding of cell-based immunization in immunogenic tumor models (Dranoff, Jaffee et al. 1993).

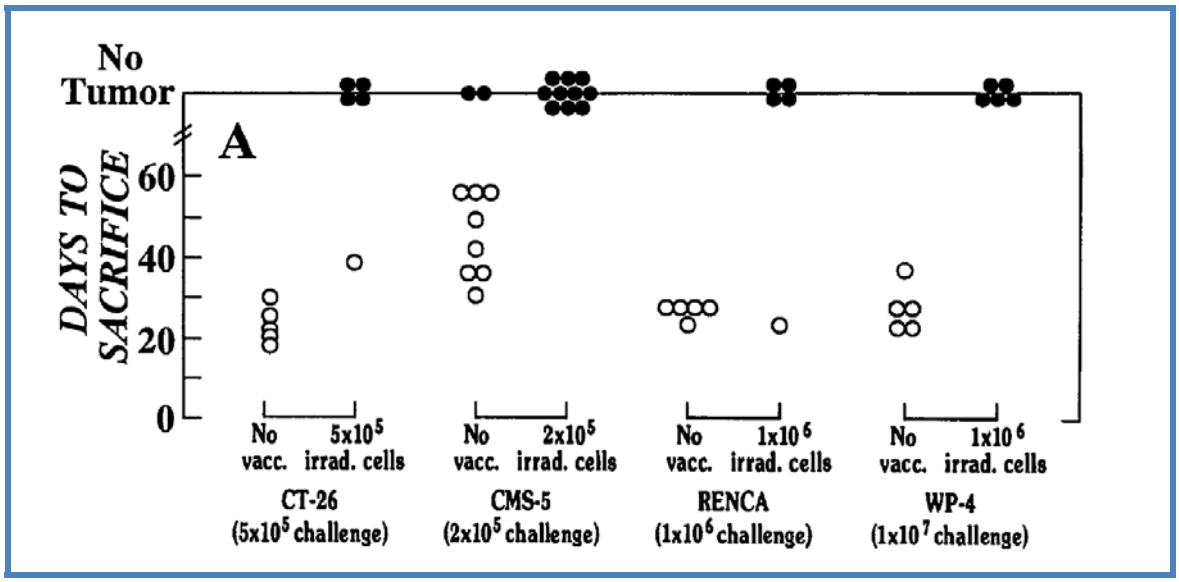

Figure 1 from Dranoff et al. (Dranoff, Jaffee et al. 1993)

Figure 1, illustrates the intrinsic immunogenicity of 4 murine tumor cell lines. Without adjuvant, immunization with irradiated un-modified cells induces protection upon tumor challenge in most animals while no protection is observed in the absence of vaccination. These tumor cells are considered strongly immunogenic; that is they harbour tumor-rejection antigen or antigen inducing tumor rejection upon rechallenge.

As discussed above, animal models often are misleading in tumor immunotherapy as the experimental setting does not reflect the biology of cancer arising in clinical oncology. Indeed immunization with inactivated cells without adjuvant has minimal or no effect in clinical trials and despite positive experimental data in selected tumor type it is very unlikely to have a meaningful effect for cancer patients. As already observed by scientists in the late 19th century, adjuvants are required to trigger and/or amplify the magnitude of the immune response. BCG, Corynebacterium Parvum, Freund' adjuvant or mixed of bacterial extract have been combined with tumors cells. Among mostly negative clinical trials, positive results have been clearly demonstrated by randomized studies and meta-analysis that local BCG instillation in localized bladder is beneficial and superior to local chemotherapy (Bohle and Bock 2004; Duchek, Johansson et al. 2010; Sylvester, Brausi et al. 2010). In addition two randomized trials in locally advanced colon cancer, stage Duke B2 and C or Stage II diseases have been published in the Journal of Clinical Oncology in 1993 and in The Lancet 2000 with evidence for both PFS and overall survival benefit of an autologous cancer cell immunization combined with BCG (Hoover, Brandhorst et al. 1993; Vermorken, Claessen et al. 1999).

Genetic modification of tumor cells

Genetic engineering of cancer cells to boost tumor immunity has been pioneered by Gansbacher at the Memorial Sloan Kettering in early 90's (Gansbacher, Zier et al. 1990). Using retroviral vector to integrate immunomodulating gene in a stable fashion in the tumor cell's genetic material represents a turning point in cell-based immunology. Indeed, the ability to genetically modified tumor cells represents a major step as it can evaluate the effect of adjuvant protein produced by the tumor cells at the immunization site.

By comparing numerous adjuvants in the same tumor cell line it allows to classify them in term of potency as immunomodulators.

It also allows a better understanding of the subtle changes in the priming phase of the immune response.

Analysis of key parameters such as antigen presenting cell recruitment, induction of humoral and/or cellular immune response can be compared between different adjuvants.

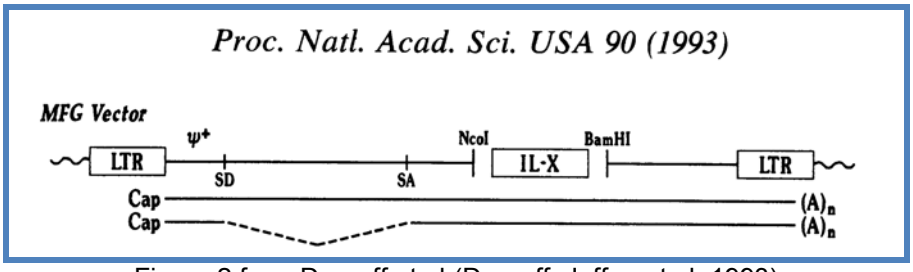

Figure 2 from Dranoff et al (Dranoff, Jaffee et al. 1993)

Schematic view of the MFG retroviral vector used to produce defective viral particles. The cDNA of interest has to be cloned in frame as shown in the figure 2.

Interleukin-2, a cytokine known to stimulate the proliferation of cytotoxic T cells, Helper T cells, NK and LAK cells was one of the obvious candidate cytokine to be tested as an adjuvant to be relased locally by tumor cells. Data from several groups reveal that IL-2 production by tumor cells abrogates tumorogenicity and, in mice surviving tumor challenge, specific, long lasting immunity was obtained. The data were mainly gathered in immunogenic, chemically induced tumor model CMS5, a fibrosarcoma in Blab/c mice (Gansbacher, Zier et al. 1990)

The conclusion by Gansbacher et al. is somewhat misleading as it has been shown by several groups including Dranoff et al that protective immunity in the CMS5 model can also be obtained with un-modified irradiated CMS5 cells (cf Figure 3 below)

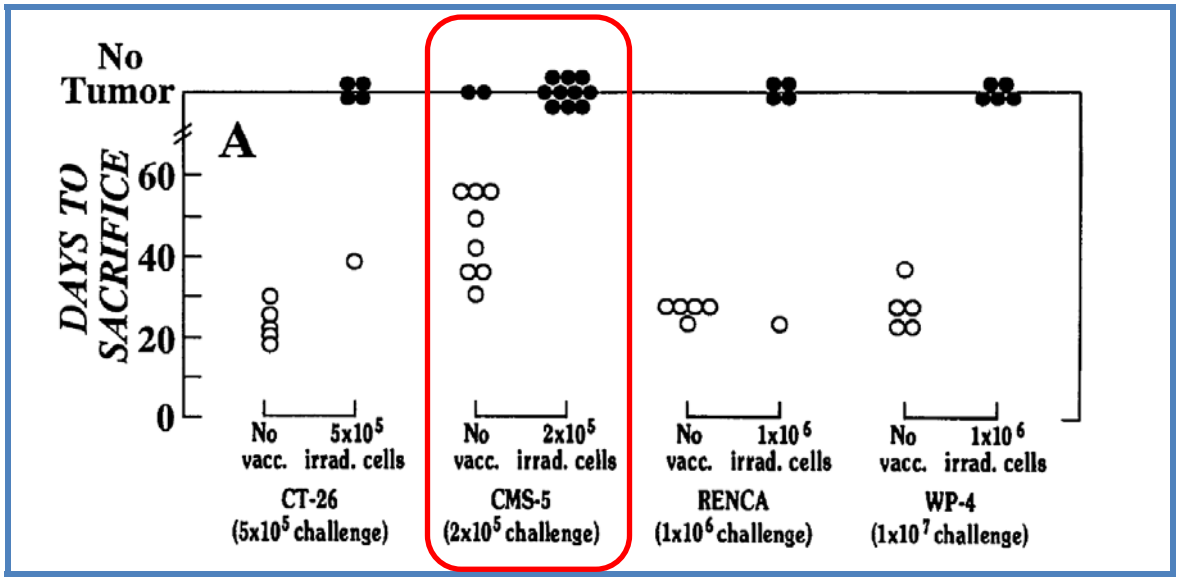

Figure 3 adapted from Dranoff et al.(Dranoff, Jaffee et al. 1993)

e) Local release of GM-CSF by engineered tumor cells

The publication by Dranoff et al. in 1993 is instrumental for a good understanding of cell-based immunotherapy and the role of adjuvant cyctokines produce at the vaccine site by engineered tumor cells. As experimental data reported in that manuscript set the bases for further clinical development, they are presented in detail below. The authors compared the efficacy of many cytokines and immunostimulatory proteins in the same experimental tumor model of poorly immunogenic murine B16 melanoma. This direct comparison set the basis for selecting the most potent adjuvants for testing in other immunization models.

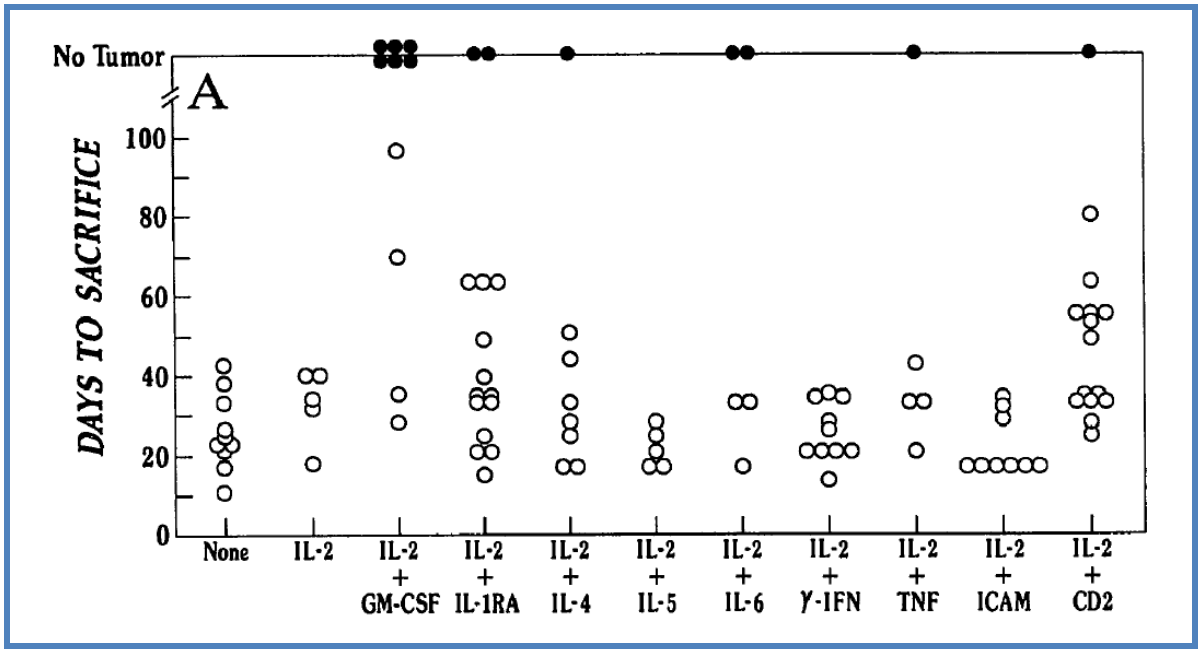

Figure 4 from Dranoff et al (Dranoff, Jaffee et al. 1993)

Comparison of several adjuvants in the poorly immunogenic B16 melanoma murine model. Figure 4, above shows tumor immunization experiments with **live** cells secreting several cytokines in the poorly immunogenic B16 melanoma model. As already shown by gansbacher at al,(Gansbacher, Zier et al. 1990) only IL-2 secreting live tumor cells are rejected, therefore the experiments tested several cytokines in addition to IL-2.
Mice were vaccinated with live wild type B16 or B16-secreting IL-2 or a combination of IL-2 and another cytokine as mentioned in the graph. 10 days after immunization, mice were re-challenge with wild type B16 cells. The data reveal that in the poorly immunogenic B16 melanoma model mice rejecting IL-2 producing tumor cells are not able to mount a protective immunity as all mice died upon rechallenge. Marginal protective immunity was observed for mice immunized with IL-2 and either INF gamma, TNF, IL-5, IL-4 or ICAM. In contrast strong protective effect was observed for mice immunized with IL-2 and GM-CSF-secreting live cells.

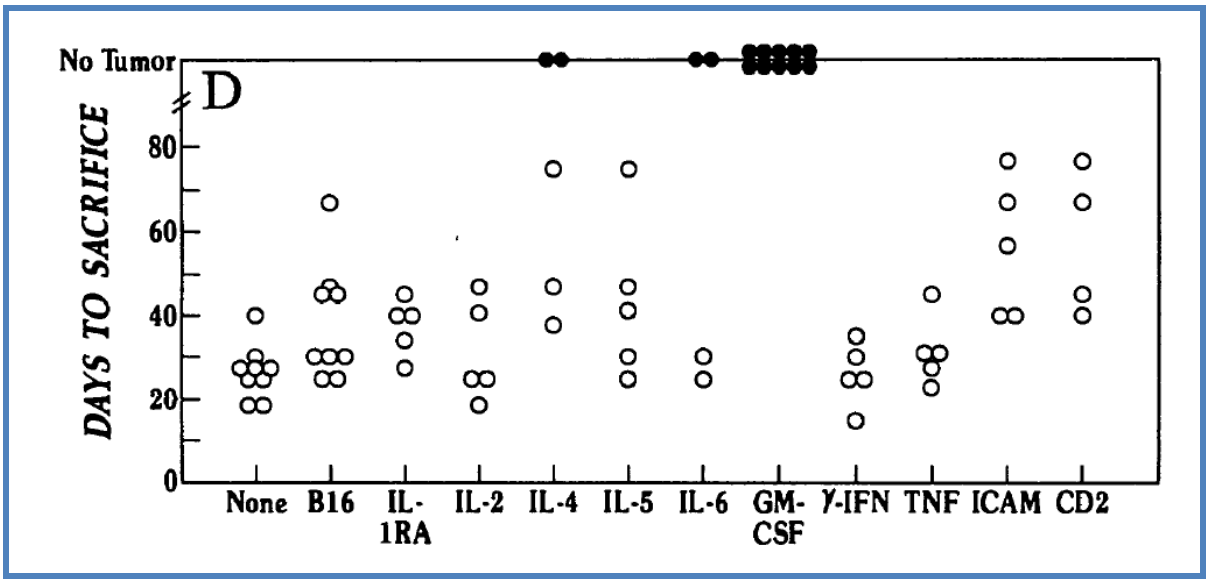

Figure 5 from Dranoff et al.(Dranoff, Jaffee et al. 1993)

Tumor immunization with **lethally irradiated** tumor cells engineered to produce the mentioned cytokine. In Figure 5, the data show the percentage of mice surviving wild type B16 challenge one week after immunization with irradiated B16 melanoma cells engineered to produce the described cytokine. B16 melanoma is poorly immunogenic as immunization with irradiated un-modified cells does not induce marked delay or prevent tumor growth upon re-challenge, all mice developing tumor. Similarly to the previous experiment GM-CSF is the most potent adjuvant cytokine in this comparative analysis. Altogether 30 potential candidate genes have been inserted in genetically modified B16 melanoma in other to evaluate their immunostimulatory effect (Dranoff 2002).

3) Irradiated genetically modified tumor cells secreting cytokines

a) Review of published experimental data in immunogenic and non- immunogenic models

The immunostimulatory effect of local production of GM-CSF by engineered tumor cells has been demonstrated in melanoma by several research groups(Armstrong, Botella et al. 1996) but is not limited to this tumor. Indeed already in its pivotal 1993 paper Dranoff et al (Dranoff, Jaffee et al. 1993) showed that in several models, poorly or highly immunogenic, the local production of GM-CSF by irradiated genetically engineered tumor cells increase protective immunity.

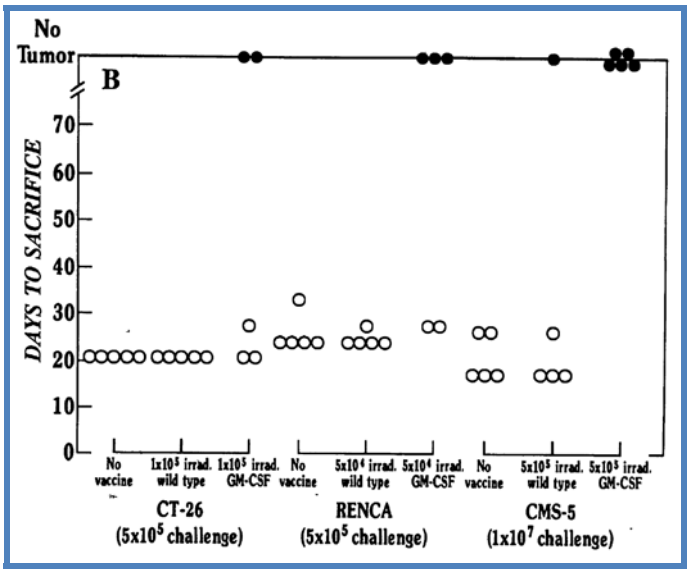

Figure 6 from Dranoff et al.(Dranoff 2002)

Figure 6 shows that immunization with irradiated GM-CSF secreting tumor cells is more potent than vaccination with non-transfected irradiated tumor cells in three different poorly immunogenic models. GM-CSF can induce protective immunity in poorly immunogenic model such as B16 melanoma but also increased immunogenicity of immunogenic tumor.
Local release of GM-CSF by irradiated tumor cells is one of the most potent adjuvant in murine tumor immunization models. Interestingly lymphocytes depletion analysis revealed that GM-CSF protective immunity is both CD4+ and CD8 + lymphocytes dependant but independent of NK cells.
Since the pivotal data from Dranoff et al in 1993, many tumor models has been tested with irradiated tumor cells genetically engineered to produce murine GM-CSF to evaluate the ability to trigger protective immunity.
Indeed positive immunostimulatory effects have been observed in most tumor models tested such as kidney, bladder, prostate, lung, breast, ovarian, colon, pancreas, liver or head and neck cancer as well as lymphoma, leukemia, neuroblastoma, melanoma, glioblastoma and sarcoma. Some of the published data are described below:

Lymphoma
Levitsky et al reported anti-tumor immunization both in a prophylactic and a therapeutic model of lymphoma using the A20 lymphoma cell-line (Levitsky, Montgomery et al. 1996).

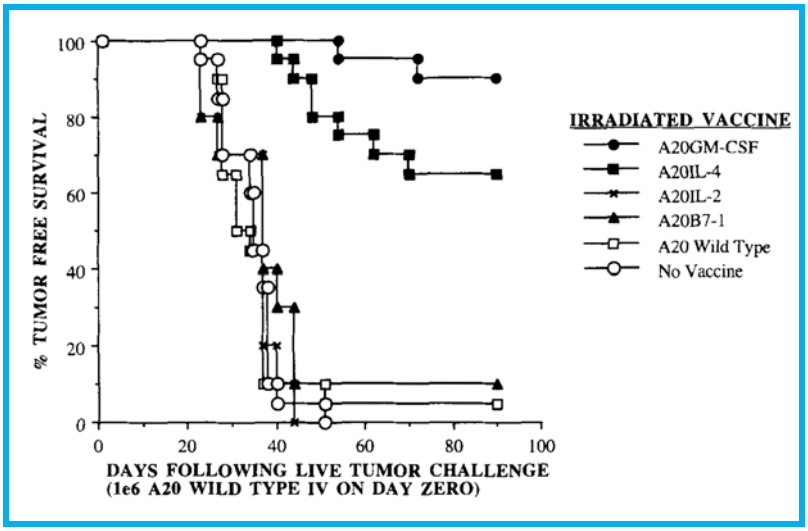

Figure 7 from Levitsky et al.(Levitsky, Montgomery et al. 1996)

Figure 7 shows the percentage of animals surviving wild type tumor challenge after vaccination with several cytokine producing irradiated tumor cells in the A20 lymphoma model. In this setting, irradiated A20 cells producing GM-CSF or IL-4 showed very good protective immunity compared to mice immunized with wild-type A-20 or tumor cells releasing IL-2 or the co-stimulatory protein B7-1

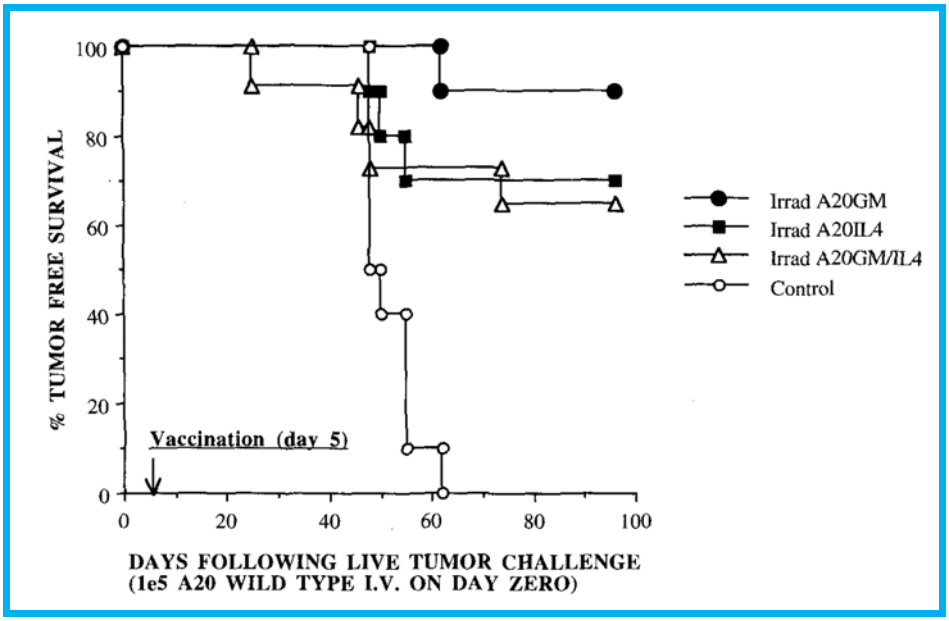

Figure 8 from Levitsky et al.(Levitsky, Montgomery et al. 1996)

In the same publication, the authors showed that in addition to the prophylactic vaccination, protective immunity was obtained in a therapeutic model where immunization is performed after live tumor challenge. Indeed, Figure 8 shows that 90% of the mice treated with irradiated A20 secreting GM-CSF experienced long time survival compared to 70% in IL-4 secreting group and 0% in the untreated group. No synergy was observed between IL-4 and GM-CSF in a group of mice immunized with irradiated tumor cells releasing both cytokines.

Neuroblastoma

In this 1996 publication, Bausero et al. studied anti-tumor immunization using a neuroblastoma cell-line genetically engineered to produce GM-CSF. Approximately 41% of mice immunized with irradiated N-2a/GM (neuroblastoma cell-line producing GM-CSF) versus 0% of those vaccinated with irradiated parental tumor survived. Long term specific immunity was confirmed by late subsequent challenge after 50 days with either wild-type neuro-2a or with the Sa1 syngeneic sarcoma. All mice survived wild-type neuro-2a challenge, whereas none survived inoculation with Sa1. Depletion experiments showed that both CD4+ and CD8+ T cells were necessary for successful immunization. Vaccination of mice with preexisting retroperitoneal tumors with irradiated N-2a/GM also improved survival. (Bausero, Panoskaltsis-Mortari et al. 1996)

Glioma

Immunization with sc injection of muGM-CSF-producing irradiated GL261 murine glioma and intracerebral implantation of live parental GL261 cells. Both preventive and therapeutic models showed delayed tumor progression compared to immunization with unmodified cells or no vaccine. These data showed that a subcutaneous immunization trigger a protective immunity with effectors mechanisms (cellular, humoral) able to operate in immune-privileged site such as brain (Herrlinger, Kramm et al. 1997) . Similar effect has been obtained with sc. immunization with GM-CSF secreting melanoma cells and subsequent intracerebral tumor challenge (Sampson, Archer et al. 1996; Lee, Wu et al. 1997).

In a subsequent paper Yu et al. (Yu, Burwick et al. 1997) showed that sc vaccination of syngeneic mice with irradiated GM-CSF-secreting B16 melanoma cells was capable of completely protecting animals against subsequent intracranial B16 tumor inoculation. In contrast, non-vaccinated animals or animals vaccinated with irradiated, nontransduced B16 cells succumbed to intracranial tumor within 3 weeks after inoculation. Treatment of established intracranial B16 melanoma tumors with subcutaneous injection of irradiated GM-CSF-secreting B16 cells significantly delayed death, as compared to injection of irradiated nontransduced B16 cells or no treatment. In addition, treatment of established intracerebral GL261 gliomas by vaccination with irradiated GM-CSF-secreting B16 cells mixed with irradiated, transduced, or nontransduced GL261 cells also extended survival. These B16/GL261 co-vaccinations also improved outcome and, in some cases, induced immunological memory that protected survivors from subsequent intracranial challenge with GL261 tumor cells. These findings indicate that peripheral vaccination with irradiated tumor cells in the presence of GM-CSF-producing cells can initiate a potent antitumor immune response against intracranial neoplasms.

Lung cancer

Therapeutic immunization with irradiated Lewis Lung Carcinoma cells producing murine GM-CSF (Lee, Wu et al. 1997). This is the first experiment using adenoviral vector rather than retroviral vector for genetically modified the parental tumor cells.

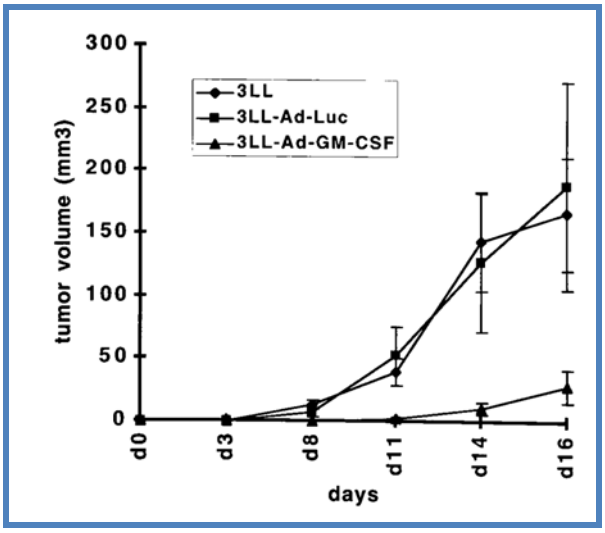

Figure 9 from Lee et al.(Lee, Wu et al. 1997)

As shown in Figure 9, in this experiment tumor volume in mice immunized with irradiated 3LL tumors cells secreting GM-CSF is much smaller compared to mice vaccinated with either un-modified 3LL or 3LL producing luciferase.

Acute leukaemia

In this publication Dunussi et al. evaluate the ability to trigger an immune response in a model of acute myeloid leukemia (Dunussi-Joannopoulos, Dranoff et al. 1998).

Comparison in a prophylactic immunization model of acute myeloid leukemia of 3 cytokine producing irradiated AML tumor cells. ■ GM-CSF, ◯ TNF-alpha, ▲ IL-4, ▨ mock-infected

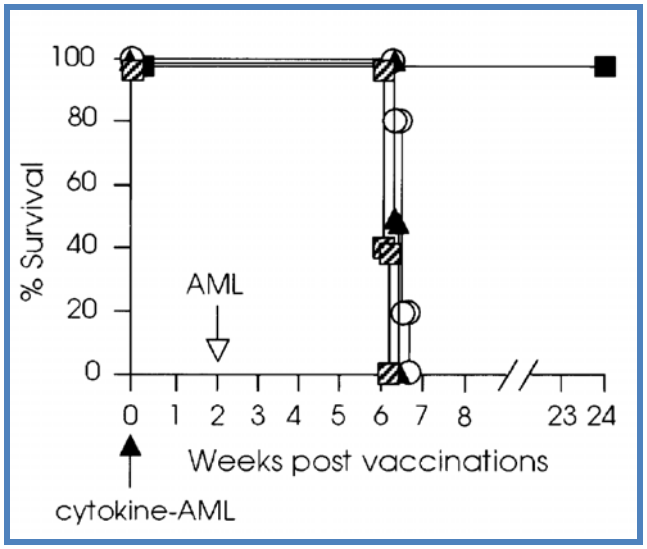

Figure 10 from Dunussi et al. (Dunussi-Joannopoulos, Dranoff et al. 1998)

This prophylactic vaccine experiment in an acute myeloid leukemia murine model shows a striking difference in tumor protection for mice immunized with irradiated leukemia cells producing GM-CSF compared to IL-4, TNF-alpha or mock-transfected cells with respectively 100%, 0%, 0% and 0% long term survival as illustrate on Figure 10.

Head and neck squamous cell carcinoma

This manuscript describes experimental data of anti-tumor immunization in a head and neck squamous cell carcinoma model (Couch, Saunders et al. 2003).

In this spontaneous murine squamous cell carcinoma, mice immunized either subcutaneously or in the mouth, with irradiated tumor cells transduced with GM-CSF were protected against wild-type challenge compared to mice treated with irradiated un-modified cells or non-treated controls. Very good tumor protection was similar in groups immunized sc(HL) or in the floor of mouth (FOM) as shown below on Figure 11.

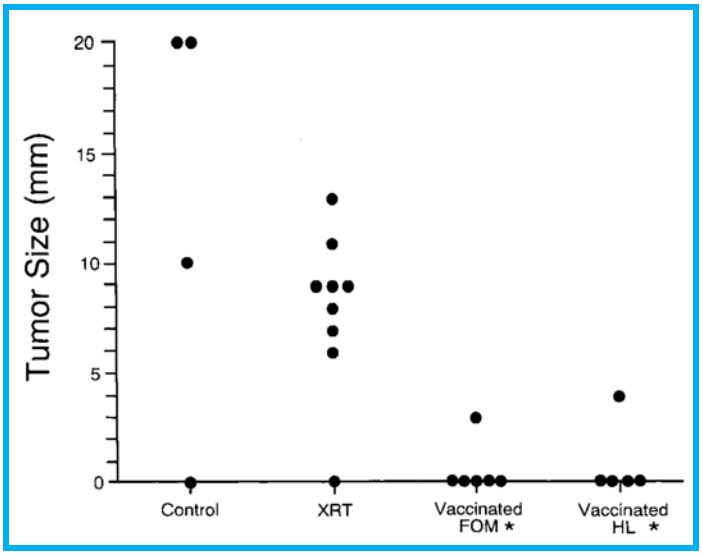

Figure 11 from Couch et al.(Couch, Saunders et al. 2003)

Prostate

At least two publications evaluated the role of GM-CSF transduced cancer cells in vaccination models of prostate cancer, both in the Dunning rat model. The study by Vieweg et al used human GM-CSF (Vieweg, Rosenthal et al. 1994) as the publication by Sanda et al used murine GM-CSF(Sanda, Ayyagari et al. 1994).

The paper by Vieweg et al. shows a better protection with genetically transduced cels compare to administration of recombinant GM-CSF protein or no vaccination. The data from Sanda et al. compared IL-2 and GM-CSF transduced cells to un-modified cells or no vaccine. As shown in Figure 12, both IL-2 and GM-CSF transfected cells induced protective immunity with a better results obtained with IL-2. All mice immunized with IL-2 producing vaccine survived compared to 40% with GM-CSF and 0% for un-modified irradiated tumor cells or unvaccinated mice.

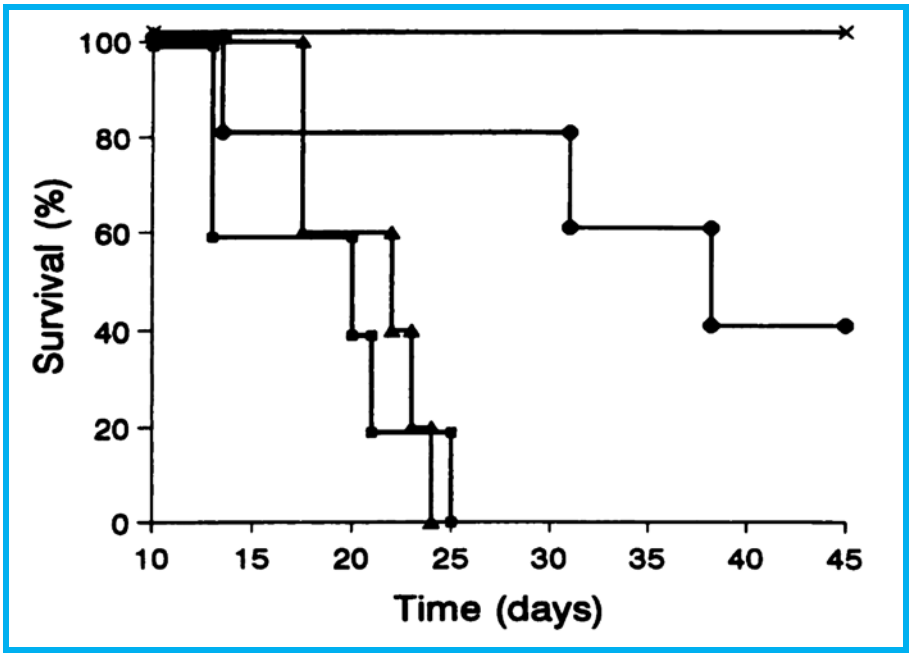

Figure 12 from Vieweg, et al. (Vieweg, Rosenthal et al. 1994)

Sc immunization with irradiated prostate tumor cells engineered to release murine GM-CSF(•), human IL-2(X), un-modified cells(▲) or no vaccination(■).

Breast Cancer

Using the murine breast cancer cell-line BalbMC, the authors evaluated the ability of irradiated cells engineered with adenoviral vector to secrete GM-CSF to protect against subsequent wild type tumor challenge. Control groups were treated subcutaneously with saline or irradiated un-modified BalbMC cells(Ogawa, Tomomasa et al. 2001).

Vaccination with irradiated GM-CSF-secreting BalbMC completely protected syngeneic mice challenged with live parental cells while the control group, vaccinated with un-modified irradiated BalbMC, showed a 60% protection, revealing the immunogenicity of this model. None of the tumor-free mice initially vaccinated with irradiated GM-CSF-producing BalbMC cells developed tumor upon repeated challenge with parental cells during the entire observation period as reported in Figure 13

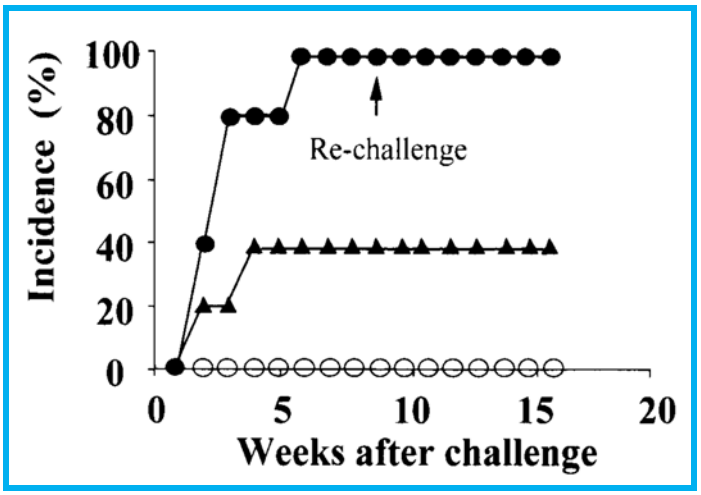

Figure 13 from Ogawa et al.(Ogawa, Tomomasa et al. 2001)

Colon cancer

At least two distinct experimental models have been used for evaluating the adjuvant effect of locally produced GM-CSF by colon cancer cell-line engineered to produce this cytokine. Kielian et al studies the CT26 colon cancer model while Ikubo et al. worked with the highly metastatic colon cell-line LM17. Both publications showed a marked increased in immunogenicity and protective vaccination in irradiated GM-CSF transduced cancer cells. Data from Kielian et al. are shown on Figure 14 and 15 (Ikubo, Aoki et al. 1999) (Kielian, Nagai et al. 1999)

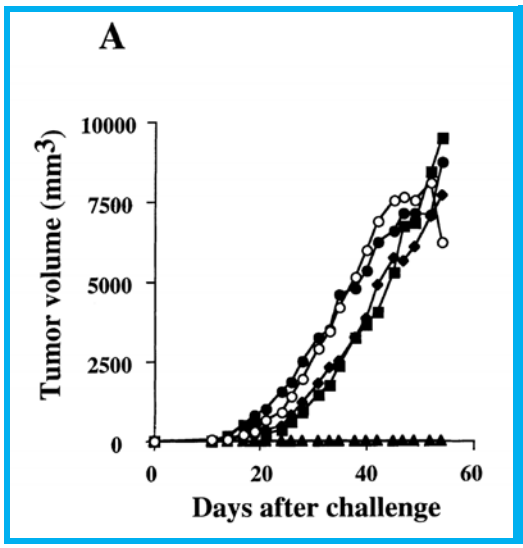

Figure 14 from Kielian et al.

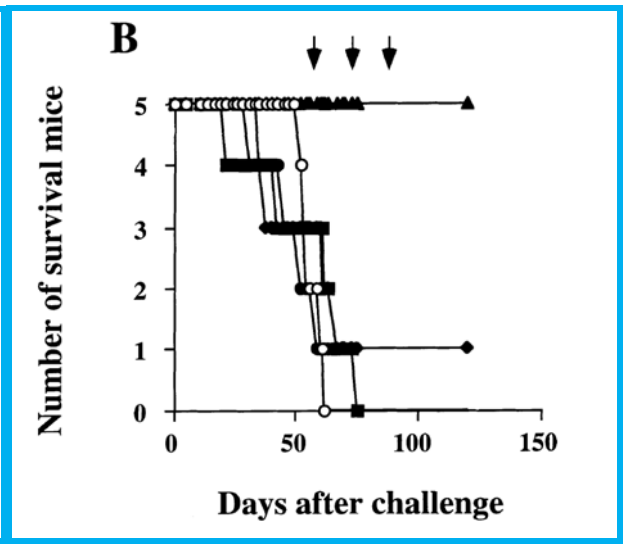

Figure 15 from Kielian et al.

In this CT26 murine colon carcinoma model, mice were immunized with either GM-CSF, MCP-1, INF-gamma, or mock transfected irradiated CT26 tumor cells and subsequently challenged with wild type CT26. Mice vaccinated with GM-CSF (▲) producing irradiated tumor cells showed a complete protection as shown on both tumor volume (Figure 14) and survival (Figure 15). All mice in GM-CSF group experienced long term tumor protection as all animals survived subsequent rechallenge (↓).

Vaccination post-syngeneic or allogeneic bone marrow transplantation (BMT)
In this set of experiments using B16 melanoma secreting GM-CSF, we evaluated the ability of transplanted mice to developed a protective immune response and if such response was related to a graft versus host disease in allogeneic recipients (Teshima, Mach et al. 2001).
In recipients of syngeneic BMT, immune reconstitution was critical for the development of antitumor activity. Vaccination did not stimulate antitumor immunity after allogeneic BMT because of the post-BMT immunodeficiency associated with graft-*versus*-host disease (GVHD). Remarkably, vaccination was effective in stimulating potent and long-lasting antitumor activity in recipients of T-cell-depleted (TCD) allogeneic bone marrow as shown in Figure 16.

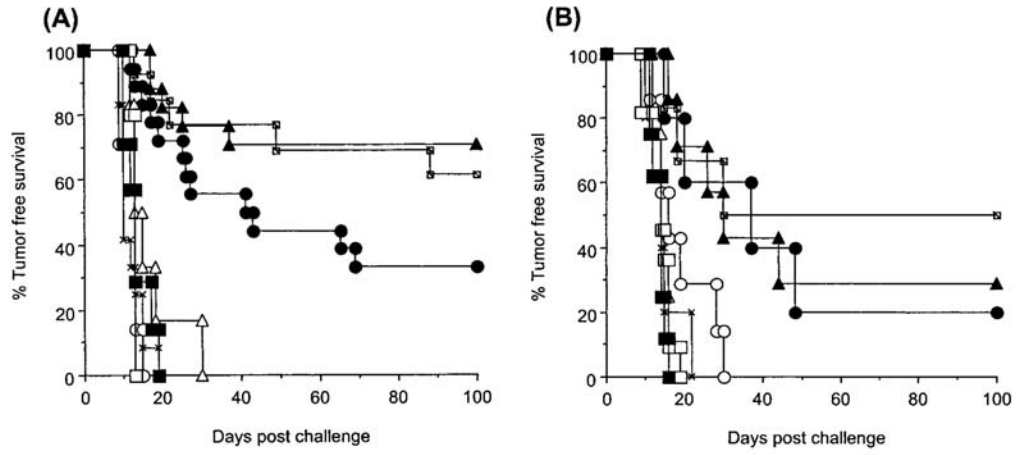

Fig. 2. Vaccination confers significant antitumor immunity after allogeneic TCD BMT but not allogeneic BMT. Lethally irradiated BMT recipients were transplanted with BM and splenic T cells from syngeneic or allogeneic donor mice. Each group of animals was divided into a vaccination group and a control group. Animals were vaccinated 6 weeks after BMT with 5×10^5 irradiated, GM-CSF-secreting B16 cells and challenged with 1×10^6 live B16 1–4 weeks later. *A*, SJL→B6SJLF1. Percentage of TFS after tumor challenge in recipients of syngeneic BMT (○, control, Syn BMT, − vax, $n = 7$; ●, vaccinated, Syn BMT, + vax, $n = 18$), allogeneic BMT (□, control, Allo BMT, − vax, $n = 5$; ■, vaccinated, Allo BMT, + vax, $n = 7$), allogeneic TCD BMT (△, control, AlloTCD BMT, − vax, $n = 6$; ▲, vaccinated, AlloTCD BMT, + vax, $n = 17$), and naive B6SJLF1 (×, control, − BMT, − vax, $n = 5$; ⧅ vaccinated, − BMT, + vax, $n = 7$). *B*, LP→B6. Percentage of TFS after tumor-challenge recipients of syngeneic BMT (○, control, $n = 7$; ●, vaccinated $n = 5$), allogeneic BMT (□, control, $n = 8$; ■, vaccinated $n = 11$), allogeneic TCD BMT (△, control, $n = 4$; ▲, vaccinated, $n = 7$), and naive B6 (×, control, $n = 8$; ⧅ vaccinated, $n = 11$). Data derive from three similar experiments.

Figure 16 from Teshimura et al. (Teshima, Mach et al. 2001)

Recipients of TCD bone marrow who showed significant immune reconstitution by 6 weeks after BMT developed B16-specific T-cell-cytotoxic, proliferative, and cytokine responses as a function of vaccination. T cells derived from donor stem cells were, therefore, able to recognize tumor antigens, although they remained tolerant to host histocompatibility antigens. These results demonstrate that GM-CSF-based tumor cell vaccines after allogeneic TCDBMT can stimulate potent antitumor effects without the induction of GVHD.

Altogether these experimental data shows the potent adjuvant effect of GM-CSF's local release by engineered tumor cells at the vaccine site in a wide range of tumor type in several animal models (Balb/c, C57Bl6 mice, Dunning rat). The immunostimulatory effect observed in these published experiments is tumor specific, long lasting and with a strong endpoint as survival is the main objective rather than tumor volume or immunological parameters such as T-cell or antibody responses. Cell-based immunization with genetically modified tumor cells is triggering the immune response in its primary phase. Indeed, the cytokine produced locally at the vaccination site by irradiated tumor cells has a biological activity limited to several days. The cytokine has no direct implication during the effector phase as it is no longer biologically active. As GM-CSF has a wide spectrum of activity on hematopoietic cells the mechanisms driving its major immunostimulatory effect in tumor vaccination models is not obvious. One of the leading hypotheses is the recruitment and/or activation of antigen presenting cells (APC) such as dendritic cells.

b) DC recruitment and tumor vaccination: direct comparison between GM-CSF and FLT3-L (Mach, Gillessen et al. 2000)

During the priming phase of the immune response APC play a critical role for processing and presenting antigen to T lymphocytes. Key elements such as MHC-TCR interaction, co-stimulation signals as well as APC maturation processes have been described in the past 20 years. As professional APC, the role of dendritic cells in subcutaneous immunization models has been well documented. As GM-CSF is a well known maturation factor for DC, inducing DC to become more efficient at presenting Ag may be an explanation for its immunostimulatory activity. GM-CSF has many other known activities such as recruitment of granulocytes, eosinophils macrophages, lymphocytes and DC when produced locally (Metcalf 2008).

As FLT3-L has been characterized and showed to be one of the most potent cytokine at inducing DC maturation, testing its ability to enhance anti-tumor immunity was obvious.

In 2000, we published the direct comparison of GM-CSF and FLT3-L in irradiated tumor cells immunization models.

Briefly, FLT3-L mRNA was extracted from murine spleen. Using RT-PCR methods cDNA was obtained and the sequence confirmed. Primers were designed in order to incorporate restriction site allowing in frame cloning into retroviral vector. The sense strand contains a *BspHI* restriction site upstream of the initiator ATG and the antisense primer incorporates a BamHI restriction site downstream of the termination codon. After PCR amplification and digestion with both *BspHI* and *BamHI*, the cDNA was subcloned into the MFG retroviral vector. pMFG-muFLT3-Lvector was transfected into packaging cells (293GPG cells) to generate high titer stocks of concentrated recombinant MMLV particles that have incorporated the vesicular stomatitis virus G protein.

B16 melanoma cells were then infected with concentrated viral particles. Southern blot analysis confirmed a 1.5 proviral copies per infected cells.

No selection marker was used and no subcloning was performed. This strategy allows minimal difference between the genetically modified cells containing FLT3-L cDNA and the parental cell line.

Biological activity of FLT3-L-producing cells was confirmed after subcutaneous injections. Mice bearing FLT3-L secreting tumors showed marked leukocytosis at day 14, similar to effects previously described with administration of recombinant FLT3-L protein (Brasel, McKenna et al. 1996). FLT3-L-secreting B16 cells also elicited marked generalized lymphadenopathy and splenomegaly with marked expansion of the marginal zones and periarteriolar T cell-rich regions.

FACS analysis using both CD11c and MHC class II molecules (dendritic cells have both receptors on their surface) revealed a dramatic increased in double positive cells population representing up to 25% of the splenocytes. This massive DC infiltration was also observed in lymphnodes.

GM-CSF secreting B16 cells also caused marked leucocytosis and splenomegaly with less DC expansion (15% of splenocytes)

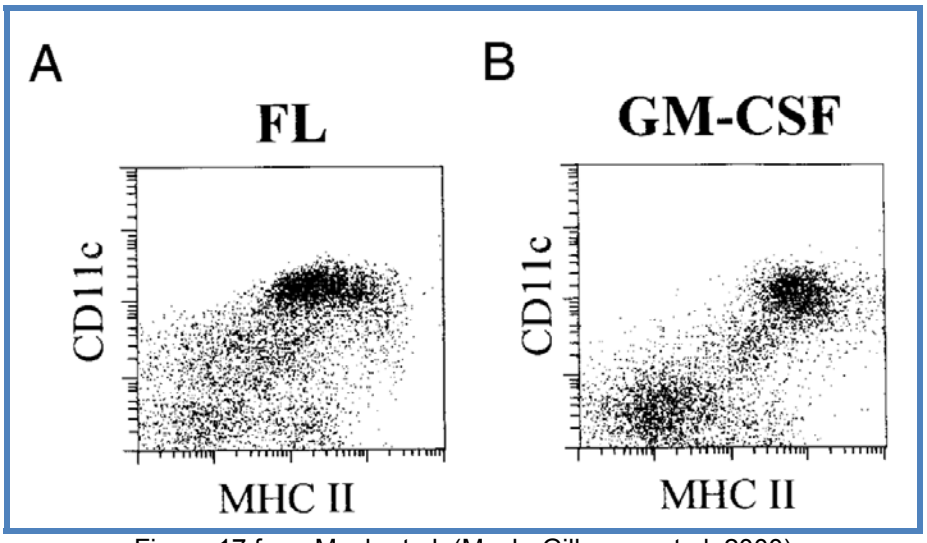

Figure 17 from Mach et al. (Mach, Gillessen et al. 2000)

The figure 17 above shows double CD11c / MHC II staining of splenocytes from C57Bl/6 mice obtained 14 days after sc injection of either B16-FLT3-L or B16-GM-CSF tumor cells. 25% and 15% of splenocytes are double positive in animals receiving respectively FLT3-L and GM-CSF secreting tumor cells.

Despite a higher capacity to recruit DC, FLT3-L secreting tumor cells are not as efficient as GM-CSF-secreting cells in tumor immunization experiments (Figure 18).
Indeed 75% of mice vaccinated with irradiated FLT3-L secreting b16 cells died upon B16 cells challenge compared to 0% of mice immunized with GM-CSF secreting B16 cells.

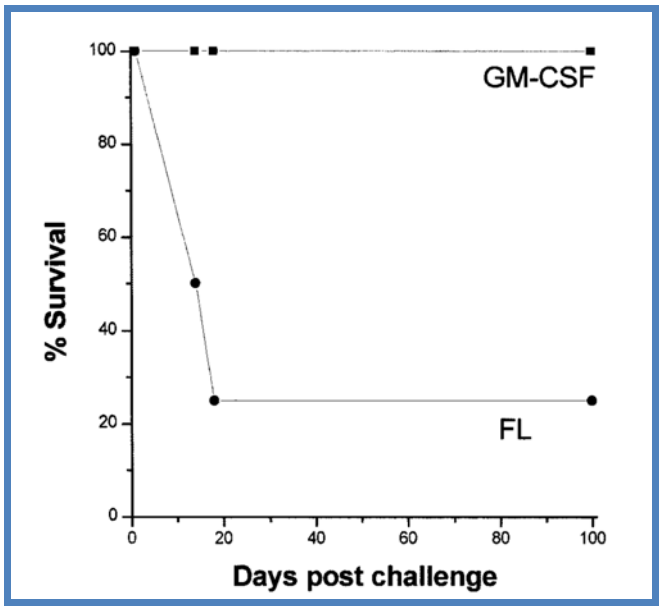

Figure 18 from Mach et al. (Mach, Gillessen et al. 2000)

GM-CSF stimulates more potent anti-tumor immunity than FLT3-L. C57Bl/6 mice were immunized sc. with $5x10^5$ irradiated, GM-CSF or FLT3-L-secreting B16 melanoma cells and were challenged 1 week later sc. With $1x10^6$ live, wild-type B16 cells. Vaccination with irradiated, wild-type B16 cells failed to elicit any tumor protection (data not shown). Similar results were found in five independent experiments. The difference observed between GM-CSF and FLT3-L groups is highly significant: $P<0.0001$ using the Fisher's exact test.

Additional experiments were performed to understand this striking decrease tumor protection despite higher DC recruitment for FLT3-L-secreting cells. Differences in cytokine release from lymphocytes vaccinated with either GM-CSF or FLT3-L-secreting tumor cells. Indeed lymphocytes from mice immunized with GM-CSF-secreting cells showed a much higher production of GM-CSF, IL-5 and INF-gamma. But the most interesting data gathered in these additional experiments came from the analysis of DC (Figure 19).

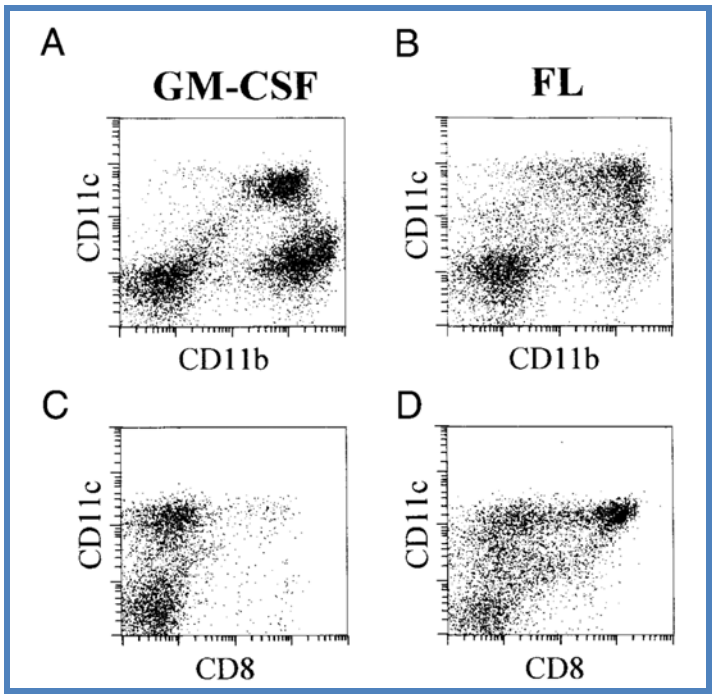

Figure 19 from Mach et al. (Mach, Gillessen et al. 2000)

Splenocytes harvested at day 14 after sc. injection of either GM-CSF or FLT3-L-secreting B16 tumor. Cells were stained with CD11c, CD11b and CD8α.
DC from mice receiving GM-CSF-secreting tumor are myeloid-type DC ($CD11^+/CD11b^+/CD8\alpha^-$) whereas DC from FLT3-L exposed mice are both lymphoid-type DC ($CD11^+/CD11b^-/CD8\alpha^+$) and myeloid.
This difference in the class of DC recruitment may explain the observed difference in tumor immunity for at least two reasons. First, myeloid DCs elicit a broader cytokine response (Th1 and Th2) whereas lymphoid-type DCs elicit a Th1 response (Maldonado-Lopez, De Smedt et al. 1999; Pulendran, Smith et al. 1999). Second, because antigen presentation stimulated by GM-CSF-based tumor cell vaccines involves cross-priming by bone marrow-derived cells, the capacity of DCc to phagocyte irradiated cells is particularly relevant. The capture of apoptotic bodies by DC infiltrating tumor cells co-expressing GM-CSF and CD40 ligand has been demonstrated (Chiodoni, Paglia et al. 1999). In this context, $CD8\alpha^-$ DCs seem to be much more effective in the ingestion of particulate antigen than $CD8\alpha^+$ DCs (Mackey, Gunn et al. 1997; Pulendran, Lingappa et al. 1997; Shen, Reznikoff et al. 1997).

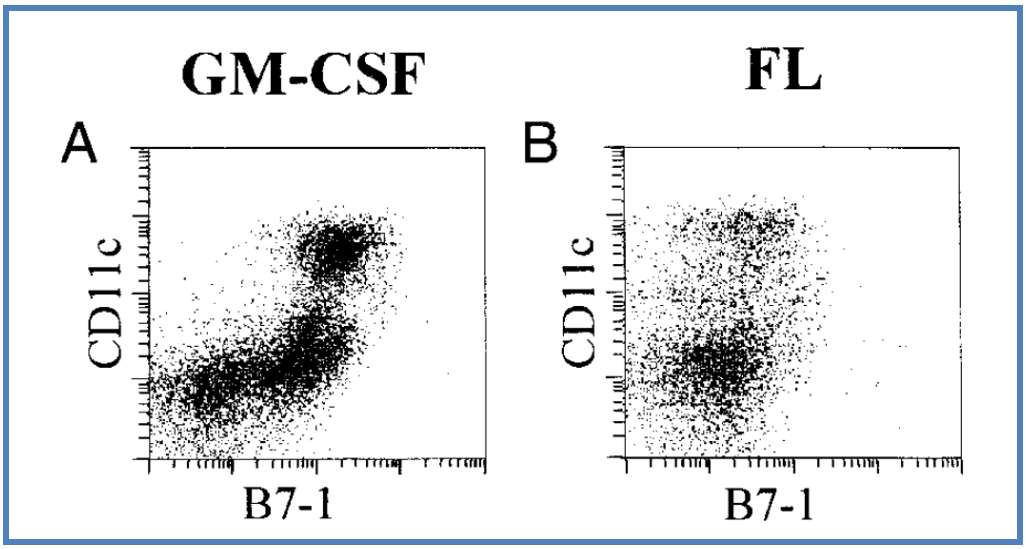

Figure 20 from Mach et al. (Mach, Gillessen et al. 2000)

The striking differences in B-71 expression by DCs generated in-vivo with GM-CSF or FLT3-L is another finding that may explain the observed tumor protection effect (Figure 20). Indeed co-stimulatory signals are critical factors for efficient priming and high B7-1 expression markedly reduces the amount of antigen necessary to trigger T-cell proliferation and expands the diversity of cytokine released (Murtaza, Kuchroo et al. 1999). Requirement for functional CD40 /CD40-L interaction in GM-CSF secreting tumor cells vaccination has already been demonstrated using DC40 knock-out mice (Mackey, Gunn et al. 1997).

c) Rational for local GM-CSF release

GM-CSF as adjuvant

GM-CSF is a monomeric protein of 127 amino acids with two glycosylation sites. The protein is synthesized as a precursor of 144 amino acids, which included a hydrophobic secretory signal sequence at the aminoterminal end. The sugar moiety is not required for the full spectrum of biological activities. Non-glycosylated and glycosylated GM-CSF show the same activities in vitro. Fully glycosylated GM-CSF is biologically more active in vivo than the non-glycosylated protein. The different molecular weight forms of GM-CSF (14 kDa, 35 kDa) described in the literature are the result of varying degrees of glycosylation. GM-CSF contains four cysteine residues (positions 54/96 and 88/121).

This protein is secreted, together with other factors, by T-cells and macrophages following cell activation by antigens or mitogens. The synthesis of GM-CSF by various other cells types, for example, endothelial cells and fibroblasts, is inducible by TNF-alpha, TNF-beta, IL1, IL2 and IFN. Some cell types express GM-CSF constitutively. Constitutive synthesis may be the result also of promoter insertion mutations. Spontaneous secretion of GM-CSF by human cancer cells has been documented for several tumor types.GM-CSF was isolated initially as a factor stimulating the growth of colonies of macrophages and granulocytes in soft agar cultures. Since it has been recognize that GM-CSF stimulates proliferation of most hematopeoitic cell lineage except megakaryocytes. GM-CSF is responsible for the growth and development of progenitors of granulocytes and macrophages. It stimulates myeloblast and monoblasts and triggers irreversible differentiation of these cells. GM-CSF synergises with Epo in the proliferation of erythroid and megakaryotics progenitors cells. In combination with another colony stimulating factor, M-CSF, one observes the phenomenon of synergistic suppression, i.e., the combination of these two factors leads to a partial suppression of the generation of macrophage-containing cell colonies.

GM-CSF is a strong chemoattractant for neutrophils. It enhances microbicidal activity, oxidative metabolism, and phagocytotic activity of neutrophils and macrophages. It also improves the cytotoxicity of these cells.
In addition, GM-CSF also enhances expression of receptors for fMLP (Formyl-Met-Leu-Phe) which is a stimulator of the activity of neutrophils.

Phagocytotic activities of neutrophil granulocytes and the cytotoxicity of eosinophyls is also enhanced considerably by GM-CSF. Since GM-CSF is produced by cells (T-Lymphocytes, tissues macrophages, endothelials cells, mast cells) present at sites of inflammatory responses it can be assumed that it is an important mediator for inflammatory reactions.
The functional state of Langerhans cells of the skin is also influenced by GM-CSF. These cells are not capable of initiating primary immune responses, for example, contact sensibilization. They are converted to highly potent immunostimulatory dentritic cells by GM-CSF. Langerhans cells therefore form an in situ reservoir for immunologically immature dentritic cells.

GM-CSF recombinant protein as adjuvant:
GM-CSF recombinant protein has been used and is used as adjuvant in many clinical trials for both infectious disease vaccination and anti-tumor immunizations.
The adjuvant effects of GM-CSF recombinant protein has been reviewed extensively in the medical literature (Disis, Bernhard et al. 1996; Somani, Lonial et al. 2002; Cruciani, Mengoli et al. 2007; Waller 2007; Spearman, Kalams et al. 2009).
Although some positive adjuvant effects has been demonstrated in hepatitis B vaccination in end stage renal patient undergoing hemodialysis, **GM-CSF is not a very potent adjuvant when given as a systemic treatment and it can even have a detrimental effect.**
The fine tuning of GM-CSF production for inducing a strong adjuvant effect for both infectious and cancer immunization has been well documented.
For infectious diseases, experimental models have shown that the local delivery of GM-CSF is efficient in bacterial, viral and parasitic diseases as systemic sc injection have little effect.
In Influenza prevention (Ramanathan, Potter et al. 2002), hepatitis (Somani, Lonial et al. 2002), in HIV (Barouch, Santra et al. 2002) (Song, Liu et al. 2006), in malaria (Weiss, Ishii et al. 1998) and in tuberculosis (Murray, Aldovini et al. 1996) (Baek, Ko et al. 2003) (Chianese-Bullock, Pressley et al. 2005; Ryan, Wozniak et al. 2007)
The effect of recombinant GM-CSF in anti-tumor immunization has not been very successful with conflicting results in peptide based vaccination trials. Some publications revealing positive effect while others showed no effect or even a detrimental effect (Chianese-Bullock, Pressley et al. 2005) (von Mehren, Arlen et al. 2001).
Comparative analysis revealed a much better effect when GM-CSF is produced locally at the vaccine site rather than sc injection of the recombinant protein (Reali, Canter et al. 2005).
GM-CSF and prostatic acid phosphatase (PAP) are the components of a fusion protein used in the first cell-based anti-cancer immunotherapy approved for commercial application. The personalized therapy developed by Dendreon is based on the in-vitro maturation of patient's peripheral mononuclear cells into educated dendritic cells recognizing prostate antigen. In this medical product GM-CSF is critical for efficient antigen processing and maturation of the dendritic cells before reinjecting the autologous cells into the patient (Higano, Schellhammer et al. 2009).

Studies in animal models nicely demonstrated that the systemic delivery of GM-CSF induces a loss of anti-tumor immunity by the induction of myeloid suppressor cells (Serafini, Carbley et al. 2004) and/or the induction of immature dendritic cells from myeloid precursors. (Gabrilovich 2004). This effect has also been found in clinical trial (Filipazzi, Valenti et al. 2007). Altogether these findings help understanding the conflicting results obtained with GM-CSF as adjuvant as reviewed by Parmiani et al (Parmiani, Castelli et al. 2007). Excessive GM-CSF production and systemic delivery induces a weak or a negative effect while local

production of GM-CSF at the immunization site is beneficial in both infectious diseases and in genetically modified tumor cell-based immunization (Jinushi, Hodi et al. 2008).
The dual effect of GM-CSF on immune mechanism is also well demonstrated in pancreatic islets functions. Lack of GM-CSF induces diabetes as shown by studies of knock-out mice. Indeed, aging mice deficient for GM-CSF and IL-3 develop insulitis, islets cells destruction and defective glucose homeostasis (Enzler, Gillessen et al. 2007). In contrast, excess of systemic recombinant GM-CSF induces diabetes in NOD mice by increasing Treg population and inducing tolerogenic DC as elegantly demonstrated by Gaudreau et al. in 2007 (Gaudreau, Guindi et al. 2007).

4) Learning from knock-out mice

Strong evidences shows that GM-CSF is very potent adjuvant, inducing major immunostimulatory effect when produced locally during the priming phase. IL-3 another hematopoietic cytokine with a broad spectrum of activities also shows an adjuvant effects in several cell-based genetically modified anti-tumor vaccination models. The putative mechanism of action form these cytokines is the generation of an optimal local environment for DC recruitment and subsequent antigen processing by APC.
As hematopoietic cytokines has overlapping roles(Metcalf 2008), the study of mice lacking either GM-CSF, IL-3, both or its receptors are of interest.

a) GM-CSF knock-out mice

GM-CSF knock-out mice have no major hematopoietic defect (Dranoff, Crawford et al. 1994), have no decrease dendritic cells numbers and are not prone to infection in usual conditions. Such lack of haematological phenotype illustrates the strong overlap between hematopoietic cytokines. Surprisingly, mice lacking GM-CSF develop progressive respiratory failure secondary to alveolar proteinosis. Defective macrophage function within the lung is responsible for this phenotype. Life span of GM-CSF-/- mice is moderately decreased because of this lung pathology. The study of aging mice also revealed auto-immune disease and subtle changes in antigen processing capacities. Studies of GM-CSF deficient mice have helped described the dual role of GM-CSF in APC activation: tolerogenic or adjuvant effect. This phenomenon has been linked to the ability of APC to process apoptotic cells in a MFG-E8 dependant manner. GM-CSF is a key regulator of MFG-E8 expression by APC leading to either GM-CSF-triggered tolerance or immunity as described by Jinushi et al. (Jinushi, Nakazaki et al. 2007). Figure 21 illustrate the mechanism involved in the tolerance/adjuvant switch as proposed by Jinushi et al.

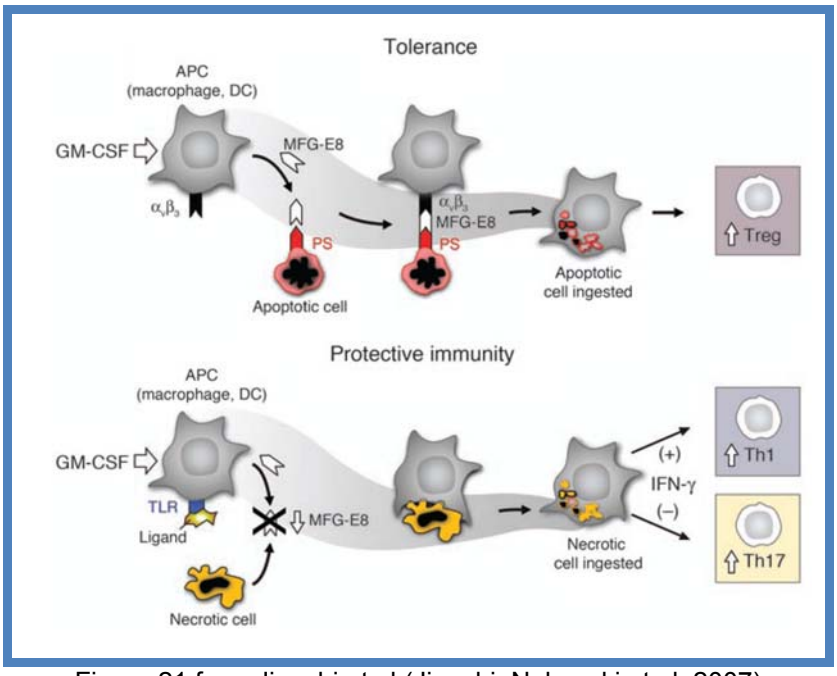

Figure 21 from Jinushi et al.(Jinushi, Nakazaki et al. 2007)

Mice lacking IL-3, a hematopoietic cytokine with similar or broader biological activity than GM-CSF were generated using classical homologous recombination techniques in embryonic stem cells (Mach, Lantz et al. 1998). Interestingly IL-3-/- mice do not present alveolar proteinosis but showed impaired contact hypersensitivity (a form of delayed type hypersensitivity directed towards a hapten-protein conjugate) and increased risk for parasitic infections.(Lantz, Boesiger et al. 1998; Mach, Lantz et al. 1998)
Surprisingly both GM-CSF knock-out and Il-3 knock-out mice showed no defect in anti-tumor immunization using irradiated tumor cells as vaccine.

b) GM-CSF & IL-3 double knock-out mice (Gillessen, Mach et al. 2001)

As GM-CSF and IL-3 share biologic activities on hematopoietic precursors, and have both immunostimulatory effect in cell-based vaccination studies, the generation and analysis of mice lacking both genes was of great interest for hematopoietic and immunological analysis.
Because GM-CSF and IL-3 are separated by only 14 kb on chromosome 11, doubly deficient mice could not be obtained by interbreeding single knockout animals. Thus, mice lacking both cytokines were generated through sequential gene targeting experiments in ES cells. A hygromycin cassette replacing exons 3 and 4 of the GM-CSF locus was introduced by homologous recombination into IL-3 heterozygous deficient ES cells as shown below on Fugure 22..

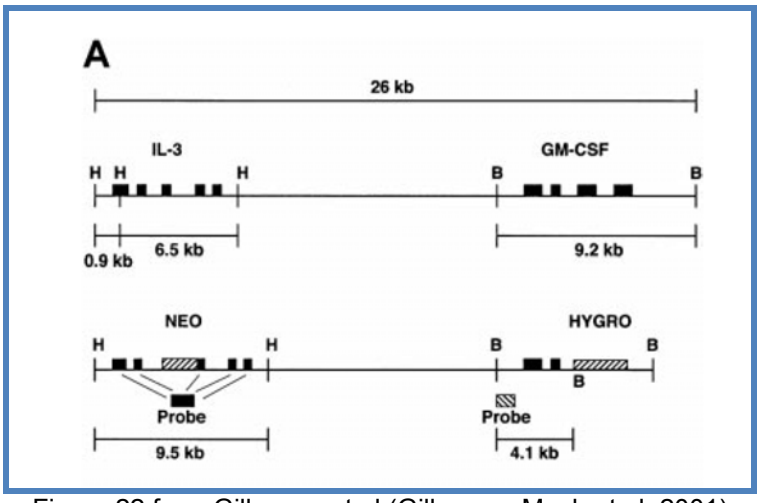

Figure 22 from Gillessen et al.(Gillessen, Mach et al. 2001)

Heterozygous mutant mice were interbred to generate homozygous GM-CSF/IL-3–deficient animals. Mutant mice were obtained at the expected frequencies, remained clinically healthy throughout 18 months of observation, and were fertile. Supernatants of concanavalin A-stimulated splenocytes from mutant animals showed no immune-reactive GM-CSF or IL-3 protein as determined by ELISA, confirming the generation of a null allele. The mutant allele was back-crossed 9 generations onto Balb/c and C57Bl/6 backgrounds for detailed analysis.
The hematocrits and total circulating white blood cell and platelet counts were normal in GM-CSF/IL-3–deficient mice. Unexpectedly, examination of stained blood smears revealed that circulating eosinophils were increased in doubly deficient mice, as compared to single knockouts and wild-type controls. In contrast, circulating neutrophils, lymphocytes, and monocytes were not affected. To characterize hematopoiesis in GM-CSF/IL-3–deficient mice further, we lethally irradiated mutant animals and transplanted them with doubly deficient marrow. GM-CSF/IL-3–deficient mice achieved reconstitution that was comparable to wild-type controls, although there was a modest delay in the kinetics of leukocyte recovery, similar to that previously observed for GM-CSF–deficient animals.
Analysis of the spleens, thymi, and lymph nodes of GM-CSF/IL-3–deficient mice similarly revealed normal numbers of both myeloid- and lymphoid-type dendritic cells.
In an effort to identify other factors that might contribute to dendritic cell development in these animals, we implanted syngeneic tumor cells engineered to secrete high levels of flt3-ligand. These cells serve as an efficient vehicle for the systemic administration of flt3-ligand, a cytokine that dramatically augments dendritic cell numbers in wild-type mice. By 14 days after injection, there was a marked increase in splenocytes staining positive for CD11c and MHC II in both mutant and wild-type animals, with an average of 25% positive cells per spleen (Figure 23 A-B below). Because injection of flt3-ligand–expressing tumor cells produced a 3- to 4-fold increase in total spleen cellularity, a nearly 100-fold expansion of dendritic cell numbers was accomplished in the absence of GM-CSF and IL-3. Flt3-ligand–secreting tumor cells stimulated the generation of both myeloid-type (CD8a-, CD11b+) and lymphoid-type (CD8a+, CD11b-) dendritic cells (Figure 23 C-F).
No differences in B7-1, B7-2, CD40, or CD1d expression were observed between doubly deficient and wild-type animals. Taken together, these results suggest that flt3-ligand may be a critical regulator of dendritic cell development in vivo.

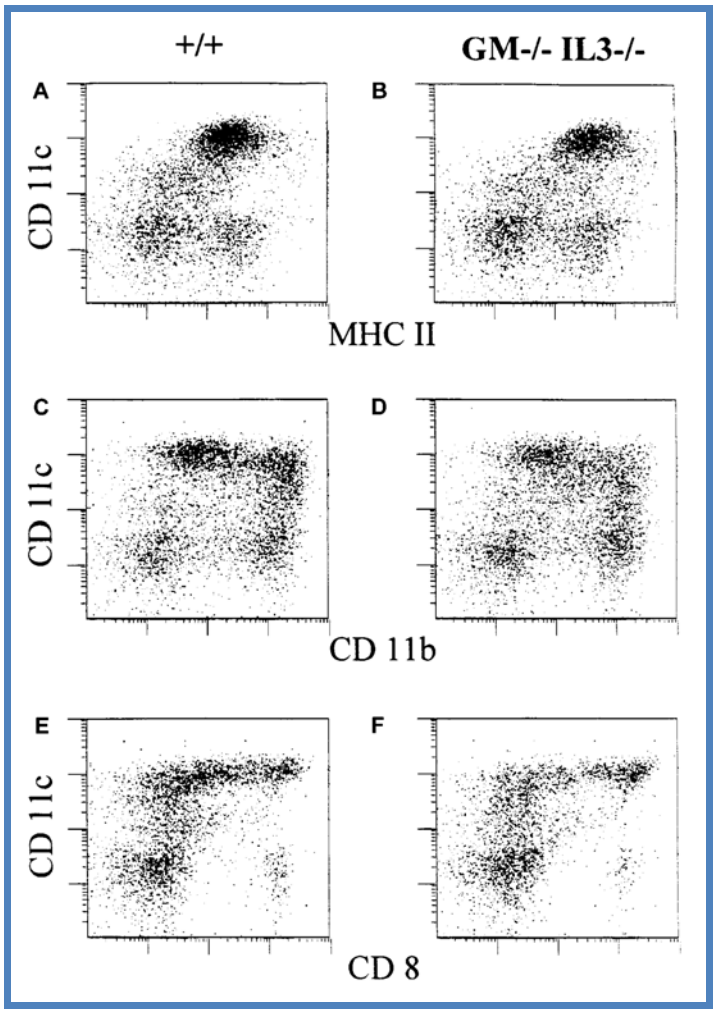

Figure 23 from Gillessen et al. (Gillessen, Mach et al. 2001)

Mice lacking both genes did not showed any defect in cell-based vaccination models compared to single knock-out or littermates wild-type controls.
The only immunological abnormality that we could observe in the double knout-mice was a decrease in the magnitude of delayed type hypersensitivity reaction as shown below (Figure 24). This defect could be corrected by the administration of recombinant proteins GM-CSF and IL-3 (Figure 25).

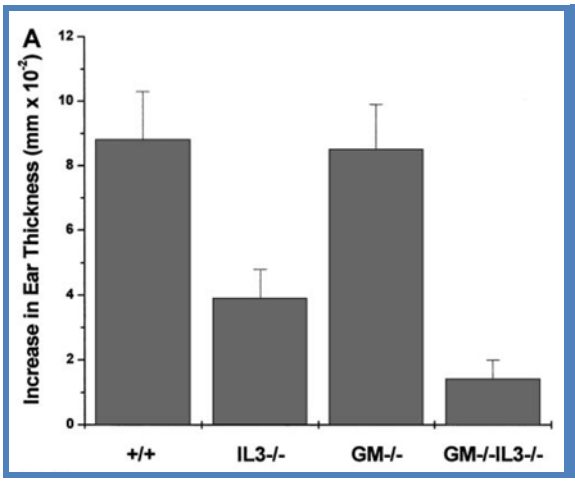

Figure 24 from Gillessen et al.

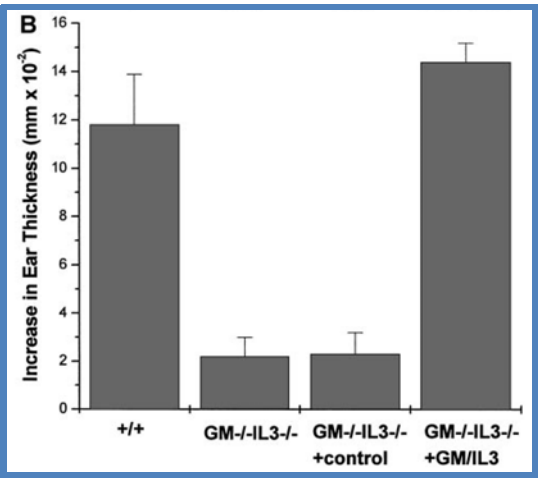

Figure 25 from Gillessen et al.

In conclusion, despite lacking both GM-CSF and IL-3, two major genes involved in the growth and differentiation of many hematopoietic cells, the mild phenotype observed in theses double knock-out mice is an increased in eosinophils and a marked defect in DTH reaction.
Double knock-out mice despite lacking two major hematopoietic cytokines showed no impairment in cell-based vaccination when immunized with either irradiated GM-CSF secreting B16 melanoma or irradiated Renca cells (spontaneously immunogenic). These mice have all the necessary machinery to mount an efficient anti-tumor immune response.

c) Mice lacking GM-CSF signaling (β common subunit receptor knock-out)

As GM-CSF, IL-3 and IL-5 share a common receptor subunit we were interested in analyzing the anti-tumor immunity of mice lacking this beta common receptor subunit (βc). Mice lacking the βc subunit are not able to signal via GM-CSF and IL-5 but have a normal IL-3 signalling as a beta IL-3 subunit receptor is functional even in the absence of βc.
βc-/- mice have been generated by two research groups (Robb, Drinkwater et al. 1995; Nishinakamura, Miyajima et al. 1996; Nishinakamura, Wiler et al. 1996; Scott, Hughes et al. 1998). As expected the phenotypes is similar to GM-CSF-/- mice (alveolar proteinosis) and IL-5-/- as described by both Kopf et al (Kopf, Brombacher et al. 1996) and Foster et al (Foster, Hogan et al. 1996) with decreased eosinophils counts but normal antibody and cytotoxic T cell response.
Comparative analysis of protective anti-tumor vaccination between mice lacking either GM-CSF (GM-/-), GM-CSF & IL-3 (GM-IL-3-/-), IL-5 (IL-5-/-), βc signalling (βc-/-) and WT mice revealed very interesting data. These results, described below, have been published in 2009 by our group in Blood (Zarei, Schwenter et al. 2009).

Comparative immunization in several knock-out mice using a poorly immunogenic model
Immunization with irradiated GM-CSF-secreting B16 melanoma cells in mice lacking either GM-CSF, Il-5, GM-CSF signalling or WT background.

The first experiment evaluate the anti-tumor vaccination in the B16 melanoma model using irradiated tumor cells engineered to produce GM-CSF as vaccination in mice lacking either GM-CSF or GM-CSF signalling. As described in Figure 26 above we can see that a very good protective immunity upon B16 wild-type challenge is observed in GM-/- and WT mice as 75% of animals do not develop tumors.

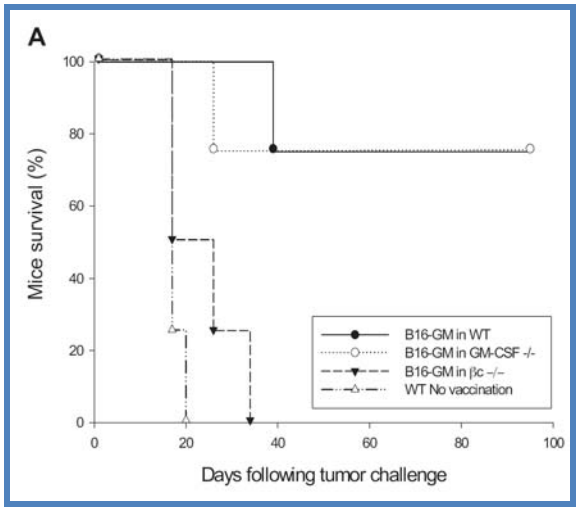

Figure 26 from Zarei et al.(Zarei, Schwenter et al. 2009)

The lack of endogenous GM-CSF in GM-CSF-/- mice (during development and adult life) does not limit the ability to mount a protective immune response. All the necessary cells can be recruited and activated in the absence of endogenous GM-CSF. In addition GM-CSF is not required during the effector phase as no GM-CSF is produced neither by the host nor the B16 melanoma cells during the challenge. The most likely hypothesis for understanding the observed tumor protection in both WT and GM-CSF-/- mice is the local production of GM-CSF by the genetically engineered irradiated tumor cells at the vaccine site.

Another interesting finding from this experiment is the complete loss of protection in βc -/- mice. Indeed, when GM-CSF signalling is lacking (in βc-/-), all mice developed rapidly growing tumor after B16 challenge. The kinetic of tumor growth in the βc-/- was somewhat similar to un-vaccinated control mice, all dying with rapid tumor growth.

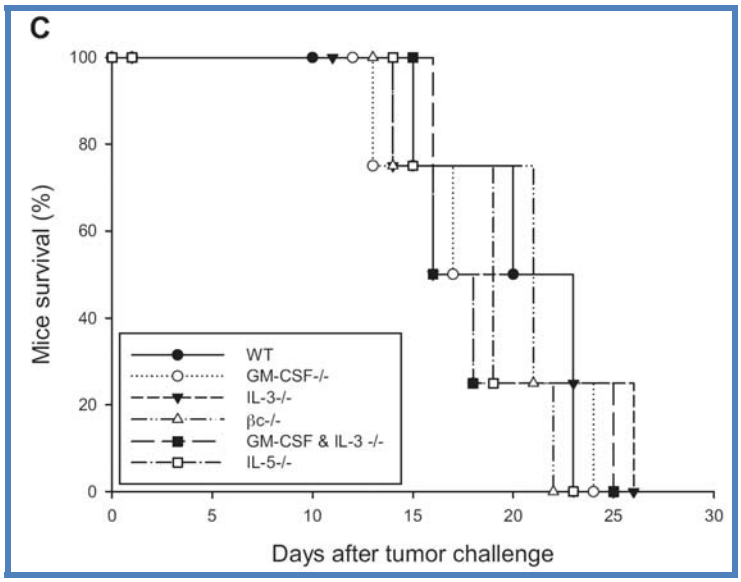

Figure 27 from Zarei et al.(Zarei, Schwenter et al. 2009)

The loss of vaccination observed in the βc-/- group is not related to increased tumorogenicity in βc-/- as shown on Figure 27. In this tumorogenicity experiment, mice lacking GM-CSF, IL-3, both GM-CSF & IL-3, IL-5, βc or wild-type controls were challenged with B16 melanoma cells. No difference was observed in tumor kinetics between the groups, all mice showing rapid tumor growth by day 10 and all mice being sacrificed by day 26.
Our hypothesis that lack of GM-CSF signalling induced the loss of immune protection is not formally confirmed by this experiment as βc-/- mice lack both GM-CSF and IL-5 signalling. A role for IL-5 signalling in the ability to trigger immunization cannot be ruled out with these findings.
To rule out the possible role of IL-5 signaling in the failure of βc-/- mice to develop a protective anti-tumor immunity in the B16-GM-CSF melanoma vaccination model, we tested IL-5 deficient mice in the same experimental model. IL-5-/- mice described in 1996 were kindly provided by M. Kopf (Kopf, Brombacher et al. 1996).

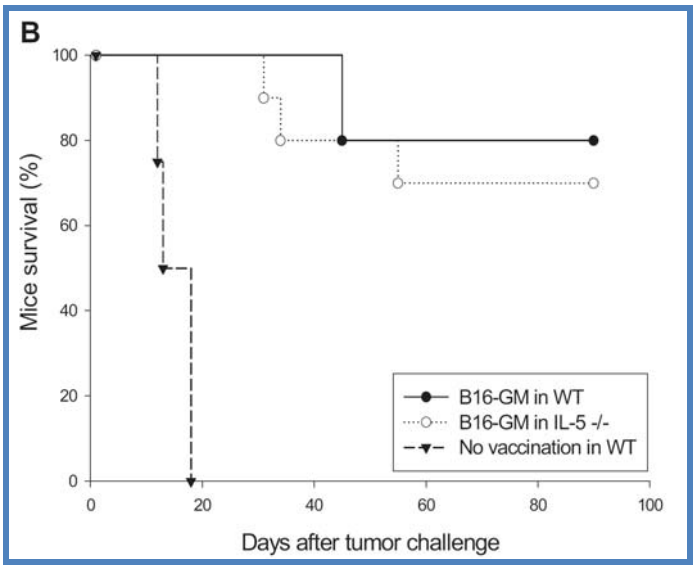

Figure 28 from Zarei et al.(Zarei, Schwenter et al. 2009)

Immunization with irradiated B16-GM-CSF cells in WT and IL-5-/- mice showed a good anti-tumor protection similar in both groups with 80% of mice showing long-term protection compared to unvaccinated control (0% survival) as shown on Figure 28.
Protective immunization in IL-5-/- mice is in accordance with our initial hypothesis on the critical role of GM-CSF signalling. Lack of IL-5 signalling during both priming and effector phase does not affect the efficacy of anti-tumor immune response in this model.

Comparative immunization in several knock-out mice using an immunogenic model
Vaccination with immunogenic Renca cells in mice lacking either GM, GM-CSF signalling or WT background.
As GM-CSF has been shown to boost anti-tumor cell-based vaccine in poorly immunogenic but also in spontaneously immunogenic tumor, we tested the ability of both GM-/- and βc-/- mice to develop a protective immune response in the spontaneously immunogenic Renca, a renal cancer cell line in Balb/c strain.

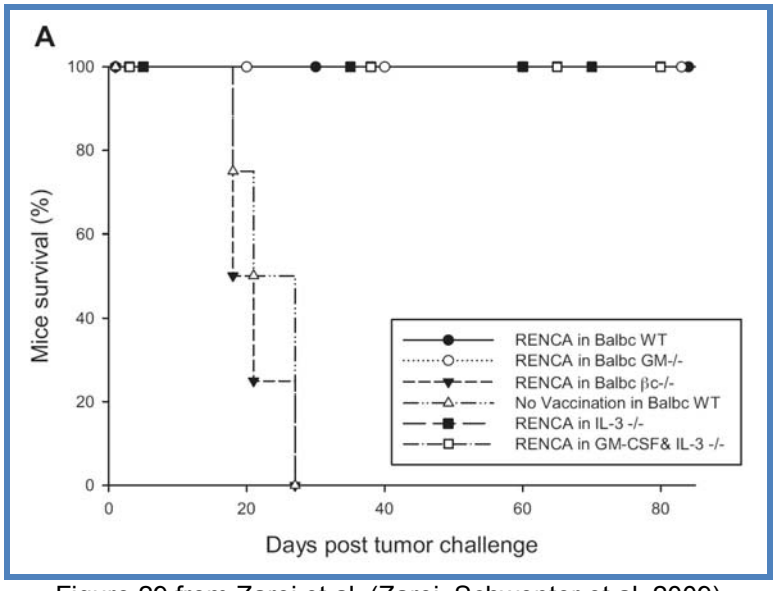

Figure 29 from Zarei et al. (Zarei, Schwenter et al. 2009)

In the Renca model, subcutaneous injection of $1x10^5$ of irradiated Renca tumor cells induces protective immunity against $1x10^6$ Renca rechallenge. As expected, similarly to the data obtained in the B16-GM melanoma model, mice lacking GM-CSF, IL-3 or both GM-CSF and

IL-3 did not showed any defect in protective immunity. Surprisingly, βc-/- had an opposite phenotype with all mice succumbing rapidly to Renca challenge with kinetics similar to un-immunized mice (Figure 29 above).

These data showed that in this immunogenic model, endogenous GM-CSF is not required but signalling through the β common receptor subunit is critical.
Although local GM-CSF production by B16-GM-CSF vaccine in the B16 melanoma model explains the tremendous difference in tumor protection between GM-/- and βc-/-, the observed data in the Renca model could not be easily explained. The loss of immunogenicity in βc-/- but not in GM-/- mice in a spontaneously immunogenic tumor was puzzling.

A novel hypothesis was raised with the observation that some human cancers such as renal cancers (Gerharz, Reinecke et al. 2001), head and neck cancers (Gutschalk, Herold-Mende et al. 2006), breast cancers (Zaks-Zilberman, Zaks et al. 2001), basal cell carcinoma (Mueller and Fusenig 1999) and many experimental tumor cell lines (Steube, Meyer et al. 1998) do spontaneously secrete GM-CSF.

Hypothesis:
In immunogenic tumor model, the spontaneous GM-CSF secretion by irradiated, unmodified cancer cells is able to trigger a protective, immune response even in the absence of endogenous GM-CSF.

Such hypothesis could explain the striking difference observed between GM-/- and β-/- in the immunogenic Renca cancer vaccination model.
If confirmed this could bring a new concept, challenging an old dogma: spontaneous immunogenicity of 'immunogenic' tumor may not only be related to strong antigens but also to the spontaneous release of potent adjuvant such as GM-CSF.

In order to confirm this hypothesis we needed to get answer to the five questions below:
1) Can other cytokine compensate for the loss of GM-CSF signalling?
2) Does Renca secrete GM-CSF spontaneously?
3) If we can abrogate GM-CSF secretion from Renca cells does it modify its immunogenicity ?
4) If the loss of spontaneous GM-CSF release by irradiated Renca cells abrogates immunization, can protective vaccination be restored by providing GM-CSF during vaccination.
5) If 1,2 and 3 are proven, can we restored protective immunity in βc-/- by vaccinating with wt antigen presenting cells (the putative cells interacting with GM-CSF during priming).

Question 1: Can other cytokine compensate for the loss of GM-CSF signalling
To evaluate this point, we used two cytokines with proven adjuvant effect in cell-based vaccination models and that are not known to require functional βc receptor subunit. We tested the ability of IL-3 secreting or FLT3-L secreting tumor cells in both Renca and B16 tumor models. The figure 30 below illustrates the finding observed with Renca-FLT3-L in the Renca model. Neither FLT3-L nor IL-3, two cytokines with known adjuvants effect could trigger a protective vaccination in βc-/- mice. **Answer: No**

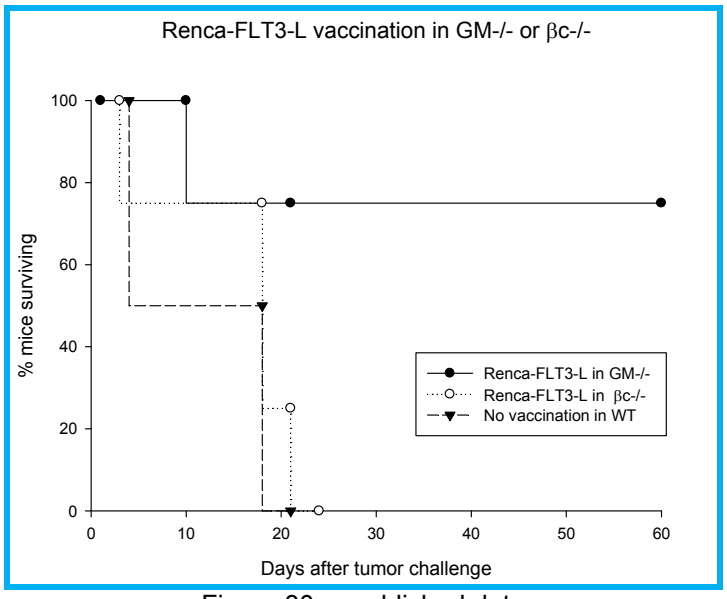

Figure 30 unpublished data

Question 2: Do Renca cells secrete GM-CSF spontaneously ?

As mentioned earlier many experimental tumor cell lines do secrete GM-CSF (Steube, Meyer et al. 1998). Renca cell's culture supernatant analysis by ELISA as well as RT-PCR analysis from Renca cells showed murine GM-CSF secretion ranging from 0.4 to 0.8ng/10^6 cells/24hrs, as illustrated below (Figure 31). Similar analyses for B16 melanoma were negative. **Answer: Yes**

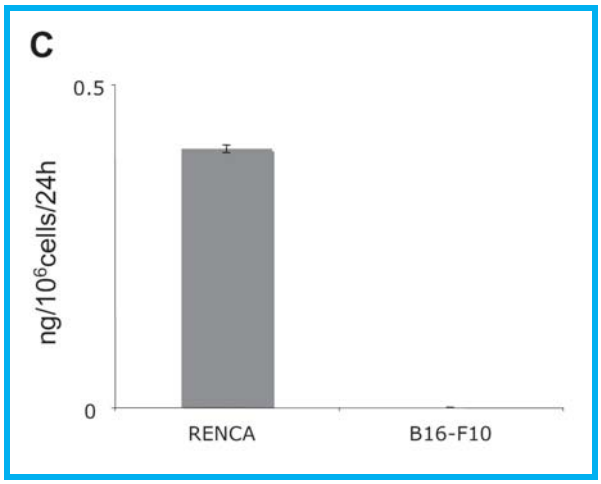

Figure 31 from Zarei et al. (Zarei, Schwenter et al. 2009)

Question 3: If we can abrogate GM-CSF secretion from Renca cells does it modify its immunogenicity ?

To answer this question we had to find a way to abrogate GM-CSF secretion from Renca cells. After unsuccessful attempts using silencing RNA we develop a modified Renca cell line with no GM-CSF release using intrakine trap strategy. This technique has been used previously to trap HIV protein as described by Chen et al. (Chen, Bai et al. 1997).

Briefly, sub-units α and βc of the GM-CSF receptors cDNA were obtained from spleen cells using RT-CPR techniques. With designed primers a KDEL tail was added to both cDNA, The KDEL motif anchored the protein within the membrane of the endoplasmic reticulum, preventing its release. The cloned proteins were inserted into retroviral vector (MFG) and transfected into packaging cells (293GPG) as described before.

The concentrated viral particles were then used to infect Renca cells.

In modified Renca cells expressing both α and βc subunits within its endoplasmic reticulum, GM-CSF remains sequestrated within the Golgi apparatus and is not released. The next figure (Figure 32) shows the GM-CSF concentration of GM-CSF in the supernatant of the various cell lines. The cell lines lacking the α subunit, the βc sub-unit or both were named Renca α-KDEL, Renca β-KDEL and Renca α/β-KDEL respectively. Supernatant from Renca α/β-KDEL cells did not contain GM-CSF as measured by ELISA.

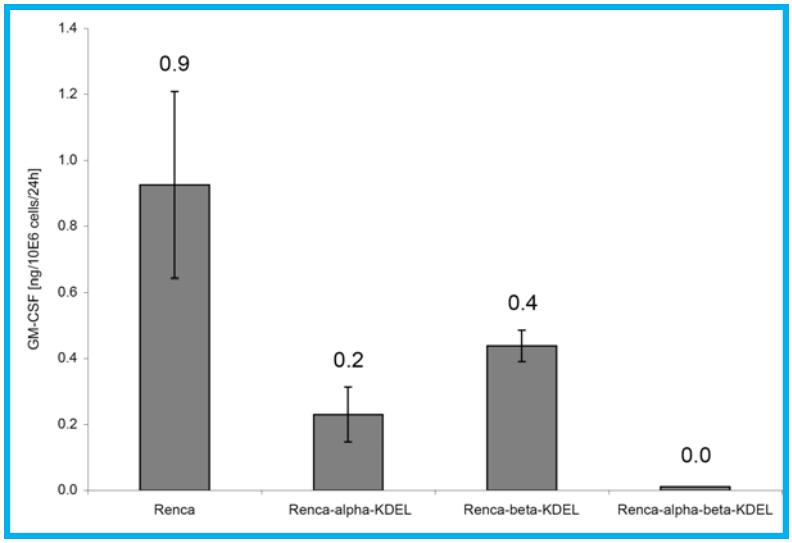

Figure 32 unpublished data

We then tested the ability of irradiated Renca wt, Renca α-KDEL , Renca β-KDEL and Renca α/β KDEL cells to induce protective immunity in Balb/c mice using the similar model as described previously.

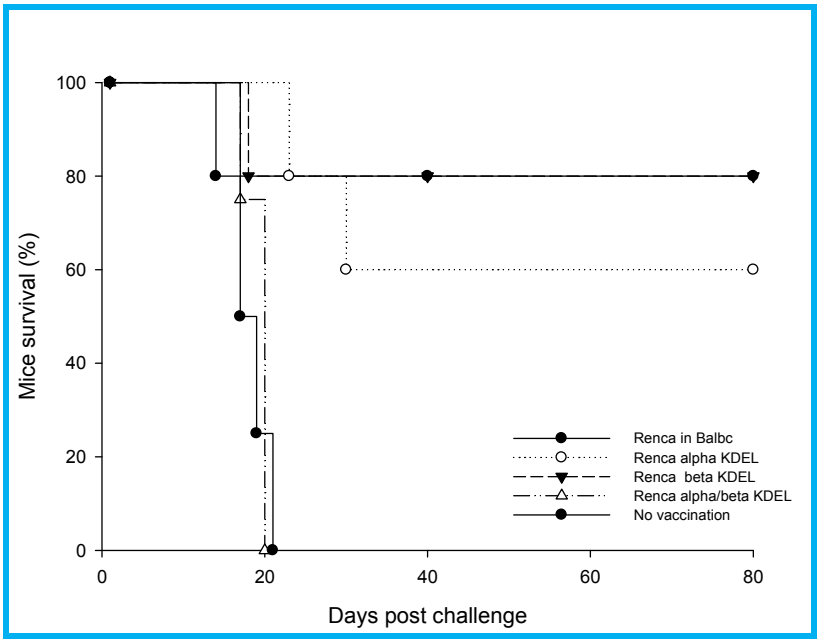

Figure 33 unpublished data

While Balb/c mice vaccinated with either Renca wt, Renca α-KDEL or Renca β-KDEL shows good protection, mice immunized with Renca α/β KDEL all succumb to tumor challenge, similar to unvaccinated mice and similar to the results observed in βc-/- (Figure 33).
These results strongly support the hypothesis that local production of GM-CSF at the vaccination site by immunogenic tumors and by tumor genetically engineered to produce GM-CSF triggers a potent immune response. **Answer: Yes**

Question 4: Can protective vaccination with Renca α/β KDEL be restored by providing GM-CSF during vaccination?

In addition to the loss of immunogenicity observed when GM-CSF is no longer produced at the vaccine site, the corrective effect of supplemented GM-CSF should bring additional evidence for its critical role. To answer this question we performed a set of experiments in which the immunization is done with a combination of two distinct irradiated cells lines. The ineffective Renca α/β KDEL cells are combined with cells from a syngeneic tumor cell-line engineered to secrete GM-CSF (CMS5-GM). One week after immunization all animals are challenged with live Renca cells and tumor formation and survival is recorded. The positive control is a vaccination performed with irradiated Renca cells and the two negative controls are vaccination with Renca α/β KDEL alone and no vaccination
As shown in the figure 34 below, the combination of Renca α/β KDEL and GM-CSF producing cells is able to restore protective immunity in 80% of the mice as all mice immunized with Renca α/β KDEL alone died from tumor growth.

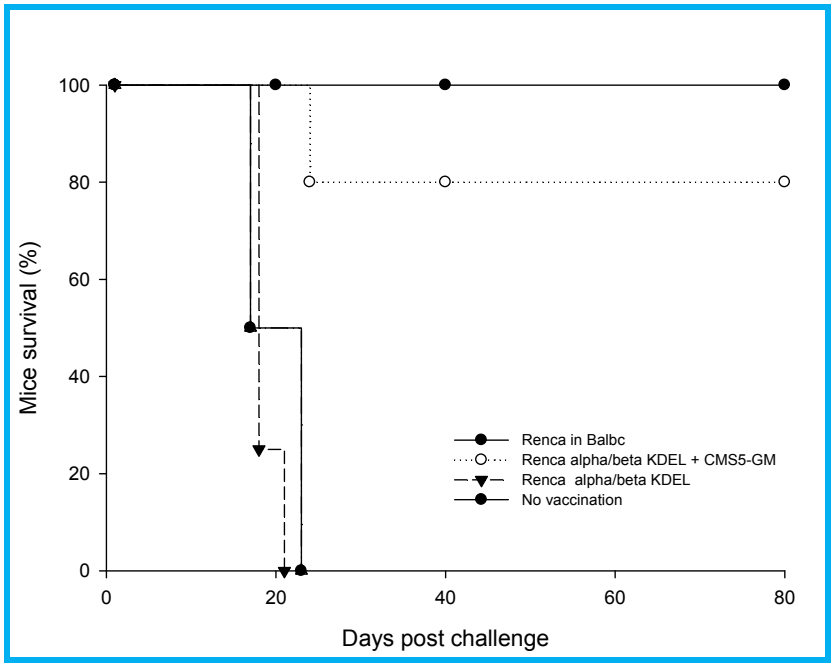

Figure 34 unpublished data

The addition of GM-CSF provided locally at the vaccination site reverses the loss of immunogenicity observed with Renca α/β KDEL in WT Balbc mice. **Answer: Yes**

Question 5. As questions 1, 2 and 3 are positively answered, can we restored protective immunity in βc-/- animals by vaccination with wild-type antigen presenting cells and Renca cells?

As hypothesized and supported by the data presented, the inability of βc-/- mice to trigger a protective immune response is most likely GM-CSF dependant. To further confirm our hypothesis we developed a novel experimental design assessing the role of dendritic cells from either wild type or βc-/- origin in cell-based tumor immunity. As dendritic cells have receptors for GM-CSF, are known to respond to GM-CSF stimulation and are critical partners in the priming phase of anti-tumor immune response we selected this type of antigen presenting cells for these experiments. Therefore we evaluated the ability to immunize βc-/- mice with immature wild-type DC combined with irradiated Renca cells producing GM-CSF.

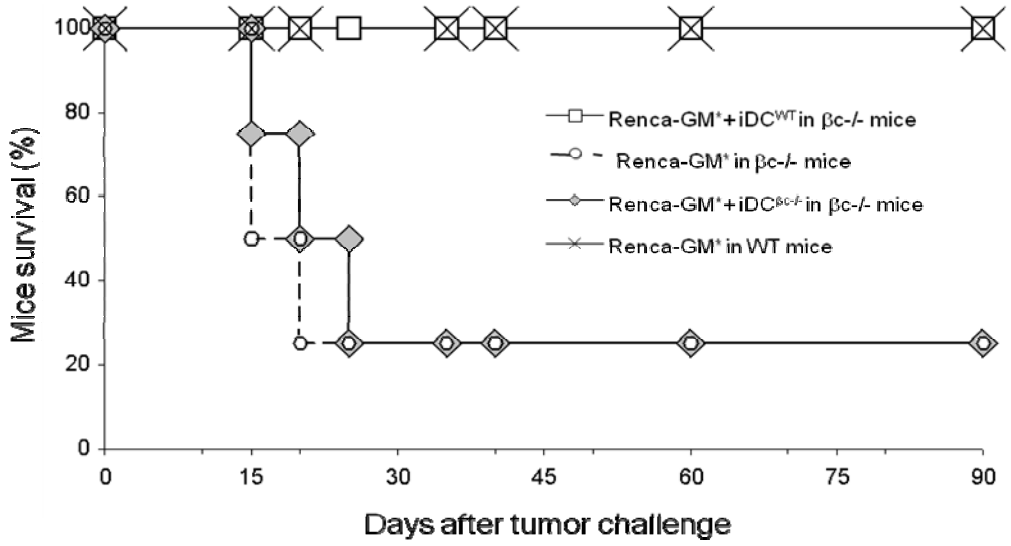

Figure 35 unpublished data

Figure 35 shows that immunization with immature DC from βc-/- mice and irradiated GM-CSF secreting Renca cells are not able to trigger a protective immunity in βc-/- mice. Contrarily, vaccination combining immature DC from WT mice and irradiated GM-CSF secreting Renca cells induces a very good immunization in βc-/- mice, all mice surviving subsequent Renca tumor challenge.
Therefore this experiments shows that vaccination with irradiated GM-CSF secreting tumor cells and immature wt DC cells reverts the loss of immunization observed previously in βc-/- mice. **Answer: Yes**

Altogether these experiments demonstrate that:

1) **GM-CSF is a strong adjuvant in experimental anti-tumor immunization models when produced locally by genetically modified cells.**
2) **Endogenous GM-CSF is not required for efficient and long lasting anti-tumor immunity as long as GM-CSF is provided locally during the priming phase.**
3) **GM-CSF signalling is critical as lack of GM-CSF signalling results in loss of immunization in the two models tested (poorly immunogenic B16 melanoma and immunogenic renca)**
4) **The observed 'spontaneous' immunogenicity of some cancer cells is, in the models tested, related to spontaneous GM-CSF production by the tumor cells. Abrogation of GM-CSF signalling results in loss of immunogenicity.**

Furthermore, the accepted dogma linking tumor immunogenicity to strong antigens may be challenged by our results as spontaneous release of GM-CSF by irradiated cells is critically needed for immunogenicity.

5) Data from published clinical trials evaluating tumor immunization with GM-CSF producing cells

As discussed in the previous sections, cell-based vaccination, using tumor cells as the source of known and unknown antigens has a good rationale. Early works in this field quickly revealed that vaccination with unmodified inactivated tumor cells was only effective in models using very immunogenic cells lines but not in poorly immunogenic setting and furthermore not efficient in clinical setting. The natural history of most patients with cancers is a process ongoing during several months during which the immune system is not able to eliminate the tumor cells and the tumor expands until it is detected by clinical examinations, imaging techniques or biological analysis.
To some extent, the field of cancer immunization has benefited from the progress done in Infectious diseases. Vaccination against infectious agents such as viruses or bacteria induces potent triggering of the immune system by recognizing foreign biological material. This is an amplification of the usual 'defensive' process of the immune system. Effort to induce an immune response towards tumour cells arising from our own self is much more complex.
Immunizations against both infectious agents and tumour can be enhanced by adjuvants. Adjuvants frequently used to potentiate immunization procedures are Freund Adjuvant (either complete or incomplete), Alun, CpG oligodeoxynucleotides, Montanide, and GM-CSF.

Based on the pre-clinical data demonstrating sustained, specific protective immunity in mice vaccinated with irradiated, genetically modified tumor cells secreting GM-CSF in all tumor type tested, pilot clinical trials using similar strategy were undertaken. As of fall 2011, more than 27 studies testing cell-based immunization with GM-CSF have been published (22) or reported in scientific meetings (5 abstracts). Altogether more than 1000 patients have been treated in cancer vaccination trials evaluating GM-CSF producing cells. In addition, 38 clinical trials are listed on clinicaltrial.org website as ongoing or planned. The next paragraphs will critically review the published data from these trials.

Retroviral gene transfer technique
The first six trials performed required retroviral gene transfer techniques. Such technology using replication-defective retroviral vectors and packaging cells is restricted to replicative cells, requiring short-term culture of tumor cells. These trials were performed in patients with advanced tumor such as melanoma, prostate and renal cancer. Technological challenges, including extensive time for vaccines preparation (from 8 to 32 weeks), are the main reasons for the low percentage of patients being treated after harvesting tumor cells for vaccine preparations in the first melanoma trial (Soiffer, Lynch et al. 1998). Indeed from 33 enrolled patients, 29 had vaccines produced and 21(63%) completed treatment schedule. Hu GM-CSF production was highly variable from one patient to the other ranging from 82 to 965 ng/10^6 cells/24hrs. All patients received 10^7 lethally irradiated genetically modified cells at three different schedules: every 28, 14 or 7 days intervals for a total of 84 days (ranging from 3 to 12 subcutaneous immunizations). Detection of Anti-melanoma T lymphocyte and antibodies were associated with tumor destruction. Analysis of metastatic tumor deposit in immunized patients revealed dense T lymphocytes and plasma cells infiltration, >80% tumor destruction, fibrosis and edema in 11 out of 16 patients studied. Strong DTH response was observed in all patients after vaccination upon sc injection of irradiated un-manipulated tumor cells. Best objective response was partial response.
In a Phase I study in renal cell carcinoma using similar technology, three dose levels were tested ranging from $4x10^6$, $4x10^7$ or $4x10^8$ irradiated autologous cells per vaccines (Simons, Jaffee et al. 1997). Vaccine preparation rate was 70, 88 and 20% respectively. GM-CSF production from transduced cells ranged from 42 to 149ng x10^6cells/24hrs. Only 18 of the 33 (54%) enrolled patients received vaccine therapy, illustrating the logistical and technological challenge despite evidence of induction of immune response in most treated patients.

In a similar phase I pilot trial, patients with resected prostate carcinoma were immunized with ex-vivo genetically modified GM-CSF secreting autologous tumor cells(Simons, Mikhak et al. 1999). Failure to expand primary culture prevented adequate dose level 2 ($5x10^7$ tumor cells). In the 8 treated subjects, secretion of GM-CSF range from 143 to 1403 ng/10^6 cells/24h. Similar to previous studies DTH reaction was observed in all but one immunized patients after 3 vaccinations.

Two other small Phase I trials have been performed with few patients. 4 Japanese patients with stage IV clear cell renal cancer (Tani, Azuma et al. 2004) and 5 patients with advanced melanoma (Chang, Li et al. 2000). All patient exhibit DTH reactions and some patients showed prolonged survival but treatment was not standardized as some patients received additional low-dose IL-2.

The only Phase I/II using retroviral gene transfer technique was performed in the Netherlands in patients with stage IV metastatic melanoma and reported in the Journal of Clinical Oncology in 2005 (Luiten, Kueter et al. 2005). Similarly to previous trials vaccine preparation could not be performed in all patients. Primary culture of the harvested tumor cells was successful in 56 of 64 tumor samples (88%). Time from tumor excision to vaccine preparation was also long, ranging from 6 to 26 weeks (median 10 weeks). As expected many patients experienced progression before treatment with only 38 patients (59%) evaluable for toxicity. 10 additional subjects developed progressive disease after the first or second immunization, preventing further experimental treatment. Therefore only 28 patients (43%) received the three planned vaccinations protocol. Two doses levels was tested $5x10^6$ and $5x10^7$ cells while GM-CSF secretion ranged from 41 to 738 ng/10^6 cells/24h. 11 and 8 of the 28 treated patients were alive at 12 months and 36 months respectively as illustrated on Figure 36, taken from the JCO publication

Table 1. Individual Data of Vaccinated Patients

Patient No.	Age (years)	Sex	Previous Therapy	Metastasis Vaccine (sites)	GM-CSF Production*	Clinical Response	Survival (months) PFS	Survival (months) OS
Patients vaccinated with the low-dose tumor-cell vaccine								
1	59	Male	—	sub	169	PD	26	
2	28	Female	—	liver	249	NA	6	21.5
6	49	Male	CT-RT	LN	125	PD	16.5	
8	27	Male	CT-IT-RT	sub	97	NA	96+	96+
10	54	Male	—	sub	46	PD	9	
11	46	Male	RT	cut, LN	124	NA	5.5	73
20	29	Female	—	sub	180	PD	6.5	
24	36	Female	—	lung	209	PD	8	
28	70	Female	RT	LN	153	NA	34	56
29	51	Male	RT	LN	125	NA	6	90+
39	71	Male	—	cut, LN	41	PD	15	
47	36	Female	RT	cut	553	PD	13	
48	48	Female	CT	sub	503	SD	6.5	16.5
55	43	Male	CT	LN	190	NA	8	24
Patients vaccinated with the high-dose tumor-cell vaccine								
18	62	Female	RT	sub	187	PD	10.5	
19	66	Male	IT	sub	128	PD	22.5	
41	65	Male	—	LN	151	PD	18	
45	45	Female	—	LN	189	PD	10.5	
46	38	Female	—	cut	265	NA, vit	84+	84+
50	39	Male	CT	LN	271	NA	4	80
53	58	Male	—	sub	187	PD	17	
57	50	Female	RT-CT	LN	235	PD	10	
58	35	Female	IT	cut	206	PD	14	
59	58	Female	CT	stomach	689	PD	8.5	
61	51	Female	—	cut, sub	738	PD	27.5	
63	46	Male	RT	LN	373	SD	6	37
64	51	Male	—	sub	515	NA, vit	67+	67+
65	63	Male	RT	sub	154	PD	20	

Abbreviations: PFS, progression-free survival; OS, overall survival; GM-CSF, granulocyte macrophage colony-stimulating factor; CT, chemotherapy; IT, immunotherapy; RT, radiotherapy; sub, subcutaneous; cut, cutaneous; LN, lymph node; PD, progressive disease; NA, nonassessable disease; SD, stable disease; vit, patient developed vitiglio after vaccination.

*GM-CSF production by the tumor cell vaccine (ng/10^6 cells/24 hours).

Figure 36 from Luiten et al.(Luiten, Kueter et al. 2005)

Analysis of these ex-vivo gene therapy trials using retroviral gene transfer technology shows that delays in vaccine production ranging from 6 to 32 weeks is not compatible with further development despite very limited grade 1-2 local toxicity and very interesting data showing enhancement of both T lymphocytes and antibody anti-tumor responses

Two strategies were developed to circumvent the technical challenges observed with retroviral gene transfer techniques:
1) Novel viral vector such as adeno or adeno-associated vectors to allow rapid gene transfer into autologous tumor cells
2) Replacement of custom-made genetic modification of autologous tumor cells by allogeneic tumor cells producing GM-CSF.

1) Adenoviral vectors and autologous tumor cells:

7 trials has been reported using ex-vivo adenoviral vector gene therapy to engineer autologous tumor cells producing huGMCSF.
2 trials in melanoma enrolling respectively 9 and 35 patients (Kusumoto, Umeda et al. 2001; Soiffer, Hodi et al. 2003).
3 lung cancer trials with 35 patients (Salgia, Lynch et al. 2003) 35pt (Nemunaitis, Sterman et al. 2004), 52pts schiller j, abstract 2005 IASLC.
1 trial in acute myeloid leukemia (Ho, Vanneman et al. 2009).
1 trial in advanced ovarian carcinoma (15 patients, abstract reported at 2002 ASCO meeting but not published)
Although adenoviral gene transfer technology does not require primary culture and thus reduced dramatically the time from tumor harvest to vaccination, variation in viability and GM-CSF secretion remains dramatic. Cell viability range from 2 to 100% and GM-CSF secretion by genetically modified cells range more than 2 logs (from 4 to 1800ng, 11 to 2600ng or 6 to 3017 ng/10^6 cells/24hrs in three separate trials)

Phase I trial in melanoma observed interesting distant tumor destruction and T lymphocytes infiltration as well as long term survival (>36 months) in more than a quarter of the enrolled patient (Soiffer, Hodi et al. 2003).

In the Phase II lung cancer trial by Nemunaitis et al. a predictive factor of response could be established. Patients immunized with cells producing more than 40ng/106 cells/24hrs demonstrated better prolongation in PFS, in survival (Figure 37) as well as isolated case with good tumor regression (Figure 38)

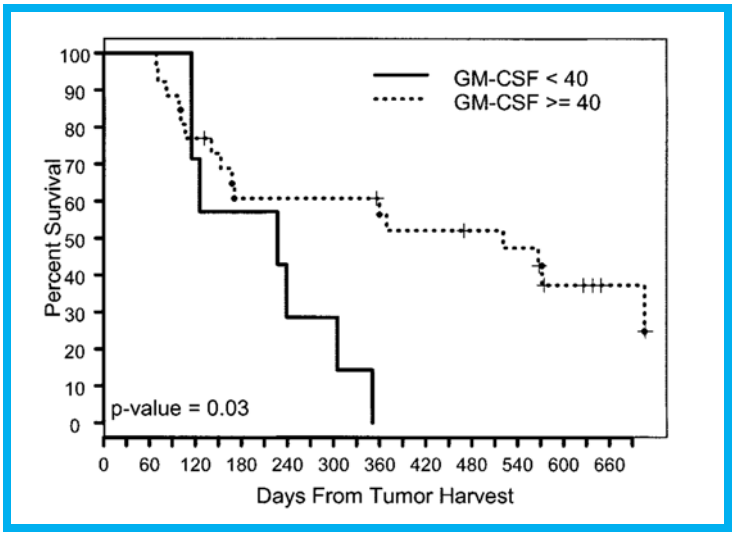

Figure 37 from Nemunaitis et al. (Nemunaitis, Sterman et al. 2004)

Statistically significant difference in patient survival related to GM-CSF release by irradiated, autologous tumour cells. Patients immunized with a vaccine secreting >40ng/10^6cells/24hr have a better outcome.

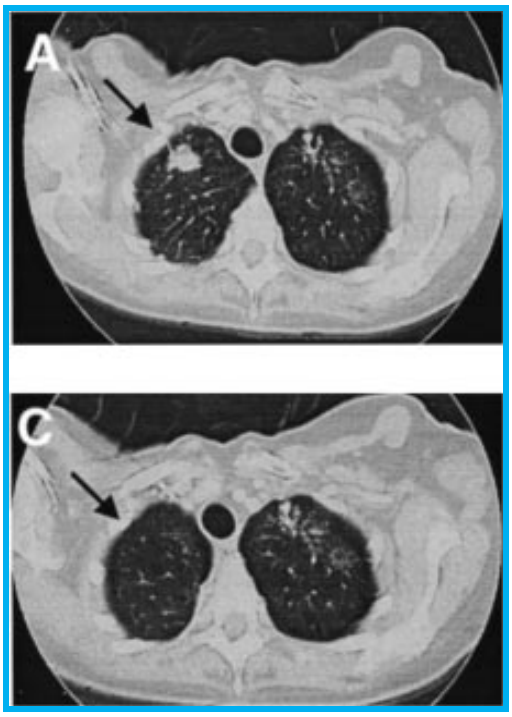

Figure 38 from Nemunaitis et al.(Nemunaitis, Sterman et al. 2004)

Panel A baseline lung tumor evaluation before treatment (metastatic bronchioalveolar carcinoma). Panel C: Imaging after completing vaccination (3 to 6 immunization, 2 weeks apart)

In addition to documented anti-tumor immunization in selected case, vaccination with cells genetically modified with an adenoviral vector induced immune responses against adenoviral protein as shown below in the lung cancer trial published by Salgia et al. All nine patients tested before treatment had detectable antibody titers against either intact (Ad) or lysed adenoviral particles (Ad Lys). Elisa data showing the elevations of adenoviral titer over time from two patients were described in the publication (Figure 39 and 40 below).

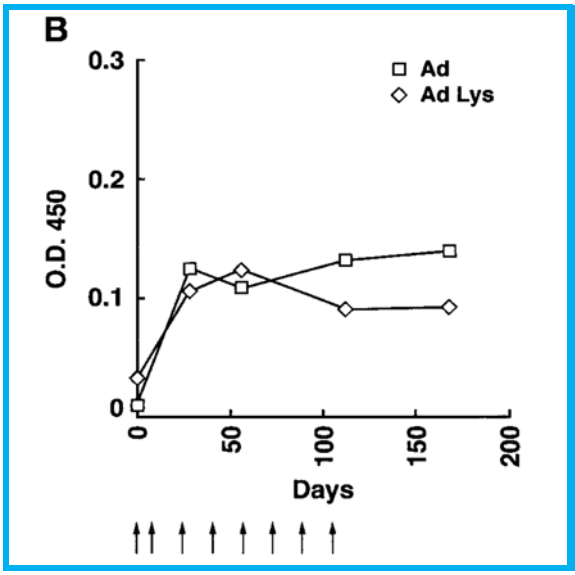

Figure 39 from Salgia et al.

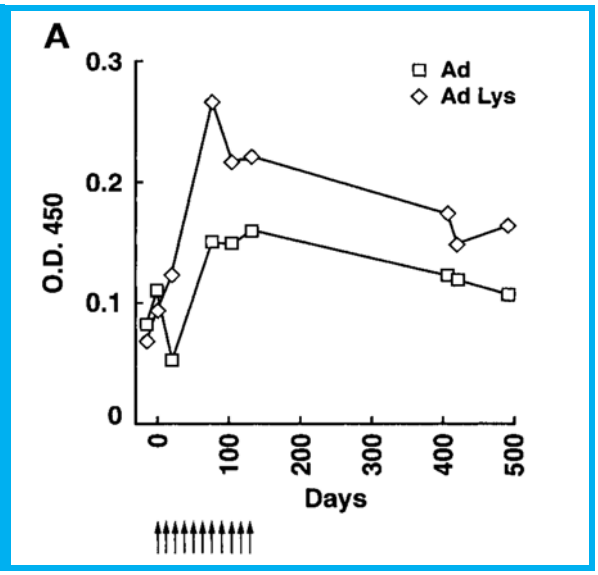

Figure 40 from salgia et al(Salgia, Lynch et al. 2003)

Such ‘skewed’ immune response toward viral epitopes of the vector may favor rapid cell destruction upon subsequent vaccination.

In the Phase 1 ovarian trial, high GM-CSF secretion was obtained (4000ng/10^6 cells/24hrs) and strong DTH reaction to untransduced autologous tumor was observed in all patients with no systemic toxicity and no Grade III-IV side-effects. Clinical endpoints were not reported in the 2002 ASCO meeting abstract but long term surviving patients from the clinical trial have been described in subsequent manuscript describing a synergistic effect between GM-CSF cell-based vaccination and CTLA-4 blockade monoclonal antibody (Hodi, Mihm et al. 2003).

Despite these interesting results including correlation between vaccination and clinical response and frequent DTH reaction, workload for custom made gene therapy for every single patient and the lack of reproducibility in GM-CSF release proved to be too difficult to overcome and genetically modified autologous tumour cells vaccination was not develop further then Phase I/II. No unexpected toxicity has been reported, most patient experiencing local grade 1-2 toxicity at the vaccinations sites.

2) Vaccination with GM-CSF producing allogeneic tumor cells.

Using allogeneic tumor cells secreting GM-CSF has many technical advantages.

- No need to harvest tumor cells from each patients
- No need to perform individualized customized gene transfer for each subject to be treated
- All the patients will be treated with the same product
- Optimization / selection of GM-CSF producing cells can be performed
- Industrialization of biological production can be foreseen

All these points are critical advantage over autologous tumor cell gene therapy.

Based on the hurdles observed with ex-vivo autologous gene transfer clinical trials (with either retroviral or adenoviral vectors) and the advantages described above academic centers and biotechnology companies have developed clinical programs based on immunization with allogeneic tumor cells engineered to produce human GM-CSF.

The fundamental hypothesis backing allogeneic cell-based cancer immunization is that both allogeneic tumor cells and the patient's cancer cells shared common antigens capable of triggering a specific anti-tumor immune response.

Such hypothesis is not yet demonstrated even if tissue specific antigens are potential antigen candidates such as prostate specific antigen in prostate carcinoma.

A major biological mechanism limiting the efficacy of allogeneic cell-based therapy is the strong reactivity against allogeneic material from both innate and adaptive immune responses.

Indeed one of requirement for efficient protective anti-tumor immune response in experimental model is the sustained released over 3-5 days of the strong adjuvant, inducing the favorable 'milieu' for optimal antigen presenting cells recruitment and function. Prolonged survival for several days of allogeneic tumor cells producing GM-CSF injected subcutaneous at regular intervals is unlikely and has not been demonstrated.

At least 9 clinical trials testing allogeneic cancer cells genetically modified to produce GM-CSF have been reported and at least 19 clinical trials are ongoing or planned (clinical trial.gov) in patients with breast, prostate, pancreas cancers and also hematological malignancies such as multiple myeloma or Hodgkin's disease.

The nine reported trials cumulate more than 700 patients, illustrating the more 'user friendly' technology compare to customized ex-vivo gene therapy.

Two Phase III randomized clinical trials have been performed in hormone refractory prostate cancer patients, experimental arms were allogeneic GM-CSF secreting prostate tumor cells in VITAL 1 and the same vaccine combined with standard chemotherapy in VITAL 2 with respectively 313 and 204 patients in each treatment groups. To maximize tumor associated antigens exposure, vaccinations were performed with a pool of 3 different prostate cancer cells lines obtained from prostate cancer, lymphnode and bone metastasis, each genetically modified to secrete high quantities of GM-CSF. VITAL-1 study, comparing vaccine to

docetaxel chemotherapy has been closed prematurely following a futility analysis by the Independent Data Monitoring Committee (IDMC) analysis. The study failed to demonstrate the planned improvement in overall survival over standard chemotherapy. Toxicity profile was very favorable for vaccine compare to chemotherapy. The VITAL-2 study, comparing vaccine plus docetaxel to docetaxel and prednisone was prematurely stopped after routine IDMC analysis reporting imbalance in death rate with excess mortality in the experimental arm. The putative explanation has been linked to the absence of prednisone in the experimental arm, increasing docetaxel associated toxicities.
A Phase I breast cancer trial combined vaccination with two breast cancer cells line secreting GM-CSF and chemotherapy with cyclophosphamide and epirubicine (Emens, Asquith et al. 2009). Described toxicity was related to chemotherapy and local inflammatory reaction at the vaccine sites. Authors described induction of Her2 specific delayed-type hypersensitivity.

In pancreatic carcinoma, allogeneic tumor cells secreting GM-CSF has been tested in the adjuvant setting, in addition to postoperative chemo-radiation (Lutz, Yeo et al. 2011). 60 patients have been treated with mild, local side-effect and no Grade III-IV toxicity.
Comparison with historical control from the same institution reveal no significant difference in the median overall survival (HR:0.96) as illustrated by the figures below.

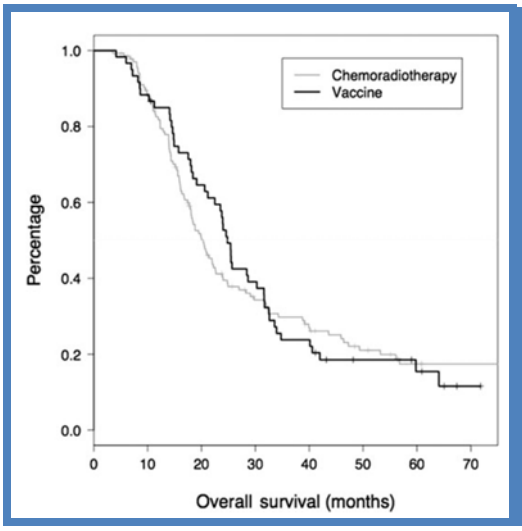

Figure 41 from lutz et al.(Lutz, Yeo et al. 2011)

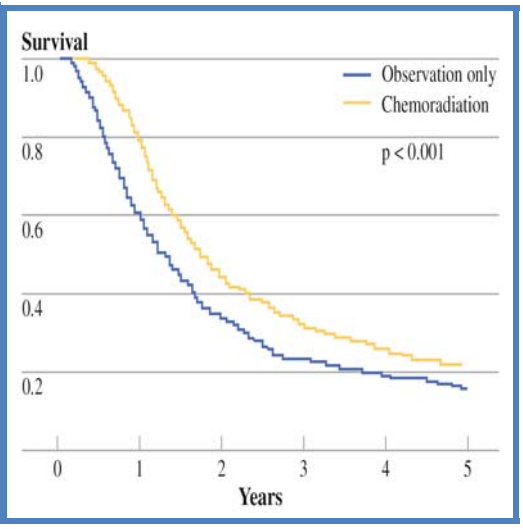

Figure 42 from hsu et al.(Hsu, Herman et al. 2010)

These data have to be compared with data of large cohort of pancreatic carcinoma treated with postoperative chemo-radiationsuch as the 1092 patient from the John Hopkins Mayo Clinic collaborative study reported by Hsu et al. (Hsu, Herman et al. 2010). A comparison with a subset of Johns Hopkins Hospital patients matched on tumor size, nodal status, and margin status produced nearly identical results and did not favor the immunotherapy group as reported by Lutz.

Altogether repeated immunization with GM-CSF-producing allogeneic tumor cells has not been successful despite the potential technical advantages. Rapid destruction of allogeneic cells, lack of sustained GM-CSF exposure at the vaccination site and lack of shared immunogenic tumor associated antigen are the most likely explanations for the negative clinical data. **Reaching a more sustained and standardized GM-CSF release is critical for optimal vaccination.**
The major challenge is to engineer an immunization strategy with standardized, sustained GM-CSF secretion but without the limitation of allogeneic cells rejections.

6) Novel cell-based immunization strategies for clinical applications

As demonstrated in the previous chapters, technical difficulties are limiting the development of customized treatment based on genetically modified autologous tumor cells and rapid destruction of allogeneic tumor cells prevent its use in cancer vaccination. Both strategies are very unlikely get beyond clinical trial testing and reach market authorization. Therefore novel cell therapy schemes have to be developed for in order to meet clinical expectation in the field of cancer immunization.
Learning from both experimental data and clinical trials results we can elaborate a list of minimal requirements for a clinically meaningful approach. The requirements are described below:

- **Standardized GM-CSF release at the vaccination site**
- **Sustained release of the adjuvant protein for up to 5 days at the immunization site**
- **Minimal or no custom made gene therapy**
- **Maximal tumor antigen exposure**
- **Minimal skewing of the immune response toward unrelated immunogenic proteins (viral proteins)**

These requirements, critical for further clinical development of cell-based anti-tumor immunotherapy, have been addressed by two distinct strategies: One by Borello and colleagues at John Hopkins Medical School and the other by our group at HUG in close collaboration with research laboratory at the Ecole Polytechnique Fédérale de Lausanne (EPFL).

Both strategies rely on an allogeneic bystander cell-line producing GM-CSF at the vaccination site to address the first 3 requirements described above. The two approaches use distinct methods to prevent early destruction of the allogeneic GM-CSF producing cell line. The John Hopkin's scheme is defined as MHC negative by-stander cell line as our stretegy relies on encapsulation cell technology.
Borello's scheme has already been tested in small Phase I-II clinical trials and results published recently. The technology developed by our group has not yet been tested in human but pre-clinical data and clinical grade material implementation are available.
Both approaches are described below with detailed experimental description of our ongoing clinical project.

a) MHC negative allogeneic by-stander GM-CSF secreting cells

The novel strategy developed by Borello et al. as early as 2000 is based on the combination of autologous tumor cells and an allogeneic GM-CSF-producing cell-line. Irradiated autologous tumor cells provide the antigenic load while the allogeneic by-stander cell line releases GM-CSF at the immunization site. To diminished alloreactivity against by-stander allogeneic cells, Borello et al. selected a cell-line lacking both MHC class I and II. Allogeneic cells lacking MHC molecules will not trigger TCR-mediated or humoral response and therefore may survive longer in a foreign host. Bystander cell-line lacking MHC molecules can be genetically engineered to secrete GM-CSF and this may allow the release GM-CSF for a longer period of time allowing the induction of an efficient immune response.
In1999, Borello et al. described the combination of irradiated lymphoma cells and a MHC class I negative melanoma cell line engineered to release mu GM-CSF (Borrello, Sotomayor et al. 1999). Surprisingly, immunization with this by-stander GM-CSF producing cell-line proved better than GM-CSF producing lymphoma cells at inducing protective immunity.

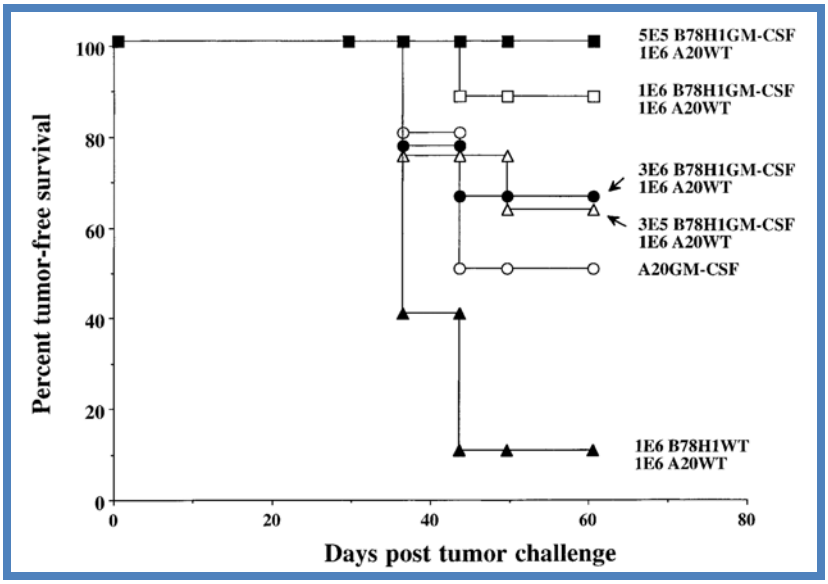

Figure 43 from Borello et al. (Borrello, Sotomayor et al. 1999)

The allogeneic cell-line B78HI was genetically engineered to secrete murine GM-CSF. The combination of various concentrations of this cell-line and irradiated A20 lymphoma cells was compared to A20 lymphoma cells engineered to produce muGM-CSF. Figure 43 shows that protective immunization depends on the concentration of the by-stander cell-line with the best results obtained with the $5x10^5$ cells doses. As lower or higher doses were less efficient, all the combined immunizations were more efficient than GM-CSF producing lymphoma cells.
No other publications have reported the use of mu-GMCSF producing MHC class I negative by-stander cell line in animal anti-tumor immunization model.

The K562 cell-line, a human erythroleukemia cell line obtained form a patient suffering from advanced chronic myeloid leukemia lacks both MHC class I and II molecules. K-562 cell line is well characterized (Lozzio, Lozzi et al. 1976; Andersson, Nilsson et al. 1979). It is used in many experimental immunological assays as a control for non-specific cell killing as K-562 is a very sensitive target for lysis by NK cells. Cytotoxicity of human effector cells against K-562 cells has been widely reported(Thranhardt, Zintl et al. 1980). Study of more than 200 volunteers shows that spontaneous or natural killer cytotoxicity of K-562 cells is high in 4 hours chromium release assays and is not modified by age, gender or smoking habits (Nagel, Collins et al. 1981). Nevertheless, K-562 cell-line has been genetically modified to produce human GM-CSF using non-viral gene transfer technique resulting in a novel cell-line, K-562-GM. The well described high sensitivity of the parental K-562 cell-line to NK cells may well decrease the ability of the new K-562-GM cell-line to survive for several days at the vaccine site and therefore diminished its ability to delivers sustained level of the transduced cytokine.

At least 5 clinical trials have been performed since 2004, evaluating subcutaneous injection of K-562-GM cells.

2004: Borello I. et al ASH meeting, abstract 440: **Autologous Tumor Combined with a GM-CSF-Secreting Cell Line Vaccine (GVAX®) Following Autologous Stem Cell Transplant (ASCT) in Multiple Myeloma.** This trial did not address cell-based immunization specifically as it combined chemotherapy, autologous bone marrow transplantation, leucocytes re-infusion and vaccinations pre and post ASCT with irradiated autologous myeloma cells and K-562-GM. DTH was observed only in 1 out of 15 patients. Toxicity of this multimodal therapy showed no side effect related to vaccination beside some local Grade I-II reactions. No efficacy could be reported from this Phase I but autologous tumor-reactive antibodies as well as antibodies reactive against CG9962 cells was observed.

2006: **Phase I/II trial of autologous tumor mixed with an allogeneic GVAX vaccine in advanced-stage non-small-cell lung cancer** Nemunaitis et al, published in Cancer Gene Therapy (Nemunaitis, Jahan et al. 2006)**.** This Phase I/II trial in advanced lung cancer patients is assessing immunization with autologous irradiated tumor cells and K-562-GM. 5 distinct doses levels were assessed with high level of K-562-GM. Measurements of serum GM-CSF in the days after immunization showed rapid declined after day 1 and further diminution of GM-CSF quantities with subsequent immunization raising the possible of a more rapid clearance of K-562-GM upon repeated injections. Compared to the good clinical data obtained in a previous study with irradiated autologous tumor cells engineered to release GM-CSF (Nemunaitis, Sterman et al. 2004), no patient experienced complete or partial response and 14% of patients presented stable disease at 12 weeks. These disappointing results in a patient's population similar in the two studies may be related to several factors including lack of sustained GM-CSF exposure.

2007: **A Phase I trial using a Universal GM-CSF-producing and Cd40L-expressing Bystander cell line in the formulation of autologous tumor cell-based vaccines for cancer patients with stage IV disease.** Dessureault et al. published in Annals of Surgical Oncology (Dessureault, Noyes et al. 2007). This Phase I trial evaluated autologous tumor cells combined with a K-562 cell line producing both GM-CSF and CD40-L. Patient with stage IV disease were immunized 3 times at 4 weeks intervals. 21 patients were analyzed, with stable disease observed in 6 of 10 melanoma patients and 4 patients developed tumor specific T-Cell responses on Elispot. No systemic toxicity was observed. Minimal increase in serum GM-CSF peaked at 24hrs after immunization. In opposition to strong DTH observed in patients treated with irradiated autologous GM-CSF secreting tumors, no DTH reaction was observed either before or after 3 immunizations.

2009: **Granulocyte-macrophage colony-stimulating factor-secreting cellular immunotherapy in combination with autologous stem cell transplantation as postremission therapy for acute myeloid leukemia.** Borrello et al. published in Blood (Borrello, Levitsky et al. 2009). This trial for acute myeloid leukemia patients did not assessed cell-based vaccination as a single treatment modality. Indeed vaccination was performed in patients before and after ASCT. Additional treatments include autologous primed T-Cell re-infusions before and after ASCT. Immunization was a mixed of irradiated autologous myeloid leukemia cells and K-562-GM. Out of 54 patient enrolled 46 (85%) achieved a complete remission and 28 (52%) received pre-transplantation immunization. Immunized patients developing a positive DTH to autologous leukemia cells showed much longer 3-years relapse free survival than patient without DTH (100% vs 48%) as shown in the figure below

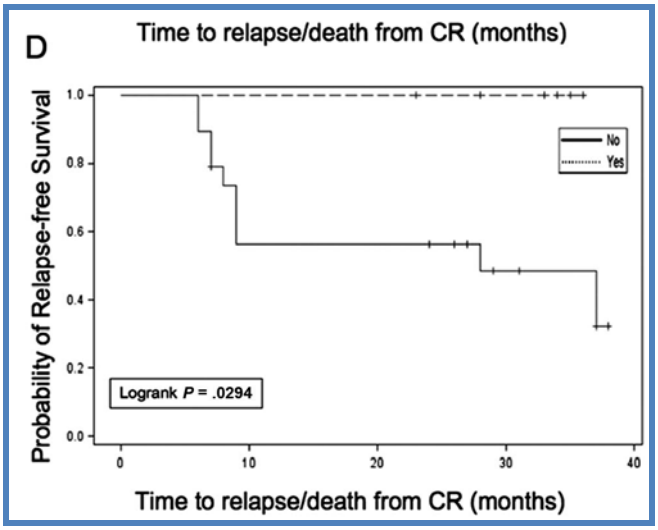

Figure 44 from Borrello et al. (Borrello, Levitsky et al. 2009)

Toxicities related to immunizations were minor local reactions and some flu-like symptoms. With a follow-up of 36 months no late side effect has been observed.

2010: **K-562-GM-CSF Immunotherapy reduces tumor burden in chronic myeloid leukemia patients with residual disease on imatinib mesylate.** Smith et al. published in Clinical Cancer Research (Smith, Kasamon et al. 2010). This trial enrolled patients with chronic myeloid leukemia on imatinib therapy with at least major cytological response and measurable disease. This trial is different from the four previously describe protocols as immunization is performed with K-562-GM alone without autologous or allogeneic tumor cells. As K-562 is a cell-line derived from a CML patient, the rationale for not providing autologous tumor cells is that shared antigens present both on K-562-GM and patient's leukemic cells are sufficient to trigger the immune response. The study has not tested immunization as a monotherapy as all patients were on imatinib treatment throughout the trial. Out of 19 patients, 13 showed a decline in tumor burden including 7 who became PCR undetectable. No severe acute or chronic side effects were observed in this study. Each vaccination was performed with 10 intradermal injections on the limbs each with $1x10^7$ K-562-GM cells. Vaccination was performed 4 times at 3 weeks interval. Analysis of both antibody and T-cell responses to known candidates antigens shared by K-562 and primary CML such as BCR-ABL fusion protein, proteinase 3, Wilm's tumor-1, surviving and PRAME were not detected after immunization with K-562-GM under imatinib therapy. In subsequent analysis the authors mentioned the ongoing characterization of more than 25 antigens expressed by both CML and K.562 cells in post-vaccines but not in pre-vaccine samples.

Although very interesting, the impact of immunization with K-562-GM is difficult to extrapolate from data reported from patients receiving multimodality treatment. In the two trials in which immunization with K-652-GM or K-652-GM+CD40L was evaluated with irradiated autologous cells as a monotherapy, reported results are disappointing compared to autologous cell engineered to produce GM-CSF.

b) Cell-Encapsulation technology

While trials published with K-562-GM bystander cells therapy did not reach the expected results, we crafted a novel immunization scheme aiming at triggering potent, long lasting specific anti-tumor response based on a distinct strategy.
Our hypothesis was driven by the published data on K-562 extreme susceptibility to NK cytotoxicity. Despite lacking MHC class I and II molecules, K-562 are rapidly eliminated in-vivo by non specific mechanisms such as NK mediated cytotoxicity.
A critical issue is to set an immunization process during which a by-stander GM-CSF producing cell line is not recognized by the host and can therefore delivered stable and sustained amount of the adjuvant at the vaccine site over days. We have seeing previously that neither allogeneic tumor cells engineered to release GM-CSF nor K-562-GM are able to performed adequately this task. In collaboration with the Swiss Technology Institute in Lausanne (EPFL) we evaluated the ability of encapsulated cells to address this critical point.

- **Biocompatible capsules** have been engineered to contain numerous products such as chemotherapy, recombinant proteins, radio-labelled materials and live cells. Cells loaded into macro-capsules can survive for long period of time in allogeneic and even xenogeneic hosts. Encapsulated cells are immuno-isolated with no contact with the host immune cells while nutrients and proteins can diffuse through the capsule's pores (schematic view on Figure 45). Both macro and micro-capsules have been design for either local or systemic delivery. Cell encapsulation has been developed primarily to supplement defective proteins by in-vivo delivery by allogeneic cells without requiring immunosuppression. Encapsulated, genetically engineered cells produce in-vivo therapeutics for systemic or local delivery.

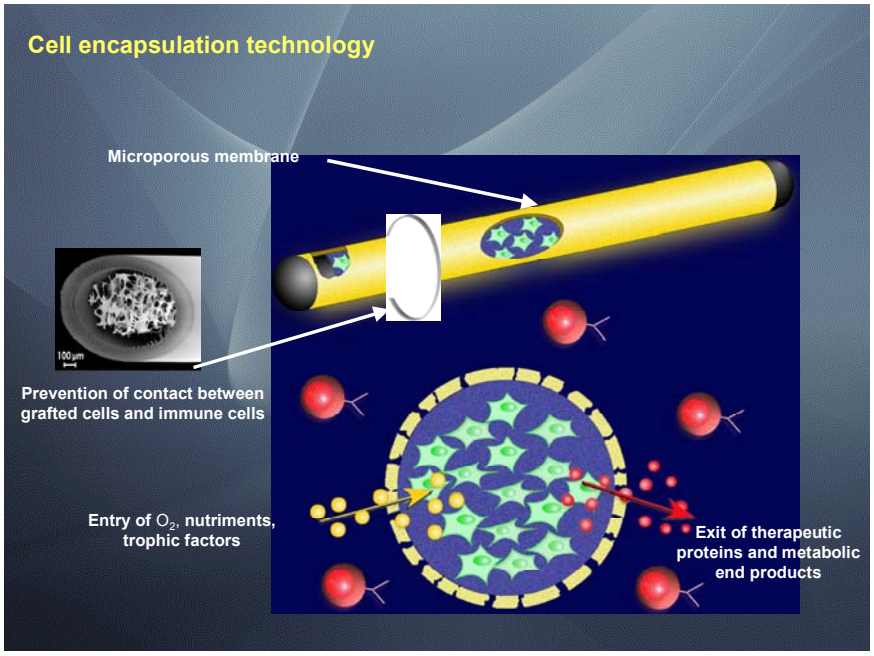

Figure 45

Encapsulation cell technology has been evaluated in experimental models, in early clinical trials using both allogeneic and xenogenic cells lines (Orive, Hernandez et al. 2003). Aebischer's lab has been developing encapsulation cell technology using macrocapsules over the last 20 years(Aebischer, Buchser et al. 1994). Intra-thecal implantation of encapsulated allogeneic cells line secreting ciliary neurotrophic factor (CNTF) have been tested in patients suffering from neurodegenerative disorders such as Huntington's disease (Bachoud-Levi, Deglon et al. 2000; Bloch, Bachoud-Levi et al. 2004) or amyotrophic lateral sclerosis(Aebischer, Schluep et al. 1996; Zurn, Henry et al. 2000). Other neurotrophic factor such as glial cell line-derived neurotrophic factor (GDNF) has been evaluated for Parkinson's disease with intraventricular implantation of capsules containing GDNF secreting cells in primates (Kishima, Poyot et al. 2004). Pre-clinical data have also been published regarding delivery of angiostatin by encapsulated cells genetically engineered to secrete this anti-angigogenic protein (Visted, Furmanek et al. 2003). The improvement in biotechnology allowing the generation of pegylated protein such as erythropoietin has somewhat limited the clinical interest of such strategies. After a successful pilot Phase I study testing the local long term delivery of CNTF by encapsulated cells into the vitreous of the eye (Sieving, Caruso et al. 2006), the positive results of a Phase II study in patients suffering from retinal degeneration has recently been published (Zhang, Hopkins et al. 2011). Clinical data with polyethersulfone macrocapsules shows good biocompatibility, little inflammatory reaction, low acute or long term toxicity.

c) Cell-Encapsulation technology in cell-based immunization models

Our working hypothesis for selecting encapsulation cell technology was that anti-tumor immunization could be achieved by the co implantation of irradiated autologous tumor cells and a macrocapsule containing immune-isolated, allogeneic cells engineered to secrete GM-CSF. The combination of irradiated autologous tumor cells and the sustained release of GM-CSF by encapsulated cells on the vaccine site should trigger the immune system as observed with genetically engineered autologous cells. The sustained local release of GM-CSF should recruit hematopoietic cells including DC and induce a local environment favourable for optimal antigen uptake from cancer cells as described in the scheme on Figure 46.

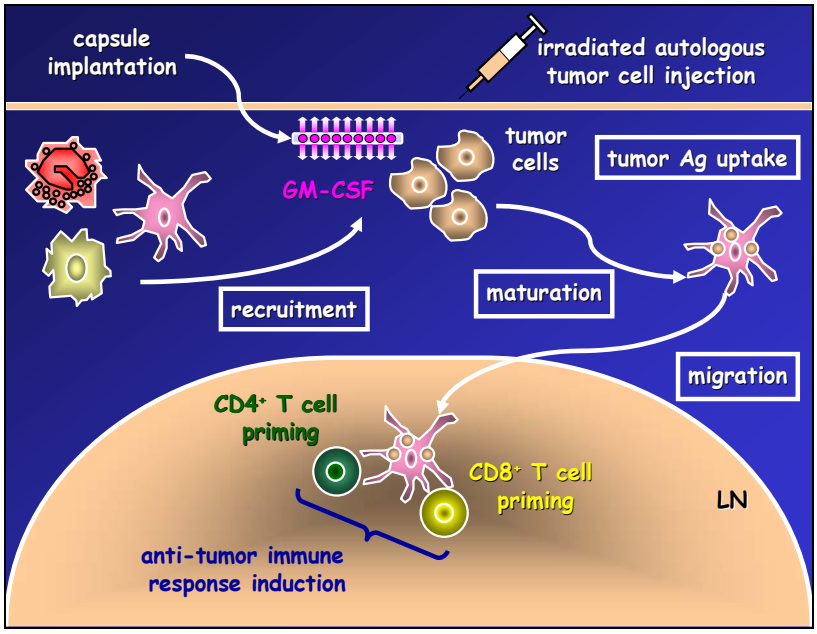

Figure 46

Such novel immunization strategy should by-pass the difficulties observed in previous trials and fulfils the requirement for clinical development as listed below:

-**best antigenic repertoire**: yes by using irradiated autologous tumor cells.

-**no need of customized gene therapy**: yes, GM-CSF is provided by a by-stander cell-line

-**no skewed immune response toward viral vector**: yes, transfection of the by-stander cell-line is not performed with viral vectors.

-**GM-secretion is sustained**: yes, encapsulated allogeneic GM-secreting cells are protected and are not quickly eliminated by the immune system (opposite to K-562-GM or non protected allogeneic cells)

-**Quantity of GM-CSF is modulable**: yes, capsule size and cell density within the capsules can be modified.

-**GM-CSF production can be standardized**: yes, all subjects will receive similar loaded capsules with known production of GM-CSF.

-**Clinical implementation is technically feasible**: yes, both components (irradiated autologous cells and encapsulated GM-secreting cells) can be frozen and stored for subsequent immunization.

In order to have a proof of concept we evaluate the ability of cell encapsulation technology to recapitulate the biological effect of GM-CSF secreting cells in our murine models.

We tested several human and murine cell lines for their ability to survive encapsulation in-vivo and in-vitro. Figure 47 illustrates in-vivo data

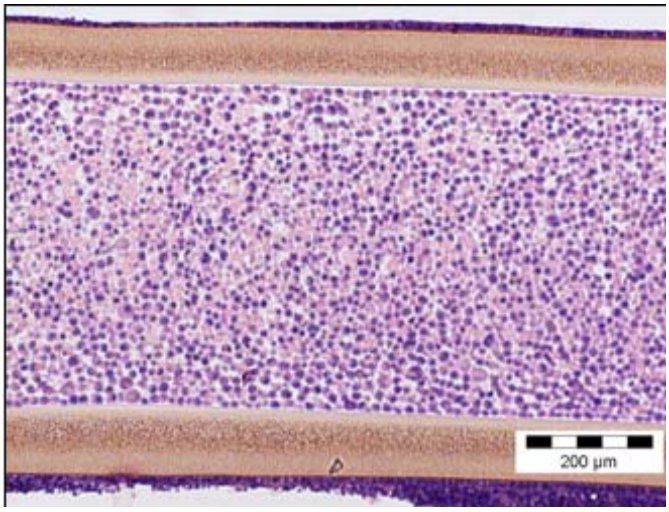

Figure 47

Magnified Cross-section view (Hematoxylin-Eosin stain) of a capsule containing muK562 cells analyzed after recovery, five days post subcutaneous implantation in a Balb/c mouse. Good viability of the human K-562 cells can be observed.

Biological activity of macrocapsule containing muGM-CSF secreting cells was subsequently analyzed by histo-pathological analysis of the subcutaneous implantation site.

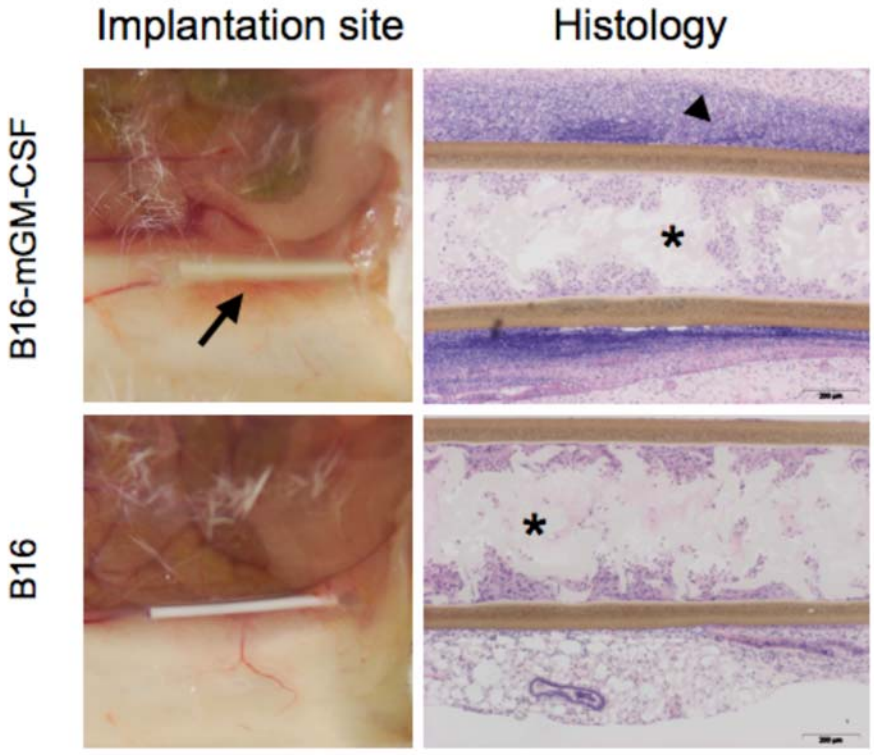

Figure 48 from Schwenter et al. (Schwenter, Zarei et al. 2011)

Implantation site and histologies

The top two panels of Figure 48 shows the inflammatory rim around the capsule (macro and microscopy)

The lower panels represent the control group, similar analysis with capsule not secreting GM-CSF. The encapsulated cells used in this experiment are adherent cells, requiring a matrix within the capsule. The PVA matrix is amorphous substance in the centre of the capsule (*).

Similar experiments in Balb/c mice reveal an even more striking inflammatory reaction around GM-CSF secreting capsules

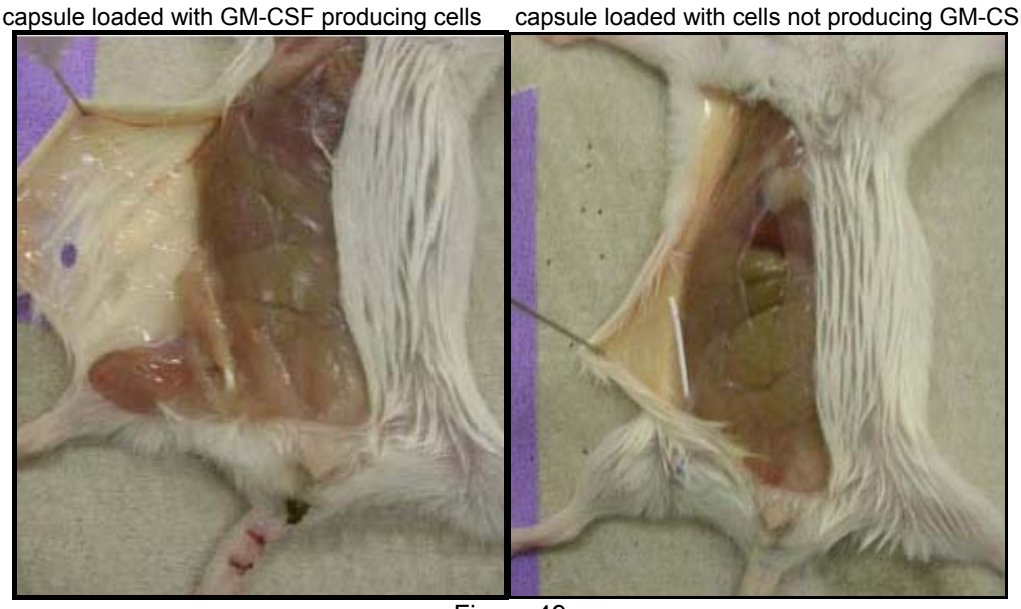

Figure 49

Striking difference between GM-secreting capsule and non-GM-secreting capsule can be observed macroscopically in Figure 49. Such inflammatory reaction is not related to traumatic implantation as non-secreting capsule did not show such infiltrate.

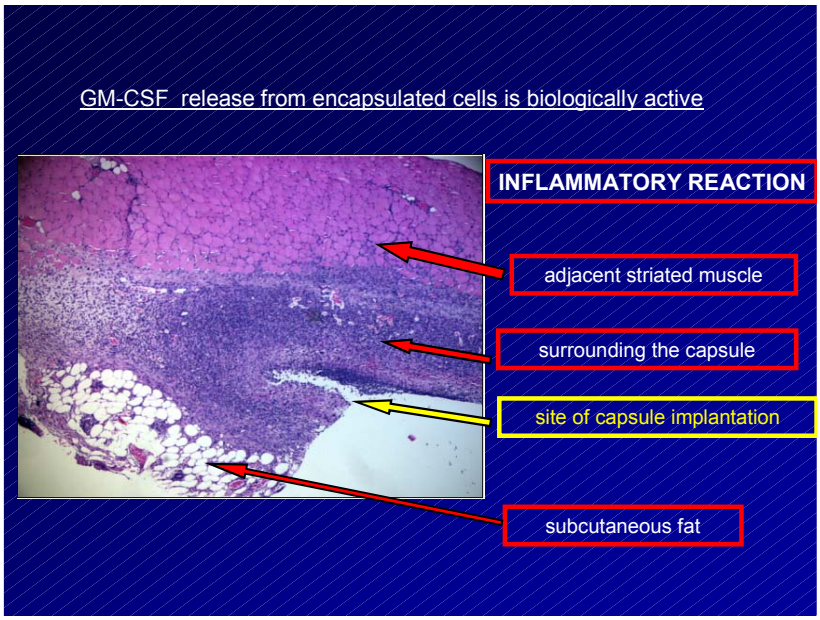

Figure 50

Histological analysis of capsule implantation site confirmed the strong inflammatory reaction with lymphocytes, macrophages, eosinophils, dendritic cells, neutrophils

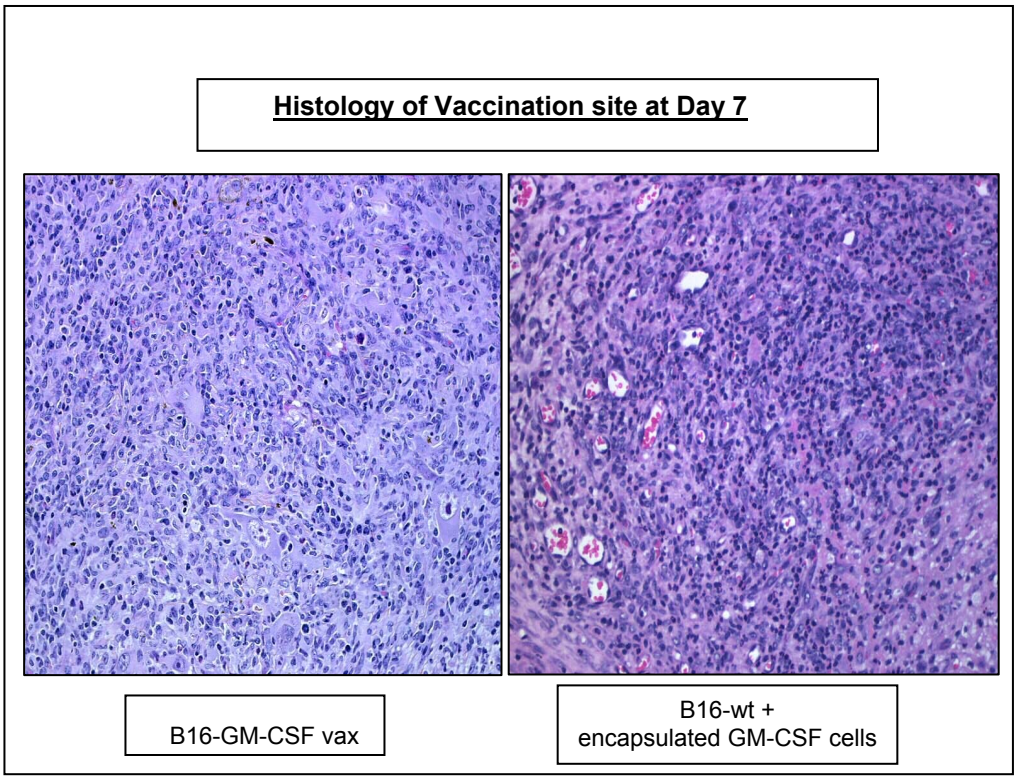

Figure 51

Histological analysis of immunization sites: Comparative analysis of irradiated GM-CSF secreting autologous tumor cells (B16-GM-CSF, left) and irradiated autologous cells combined with a capsule containing GM-CSF secreting cells (right). Similar strong infiltration with pleomorphic inflammatory cells can be observed in both immunization sites (Figure 51). These analyses suggest a similar biological activity at the implantation site.

With good evidence for biological activity of encapsulated GM-CSF secreting cells implanted subcutaneously, we evaluated the anti-tumor activity of the vaccine formulation combining irradiated tumor cells and GM-CSF containing capsule co-implanted subcutaneously.

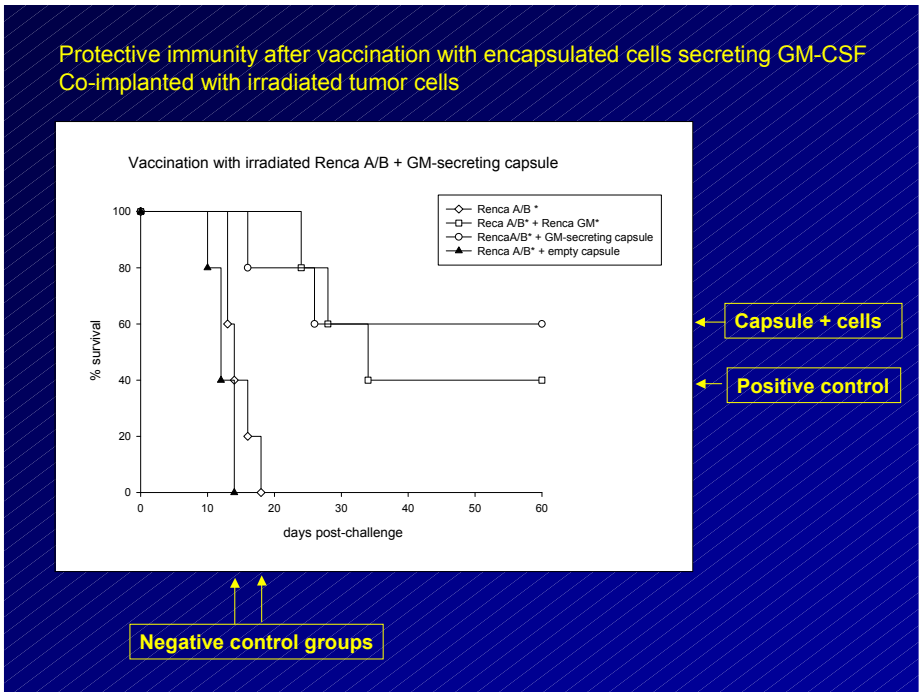

Figure 52

Figure 52 shows a good protective immunity in the modified Renca model. Mice were immunized with Renca α/β KDEL to prevent any GM-CSF production by tumor cells. The only source of GM-CSF is provided by the encapsulated cells.
Protective immunity induced with GM-CSF-secreting encapsulated cells combined with irradiated tumor cells in the Renca murine model in similar or even better than the control group in which GM-CSF is provided by non encapsulated cells.

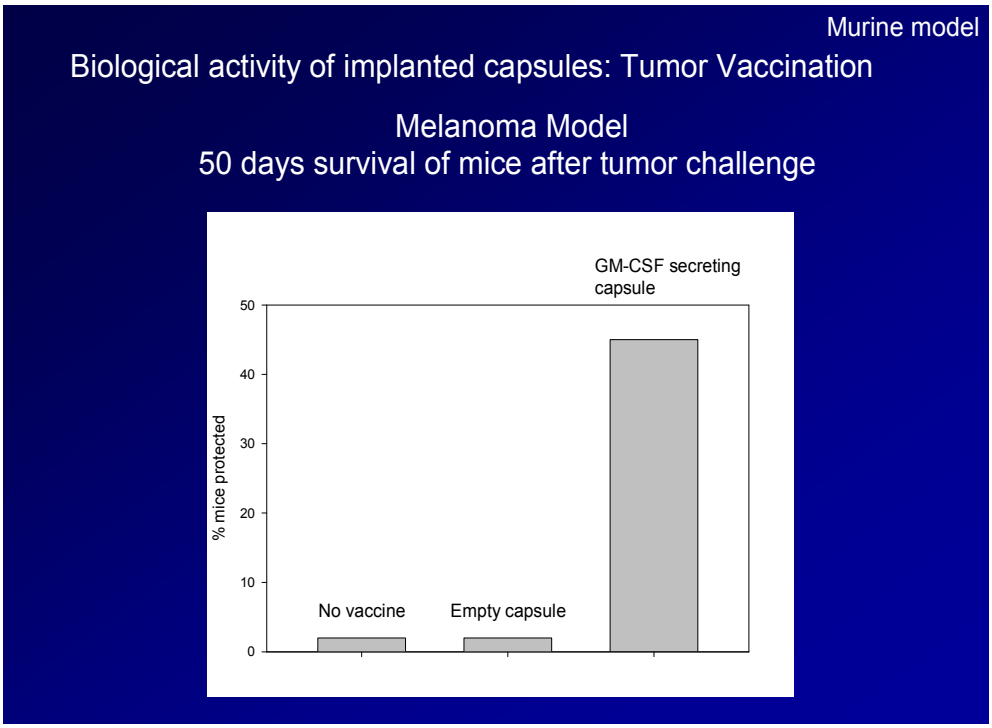

Figure 53

Similar experiments in the B16 melanoma murine model showed a marked protective immunity

9) Perspectives and Conclusions

a) Clinical development of a novel anti-tumor immunization strategy combining encapsulation cell technology and autologous tumor cells.

Based on our encouraging experimental data, integrating both the positive and negative results published in the past years, we decided to test the ability of GM-CSF-secreting cells to undergo encapsulation and release GM-CSF in a stable and sustained manner before foreseeing a proof of concept, first in human, pilot clinical trial.

Cell survival and GM-CSF secretion of encapsulated genetically modified cells:
In-vitro experiment assessing the ability of GM-CSF secreting cells lines to undergo macroencapsulation and release stable of GM-CSF. Planning for potential clinical application we decided to test an already certified clinical grade GM-CSF secreting cell-line. In collaboration with the group of Richard Mulligan (Harvard Medical School) we agreed to test a novel clinical grade K-562-GM cell line engineered by the Harvard Gene Therapy Initiative. Most of data presented below have been published recently by our group (Schwenter, Zarei et al. 2011).

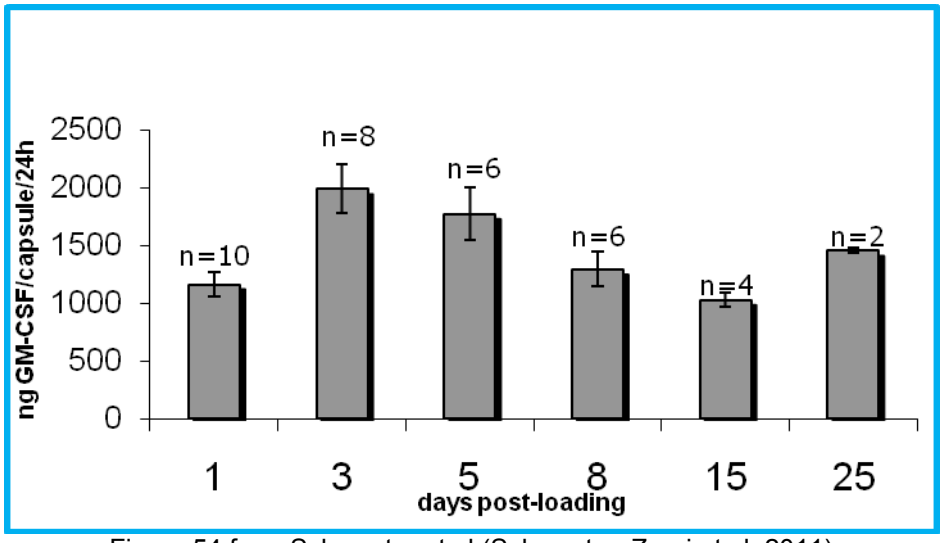

Figure 54 from Schwenter et al.(Schwenter, Zarei et al. 2011)

Time course of GM-CSF secretion from capsules containing K-562-hGM-CSF loaded at a density of 10^5 per capsule

Freezing/Thawing experiments:
Storage is a critical step in the development of a clinically meaningful cell-therapy product. Indeed, as cell banking of irradiated autologous tumor cells has already been address, freezing and thawing macrocapsules containing genetically engineered cells is a more difficult task. Figure 54, below, illustrates the modification of GM-CSF secretion release by loaded capsule before and after freezing/thawing procedure under distinct conditions.

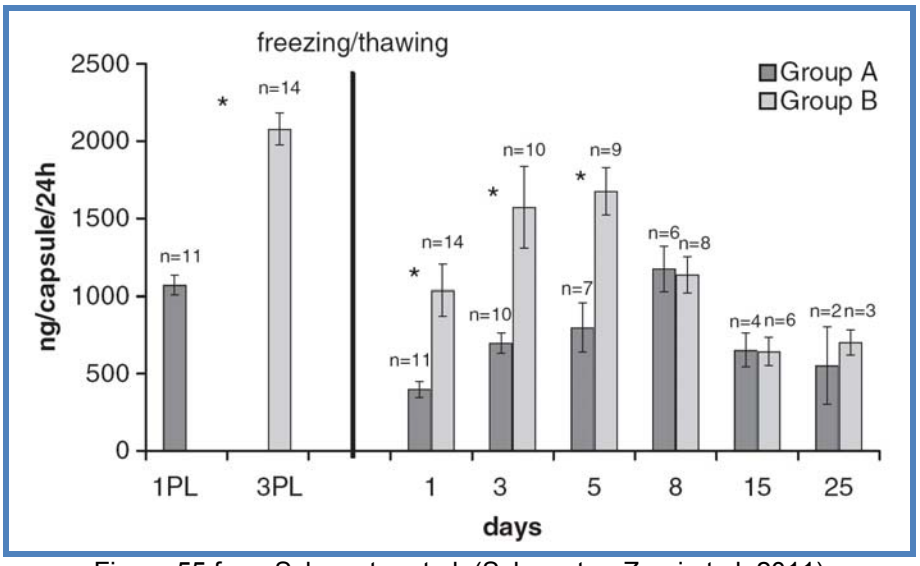

Figure 55 from Schwenter et al. (Schwenter, Zarei et al. 2011)

Time course of GM-CSF secretion from K-562-hGM-CSF capsules after freezing and thawing. Capsules were frozen 1 day (Group A) or 3 days (Group B) after cell loading. 1PL=1 day post-loading:GM-CSF secretion from group A before capsule freezing. 3PL=3 days post-loading: GM-CSF secretion from group B before capsule freezing.

Histological analysis of loaded capsules at different time point with or without freezing/thawing procedure:

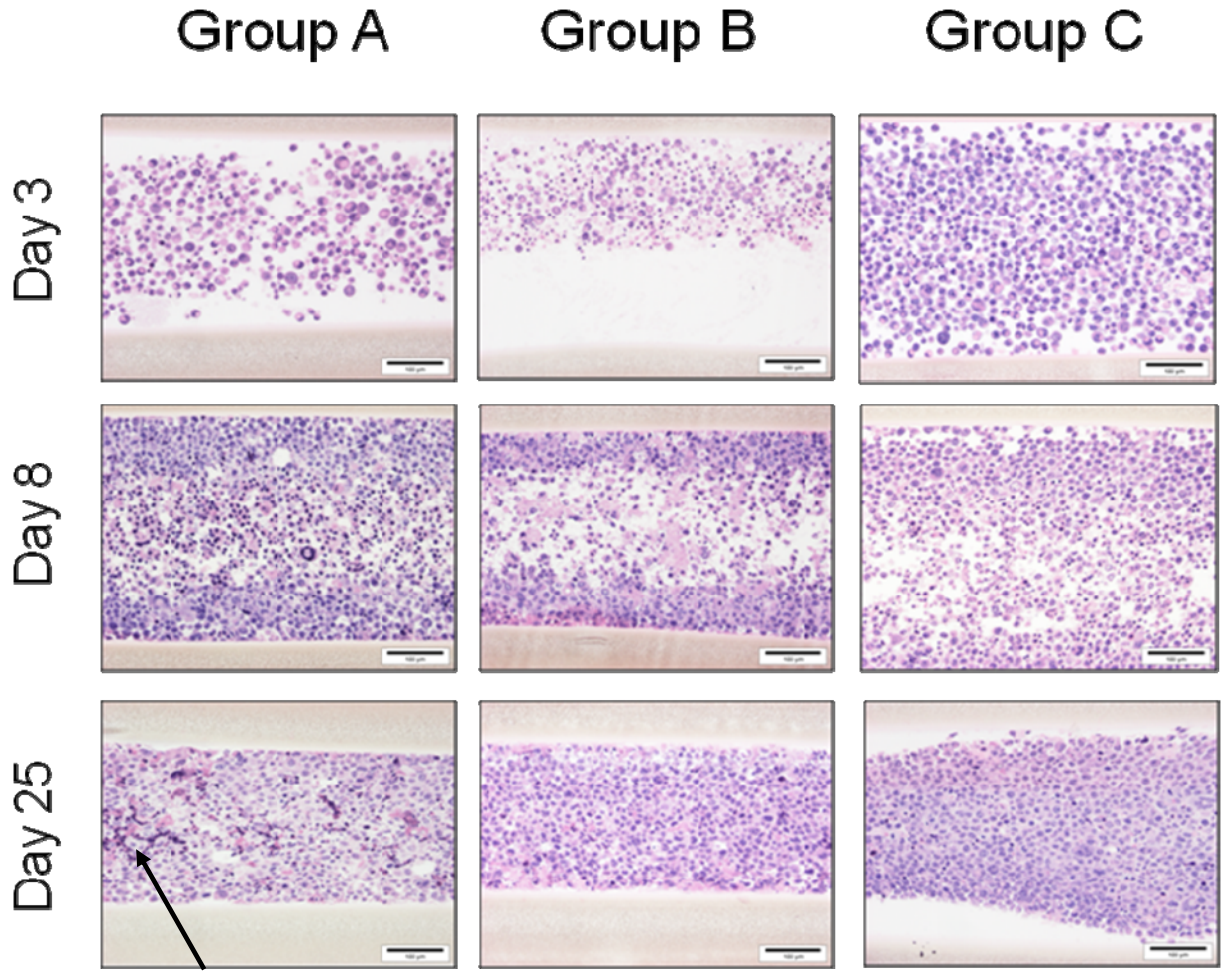

Figure 56 from Schwenter et al. (Schwenter, Zarei et al. 2011)

Histologies of capsules cultured in-vitro for 3, 8 and 25 days after thawing are shown on Figure 56. Capsules were frozen 1 day (group A) or 3 days (group B) after cell loading. Group C represents capsules that were not frozen. Arrow on Group A at day 25 shows necrotic debris, the upper and lower part of the picture represents the polyethersulfone semi-permeable membrane

Based on Elisa results and histological analysis, freezing 3 days post-loading was selected as the best strategy for sustained, reliable GM-CSF release after thawing procedure.

Additional sets of experiments were performed to select the best timing for lethally irradiating the loaded capsules as this additional safety step maybe required by health authorities. The Figures 57 and 58 reveal that better GM-CSF level can be achieved when irradiation is after performed freezing/thawing rather than before.

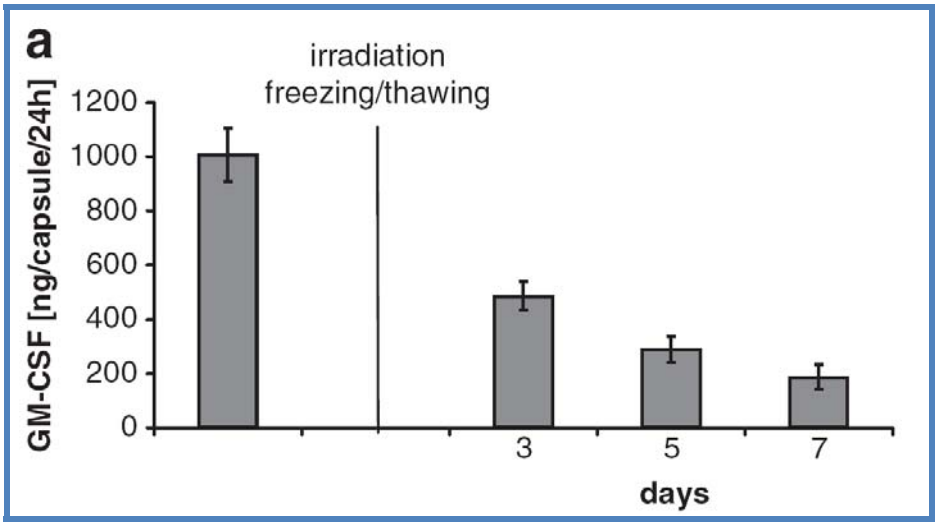

Figure 57 from Schwenter et al.(Schwenter, Zarei et al. 2011)

Time course of GM-CSF secretion from encapsulated K-562-hGM-CSF cells. Irradiation with 10000 rads is followed by freezing thawing (n=3)

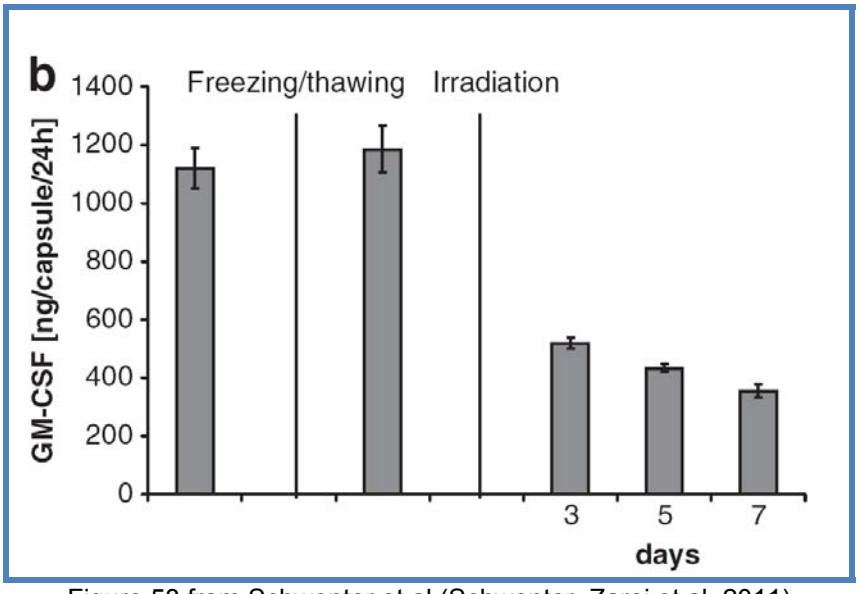

Figure 58 from Schwenter et al.(Schwenter, Zarei et al. 2011)

Time course of GM-CSF secretion from encapsulated K-562-hGM-CSF cells. Freezing and thawing is followed by irradiation with 10000 rads (n=6)

After the confirmation that K-562-GM can undergo encapsulation, freezing/thawing procedure while producing sustained and stable level of GM-CSF, testing was performed with clinical grade macrocapsule as described below. Clinical grade capsules are longer and have been designed with clinical grade components and contain a coil within the capsule that prevents bending, kinking and provide additional strength. Furthermore, clinical grade capsules contain a hook that allows easy recovery of the device when required. The following figure gives a schematic view of the clinical grade capsule.

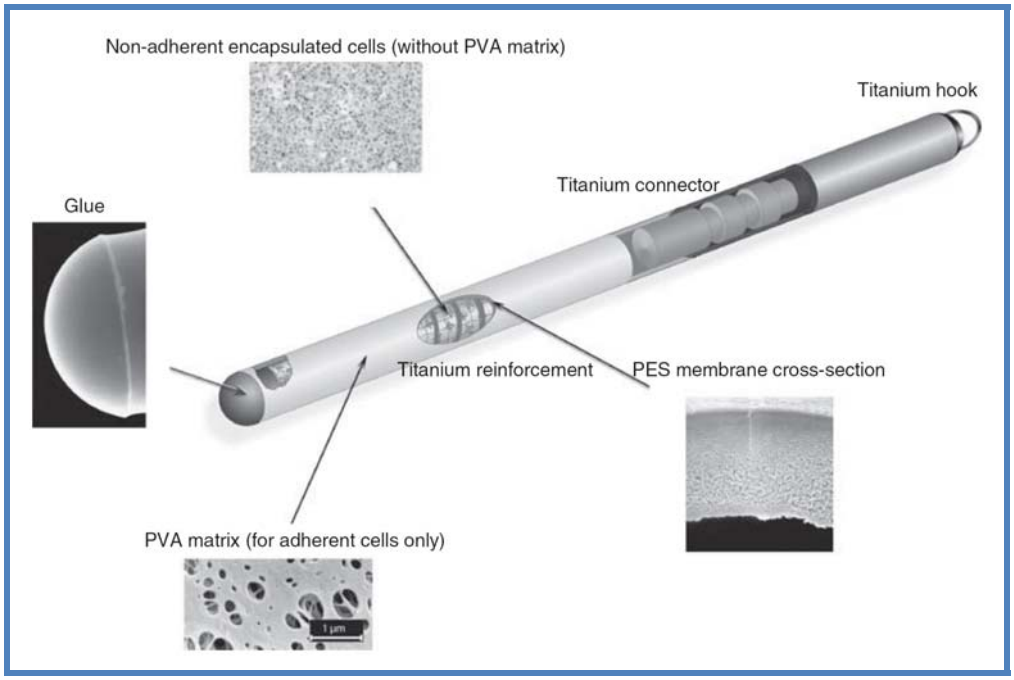

Figure 59
Illustration of the capsule developed for human application showing the titanium coil inserted into the hollow polyethersulfone fibre.

The next graph (Figure 60) shows the GM-CSF release from loaded clinical grade capsules at different time points before freezing/thawing, irradiation procedures. It shows that for macrocapsules loaded with $2x10^5$ cells, more than 150ng of GM-CSF is release in the supernatant/24hr after thawing and irradiation.

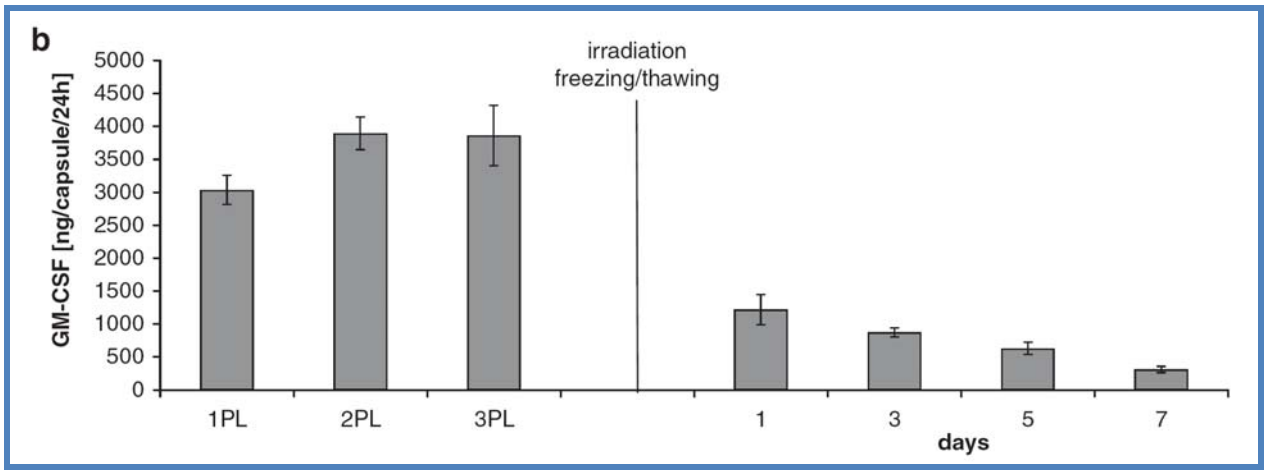

Figure 60 from Schwenter et al. (Schwenter, Zarei et al. 2011)

Time course of GM-CSF secretion from 2-cm-long capsules reinforced with a titanium coil and containing K562-hGM-CSF loaded at a density of $2x10^5$ cells per capsule. Capsule were irradiated with 10000rads before freezing and thawing (n=4).

As the results of GM-CSF release presented above shows good biodelivery of GM-CSF from encapsulated K-562-hGM-CSF cells, we also demonstrated good feasibility and reproducibility for up to 14 days (Schwenter, Zarei et al. 2011). With these convincing and robust animal vaccination data and in-vitro data regarding sustained and stable release a first clinical trial can be foreseen.

Planning this first clinical trial requires expertise from HUG, EPFL, public and private funding as well as top quality infrastructures at the HUG such at the Cell Therapy Laboratory and the Clinical Research Unit of the Fondation Dr.Henri Dubois-Ferrière Dinu Lipatti. In the past 24 months, we have engineered the clinical grade material, including a clinical grade genetically modified cell-line producing human GM-CSF, develop all the standard operating procedures according to GMP, GCP and ICH guidelines and wrote the protocol as well as the investigator's brochure.

The following figures shows the the overall setting of the future clinical protocol as well as technical details of the encapsulation device.

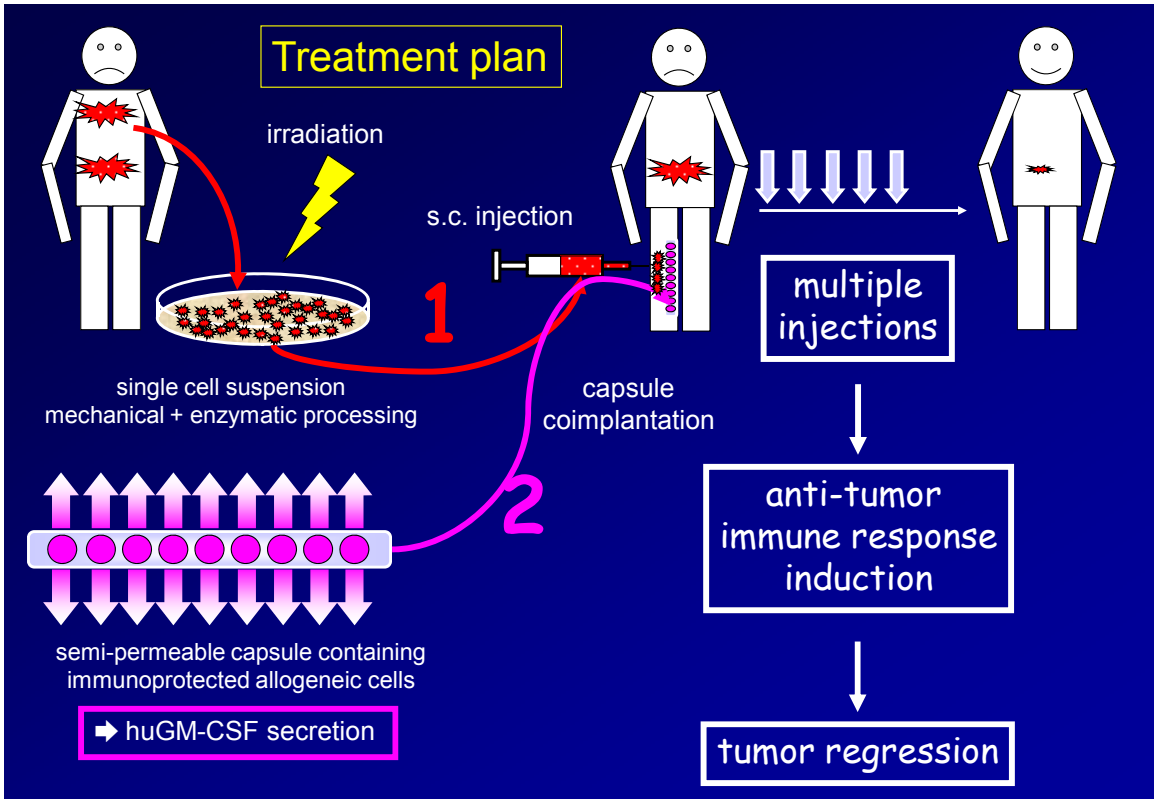

Figure 61

Figure 61 illustrate the two components immunization scheme that will be implanted sc in site distant from the tumor deposits. Six vaccinations will be performed, four at weekly intervals followed by two immunizations two weeks apart. Primary endpoints are feasibility and toxicity. Secondary endpoints include classical clinical response parameters but also specific parameters selected specifically for cancer immunotherapy (Wolchok, Hoos et al. 2009) and extensive immunological analysis such as DTH reaction, circulating lymphocytes and tumor infiltrating lymphocytes characterization and culturing to assessed changes in cancer cells destruction.

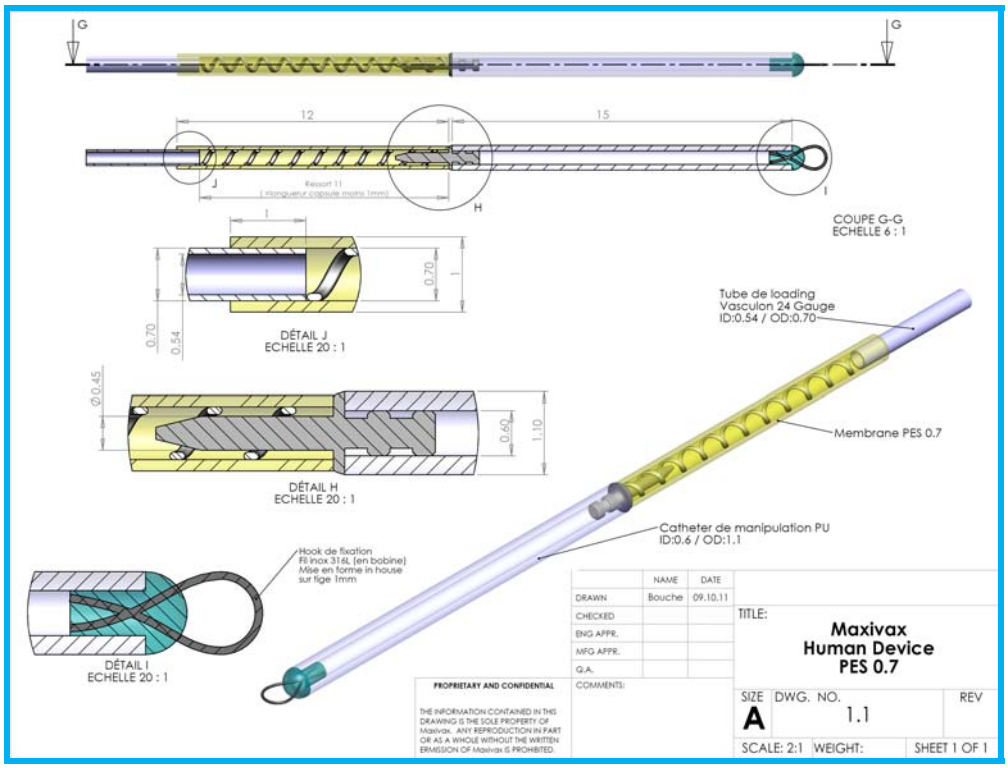

Figure 62

Technical description of the biocompatible macrocapsule of the future clinical trial.

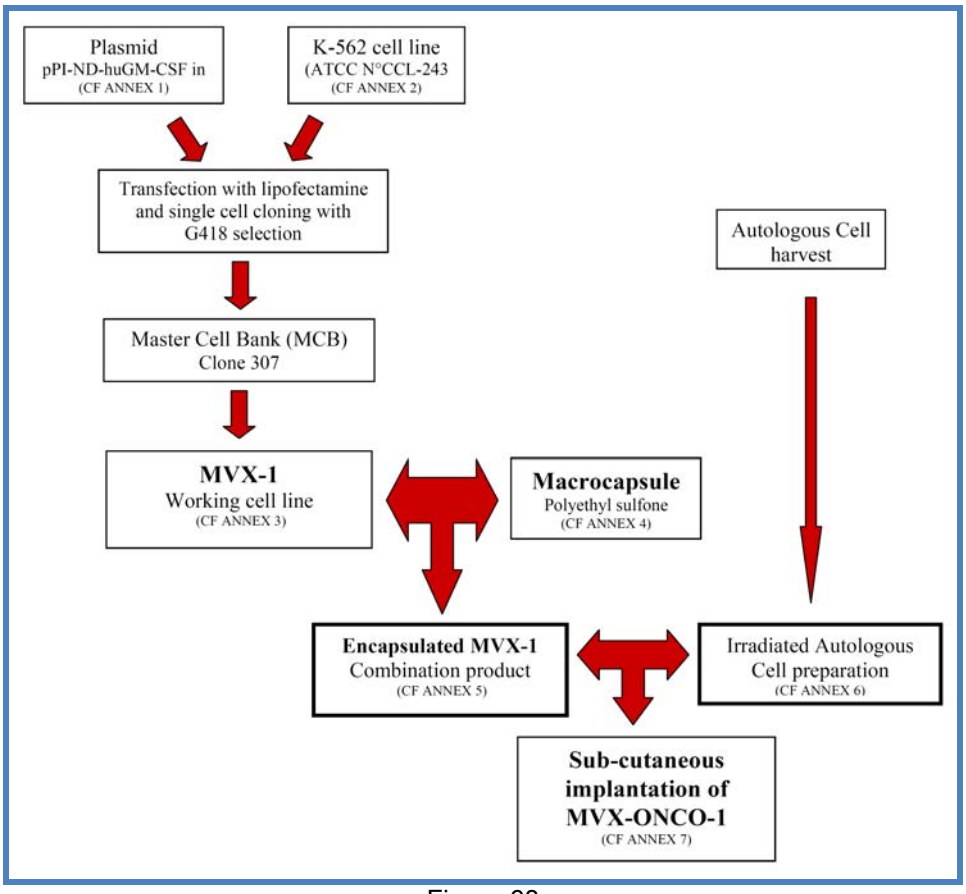

Figure 63

Diagram illustrating the components of the planned Phase I clinical trial

The clinical protocol for the First in Human Phase I feasibility/safety study has been recently approved, by the ethical committee of the Geneva University Hospital. Authorization by the Swiss health regulatory authority (SwissMedic) is pending.

The study will enrol 15 patients with advanced solid malignancies refractory to currently available therapies and hopefully patient's inclusion will start by Q3-Q4 2012. Extensive monitoring is planned for feasibility, toxicity but also clinical and immunological parameters.

b) Conclusion

After decades of frustration from both scientists and medical oncologists for not being able to translate tremendous knowledge in tumor immunology into clinical applications, innovation in biotechnology give us opportunities to test novel vaccination strategies. Major progresses have been made in the last ten years bringing renewed interest in cancer immunotherapy with special interest in cell therapy. Patient specific therapies have been developed in the past years and now the first personalized cell therapy has been granted market approval in the US for metastatic prostate cancer.
This review has integrated scientific and clinical information published by many groups working in this field and also data from our research group. It is not covering all the aspects of cell-based anti-tumor immunization as it focuses mainly on immunostimulation by genetically modified cells with specific emphasis on GM-CSF. Learning from positive preclinical results but also from many negative clinical trials helped us crafting a novel cell therapy strategy with meaningful clinical endpoints. As the first clinical trial has not yet started, upgrade of the current cell-therapy platform can already be foresee such as cytokine combination, different antigen formulation, Treg depletion and synergy with other stimulatory molecules such as CTLA-4 blockade.

From all the data presented in this review, the following key messages should remain.

1 Single antigen-based immunizations against cancer has not been very successful
2 Immunization against antigen arising from autologous cancer cells is difficult
3 Cell-based immunotherapy needs potent adjuvant
5 GM-CSF is one of the most potent immunostimulatory cytokine
6 Tumor immunity does not require endogenous GM-CSF but GM-CSF signaling
7 Irradiated cancer cells engineered to secrete GM-CSF stimulate tumor immunity in all murine models tested
8 Local production of GM-CSF at the vaccine site recruits potent dendritic cells
9 'Spontaneous' tumor immunogenicity may be related to GM-CSF release
10 Fine tuning in GM-CSF's delivery is critical as it can have tolerogenic effects
11 Allogeneic tumor cells are good providers of neither antigen nor GM-CSF
12 Autologous cancer cells still are the best sources of tumor associated antigens
13 Personalized genetic engineering of cancer cells is not yet clinically meaningful
14 Progress in biotechnology, cell engineering and biomaterials brings new tools
15 Novel strategies in cell therapy may overcome the hurdles observed in previous trials
16 Only clinical research will be able to test and validate successful approaches

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9) Annexes

Seven manuscripts by the author, from which either Figures, Data or discussion were extracted for this review.
The documents are listed by date of publication

Lantz, C. S., J. Boesiger, et al. (1998). "Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites." Nature **392**(6671): 90-93.

Mach, N., C. S. Lantz, et al. (1998). "Involvement of interleukin-3 in delayed-type hypersensitivity." Blood **91**(3): 778-783.

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Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites

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The cytokine interleukin-3 (IL-3), which can be derived from T cells and other sources, is a potentially important link between the immune and haematopoietic systems[1]. IL-3 may be particularly critical for the development, survival and function of tissue mast cells[1–6] and blood basophils[7,8], which are thought to be important effector cells in immunity to parasites and other immunological responses, such as allergic reactions[9]. Here we show, using IL-3-deficient mice[10], that IL-3 is not essential for the generation of mast cells or basophils under physiological conditions, but that it does contribute to increased numbers of tissue mast cells, enhanced basophil production, and immunity in mice infected with the nematode *Stronglyoides venezuelensis*. Parasite expulsion and mast-cell development are impaired even more severely in IL-3-deficient mice that also show a marked reduction in signalling by c-kit. These findings establish a role for IL-3 in immunity to parasites and indicate that one of the functions of IL-3 in host defence against infection is to expand populations of haematopoietic effector cells.

Mice lacking IL-3 (IL-3$^{-/-}$ mice) were produced using gene targeting in embryonic stem cells[10]. IL-3$^{-/-}$ mice are healthy and fertile and, like mice that carry an inactivating mutation in the α-chain of the heterodimeric IL-3 receptor[11] or that lack both IL-3 and the common β-subunit of the receptors for IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor[12], show no detectable abnormalities in multiple aspects of haematopoiesis *in vitro* or *in vivo*[10]. However, the c-kit ligand, stem cell factor (SCF)[13], induced fewer mast cells to develop from bone-marrow cells of IL-3$^{-/-}$ mice than were induced from bone-marrow cells of wild-type mice (Fig. 1a, b). In contrast, markedly higher numbers of mast cells developed when bone-marrow cells from either IL-3$^{-/-}$ or wild-type mice were maintained in exogenous SCF plus IL-3 (Fig. 1b).

The findings shown in Fig. 1a, b concur with previous work indicating that exogenous IL-3 can augment SCF-dependent mast-cell development *in vitro*[6,13,14]. Although numbers of mast-cell progenitors[6,13–15] may differ in IL-3$^{-/-}$ and IL-3$^{+/+}$ mice *in vivo*, our data show that similar numbers of mast cells can be generated when bone-marrow cells from either IL-3$^{-/-}$ or wild-type mice are maintained in SCF plus IL-3 *in vitro* (Fig. 1b). Moreover, limited

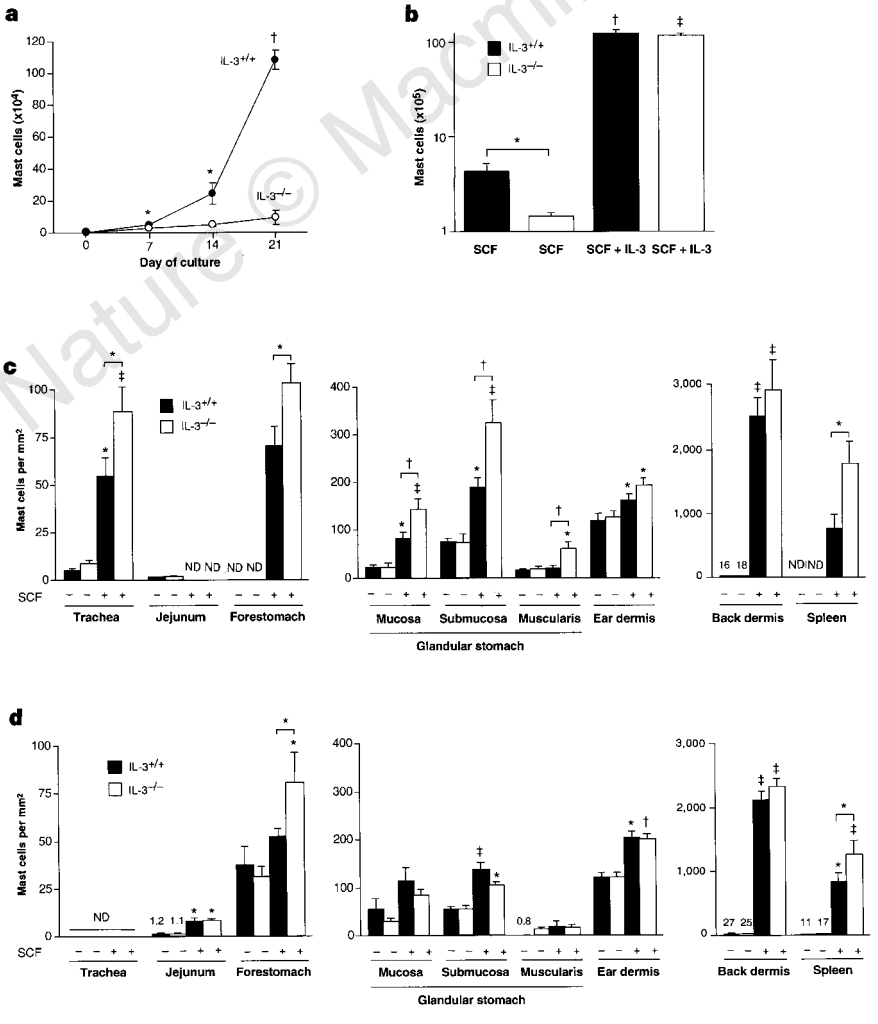

Figure 1 Differential requirements for IL-3 in SCF-induced mast-cell development. **a**, **b**, *In vitro*; **c**, **d**, *in vivo*. **a**, Development of mast cells from BALB/c IL-3$^{-/-}$ mouse bone-marrow cells cultured *in vitro* with recombinant rat SCF (rrSCF) is markedly decreased compared with the development of mast cells from BALB/c IL-3$^{+/+}$ mouse bone-marrow cells. All values are mean ± s.e.m. ($n = 3$) per 10^6 bone-marrow cells plated and are representative of the results of 3 separate experiments. Similar results were obtained using 129Sv × C57BL/6 IL-3$^{+/+}$ and IL-3$^{-/-}$ mice. Asterisk, $P < 0.05$; dagger, $P < 0.001$ versus corresponding values for IL-3$^{-/-}$ cells. **b**, Exogenous IL-3 enhances mast-cell development in cultures of BALB/c IL-3$^{+/+}$ or IL-3$^{-/-}$ bone-marrow cells maintained for two weeks in rrSCF. All results are mean ± s.e.m. ($n = 3$–4) per 10^6 bone-marrow cells plated. Asterisk, $P < 0.05$; dagger, $P < 0.001$; double dagger, $P < 0.0001$ versus corresponding values for cells cultured with SCF alone or versus values indicated by the square bracket. **c**, **d**, Numbers of mast cells in various tissues of (**c**) rrSCF-treated or untreated 129Sv × C57BL/6 IL-3$^{-/-}$ and IL-3$^{+/+}$ mice or (**d**) rrSCF-treated or vehicle-treated BALB/c IL-3$^{-/-}$ and IL-3$^{+/+}$ mice. Mice were killed to assess mast-cell numbers at baseline (**c**) or 24 h after the last of 21 daily subcutaneous injections of rrSCF (100 μg kg^{-1} d^{-1}, **c**, **d**) or vehicle (**d**). All results in (**c**, **d**) are expressed as mean ± s.e.m. ($n = 4$–11 mice per group). Statistical significance was determined by Student's *t*-test (two-tailed) or one-way analysis of variance. Asterisk, $P < 0.05$; dagger, $P < 0.001$; double dagger, $P < 0.0001$ versus corresponding values for untreated or vehicle-treated mice of the same genotype or (as indicated by square brackets) versus values for mice of the other genotype. ND, not done. +, plus SCF; –, vehicle-treated or untreated.

SCF-dependent mast-cell development can occur *in vitro* in the absence of exogenous or endogenous IL-3 (Fig. 1a, b).

To assess the role of IL-3 in mast-cell development *in vivo*, we counted mast cells in the tissues of IL-3$^{-/-}$ and wild-type mice at baseline or after 21 daily subcutaneous injections of recombinant rat SCF (rrSCF, at 100 μg kg^{-1} d^{-1}) or vehicle alone (Fig. 1c, d). The results of these experiments show that, *in vivo*, endogenous IL-3 is not essential either for the development of mast cells under physiological conditions or for rrSCF-induced mast-cell hyperplasia (overproduction). Indeed, in certain tissues, mast-cell levels after rrSCF-treatment were significantly greater (by up to 140%) in IL-3-deficient mice than in the corresponding wild-type mice (Fig. 1c, d).

We then counted mast cells and basophils, and assessed immunity to parasites, in IL-3$^{-/-}$ and wild-type mice that had been infected with the intestinal nematode *Strongyloides venezuelensis*, which is a naturally occurring parasite of murine rodents. It is rejected by a T-cell-dependent immune response, which is associated with extensive mast-cell hyperplasia in the intestinal mucosa[16,17]. In three separate experiments, we found that IL-3$^{-/-}$ mice inoculated with 2,000 *S. venezuelensis* third-stage infective larvae (L3), in comparison to corresponding wild-type mice, exhibited both significantly delayed expulsion of the adult worms (data not shown) and significantly prolonged production of the parasite's eggs (Fig. 2a, b).

In addition, the IL-3$^{-/-}$ mice that were infected with *S. venezuelensis* exhibited two striking abnormalities in their haematopoietic effector-cell response to the parasite. First, although baseline percentages of bone-marrow basophils were essentially identical in IL-3$^{-/-}$ and wild-type mice, *S. venezuelensis* infection induced a significant increase in basophil levels in the wild-type mice but not in the IL-3$^{-/-}$ mice (Fig. 2c). These findings confirm the hypothesis, which had been based mainly on analyses of effects of recombinant IL-3 (refs 7, 8), that endogenous IL-3 can expand basophil populations *in vivo*.

Second, endogenous IL-3 was required for a substantial proportion (~76%), but not all, of the mast-cell hyperplasia that occurred in C57BL/6 mice near the main site of *S. venezuelensis* infection, the jejunum (Fig. 2d–f). IL-3 contributed less to jejunal mast-cell hyperplasia during *S. venezuelensis* infection of BALB/c mice (compare Fig. 2f with 2g), perhaps reflecting strain-dependent differences in levels of other cytokines that can influence mast-cell development in mice[3–5,9,13–15]. In contrast, IL-3 was required for nearly all of the increases in the number of mast cells that developed in the ileum or spleen of *S. venezuelensis* infected C57BL/6 or BALB/c mice (Fig. 2f, g). In additional experiments with female IL-3$^{-/-}$ or IL-3$^{+/+}$ C57BL/6 mice, mice infected with 400 *S. venezuelensis* L3 showed no detectable egg production or changes in basophil percentages or mast-cell numbers, whereas the effects seen in mice infected with 10,000 *S. venezuelensis* L3 were similar to those shown in Fig. 2a, c, d, f for mice infected with 2,000 *S. venezuelensis* L3.

Host immunity to *S. venezuelensis* is also impaired in *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice[17]. Because the c-*kit* mutations in these mice result in markedly reduced c-kit/SCF signalling[18,19], *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice are profoundly mast-cell-deficient[20]. But *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice have been reported to have normal levels of blood basophils[21], as well as apparently adequate T-cell function[22]. Moreover, IL-3 can induce mast-cell development in *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice[23], which may partly account for the modest numbers of mast cells that develop in the intestines of

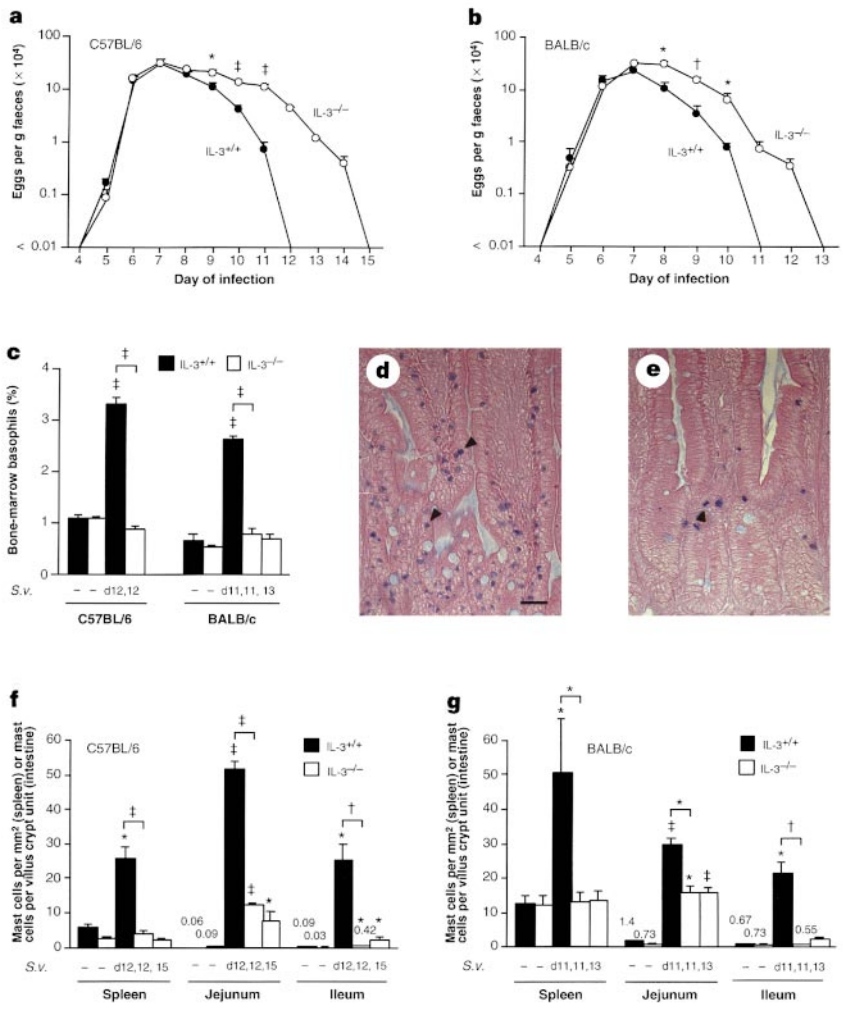

Figure 2 Defective parasite immunity and parasite-enhanced basophil and mast-cell development in IL-3$^{-/-}$ mice compared with IL-3$^{+/+}$ mice. **a**, **b**, Kinetics of *S. venezuelensis* (*S.v.*) egg production in male (**a**) C57BL/6 or (**b**) BALB/c IL-3$^{-/-}$ and IL-3$^{+/+}$ mice inoculated with 2,000 *S.v.* L3. All data are mean ± s.e.m. (n = 4–11 mice per point). Asterisk, $P < 0.05$; dagger, $P < 0.001$; double dagger, $P < 0.0001$ versus corresponding values for IL-3$^{+/+}$ mice. Mice of any one given genotype in **a**, **b** all cleared the infection on the same day. Similar results were obtained in another experiment with C57BL/6 IL-3$^{-/-}$ and IL-3$^{+/+}$ mice. **c**, Percentage of bone-marrow basophils at baseline (–) or at various days (d) after *S.v.* infection in the IL-3$^{+/+}$ and IL-3$^{-/-}$ mice shown in (**a**, **b**). All data are mean ± s.e.m. (n = 3–6 per group). Double dagger, $P < 0.0001$ versus corresponding baseline values or (as indicated by square brackets) versus corresponding values for mice of the other genotype. **d**, **e**, Mast cells (arrowheads) are much more abundant in the jejunal mucosa of *S.v.*-infected (d12) C57BL/6 IL-3$^{+/+}$ mice (**d**) than in the corresponding tissue of the *S.v.*-infected (d12) C57BL/6 IL-3$^{-/-}$ mice (**e**). Scale bar in **d** represents 50 μm. **f**, **g**, Numbers of mast cells in the spleen, proximal jejunum and ileum at baseline (–) or at various days after *S.v.* infection in the IL-3$^{+/+}$ and IL-3$^{-/-}$ mice shown in **a**, **b**, respectively. All data are mean ± s.e.m., except that only mean values are given for very low values. (n = 3–6 per group). Asterisk, $P < 0.05$; dagger, $P < 0.001$; double dagger, $P < 0.0001$ versus corresponding baseline values or (as indicated by square brackets) versus corresponding values for mice of the other genotype.

these animals during infections with some parasites, including *S. venezuelensis*[17]. Finally, studies using a neutralizing antibody against SCF indicate that adequate SCF/c-kit signalling is required for the intestinal mast-cell hyperplasia induced by *Trichinella spiralis*, as well as for normal immunity to this helminth[24].

To examine *S. venezuelensis* infection of mice that have few mast cells and cannot make IL-3, we produced $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice. Adult $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice were clinically healthy and resembled $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ mice in haematocrit and percentage of bone-marrow basophils at baseline (Fig. 3d). However, $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice exhibited a more pronounced defect in their ability to reject *S. venezuelensis* than either $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ mice (Fig. 3a, b) or *Kit* +/+, IL-$3^{-/-}$ mice (compare Fig. 3b with Fig. 2a, b). $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice, unlike $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ or wild-type mice, showed little or no enhancement of bone-marrow basophil production during *S. venezuelensis* infection (Fig. 3d). Moreover, $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice infected with *S. venezuelensis* showed levels of histologically detectable mast cells in the jejunum, ileum and spleen that were even more reduced (compared with corresponding levels in wild-type mice) than those in the corresponding tissues in *S. venezuelensis*-infected $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ mice (Fig. 3e, f).

Abnormalities in addition to those affecting their mast-cell and basophil responses may have contributed to the delayed clearance of *S. venezuelensis* infections in IL-$3^{-/-}$ or $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice. The expression of contact hypersensitivity reactions (but not T-cell-dependent immunity to tumour cells) is moderately reduced in IL-$3^{-/-}$ mice[10], indicating that IL-$3^{-/-}$ mice may express defects in some T-cell-dependent responses that are not due solely to problems with mast-cell or basophil mobilization or function. In addition, $Kit^{W}/Kit^{W\text{-}v}$ mice virtually lack interstitial cells of Cajal, which generate gut electrical pacemaker activity[25], and can exhibit reduced numbers of γδ T cells in the gastrointestinal tract[26]. Nevertheless, our findings clearly show that IL-3 contributes to the mast-cell hyperplasia and enhanced basophil development observed in mice during infection with *S. venezuelensis*. IL-3 is also needed for normal host immune responses to this nematode. Our data also indicate that IL-3 and SCF may have overlapping and/or synergistic roles in maintaining an adequate immune response to this parasite.

Note added in proof: We recently found that, compared with IL-$3^{+/+}$ mice, IL-$3^{-/-}$ mice infected with the parasite *Nippostrongylus brasiliensis* exhibited marked reductions in both hyperplasia of tissue mast cells and enhancement of bone-marrow basophil numbers, but no impairment of parasite expulsion. These findings suggest that the importance of IL-3 in immunity to parasites may vary according to the species of parasite. □

Methods

Generation of transgenic mice. IL-$3^{-/-}$ mice[10] were of the 129Sv × C57BL/6 background or were fourth backcross generation in a BALB/c or C57BL/6 background. Because $Kit^{W}/Kit^{W\text{-}v}$ mice are sterile, $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice were produced by first crossing C57BL/6 IL-$3^{-/-}$ mice (fourth backcross generation)

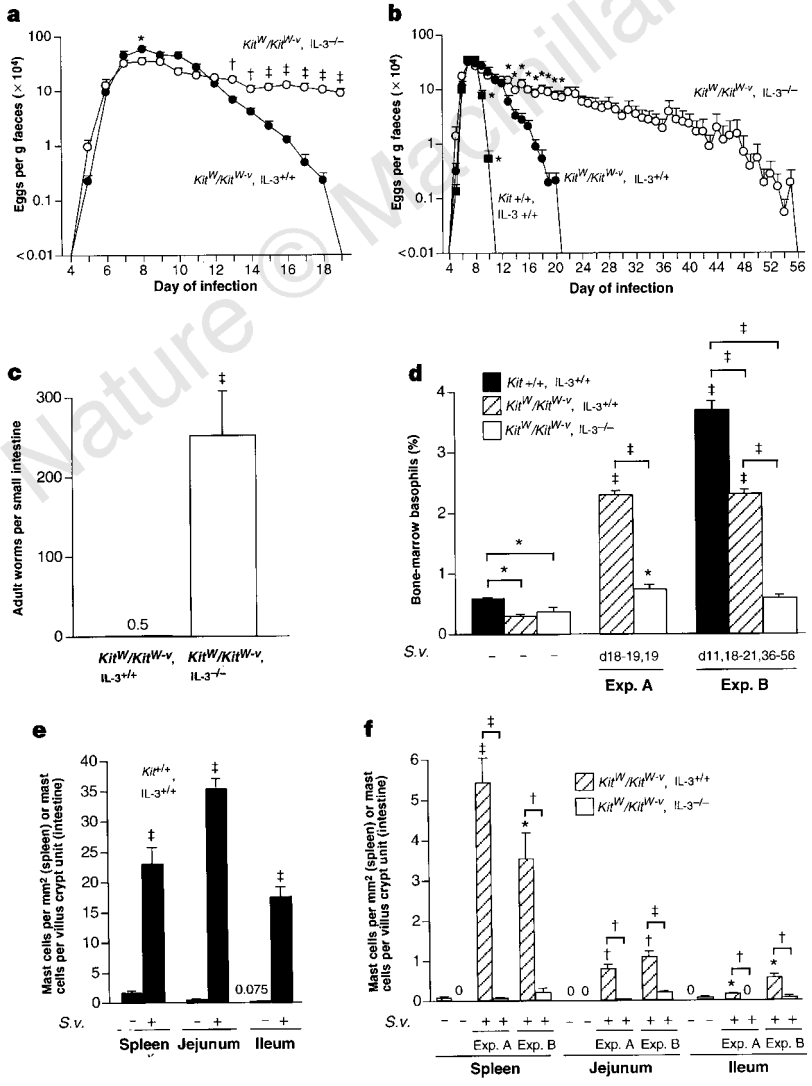

Figure 3 Markedly defective responses to *Strongyloides venezuelensis* infection are seen in $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice. Kinetics of *S.v.* egg production in (**a**) nine $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ mast-cell-deficient mice (five male, four female), six $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice (three male, three female) (all killed on d 18 or 19 of infection) and (**b**) groups of six male WBB6F$_1$-$Kit^{+/+}$, IL-$3^{+/+}$ wild-type mice, WBB6F$_1$-$Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ mice or $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice inoculated with 2,000 *S.v.* L3 (all killed on the day of clearance of infection). All data are mean ± s.e.m. Asterisk, $P < 0.05$; dagger, $P < 0.001$; double dagger, $P < 0.0001$ versus corresponding values for WBB6F$_1$-$Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ mice. **b**, The day parasite egg production fell to zero was d 11 for all the wild-type mice, d 18 (two mice) and d 21 (four mice) for $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ mice, and d 36 (one mouse), d 38 (two mice), d 48 (one mouse) and d 56 (two mice) for $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{-/-}$ mice. Similar results were obtained in another experiment. **c**, Numbers of adult *S.v.* per small intestine on d 18 or 19 of infection in the mice shown in experiment (**a**). Double dagger, $P < 0.0001$ versus value for $Kit^{W}/Kit^{W\text{-}v}$, IL-$3^{+/+}$ mice. **d**, Percentage of bone-marrow basophils at baseline ($n = 3$–7 mice) or at various days after infection in the mice shown in (**a**) (experiment A) and (**b**) (experiment B). Asterisk, $P < 0.05$; double dagger, $P < 0.0001$ versus corresponding baseline values for mice of the same genotype or versus values indicated by the square brackets. **e**, **f**, Numbers of mast cells in the spleen, proximal jejunum, and ileum at baseline ($n = 3$ or 4 mice) or at various days after *S.v.* infection in the mice shown in (**a**, **b**; experiments A and B). Asterisk, $P < 0.05$; dagger, $P < 0.001$; double dagger, $P < 0.0001$ versus corresponding values for uninfected mice of the same genotype or versus values indicated by the square bracket. **a**–**f**, All data are mean ± s.e.m., except that only mean values are shown for very low values.

with WBB6F$_1$-*Kit*^{W}/+ and *Kit*$^{W\text{-}v}$/+ mice (Jackson Laboratory) to generate *Kit*^{W}/+, IL-3$^{+/-}$ mice (50% of offspring) and *Kit*$^{W\text{-}v}$/+, IL-3$^{+/-}$ mice (50%). These mice were bred with the IL-3$^{-/-}$ mice to generate *Kit*^{W}/+, IL-3$^{-/-}$ and *Kit*$^{W\text{-}v}$/+, IL-3$^{-/-}$ mice (each 25% of offspring), which were bred to produce *Kit*^{W}/*Kit*$^{W\text{-}v}$, IL-3$^{-/-}$ mice (25% of offspring). The IL-3 genotype of mice used for breeding was determined by Southern blotting (as in ref. 10), whereas the c-*kit* genotype was determined on the basis of coat colour and white-spotting appearance. All mouse experiments were conducted according to guidelines of the AAALAC-accredited BIDMC IACUC and NIH.

Bone-marrow culture. Femoral bone-marrow cells from individual mice (Fig. 1a) or pooled from five mice (Fig. 1b) 8–12 weeks of age were placed (initial density of 1×10^6 cells ml^{-1}) in DMEM containing 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, and 2 mM glutamine supplemented with rrSCF^{164} (50 ng ml^{-1}, Amgen) ± IL-3 (50 units ml^{-1}, Genzyme) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air[27]. Non-adherent cells were transferred to new culture flasks containing 50% fresh medium one to two times per week and mast cells were counted at weekly intervals by examining cytocentrifuge slide preparations stained with Toluidine blue or May-Grünwald-Giemsa.

Treatment with SCF *in vivo*. Mice of both sexes (all at least six weeks old at the beginning of the experiment) received daily subcutaneous injections for 21 d (at the same back-skin site) of either polyethylene-glycol-conjugated rrSCF^{164} (100 μg kg^{-1} d^{-1}; Amgen) or vehicle; 4 μm paraffin sections of Carnoy's-fixed tissues were stained with safranin and 1.0% alcian blue at pH 1.0 (or, for intestines, pH 0.3) and mast cells were counted as the number per mm^2 of tissue (using the Bioquant Morphometric System, R & M Biometrics), or, for jejunum and ileum, as the number per villus crypt unit[28].

Infection with *S. venezuelensis*. *S. venezuelensis* were maintained by serial passage in male Wistar rats, and infectious L3 were obtained by faecal culture on filter paper[29]. Mice (all at least six weeks old at the beginning of the experiment) were infected by subcutaneous inoculation with 400, 2,000 or 10,000 L3. The degree of infection of individual mice was monitored by counting the numbers both of eggs excreted daily (eggs per g faeces) and, at the time of killing, of adult intestinal worms. Bone-marrow basophils were identified and quantified by flow cytometry[30] and tissue mast cells were counted as described above.

Received 30th July; accepted 25th November 1997.

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Acknowledgements. We thank Z.-S. Wang, M. Yamaguchi, L. Fox and S. Fish for experimental assistance. This work was supported by USPHS grants (S.J.G.), the Claudia Adams Barr Foundation, a Young Markey Scientist Award and the Cancer Research Institute/Partridge Foundation (G.D.), the Roche Research Foundation (J.B.), the Korea Science and Engineering Foundation (C.H.S.) and the Swiss National Science Foundation (N.M.)

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Regulated nuclear import of Rel proteins in the *Drosophila* immune response

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The *Drosophila* immune response uses many of the same components as the mammalian innate immune response, including signalling pathways that activate transcription factors of the Rel/NK-κB family[1–4]. In response to infection, two Rel proteins, Dif and Dorsal, translocate from the cytoplasm to the nuclei of larval fat-body cells[1,2,5]. The Toll signalling pathway, which controls dorsal–ventral patterning during *Drosophila* embryogenesis[6], regulates the nuclear import of Dorsal in the immune response[2,7], but here we show that the Toll pathway is not required for nuclear import of Dif. Cytoplasmic retention of both Dorsal and Dif depends on Cactus protein; nuclear import of Dorsal and Dif is accompanied by degradation of Cactus. Therefore the two signalling pathways that target Cactus for degradation must discriminate between Cactus–Dorsal and Cactus–Dif complexes. We identified new genes that are required for normal induction of transcription of an antibacterial peptide during the immune response. Mutations in three of these genes prevent nuclear import of Dif in response to infection, and define new components of signalling pathways involving Rel. Mutations in three other genes cause constitutive nuclear localization of Dif; these mutations may block Rel protein activity by a novel mechanism.

Nuclear localization of Dorsal (Fig. 1) in the fat body depends on cytoplasmic components of the Toll signalling pathway[2], but the pathway that regulates Dif has not been defined. We found that Dif is properly translocated from fat-body cytoplasm to nuclei in response to infection in *Toll*$^{-/-}$ and *pelle*$^{-/-}$ larvae (Fig. 2a–c); the Toll pathway is therefore not required for the nuclear import of Dif in fat-body cells. The *cactus* gene encodes a member of the 1κB

blood 1998 91: 778-783

Involvement of Interleukin-3 in Delayed-Type Hypersensitivity

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

RAPID COMMUNICATION

Involvement of Interleukin-3 in Delayed-Type Hypersensitivity

By Nicolas Mach, Chris S. Lantz, Stephen J. Galli, Glen Reznikoff, Martin Mihm, Clayton Small, Richard Granstein, Stefan Beissert, Michel Sadelain, Richard C. Mulligan, and Glenn Dranoff

The in vivo functions of interleukin-3 (IL-3) were investigated by generating IL-3–deficient mice. Although hematopoiesis was unimpaired in homozygous mutant animals, contact hypersensitivity reactions were compromised. IL-3 was required for efficient priming of hapten-specific contact hypersensitivity responses, but was dispensable for T-cell–dependent sensitization to tumor cells. These findings reveal a critical role for IL-3 in some forms of delayed-type hypersensitivity.

INTERLEUKIN-3 (IL-3) is a 28-kD glycoprotein initially identified by its ability to induce the expression of 20α-hydroxysteroid dehydrogenase in cultures of nude mouse spleen cells.[1] Subsequent work showed that the cytokine can promote the in vitro differentiation and proliferation of hematopoietic progenitors, yielding multipotential blast cells, mast cells, basophils, neutrophils, macrophages, eosinophils, erythrocytes, megakaryocytes, and dendritic cells.[2-4] Administration of IL-3 to mice, monkeys, and humans can stimulate hematopoiesis in vivo as well.[5-8] IL-3 can also enhance antigen presentation for T-cell–dependent responses, augment macrophage cytotoxicity and adhesion, and promote the function of eosinophils, basophils, and mast cells.[9-13]

Despite these numerous activities, the functions of IL-3 in vivo remain unclear. Mice carrying an inactivating mutation in the α-chain of the heterodimeric IL-3 receptor are apparently normal, and hematopoiesis can occur in vitro in the absence of IL-3.[14,15] While T lymphocytes and mast cells can produce IL-3 in culture, the sources and circumstances in which IL-3 is expressed in vivo are not fully defined.[16,17] To elucidate further the in vivo roles of this molecule, we generated mice lacking IL-3 by homologous recombination in embryonic stem (ES) cells.

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Submitted September 18, 1997; accepted November 14, 1997.

Supported by the Claudia Adams Barr Foundation, a Young Markey Scientist Award, the Cancer Research Institute/Partridge Foundation (G.D.), the Swiss National Science Foundation (N.M.), CA72074 and AI23990 (S.J.G.), and AR40667 (R.G.).

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0006-4971/98/9103-0049$3.00/0

MATERIALS AND METHODS

Generation of IL-3–deficient mice. A 9.1-kb *Xba* I fragment and 3.1-kb *Bam*HI-*Eco*RI fragment spanning the murine IL-3 locus were isolated from a 129S ES cell genomic library and inserted into the targeting vector pPNT.[18] The *Hin*dIII site in exon 1 was destroyed during the construction. This vector was electroporated into D3 ES cells[19] and clones resistant to G418 and ganciclovir were analyzed by Southern analysis as previously described.[18] The 600-bp probe indicated in Fig 1 was used to identify the 6.5-kb wild type and 9.5-kb targeted fragments after *Hin*dIII digestion. Targeted clones were injected into C57BL/6 blastocysts as described to generate chimeric animals transmitting the mutant allele through the germ line.[20] Heterozygous mice were mated to generate mice homozygous for the targeted mutation. For genotyping of animals, tail DNA was digested with *Hin*dIII and probed as above. The IL-3 mutation was backcrossed four generations onto both the BALB/c and C57BL/6 strains.

Hematologic evaluation. Peripheral blood was analyzed for hematocrit, total and differential white blood cell counts, and platelet counts, and bone marrow cells and splenocytes were assayed for CFU-G, CFU-M, CFU-GM, and CFU-GEMM as previously described.[21] For bone marrow transplantation experiments, 5×10^6 nucleated blood cells harvested from donor femurs were injected into lethally irradiated recipients (1,100 rads in two doses). Peripheral blood counts were determined at days 9, 23, 64, and 100 posttransplantation.

Contact hypersensitivity. Mice at least 6 weeks of age were sensitized on day 0 with 50 μL of 4% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone; Sigma, St Louis, MO) in acetone/olive oil (4/1) painted onto the shaved abdomen and were challenged on day 5 with 20 μL of 0.5% oxazolone or carrier only painted on the left and right ear, respectively. To assess responsiveness to 2,4-dinitrofluorobenzene (DNFB; Sigma), mice were sensitized on days 0 and 1 with 20 μL of 0.5% DNFB in acetone/olive oil (4/1) and then challenged on day 5 with 20 μL with 0.2% DNFB. To assess responsiveness to fluorescein isothiocyanate (FITC; Sigma), mice were sensitized on day 0 with 400 μL of 0.5% FITC in acetone/dibutyl pthalate (1/1) and challenged on day 6 with 20 μL of 0.5% FITC. The hapten-specific increase in ear thickness at 24 hours was determined with a micrometer. For analysis of fibrin deposition, ^{125}I-labeled guinea pig fibrinogen was injected intravenously 10 minutes before secondary oxazolone challenge in sensitized mice. Twenty-four hours later ears were removed and the urea insoluble extract (cross-linked fibrin) assayed for ^{125}I as previously described.[22]

Tumor vaccinations. Female mice (on the BALB/c background) were immunized subcutaneously on the abdomen with 5×10^5 irradiated (3,300 rads) RENCA carcinoma cells (cultured in DME plus 10% fetal calf serum and antibiotics) and challenged 7 days later with 1×10^7 live RENCA cells subcutaneously on the back. Mice were killed when challenge tumors reached 2 cm in diameter. RENCA cells do not secrete detectable IL-3 as measured by enzyme-linked immunosorbent assay (ELISA) with a sensitivity of 25 pg/mL. For evaluation of delayed-type hypersensitivity to tumor cells, female BALB/c mice were immunized subcutaneously on the abdomen with 5×10^5 irradiated

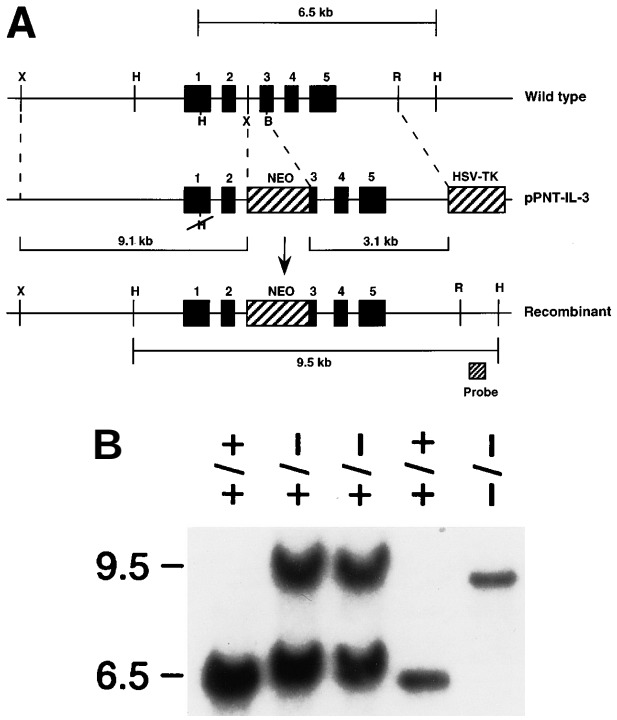

Fig 1. Generation of IL-3–deficient mice. (A) Structure of IL-3 targeting vector and disrupted IL-3 gene. The 600-bp probe indicated identifies the 6.5-kb wild-type and 9.5-kb targeted fragments after *Hin*dIII digestion as shown. X, *Xba* I; H, *Hin*dIII; B, *Bam*HI; R, *Eco*RT. (B) Genotype of mutant animals. Tail DNA was digested with *Hin*dIII and probed as above. Molecular sizes are indicated on the left (in kilobases).

RENCA carcinoma cells and 7 days later were injected in the footpads with 5×10^6 irradiated RENCA cells. Tumor-induced footpad swelling at 24 hours was determined with a micrometer. For haptenized tumor cell experiments, irradiated RENCA cells were incubated for 15 minutes at 37°C in 70 mmol/L oxazolone (dissolved in Hanks' balanced salt solution [HBSS] and ethanol, pH 7). The cells were then extensively washed with HBSS and injected subcutaneously for sensitization.

Cytokine mRNA expression. Total RNA was obtained with TRIZOL (GIBCO-BRL, Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized using oligo-dT primers and MMLV reverse transcriptase (GIBCO-BRL). Polymerase chain reaction (PCR) was performed using published IL-3 primers.[23] Amplified bands were confirmed as IL-3 by Southern blotting using IL-3 cDNA as a probe.

RESULTS AND DISCUSSION

Development of mice lacking IL-3. To generate a null allele of the IL-3 gene, a neomycin-resistance cassette was introduced by homologous recombination into the third exon of the IL-3 locus (Fig 1A). Targeted clones were injected into C57BL/6 blastocysts to yield chimeric animals, which were then mated with C57BL/6 mice to obtain germline transmission of the mutant allele. Heterozygous mutant animals were interbred to generate homozygous IL-3–deficient animals (Fig 1B). Mutant mice were obtained at the expected frequencies, remained clinically healthy throughout 18 months of observation, and were fertile. Supernatants of concanavalin A–stimulated splenocytes from mutant animals showed no immunoreactive or bioactive IL-3, as determined by both proliferative studies with the 32D myeloid cell line[24] and ELISA (not shown).

Complete pathologic examination of IL-3–deficient animals showed no abnormalities. Analysis of steady-state hematopoiesis demonstrated normal numbers of peripheral blood cells, bone marrow and splenic hematopoietic progenitors (as measured by colony-forming unit assays), and tissue hematopoietic populations. Bone marrow obtained from IL-3–deficient mice reconstituted lethally irradiated IL-3–deficient recipients with comparable kinetics as wild-type marrow transplanted into wild-type recipients. These results, which are consistent with recently reported findings,[25] show that IL-3 is dispensable for normal hematopoiesis in vivo.

Impaired contact hypersensitivity reactions in IL-3–deficient mice. To evaluate the potential role of IL-3 in T-cell–dependent immunity, wild-type and mutant animals were tested for the development of contact hypersensitivity to epicutaneously applied oxazolone. Contact hypersensitivity is a form of delayed-type hypersensitivity in which hapten-protein conjugates formed in the skin are presented by epidermal Langerhans cells, following their migration to regional lymph nodes, to hapten-specific $CD4^+$ and $CD8^+$ T lymphocytes.[26-28] Sensitized T cells initiate a local inflammatory response in the skin upon secondary hapten challenge. Although IL-3–deficient mice were indistinguishable from wild-type littermates in the magnitude of their immunologically nonspecific ''irritant'' response to initial hapten challenge (data not shown), they exhibited significantly compromised reactivity upon secondary hapten challenge, as measured by ear swelling (Fig 2A). Impairment was evident in nine experiments with IL-3–deficient mice in the C57BL/6 background and seven experiments in the BALB/c background, and was also observed in four other experiments in which 2,4-dinitrofluorobenzene or fluorescein isothiocyanate were used as haptens.

To examine the defective contact hypersensitivity reaction in more detail, ^{125}I-fibrinogen was injected systemically into IL-3–deficient and wild-type control mice at the time of secondary oxazolone challenge.[22] The conversion of fibrinogen to cross-linked fibrin at the challenge site results in the induration characteristic of cutaneous delayed-type hypersensitivity responses. Significantly less cross-linked fibrin was present in the ears of IL-3–deficient mice as compared with wild-type littermates (Fig 2B), confirming a marked reduction in the magnitude of the hapten-specific immune response.

Although no histolopathologic differences between IL-3–deficient and wild-type mice were noted in untreated skin or in skin at the sensitization site, marked differences were apparent in the challenge site (Fig 3B and C). In wild-type animals, the inflammatory response was characterized by an intense cellular infiltrate consisting predominantly of neutrophils, lymphocytes, and eosinophils, as well as substantial dermal edema, hyperkeratosis, and focal intraepidermal abscesses. IL-3–deficient animals, in contrast, developed a dramatically less intense cellular infiltrate, although the cellular composition was similar to that of wild-type animals. IL-3–deficient mice also demonstrated less edema, fewer and smaller intraepidermal abscesses, and little keratinocyte activation. The number of Langerhans cells in the skin of unmanipulated IL-3–deficient mice, as determined by immunofluorescence staining of major histocompatibility

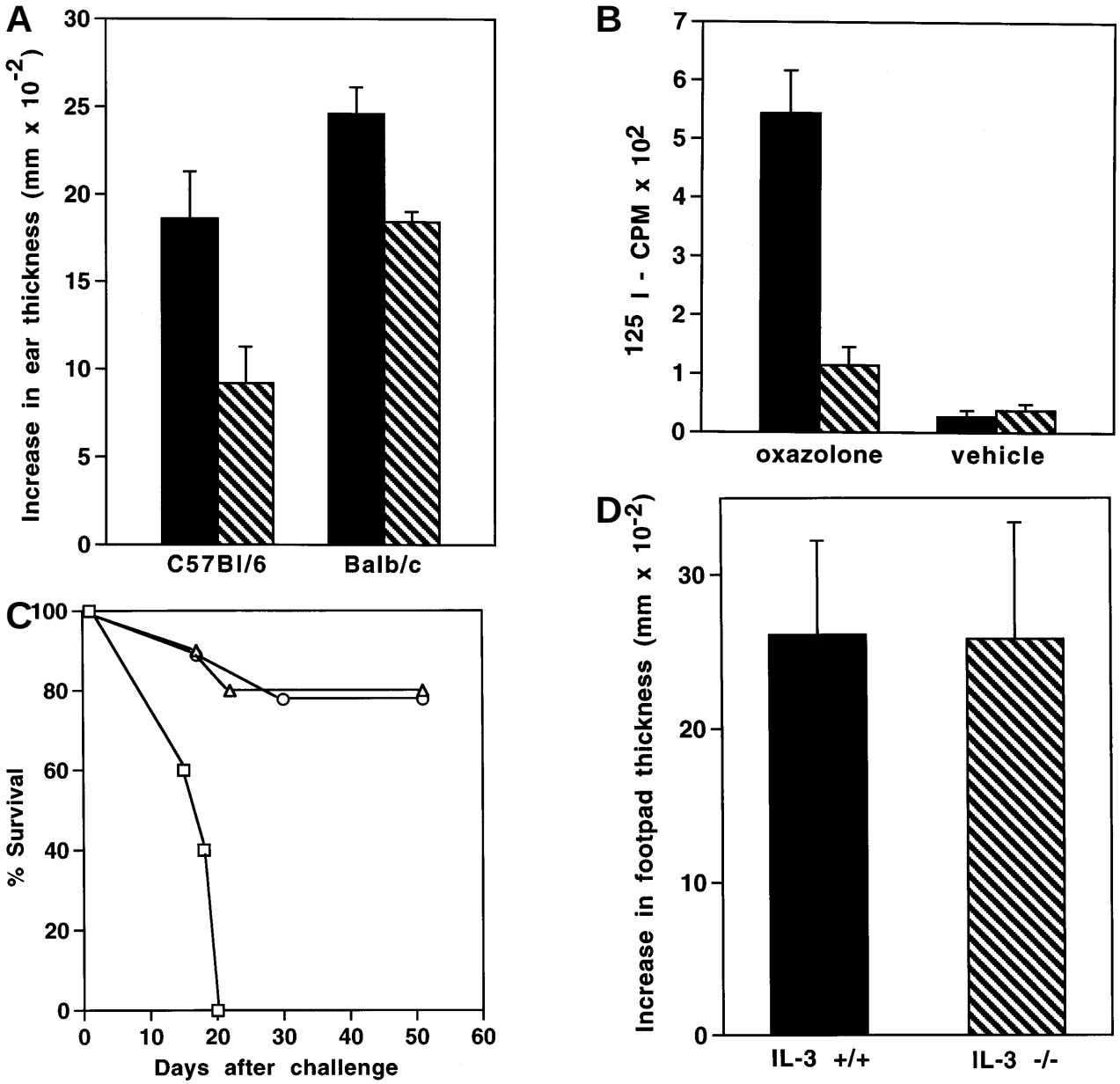

Fig 2. Delayed-type hypersensitivity reactions. (A) Contact hypersensitivity reactions to oxazolone in IL-3–deficient (▨) and wild-type littermates (■). Values (n = 5) are mean ± SEM: C57BL/6, $P = .025$; BALB/c, $P = .014$. (B) Fibrin deposition during contact hypersensitivity to oxazolone (C57BL/6 background). $P < .001$ for oxazolone challenge. Plasma values for ^{125}I-labeled fibrinogen 24 hours after injection were 1,948 ± 61.7 for +/+ mice and 1,879 ± 53.3 cpm for −/− animals. (C) Tumor protection in immunized female IL-3–deficient (△) and wild-type littermates (○) (BALB/c background). Wild-type controls, no vaccine (□). All surviving animals at day 55 were tumor free. Pooled results from two independent experiments (10 mice per group). (D) Delayed-type hypersensitivity to irradiated tumor cells inoculated in the footpads of immunized female IL-3–deficient (▨) and wild-type littermate mice (■) (BALB/c background).

complex class II positive cells in epidermal ear sheets, however, was comparable to that of wild-type littermate controls (1,663 ± 362 *v* 1,433 ± 271/mm^2 of epidermal surface for IL-3–deficient and wild-type animals).

Tumor vaccination responses in IL-3–deficient mice. To address the potential involvement of IL-3 in another type of cutaneous delayed-type hypersensitivity reaction, we evaluated the ability of IL-3–deficient mice to generate antitumor immunity after vaccination with irradiated tumor cells. Tumor immunization in this system, like contact hypersensitivity,[28] is dependent on CD4$^+$ and CD8$^+$ T cells (J. Donahue and G. Dranoff, manuscript in preparation). Nonetheless, IL-3–deficient mice showed no impairment in tumor vaccination, as measured by the ability to reject a secondary challenge of live tumor cells, under conditions where immunized wild-type mice demonstrated only partial protection against tumor challenge (Fig 2C). Moreover, tumorigenicity in naive IL-3–deficient mice was indistinguishable from wild-type littermates. Patho-

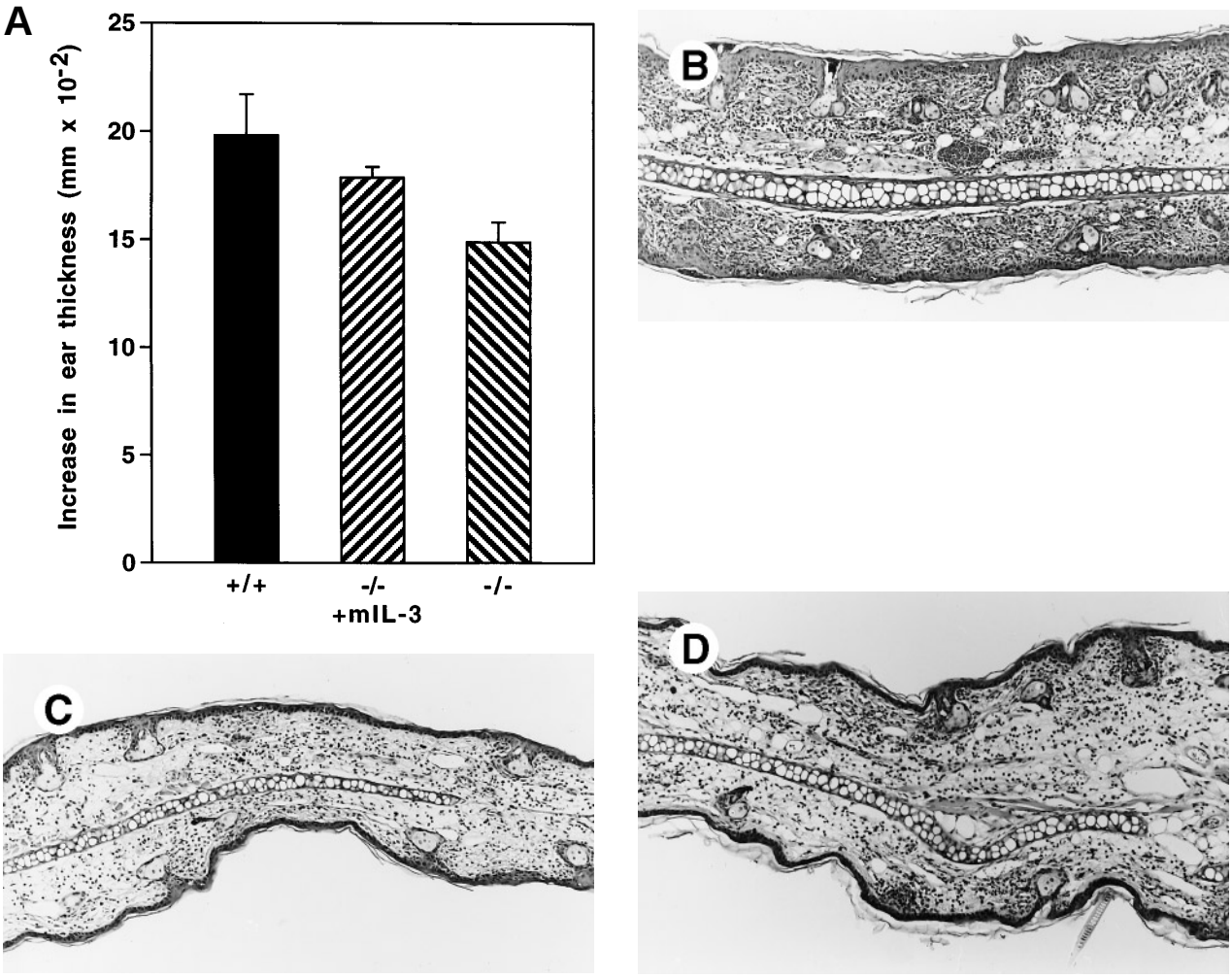

Fig 3. IL-3 is required for hapten specific priming. (A) IL-3–deficient (▨) and wild-type littermate controls (■) (BALB/c) were tested for contact hypersensitivity to oxazolone. One hundred nanograms of murine IL-3 (in 1% mouse serum) (▨) or vehicle only was administered intraperitoneally and subcutaneously (abdomen) 4 hours before, at the time of sensitization, and 6 hours afterwards. +/+ versus −/−, $P = .03$. −/− plus murine IL-3 versus −/−, $P = .008$. Murine IL-3 administered to unsensitized mice had no effect on secondary challenge. (B through D) Histopathology (tissues were formalin-fixed, paraffin-embedded, and stained with hematoxylin and eosin) of secondary oxazolone challenge sites in ears from mice (BALB/c background) killed 24 hours after challenge. (B) Ear reaction of a sensitized wild-type mouse. (C) Ear reaction of a sensitized IL-3–deficient mouse. (D) Ear reaction of a sensitized IL-3–deficient mouse administered IL-3 protein at the time of priming.

logic examination of tumor rejection sites in immunized animals showed no significant differences between IL-3–deficient and wild-type mice. The generation of antitumor immunity was also assessed by injecting irradiated tumor cells into the footpads of previously vaccinated animals. No differences were observed between IL-3–deficient and wild-type mice, as measured by tumor-induced footpad swelling (Fig 2D).

IL-3 expression during delayed-type hypersensitivity. To investigate the potential basis for the differing requirements for IL-3 in the two forms of delayed-type hypersensitivity, the expression of IL-3 in the skin during the priming phases of the two responses was examined by reverse transcriptase (RT)-PCR (Table 1). Although IL-3 transcripts were not detected in unmanipulated or shaved skin, or in skin treated only with diluent, the application of hapten rapidly induced IL-3 transcripts in wild-type, but not IL-3–deficient, animals. Expression was detected as early as 1 hour after hapten painting and

Table 1. IL-3 Expression During Delayed-Type Hypersensitivity Responses

Antigen	Time After Priming (h)					
	0	1	12	24	48	96
None	0*/8†	—	—	0/8‡	—	—
Vehicle only§	—	—	0/5	0/5	—	—
Oxazolone	—	4/4	4/4	8/10	4/10	3/8
Tumor cells‖	—	—	—	0/4	0/4	—

Wild-type mice (BALB/c) were sensitized with the indicated antigen, and the presence of IL-3 transcripts at varying times in the skin at the priming site was evaluated by RT-PCR. No message was detectable in IL-3–deficient mice.

*Number of mice positive for IL-3 transcripts.

†Number of mice tested.

‡Shaved only.

§Acetone/olive oil.

‖Irradiated RENCA carcinoma cells (3,300 rads).

persisted for up to 4 days. IL-3 transcripts were also found in the draining lymph node of the sensitization site 24 hours after hapten application. In contrast to these findings, IL-3 transcripts were not detected in the skin of wild-type mice after vaccination with irradiated tumor cells.

To evaluate the potential role of IL-3 during the priming phase of contact hypersensitivity, IL-3 protein was administered subcutaneously and intraperitoneally at the time of sensitization to IL-3–deficient animals. This resulted in partial correction of the impaired response, as measured by ear swelling (Fig 3A). Moreover, pathologic analysis showed a significant increase in the intensity of the inflammatory response in comparison to untreated mutant animals, but the reaction did not reach wild-type levels (Fig 3D). The inability to correct completely the defective response in IL-3–deficient mice could be due to either pharmacologic limitations in the delivery of IL-3 protein or a second role for IL-3 during the elicitation phase. Indeed, IL-3 transcripts were also detected in the skin upon secondary hapten challenge, and previous work has shown that administering neutralizing antibodies to both IL-3 and granulocyte-macrophage colony-stimulating factor during the elicitation phase can reduce the intensity of the reaction.[29] However, attempts to correct the impaired response in IL-3–deficient mice by providing IL-3 at the time of elicitation were unsuccessful.

The requirement for IL-3 during delayed-type hypersensitivity to haptens, but not tumor cells, demonstrates that distinct pathways underlie the generation of T-cell immunity in the skin. In this context, sensitization with haptenated tumor cells was also compromised in IL-3–deficient mice (data not shown), suggesting that intact priming to tumor cells could not overcome the IL-3–associated impairment in hapten-specific responses. Further studies will be necessary to delineate whether different antigen presenting cells or functions are involved in the responses to haptens and tumor cells, and whether other techniques of hapten administration can bypass the defect shown here or whether other techniques of tumor vaccination are dependent on IL-3. The sources of IL-3 production in normal skin remain to be clarified as well, although Langerhans cells, mast cells, and keratinocytes are possibilities. Finally, our results suggest that IL-3 antagonists might be effective therapies for contact dermatitis in humans.

ACKNOWLEDGMENT

All mouse experiments were approved by the AAALAC-accredited DFCI IACUC. We thank C. Crawford, A. Crawford, B. Ream, K. Edelman, R. Bronson, and V. Tybulewicz for their excellent help. We thank the staff of the Redstone Animal Facility for their excellent maintenance of the mouse colony.

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[CANCER RESEARCH 60, 3239–3246, June 15, 2000]

Differences in Dendritic Cells Stimulated *in Vivo* by Tumors Engineered to Secrete Granulocyte-Macrophage Colony-stimulating Factor or Flt3-Ligand[1]

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ABSTRACT

Both granulocyte-macrophage colony-stimulating factor (GM-CSF) and flt3-ligand (FL) induce the development of dendritic cells (DCs). To compare the functional properties of DCs stimulated by these cytokines *in vivo*, we used retroviral-mediated gene transfer to generate murine tumor cells secreting high levels of each molecule. Injection of tumor cells expressing either GM-CSF or FL resulted in the dramatic increase of $CD11c^+$ cells in the spleen and tumor infiltrate. However, vaccination with irradiated, GM-CSF-secreting tumor cells stimulated more potent antitumor immunity than vaccination with irradiated, FL-secreting tumor cells. The superior antitumor immunity elicited by GM-CSF involved a broad T cell cytokine response, in contrast to the limited Th1 response elicited by FL. DCs generated by GM-CSF were $CD8\alpha^-$ and expressed higher levels of B7–1 and CD1d than DCs cells generated by FL. Injection sites of metastatic melanoma patients vaccinated with irradiated, autologous tumor cells engineered to secrete GM-CSF demonstrated similar, dense infiltrates of DCs expressing high levels of B7–1. These findings reveal critical differences in the abilities of GM-CSF and FL to enhance the function of DCs *in vivo* and have important implications for the crafting of tumor vaccines.

INTRODUCTION

There is compelling evidence that DCs[4] play a decisive role in the priming of immune responses (1). DCs acquire antigens in peripheral tissues and migrate to organized lymphoid structures to stimulate antigen-specific CD4- and CD8-positive T lymphocytes and B cells. DCs are specialized to initiate immunity because of their abilities to process antigens efficiently into both MHC class I and II pathways and their high level expression of costimulatory molecules (2).

The central importance of DCs in priming immune responses has generated substantial interest in manipulating these cells for the induction of antitumor immunity. The development of *in vitro* methods to propagate large numbers of DCs from hemopoietic progenitors (3–6) has led to several studies that indicate that DCs can dramatically enhance antitumor immunity (7). DCs pulsed with tumor antigen-derived peptides or whole tumor cell lysates and DCs genetically modified to express tumor antigens elicit striking antitumor effects in murine model systems (8–11). Initial clinical testing of DC-based vaccines has revealed the induction of tumor destruction in cancer patients as well, although the underlying effector mechanisms remain to be clarified (12, 13).

In contrast to these cancer vaccination strategies that involve the *ex vivo* manipulation of DCs, other approaches attempt to enhance DC function *in vivo*. The systemic administration of recombinant FL protein results in the marked expansion of both myeloid- and lymphoid-type DCs in many tissues (14–17) and induces impressive antitumor effects in several murine models (18, 19). Tumor cells engineered to secrete FL also demonstrate reduced tumorigenicity (20). These studies suggest that DCs can infiltrate implanted tumors and initiate processing of tumor antigens; however, nonspecific mechanisms are induced by FL as well, because antitumor effects are only partially compromised in SCID mice (18).

We have demonstrated that vaccination with irradiated tumor cells engineered to secrete GM-CSF stimulates potent, specific, and long-lasting antitumor immunity in multiple murine tumor models (21). Recently, we have extended these findings to patients with metastatic melanoma; as a consequence of vaccination, patients consistently develop intense CD4- and CD8-positive T lymphocyte and plasma cell infiltrates in metastatic lesions (22). These reactions result in extensive tumor necrosis, fibrosis, and edema. Pathological analysis of the vaccination sites reveals a dense infiltrate of DCs, macrophages, eosinophils, and T lymphocytes in the dermis and s.c. tissues.

The abilities of several vaccination strategies involving DCs to enhance antitumor immunity raises the intriguing question of whether distinct or overlapping mechanisms underly the various approaches. To begin to address this issue, we used the poorly immunogenic B16 melanoma model to compare the effects of GM-CSF and FL on DC function and the concomitant induction of antitumor immunity. Although B16 cells engineered to secrete either cytokine stimulated the marked expansion of $CD11c^+$ DCs both locally and systemically, GM-CSF-expressing cells were more effective in eliciting systemic antitumor immunity. The superior vaccination activity triggered by GM-CSF involved the high level expression of B7–1 and CD1d on $CD8\alpha^-$ DCs.

MATERIALS AND METHODS

Mice. Adult female C57Bl/6 and BALB/c mice, 8–12 weeks of age, were purchased from Taconic Farms Inc. (Germantown, NY). All mouse experiments were approved by the AAALAC-accredited Dana-Farber Cancer Institute IACUC.

Recombinant Retroviruses. Total RNA was obtained from C57Bl/6 spleens using TRIZOL (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized using oligo-dT primers and MMLV reverse transcriptase (Life Technologies, Inc.). A PCR was performed to obtain cDNA encoding murine FL. The primers used were: sense strand 5′ CATATC*ATG*ACAGTGCTGGCGCCAGCC and antisense strand 5′ GTAAGGATCCTAGG*GAT*GGGAGGGGAGG, derived from the published sequence (23). The sense strand primer incorporates a *Bsp*HI restriction site upstream of the initiator ATG, and the antisense primer incorporates a *Bam*HI restriction site downstream of the termination codon. The conditions of the PCR were: 30 cycles of 96°C for 30 s, 50°C for 50 s, and 72°C for 3 min. The 711-bp amplified fragment was sequenced to confirm the integrity of the cDNA, digested with *Bsp*HI and *Bam*HI, and subcloned into pMFG, as described previously (21). The pMFG vector uses the MMLV long terminal repeat sequences to generate both a full-length viral RNA (for encapsidation into viral particles) and a subgenomic RNA that is responsible for expression of inserted sequences. pMFG-FL and pMFG-murine GM-CSF

Received 12/21/99; accepted 4/14/00.

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[1] Supported by the Swiss National Science Foundation (to N. M. and S. G.), the Swiss Cancer League (to S. G.), NIH Grant AI45051 (to S. B. W.), the Cancer Research Institute/Partridge Foundation, and National Cancer Institute Grant CA74886 (to G. D.).

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[4] The abbreviations used are: DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; FL, Flt3-ligand; MMLV, Moloney murine leukemia virus; IL, interleukin.

(21) vectors were transfected into 293GPG cells to generate high titer stocks of concentrated recombinant MMLV particles that have incorporated the vesicular stomatitis virus G protein (24).

Tumor Models. B16-F10 melanoma cells (syngeneic to C57Bl/6 mice) were maintained in DMEM containing 10% (vol/vol) FCS and penicillin/streptomycin. B16 cells were infected in the presence of polybrene (Sigma Chemical Co.), and unselected populations were used for study, as described previously (21). The proportion of tumor cells transduced with the retroviral vector (which contains no selectable marker) was determined by Southern analysis. GM-CSF secretion was determined by ELISA, as described (21). No replication competent retrovirus is generated in this system, as determined by the histidine mobilization assay (25). For tumorigenicity experiments, 5×10^5 live, wild-type, or cytokine-secreting B16 cells were injected s.c. in Hank's balanced saline solution (Life Technologies, Inc.); mice were sacrificed when tumors reached 1.5–2 cm in longest diameter. For vaccination experiments, mice were immunized s.c. on the abdominal wall with 5×10^5 irradiated (3500 rads), cytokine-secreting B16 cells and 7 days later were challenged with 1×10^6 live, wild-type B16 cells injected s.c. on the back.

Antibodies. Fluorescence-activated cell sorting of splenocyte populations (depleted of erythrocytes with ammonium chloride) were performed using FITC- or phycoerythrin-conjugated monoclonal antibodies to CD11c, CD11b, I-A^b, CD8α, CD1d, CD3ϵ, CD4, NK1.1, B7–1, B7–2, and CD40 in the presence of blocking antibodies against the FcγIII/II receptors (PharMingen).

Cellular Assays. Mixed leukocyte reactions were performed by culturing 2×10^4 irradiated splenocytes (harvested 14 days after injection of live, cytokine-secreting tumor cells and depleted of erythrocytes) with 2×10^4 nylon wool purified BALB/c T cells in RPMI supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 1% penicillin/streptomycin, 0.1 mM nonessential amino acids, 1% sodium pyruvate, and 5×10^{-5} M 2-mercaptoethanol (complete medium). After 4 days, [^{3}H]thymidine was added to the culture and incorporation was measured after 8 h with liquid scintillation counting. For the measurement of tumor-induced T cell cytokine production, splenocytes were harvested 7 days after vaccination with irradiated, cytokine-producing tumor cells, depleted of erythrocytes, and cultured (1×10^6 cells) with irradiated (10,000 rads) B16 cells (2×10^4) 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-4, IL-5, and IFN-γ by ELISA using the appropriate monoclonal antibodies (Endogen; PharMingen).

Histology. Tissues for pathological examination were fixed in 10% neutral buffered formalin, processed to paraffin embedment, and stained with H&E. In some cases, tissues were snap-frozen in liquid nitrogen and sections were immunostained for protein expression using monoclonal antibodies to CD11c, B7–1, CD3, and CD1a (PharMingen; DAKO). Isotype-matched controls were included for each primary antibody. Briefly, 4-μm sections were air-dried overnight and fixed in acetone at 4°C. After incubation with hydrogen peroxide, biotin, and Fc receptor blocking reagents, appropriate primary or isotype-matched control antibodies were applied. The peroxidase- and alkaline phosphatase-labeled streptavidin-biotin indirect methods were combined with the appropriate substrate-chromogen, resulting in either a brown or red precipitate at the antigen site. Finally, sections were counterstained with hematoxylin and evaluated using light microscopy. Human samples were obtained from vaccinated metastatic melanoma patients, as reported previously (22).

RESULTS

Generation of Cytokine-secreting Tumor Cells. To study the effects of GM-CSF and FL on DC function *in vivo*, we used retroviral-mediated gene transfer to engineer B16 melanoma cells to secrete high levels of each cytokine. High titer replication-defective viral stocks were prepared using the MFG retroviral vector and 293GPG packaging cells. B16 cells were infected with these viral stocks, resulting in 1.5 proviral copies per infected cell as determined by Southern analysis (data not shown).

Bioactivity of Cytokine-secreting Tumor Cells. GM-CSF-secreting B16 cells generated approximately 300 ng/10^6 cells/48 h of bioactive protein, as determined by ELISA (21). Because monoclonal antibodies to FL were not available to us, we evaluated the production of bioactive FL protein by analyzing the stimulation of hematopoiesis in C57Bl/6 mice that received injections of live, FL-secreting B16 cells.

Although the injection of wild-type B16 cells into C57Bl/6 mice resulted in only minimal changes in peripheral blood counts and splenocyte populations (data not shown), the injection of FL-secreting B16 cells produced dramatic alterations in hematopoiesis. FL-secreting B16 cells displayed only modest reductions in tumorigenicity (likely due to the poor immunogenicity of this tumor model) and, thus, constitutively released FL into the circulation. This cytokine production stimulated a marked leukocytosis, with total WBC counts reaching up to 17,000 ($\times 10^{-3}$/ml) by day 14 after injection, similar to effects previously described with administration of recombinant human FL protein (26). FL-secreting B16 cells also elicited marked generalized lymphadenopathy and splenomegaly. Pathological analysis of the splenic architecture revealed marked expansion of the marginal zones and periarteriolar T cell-rich regions and blurring of the red/white pulp boundaries (data not shown), alterations similarly induced by recombinant human FL protein (15).

To determine whether FL-secreting B16 cells stimulated the expansion of DCs systemically, as has been reported with recombinant FL protein (14), we analyzed splenocyte populations for cells expressing high levels of CD11c and MHC class II molecules. As shown in Fig. 1*A*, by 14 days after injection, FL-secreting B16 cells produced a marked increase in cells staining positive for both markers, with an average of 25% positive cells per spleen. Cytospin preparations revealed substantial numbers of cells with dendritic morphology (data not shown). In contrast, injection of wild-type B16 cells did not alter spleen cellularity or DC numbers (data not shown). Because injection of FL-secreting B16 cells led to a 3–4-fold increase in total spleen cellularity, overall this tumor line induced a nearly 100-fold increase in DC numbers. Moreover, these DCs functioned efficiently as stimulators in mixed leukocyte reactions (data not shown). Together, these findings demonstrate that FL-secreting B16 cells elicit comparable effects on hematopoietic populations as the injections of recombinant FL protein (14).

GM-CSF-secreting B16 Cells Stimulate DC Expansion *in Vivo*. GM-CSF-secreting B16 cells were shown previously to induce a profound leukocytosis (WBC counts of 100,000 $\times$ 10^{-3}/ml) and splenomegaly in syngeneic C57Bl/6 mice (21). To evaluate whether GM-CSF-secreting B16 cells also stimulated DC production, we again analyzed splenocyte populations for cells expressing high levels of both CD11c and MHC class II molecules. As shown in Fig. 1*B*, by 14 days after injection, GM-CSF-secreting B16 cells also induced a marked increase in cells staining positive for both markers, with an average of 15% positive cells per spleen. Cytospin preparations revealed substantial numbers of cells with dendritic morphology (data

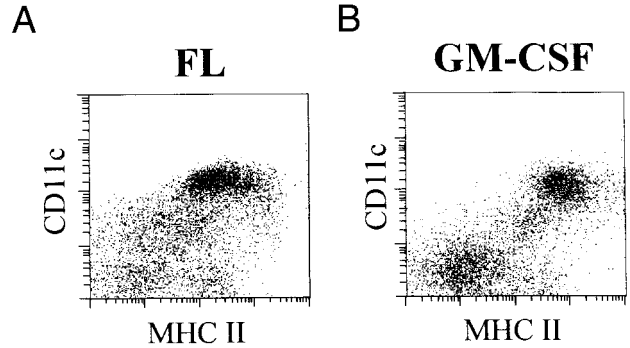

Fig. 1. GM-CSF and FL increase splenic DCs. Fourteen days after injection of live, GM-CSF- or FL-secreting B16 tumor cells, splenocytes were harvested and stained for CD11c and MHC II. Injection of wild-type B16 cells did not increase splenic DCs (data not shown). *A*, FL. *B*, GM-CSF.

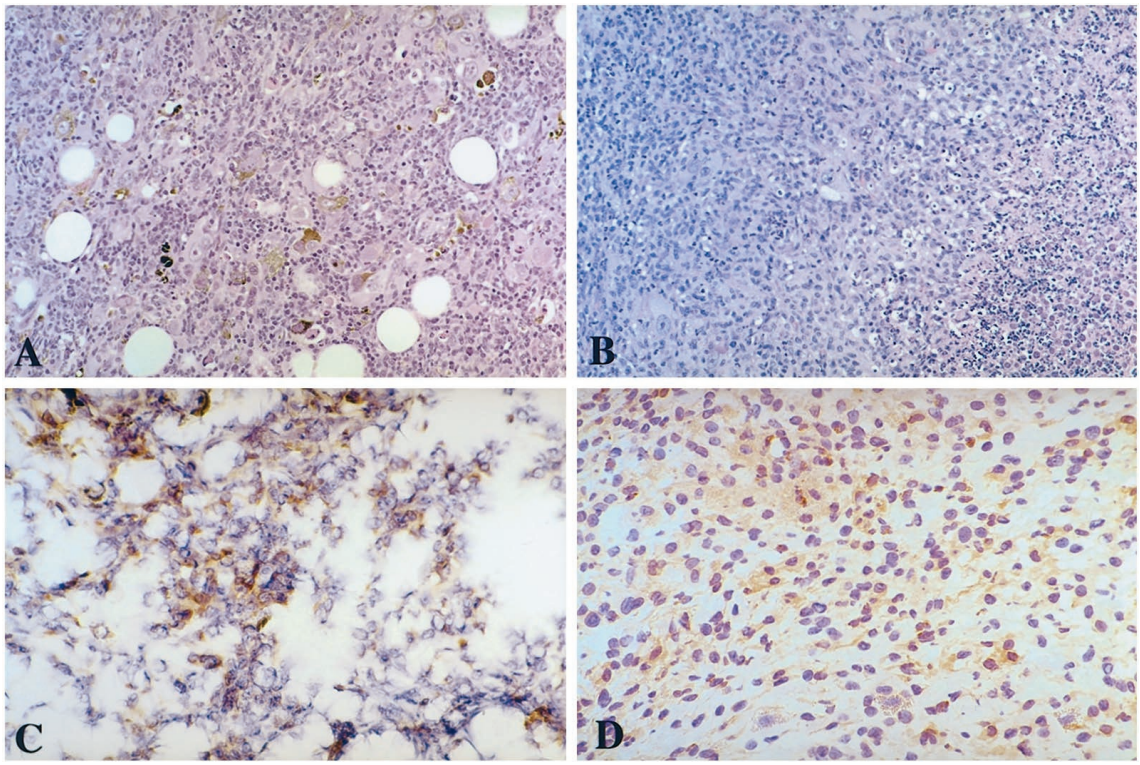

Fig. 2. GM-CSF and FL increase tumor-infiltrating DCs. Vaccination sites were examined 5 days after the injection of irradiated, GM-CSF- or FL-secreting B16 cells. Injection of irradiated, wild-type B16 cells elicited minimal infiltrates (data not shown). *A*, FL (H&E stain, ×200). *B*, GM-CSF (H&E stain, ×200). *C*, FL (CD11c stain, ×400). *D*, GM-CSF (CD11c stain, ×400).

not shown). Since the total spleen cellularity increased by 3-fold, this represented a ~40-fold increase in DC numbers. Moreover, these cells functioned efficiently as stimulators in mixed leukocyte reactions as well (data not shown). These results demonstrate that both GM-CSF and FL stimulate DC expansion *in vivo*.

Irradiated, Cytokine-secreting Tumor Cells Elicit Local DC Accumulation. Because both GM-CSF- and FL-secreting B16 cells formed tumors in syngeneic hosts, we also examined the consequences of injecting irradiated, cytokine-secreting tumor cells. Although irradiation induces cell cycle arrest, it fails to inhibit cytokine production *in vitro* for at least 7 days (21). Whereas implantation of irradiated, wild-type B16 cells evoked only a scant infiltrate (data not shown), implantation of irradiated, FL-secreting B16 cells elicited an intense local reaction composed primarily of lymphocytes and DCs (Fig. 2*A*). Strong staining for CD11c was demonstrable in these infiltrates (Fig. 2*C*).

Injection of irradiated, GM-CSF-secreting B16 cells elicited a striking local reaction as well, which was characterized by an admixture of DCs, eosinophils, neutrophils, and macrophages (Fig. 2*B*). Strong staining for CD11c was also evident in these infiltrates (Fig. 2*D*). Together, these findings indicate that both FL- and GM-CSF-secreting B16 cells markedly increase DC numbers locally.

Generation of Protective Antitumor Immunity. Because GM-CSF- and FL-secreting B16 cells both stimulated the generation of DCs *in vivo*, we compared the relative abilities of these cytokines to enhance the generation of antitumor immunity. For these experiments, mice received immunizations s.c. with irradiated, GM-CSF- or FL-secreting B16 cells and were challenged 1 week later with live, wild-type B16 cells. As shown in Fig. 3, vaccination with irradiated, GM-CSF-secreting B16 cells stimulated higher levels of protective antitumor immunity than vaccination with irradiated, FL-secreting B16 cells. Similar results were found in five independent experiments.

Metastatic melanoma patients vaccinated with irradiated, autologous melanoma cells engineered to secrete GM-CSF develop tumor-infiltrating lymphocytes that secrete a broad range of cytokines, including IL-5, IFN-γ, and GM-CSF (22). To compare the relative abilities of irradiated, GM-CSF- or FL-secreting B16 cells to induce

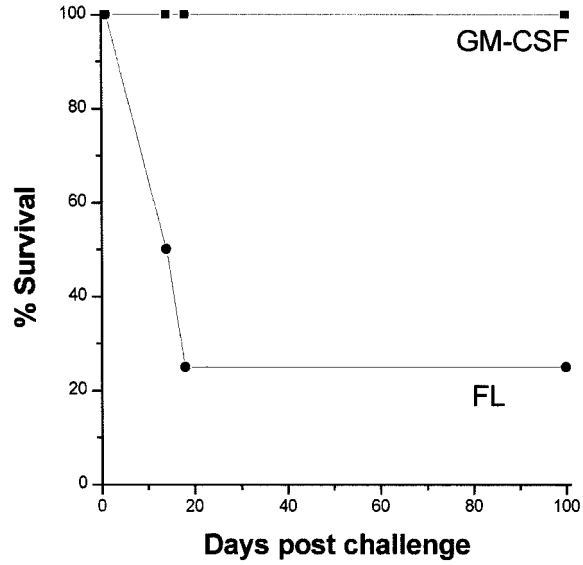

Fig. 3. GM-CSF stimulates more potent antitumor immunity than FL. C57Bl/6 mice were immunized s.c. with 5×10^5 irradiated, GM-CSF- or FL-secreting B16 cells and were challenged 1 week later s.c. with 1×10^6 live, wild-type B16 cells (four mice per group). Vaccination with irradiated, wild-type B16 cells (or B16 cells infected with a β-galactosidase-expressing vector) failed to elicit any tumor protection (data not shown). Similar results were found in five independent experiments. The difference observed between GM-CSF and FL was highly significant: $P < 0.0001$ using the Fisher's exact test.

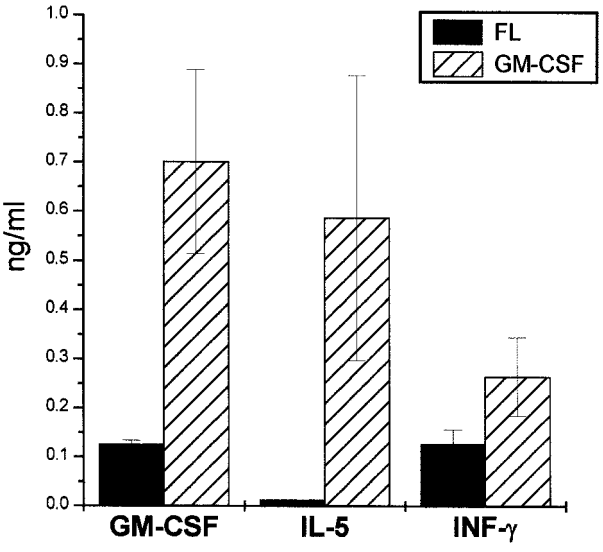

Fig. 4. Tumor-specific cytokine production stimulated by GM-CSF or FL. Splenocytes (two mice per group) were harvested 1 week after vaccination, cocultured with irradiated, IFN-γ-treated B16 cells for 5 days, and supernatants analyzed by ELISA. Similar results were found in four independent experiments.

tumor-specific cytokine production in the mouse, we harvested splenocytes 7 days after vaccination, cultured them for 5 days with IFN-γ-treated, irradiated B16 cells, and analyzed the supernatants by ELISA. As shown in Fig. 4, mice that received immunizations of irradiated, GM-CSF-secreting B16 cells developed T lymphocytes that produced high levels of IL-5, IFN-γ, and GM-CSF. In contrast, vaccination with irradiated, FL-secreting B16 cells resulted in weaker production of IFN-γ and GM-CSF and minimal amounts of IL-5. IL-4 was not detected in either group.

GM-CSF-secreting B16 Cells Stimulate the Functional Maturation of Splenic DCs. To explore the mechanism underlying the different abilities of GM-CSF and FL to stimulate antitumor immunity, we characterized the DCs stimulated by GM-CSF and FL in more detail. As shown in Fig. 5, *A* and *C*, GM-CSF-secreting B16 cells produced DCs almost exclusively of the myeloid type, which expressed high levels of CD11b and did not express CD8α (15, 27). In contrast, FL-secreting tumor cells produced the expansion of both lymphoid- ($CD8\alpha^+$, $CD11b^-$) and myeloid-type DCs (Fig. 5, *B* and *D*), as was reported following the administration of recombinant human FL protein (14, 15). No differences were observed in the DC expression of CD4 following injection of GM-CSF- or FL-secreting B16 cells (data not shown).

DCs have been shown to undergo functional maturation *in vitro* characterized by the increased expression of costimulatory molecules and the down-regulation of phagocytic capacities (28). To compare the functional maturation of DCs stimulated *in vivo* by either GM-CSF- or FL-secreting B16 cells, we examined the expression of critical costimulatory molecules on $CD11c^+$ splenocytes. The level of B7–1 expression was dramatically increased on DCs stimulated by GM-CSF as compared with FL (Fig. 6, *A* and *B*). GM-CSF also stimulated more uniform, high level expression of B7–2, CD40, and MHC class II molecules than FL, although these differences were less striking (Fig. 6, *C—F*, and Fig. 1, *A* and *B*).

A critical role for NKT cells in the generation of antitumor immunity recently has been delineated (29). Because NKT cells respond to glycolipid antigens presented by CD1d molecules (30, 31), we examined the expression of CD1d on $CD11c^+$ cells stimulated by the cytokine-secreting B16 cells. The level of CD1d expression was dramatically increased on DCs elicited by GM-CSF as compared with FL (Fig. 6, *G* and *H*). Although previous studies using recombinant FL protein had suggested that CD1d expression was restricted to $CD8\alpha^+$ DCs (15), these findings reveal that GM-CSF induces the expression of this molecule on $CD8\alpha^-$ DCs.

GM-CSF Activates DCs Locally. To test whether the differences observed between GM-CSF- and FL-activated DCs in the spleen were also demonstable locally, we compared the expression of B7–1 in the infiltrates elicited by irradiated, cytokine-secreting B16 cells. As shown in Fig. 7*A*, GM-CSF-secreting B16 cells induced a high level of B7–1 staining at the immunization site, whereas little B7–1 staining was found in the FL-elicited infiltrate (Fig. 7*B*).

To examine whether GM-CSF stimulates the functional maturation of DCs in humans as well, we studied the vaccination sites of metastatic melanoma patients treated with irradiated, autologous, GM-CSF-secreting melanoma cells. This immunization strategy consistently generates tumor-specific CD4- and CD8-positive T cells and plasma cells that mediate extensive tumor destruction without the induction of autoimmunity (22). Vaccination reactions were composed of dense admixtures of DCs, macrophages, and eosinophils (Fig. 7*C*), similar to those observed in the murine studies (Fig. 2*B*). Abundant CD1a staining of cells with dendritic morphology was evident (Fig. 7*D*), and these DCs expressed high levels of B7–1 (Fig. 7*E*).

DISCUSSION

The studies presented here demonstrate that tumor cells engineered to secrete GM-CSF stimulate the *in vivo* expansion and maturation of DCs. Because DCs play pivotal roles in the initiation of antigen-specific T- and B-cell immunity (1), our findings imply that the ability of GM-CSF to generate $CD8\alpha^-$ DCs that express high levels of B7–1 and CD1d is critical to the potent antitumor activity of this cancer vaccination strategy in mice and humans.

Although many investigations have established that GM-CSF can induce DC development from hemopoietic progenitors *in vitro* (3–6), the capacity of this cytokine to enhance DC development *in vivo* has

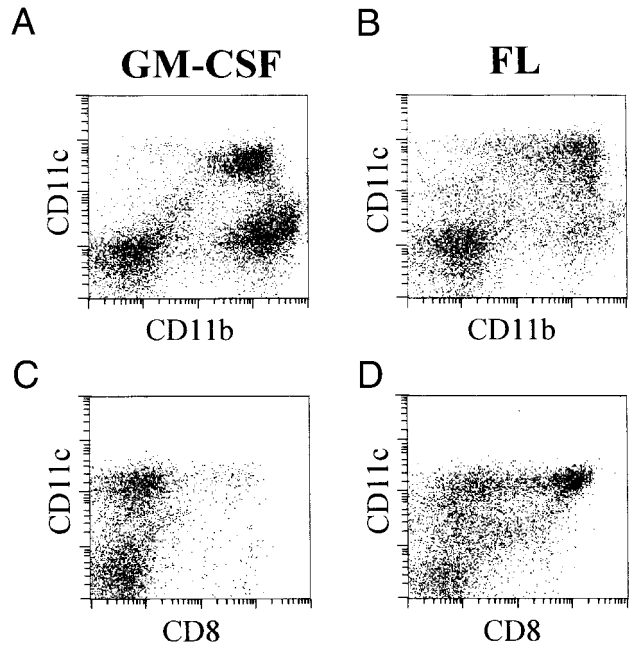

Fig. 5. GM-CSF stimulates myeloid-type DCs, whereas FL stimulates myeloid- and lymphoid-type DCs. Splenocytes were harvested 14 days after injection of live, GM-CSF- or FL-secreting B16 cells and stained for CD11c, CD11b, and CD8α. *A*, GM-CSF, CD11b. *B*, FL, CD11b. *C*, GM-CSF, CD8α. *D*, FL, CD8α.

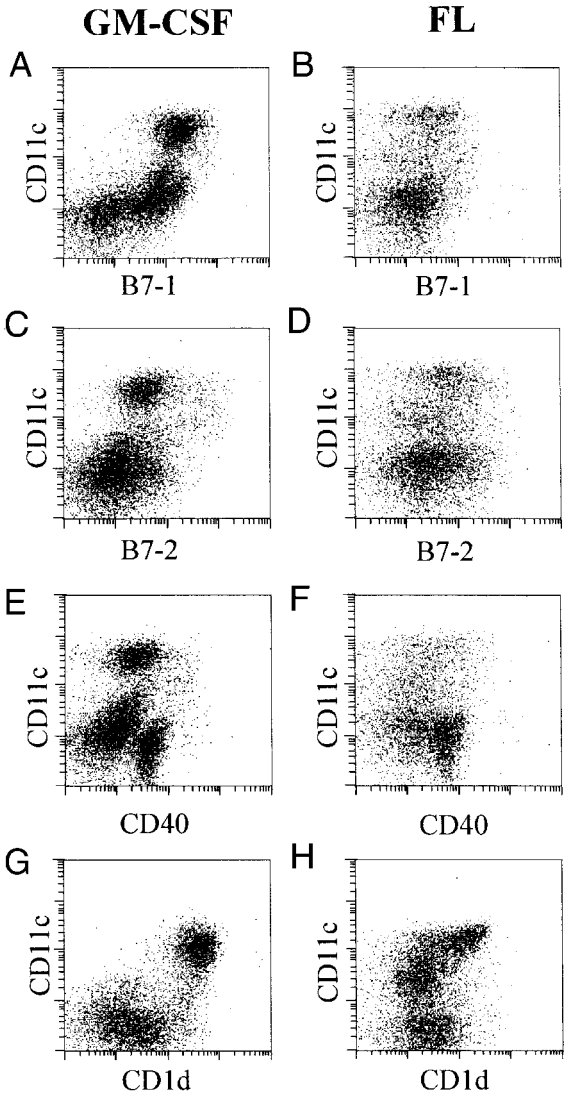

Fig. 6. GM-CSF stimulates the functional maturation of DCs. Splenocytes were harvested 14 days after injection of live, GM-CSF- or FL-secreting B16 cells and stained for CD11c, B7–1, B7–2, CD40, and CD1d. *A*, GM-CSF, B7–1. *B*, FL, B7–1. *C*, GM-CSF, B7–2. *D*, FL, B7–2. *E*, GM-CSF, CD40. *F*, FL, CD40. *G*, GM-CSF, CD1d. *H*, FL, CD1d.

been less clearly defined. The systemic administration of recombinant murine GM-CSF protein elicted only minimal effects on splenic DC populations (14), and GM-CSF transgenic mice did not manifest increased numbers of lymphoid tissue DCs (32). The intradermal administration of recombinant GM-CSF protein to patients with leprosy also evoked only moderate, local DC accumulation (33).

In contrast to these findings, our studies illustrate that the injection of GM-CSF-secreting tumor cells results in a dramatic expansion of DCs both locally and systemically. This stimulation likely reflects the efficient and stable production of GM-CSF protein by the MFG retroviral vector. Similar effects have been achieved recently with polyethylene glycol-modified recombinant GM-CSF protein (17). Despite the impressive increase in DCs elicited by the pharmacological delivery of GM-CSF, we and others demonstrated that GM-CSF is dispensable for steady-state DC generation *in vivo* (34, 35).

Although several stimuli for DC activation *in vitro* have been identified, including GM-CSF, monocyte-conditioned medium, tumor necrosis factor, and CD40 ligand (36–39), less is known concerning the signals necessary for DC activation *in vivo*. Injection of lipopolysaccharide or extracts of *Toxoplasma gondii* has been shown, however, to evoke DC migration and maturation (40, 41), in part through the induction of IL-12, tumor necrosis factor, IL-1, and secondary lymphoid organ chemokine (42, 43). The experiments presented here establish that GM-CSF is a critical regulator of DC activation *in vivo* as well.

Because tumor cells secreting GM-CSF or FL both induce the marked expansion of DCs *in vivo*, our system rendered it possible to compare the functions of these cells in the development of antitumor immunity. Although other experiments indicate that both recombinant FL and GM-CSF protein can serve as effective adjuvants for soluble proteins antigens (17, 44–47), the data presented here reveal that vaccination with irradiated tumor cells secreting GM-CSF is more potent than vaccination with irradiated tumor cells secreting FL.

The superior antitumor immunity stimulated by GM-CSF was associated with the induction of a broad T cell cytokine response, in contrast to the limited Th1 response induced by FL. Previously, we found similar, broad cytokine profiles in tumor-infiltrating lymphocytes derived from melanoma patients vaccinated with irradiated, autologous tumor cells engineered to secrete GM-CSF (22). These observations, taken together with studies examining the efficacy of GM-CSF-based tumor vaccines in cytokine-deficient mice (48), reveal important roles for both Th1 and Th2 cytokines in mediating tumor rejection.

The exclusive generation of myeloid-type DCs ($CD8\alpha^-$ and $CD11b^+$) by GM-CSF-secreting tumors, in contrast to the generation of both lymphoid- ($CD8\alpha^+$ and $CD11b^-$) and myeloid-type DCs by FL-secreting tumors, helps to explain the greater vaccination activity associated with GM-CSF in two ways. First, recent studies indicate that myeloid-type DCs elicit a broad cytokine response, whereas lymphoid-type DCs elicit a Th1 response (17, 49), perhaps via antigen transfer (50). Second, because antigen presentation stimulated by GM-CSF-based tumor cell vaccines involves cross-priming by bone marrow-derived cells (51), the capacity of DCs to phagocytose-irradiated cells (52–54) is particularly relevant; the capture of apoptotic bodies by DCs infiltrating tumor cells coexpressing GM-CSF and CD40 ligand has been demonstrated (55). In this context, $CD8\alpha^-$ DCs seem to be much more effective in the ingestion of particulate antigens than $CD8\alpha^+$ DCs (15, 56).

The comparison of DCs generated *in vivo* by GM-CSF and FL also revealed a striking difference in B7–1 expression. Whereas earlier work documented the capacity of GM-CSF to up-regulate B7–1 on cultured DCs (57), the findings presented here illustrate that GM-CSF is more powerful than FL in augmenting B7–1 expression *in vivo*. This increase in B7–1 is likely to be important for the development of antitumor immunity, because recent work using T-cell clones has indicated that high level B7–1 expression markedly reduces the amount of antigen necessary to trigger T-cell proliferation and expands the diversity of cytokines released (58). Experiments delineating the efficacy of GM-CSF-based tumor cell vaccines in B7–1 knockout mice (59) will help test this idea more thoroughly. A requirement for costimulatory function in antitumor immunity already has been established by demonstrating that vaccination with irradiated, GM-CSF-secreting tumor cells fails to induce protection against tumor challenge in CD40-deficient mice (60).

Our comparative analysis also revealed a dramatic difference between GM-CSF- and FL-generated DCs in the expression of CD1d. Although previous studies suggested that CD1d was largely restricted to the $CD8\alpha^+$ population (15), the experiments presented here show that CD1d can be expressed at very high levels by $CD8\alpha^-$ DCs. Concomitant with the induction of CD1d in animals injected with GM-CSF-secreting tumors was a significant increase in the numbers of splenic NKT cells (date not shown). Because activated NKT cells release large amounts of cytokines (61), their stimulation by $CD1d^+$

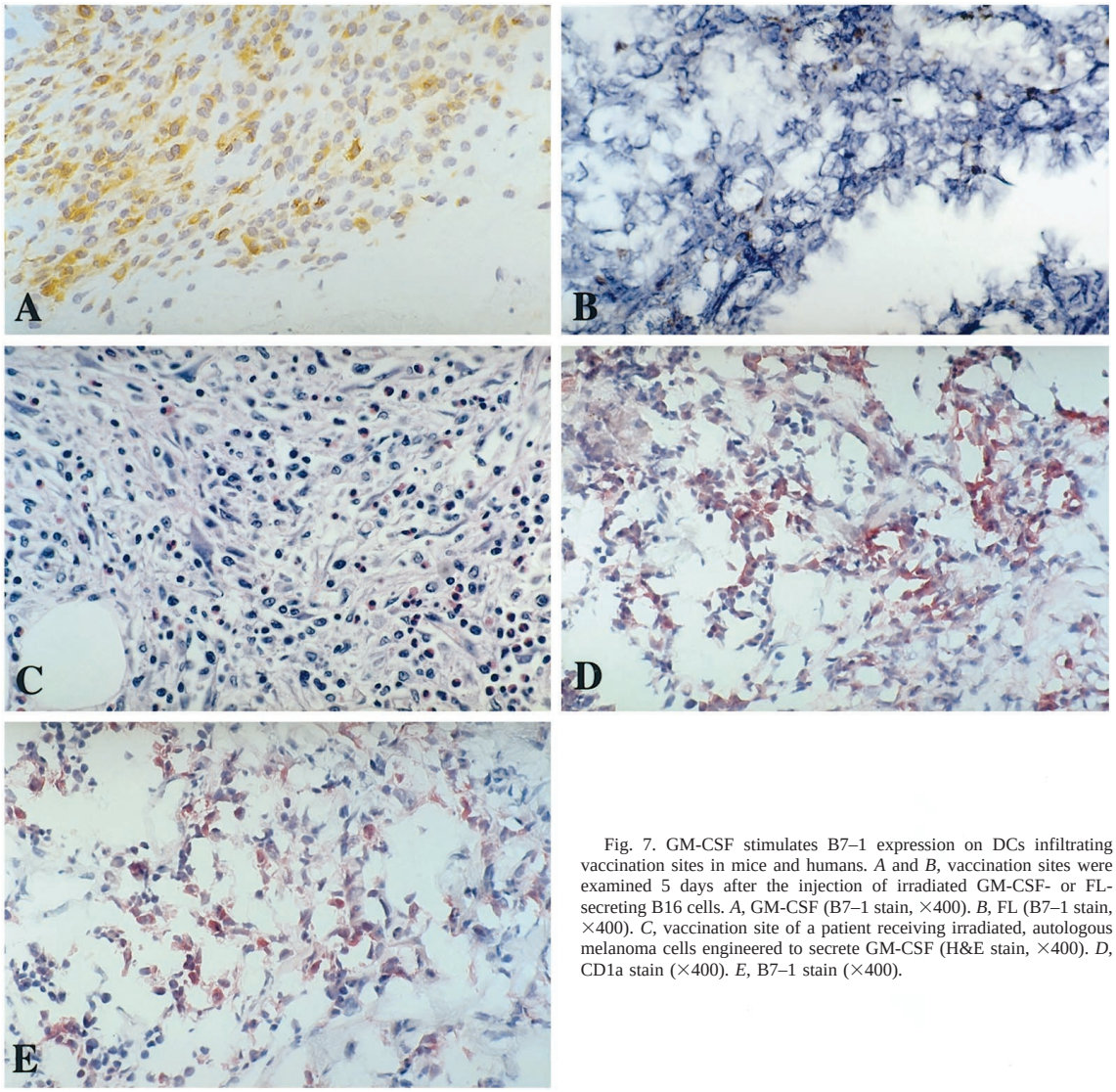

Fig. 7. GM-CSF stimulates B7–1 expression on DCs infiltrating vaccination sites in mice and humans. *A* and *B*, vaccination sites were examined 5 days after the injection of irradiated GM-CSF- or FL-secreting B16 cells. *A*, GM-CSF (B7–1 stain, ×400). *B*, FL (B7–1 stain, ×400). *C*, vaccination site of a patient receiving irradiated, autologous melanoma cells engineered to secrete GM-CSF (H&E stain, ×400). *D*, CD1a stain (×400). *E*, B7–1 stain (×400).

DCs may be essential for amplifying the nascent antitumor immune response and establishing the broad T cell cytokine profile. Indeed, other work has shown that NKT cells are essential for the antitumor effects of IL-12 (29). Experiments testing the activities of GM-CSF-based vaccines in CD1d knockout mice (62–64) should further clarify the role of NKT cells in antitumor immunity.

Lastly, our identification of a DC phenotype that results in the generation of potent antitumor immunity *in vivo* has important implications for the use of DCs in cancer vaccination strategies. It is of interest that many protocols involving the *ex vivo* expansion of DCs rely on the addition of monocyte-conditioned medium to produce functionally mature DCs (65); this requirement stems, in part, from the prior depletion of monocytes and granulocytes from the culture. An intriguing question raised by these observations is why hematopoietic progenitors capable of giving rise to granulocytes, macrophages, and DCs exist at all (66). Examination of the vaccination sites of GM-CSF-secreting tumor cells reveals the marked accumulation of each of these cell types. It is, thus, tempting to speculate that the coordinated activation of DCs, macrophages, and granulocytes by GM-CSF is intricately linked to the development and differentiation of DCs *in vivo*; this culminates in the efficient priming of antigen-specific immune responses. This perspective suggests that appropriate pharmacological delivery of GM-CSF may have broad use for vaccination strategies.

ACKNOWLEDGMENTS

We thank Esther Brisson (Albany Medical College, Albany, NY) for excellent help with the histological specimens, Susan Lazo-Kallanian and John Daley for excellent help with the fluorescence-activated cell-sorting studies, Patricia Bernardo for excellent help with the statistics, and the staff of the Redstone Animal Facility (Dana-Farber Cancer Institute, Boston, MA) for excellent help with maintenance of the mouse colony.

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[CANCER RESEARCH 61, 162–171, January 1, 2001]

Tumor Cell Vaccine Elicits Potent Antitumor Immunity after Allogeneic T-Cell-depleted Bone Marrow Transplantation[1]

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ABSTRACT

Allogeneic bone marrow transplantation (BMT) is currently restricted to hematological malignancies because of a lack of antitumor activity against solid cancers. We have tested a novel treatment strategy to stimulate specific antitumor activity against a solid tumor after BMT by vaccination with irradiated tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF). Using the B16 melanoma model, we found that vaccination elicited potent antitumor activity in recipients of syngeneic BMT in a time-dependent fashion, and that immune reconstitution was critical for the development of antitumor activity. Vaccination did not stimulate antitumor immunity after allogeneic BMT because of the post-BMT immunodeficiency associated with graft-*versus*-host disease (GVHD). Remarkably, vaccination was effective in stimulating potent and long-lasting antitumor activity in recipients of T-cell-depleted (TCD) allogeneic bone marrow. Recipients of TCD bone marrow who showed significant immune reconstitution by 6 weeks after BMT developed B16-specific T-cell-cytotoxic, proliferative, and cytokine responses as a function of vaccination. T cells derived from donor stem cells were, therefore, able to recognize tumor antigens, although they remained tolerant to host histocompatibility antigens. These results demonstrate that GM-CSF-based tumor cell vaccines after allogeneic TCD BMT can stimulate potent antitumor effects without the induction of GVHD, and this strategy has important implications for the treatment of patients with solid malignancies.

INTRODUCTION

Intensive chemo-radiotherapy alone mediates the antitumor effects of autologous BMT,[3] but the conditioning regimen together with additional graft-*versus*-tumor effects help to eliminate malignancy after allogeneic BMT (1, 2). However, relapse after BMT remains a major clinical problem, and because residual disease after BMT is frequently resistant to cytotoxic therapies, improved patient outcomes will likely require novel treatment approaches (3, 4).

Recently, a number of promising cancer vaccination strategies have been developed that significantly augment antitumor immunity in multiple rodent tumor systems (5, 6). Vaccination with modified whole tumor cells as the antigen source has been explored as a means to prime systemic antitumor immunity. Among the various schemes tested, we have shown that vaccination with irradiated tumor cells engineered to secrete murine GM-CSF elicits potent, specific, and long-lasting antitumor immunity in murine models of melanoma, sarcoma, colon carcinoma, renal cell carcinoma, and lung carcinoma (7). The efficacy of GM-CSF-secreting vaccines has also been observed in rodent models of prostate carcinoma, bladder carcinoma, metastatic and primary brain cancer, myeloma, lymphoma, and acute leukemia (4, 7–15). GM-CSF-based vaccines require the participation of both CD4- and CD8-positive T lymphocytes and likely involve improved tumor antigen presentation by host macrophages and dendritic cells (7). The principles delineated in these preclinical studies have proven relevant to patients with advanced renal cell carcinoma or malignant melanoma (16, 17). In a recent Phase I study of 21 metastatic melanoma patients, vaccination with irradiated, autologous tumor cells that were engineered to secrete GM-CSF consistently stimulated the development of tumor-specific $CD4^+$ and $CD8^+$ T lymphocytes and plasma cells that induced extensive tumor necrosis, fibrosis, and edema (17).

The efficacy of any cancer immunotherapy is likely related to the overall tumor burden (18). A previous investigation of vaccination with irradiated leukemia cells engineered to express CD86 demonstrated that therapeutic outcomes could be improved by first reducing the tumor burden with chemotherapy (19). These observations suggest that definitive clinical testing of cancer vaccines should be attempted in the setting of minimal residual disease, which could be achieved by autologous or allogeneic BMT. Although the ability of BMT to induce minimal residual disease has been well documented, relatively little attention has been directed to studying tumor vaccination in this context. This situation likely reflects the finding that BMT results in a significant immunodeficiency that may compromise the efficacy of vaccination. Immune reconstitution after BMT is characterized by a recapitulation of lymphoid ontogeny and a lack of sustained transfer of clinically significant donor T- and B-cell immunity (18, 20). Multiple quantitative and qualitative T- and B-cell defects have been described after both autologous and allogeneic BMT (18, 21), although, with the passage of sufficient time, most abnormalities resolve, except in the presence of chronic GVHD which is associated with immunosuppression in both humans and mice (21–23).

Despite the delay in immune reconstitution after BMT, some evidence suggests that vaccination may still be possible in this setting. Effective immunization with a live attenuated vaccine against measles, mumps, and rubella has been reported 2 years after BMT (24). Vaccination of both the donor and recipient against hepatitis B and tetanus has resulted in enhanced immunity in BMT recipients (25, 26). Immunization of a donor with a myeloma-associated paraprotein resulted in a tumor-specific immunity to the allogeneic BMT recipients (27). Collectively, these findings suggest that the development of antitumor immunity post-BMT may be feasible.

To investigate whether whole tumor cell vaccination strategies can be efficaciously used in combination with BMT to stimulate an antitumor effect, we have examined the ability of immunization with irradiated, GM-CSF-secreting B16 murine melanoma cells to generate specific antitumor immunity after BMT. Our findings establish that this vaccination scheme elicits potent antitumor effects after T-cell-depleted allogeneic BMT without the induction of GVHD.

Received 6/2/00; accepted 11/1/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grants CA39542 and HL55162 (to J. L. M. F.), NIH Grant CA74886 and the Cancer Research Institute/Partridge Foundation (to G. D.), the Swiss National Science Foundation (to N. M.), and the Swiss Cancer League (S. G.).

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[3] The abbreviations used are: BMT, BM transplantation; BM, bone marrow; GM-CSF, granulocyte-macrophage colony-stimulating factor; GVHD, graft-*versus*-host disease; TBI, total body irradiation; MoAb, monoclonal antibody; TCD, T-cell depleted/depletion; IL, interleukin; TFS, tumor-free survival; MiHA, minor histocompatibility antigen; ConA blast, concanavalin A-stimulated lymphocyte.

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6, H-2^b, CD45.2^+), SJL (H-2^s, CD45.1^+), B6SJLF1 (H-$2^{b/s}$, CD45.$1^+/2^+$), LP/J (H-2^b, CD45.2^+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The age of mice used as BMT recipients ranged between 11 and 16 weeks. Mice were housed in sterilized microisolator cages and received filtered water and normal chow and autoclaved hyperchlorinated drinking water for the first 3 weeks after BMT.

BMT. Mice were transplanted according to a standard protocol as described previously (28). Briefly, on day 0, mice received 11 Gy total body irradiation (TBI; ^{137}Cs source), split into two doses separated by 3 h to minimize gastrointestinal toxicity. BM cells (5×10^6) and 1–2 $\times$ 10^6 nylon wool purified splenic T cells were resuspended in 0.25 ml of Leibovitz's L-15 media (Life Technologies, Inc., Gaithersburg, MD) and injected i.v. into recipients. SJL and LP were used as donors in allogeneic BMT models. In some experiments, allogeneic BM was depleted of T cells (TCD) by incubating cells with anti-Thy-1.2 MoAbs at 4°C for 30 min followed by low-toxicity rabbit complement treatment for 40 min at 37°C. This two-round TCD procedure resulted in less than 0.01% T cell in the BM. This protocol provides complete donor myelopoiesis after TCD BMT when donor and recipient differ at multiple minor histocompatibility loci (29, 30). No evidence of GVHD after TCD BMT is seen by histological examination, as published previously (31). Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly by a scoring system that sums changes in five parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as described previously (32). Scores of less than 1.0 are not specific and do not indicate clinically significant GVHD.

Tumor Vaccination and Challenge. B16-F10 melanoma cells (H-2^b), syngeneic to B6 mice, were maintained in DMEM containing 10% FCS, 50 units/ml penicillin, and 50 mg/ml streptomycin. GM-CSF- secreting B16 cells (300 ng/10^6 cells/24 h) were generated using the retrovirus vector MFG as described previously (7). No replication component retrovirus is generated with this system, as determined by the *his* mobilization assay (33). Mice were immunized s.c. on the abdomen with 5×10^5 irradiated (33 Gy), GM-CSF-secreting or wild-type B16 cells in HBSS (Life Technologies, Inc.) and challenged 1 week later with 1×10^6 live, wild-type B16 cells s.c. on the back. Irradiation of GM-CSF-secreting B16 cells did not abrogate production of GM-CSF *in vitro* over the course of 7 days (7). Tumor growth was monitored every other day, and mice were killed when challenge tumors reached 1 cm in longest diameter. In some experiments, 10^5 irradiated (50Gy) B16 cells were s.c. injected into recipients on days 0, 7, 14, and 21 after BMT.

FACS Analysis. FITC-conjugated MoAbs to mouse CD45.2, CD4, CD11b, Gr-1, and PE-conjugated CD45.1, CD8, B220, NK1.1, DX5 were purchased from PharMingen (San Diego, CA). Cells were first incubated with MoAbs 2.4G2 (rat antimouse FcγR MoAbs) for 15 min at 4°C to block nonspecific FcγR binding of labeled antibodies, then with the relevant MoAbs for 30 min at 4°C. Finally, cells were washed twice with 0.2% BSA in PBS, fixed with 1% paraformaldehyde in PBS, and analyzed by FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). Irrelevant IgG2a/b MoAbs were used as a negative control. Ten thousand live events were acquired for analysis. Donor T-cell engraftment was determined by the percentages of CD45.1^+/CD45.2^- cells among CD3^+ cells in 3 mice per group (SJL: CD45.1^+/CD45.2^-; B6SJLF1: CD45.1^+/CD45.2^+).

Cell Culture and Analysis of T-Cell Proliferative Response. Splenocytes were harvested from animals 7 days after vaccination and three spleens combined from each group. All of the media and culture conditions were as described previously (34). After lysis of erythrocytes with ammonium chloride, cells were washed twice and resuspended in supplemented 10% FCS in DMEM. The percentage of CD4^+ and CD8^+ T cells in this fraction were estimated by FACS analysis and were normalized for CD4^+ plus CD8^+ T-cell numbers. The percentages of CD4^+ and CD8^+ T cells in the spleens of vaccinated and control group did not differ significantly. For the measurements of T-cell proliferation to B16 cells, 2×10^5 splenic T cells were plated in 96 flat-bottomed plates and cultured for 5 days with 2×10^4 B16 stimulators in 200 μl of supplemented 10% FCS in DMEM. Wild-type B16 cells were treated with IFN-γ for 24 h to increase expression of MHC class I and II molecules on their surface (35), washed twice, and irradiated (100 Gy). After 4 days of culture, supernatants were harvested from the culture for cytokine measurements, and cells were then pulsed with [^{3}H]thymidine (1 μCi per well) for an additional 16 h. Proliferation was determined on a 1205 Betaplate reader (Wallac, Turku, Finland). For the measurements of T-cell proliferative responses to alloantigens or anti-CD3 MoAbs, splenocytes were cultured with plate-bound anti-CD3 MoAbs (5 μg/ml; PharMingen) for 3 days or with 10^5 irradiated (20 Gy) peritoneal cells for 5 days.

ELISA. ELISA for GM-CSF, IFN-γ, IL-2, IL-4, IL-5, and IL-10 were performed according to the manufacturer's protocol (PharMingen). Briefly, samples were diluted 1:1 to 1:4, and each cytokine was captured by the specific primary MoAbs and detected by biotin-labeled secondary MoAbs. Assays were developed with streptavidin and substrate (KPL, Gaithersburg, MD). Plates were read at 450 nm using a microplate reader (Bio-Rad Labs, Hercules, CA). Samples and standards were run in duplicate, and the sensitivity of the assays was 5 pg/ml for GM-CSF, 0.1 units/ml for IFN-γ and IL-2, 10 pg/ml for IL-4, 4–8 pg/ml for IL-5, and 62.5 pg/ml for IL-10.

^{51}Cr Release Assays. Responder splenocytes (1×10^6 T cells/ml) were cultured with B16 stimulators (10^5/ml) in 24-well culture plate (Costar, Cambridge, MA) in the presence of 10 units/ml human IL-2 (Pharmacia Diagnostics Inc., Silver Spring, MD) for 5 days. Cells were then layered over Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) and centrifuged at 800 $\times$ *g* for 15 min. Cells were collected from the interface and washed twice before suspension in supplemented 10% FCS in RPMI medium. The percentage of CD8^+ T cells was estimated by FACS analysis, and the counts were normalized for CD8^+ T-cell numbers. IFN-γ-treated B16 targets (2×10^5) or 2×10^6 ConA blasts prepared from murine splenocytes were labeled with 100 μCi of ^{51}Cr for 2 h and plated at 10^3 or 10^4 cells per well in U-bottomed 96-well plates (Costar). Effector cells were added in quadruplicate at varying E:T ratios. ^{51}Cr activity in supernatants taken 4 h later was measured in a auto-gamma counter (Packard Instrument Company, Meriden, CT). Maximal and background release were determined by the addition of 2% Triton X-100 or media to the targets. The percentage of specific ^{51}Cr release (%) was calculated as 100 $\times$ (sample count $-$ background count)/(maximal count $-$ background count).

Statistical Analysis. Survival curves were plotted using Kaplan-Meier estimates. The Mann-Whitney *U* test was used for the statistical analysis of *in vitro* data and clinical scores, and the Mantel-Cox log-rank test was used to analyze survival data. $P < 0.05$ was considered statistically significant.

RESULTS

Immune Reconstitution Is Critical for the Induction of Antitumor Immunity Elicited by GM-CSF Tumor Cell Vaccine. To determine the relationship between immunological reconstitution and responsiveness to vaccination, we performed a time course analysis of vaccination after syngeneic BMT. B6 recipients were transplanted with 5×10^6 BM from syngeneic B6 donor mice after 11 Gy TBI. BMT recipients were then immunized with irradiated, GM-CSF-secreting or wild-type B16 cells at either 4 or 6 weeks after BMT. Mice were challenged with live B16 cells 1 week after immunization. As expected, tumor challenge was uniformly lethal in control animals vaccinated with irradiated, wild-type B16 cells; the kinetics of tumor development was similar between transplant recipients and naive mice (Fig. 1*A*). By contrast, vaccination with GM-CSF-secreting B16 cells resulted in substantial antitumor immunity at both 4 and 6 weeks after BMT ($P < 0.001$). Antitumor immunity was greater at 6 than at 4 weeks (TFS, 77 *versus* 39%; $P < 0.05$) and was as potent at 6 weeks as in naive animals (TFS, 79%). Immunophenotyping of splenocytes revealed that numbers of CD4^+, CD8^+, and B220$^+$ cells at 6 weeks after BMT were significantly greater than at 4 weeks ($P < 0.01$), but were comparable with numbers at 8 weeks after BMT (Fig. 1*B*). CD4^+ T-cell number returned to normal level by 6 weeks post-BMT, whereas CD8^+ cell counts remained below normal at all time points. B-cell numbers recovered to normal by 4 weeks and reached supranormal levels at 6 weeks. These results suggested that immune reconstitution of T cells was critical for the generation of antitumor immunity post-BMT.

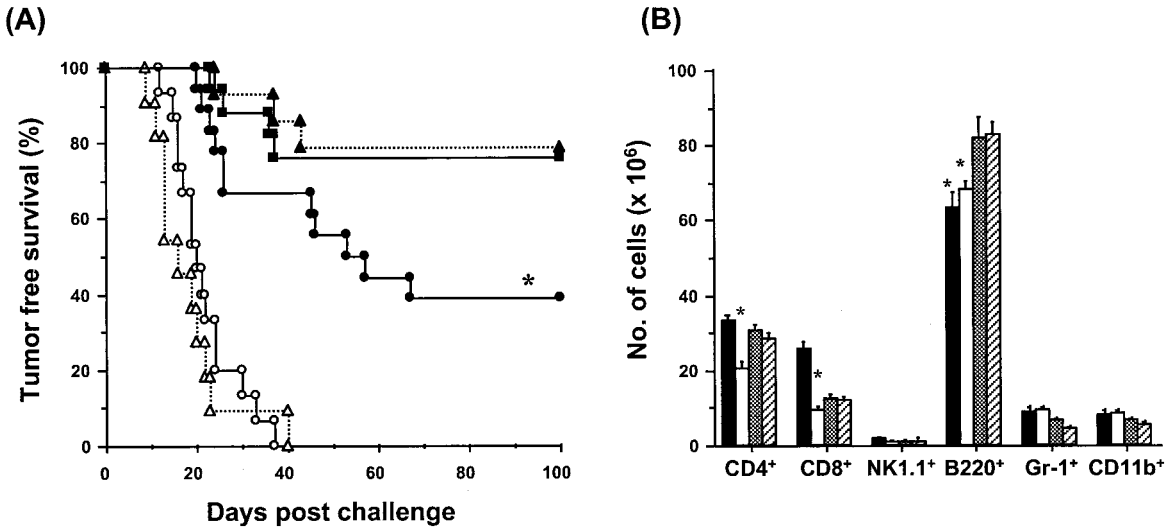

Fig. 1. Immune reconstitution is critical for the induction of antitumor immunity elicited by GM-CSF tumor cell vaccine. B6 mice were transplanted with 5×10^6 BM from syngeneic B6 donor mice after 11 Gy of TBI. *A*, B6 recipients were immunized with 5×10^5 irradiated, wild-type (○, SynBMT, wild-type vaccine; $n = 15$) or GM-CSF-secreting B16 cells 4 weeks (●, SynBMT (4w), GM-CSF vaccine; $n = 18$) or 6 weeks (■, SynBMT (6w), GM-CSF vaccine; $n = 17$) after syngeneic BMT and challenged 1 week later with 1×10^6 live B16 cells. Naive B6 mice were also immunized with 5×10^5 irradiated, wild-type B16 cells (△, No BMT, wild-type vaccine; $n = 11$) or GM-CSF-secreting B16 cells (▲, No BMT, GM-CSF vaccine; $n = 14$). Tumor growth was monitored up to day 100. and mice were killed when tumors reached 1 cm in longest diameter. Data represent results from two similar experiments. SynBMT, syngeneic BMT. ∗, $P < .05$ *versus* 4 weeks. *B*, immune reconstitution of the spleen after BMT ($n = 5$/group). ■, naive; □, 4w post-BMT; ▩, 6w post-BMT; ▨, 8w post-BMT. Data represent mean ± SD. ∗, $P < .01$ compared with 6 weeks.

GM-CSF-based Tumor Cell Vaccines Did Not Elicit Antitumor Immunity 6 Weeks after Allogeneic BMT. Allogeneic BMT is an effective form of immunotherapy for a number of hematological malignancies. To determine whether our tumor vaccination strategy could be used in the context of allogeneic BMT, we first tested whether B16 cells, which express low levels of MHC class I and II molecules *in vitro* (35), could induce tumor-specific immune responses. Naive B6 (H-2^b), B6SJLF1 (H-$2^{b/s}$), LP (H-2^b), and SJL mice (H-2^s) were challenged with 1×10^6 live B16 cells. None of the B6 or B6SJLF1 animals rejected B16 tumors, whereas the majority of LP and SJL mice rejected them, which demonstrated that B16 cells expressed sufficient amounts of MHC and/or MiHAs to induce an allogeneic immune response (Table 1). As expected, vaccination further enhanced the ability of nontransplanted SJL and LP mice to reject B16 cells (100% rejection after vaccination).

We then tested whether donor T cells from SJL could reject B16 cells after allogeneic BMT. B6SJLF1 and B6 recipients were transplanted after 11 Gy TBI with 5×10^6 BM and $1–2 \times 10^6$ splenic T cells from SJL and LP donors, respectively. After allogeneic BMT using these strain combinations, significant GVHD was developed. Nonetheless, none was able to reject the B16 melanoma when injected 1 or 7 weeks after BMT (Table 1).

Table 1 *Tumorigenicity of B16 cells in naive mice and recipients of allogeneic BMT*

Animals with or without vaccination by 5×10^5 irradiated, GM-CSF-secreting B16 cells were challenged 1 week later with 1×10^6 live B16 cells. The percentage of mice rejecting tumor challenge at day 100 is shown.

Strain	Tumor-free survivors at day 100	
	No vaccine	Vaccine
Naive		
B6	0/16 (0%)	13/19 (69%)
B6SJLF1	0/12 (0%)	8/12 (67%)
LP	6/8 (75%)	8/8 (100%)
SJL	7/11 (64%)	8/8 (100%)
BMT recipient		
LP→B6 (7w post-BMT)[a]	0/8 (0%)	
SJL→B6SJLF1		
(1w post-BMT)[a]	0/6 (0%)	
(7w post-BMT)[a]	0/5 (0%)	

[a] BMT recipients of bone marrow and T cells from allogeneic SJL donors were challenged at either 1 or 7 weeks post-BMT.

Vaccine efficacy was then assessed after allogeneic BMT. Recipients were immunized 6 weeks after BMT with GM-CSF-secreting B16 cells. Controls were not vaccinated. Mice were challenged with live, parental B16 cells 1–4 weeks later, which were lethal in control animals. Vaccination resulted in substantial antitumor immunity in both nontransplanted animals (TFS, 61.5 *versus* 0%; $P < 0.0001$) and in recipients of syngeneic BMT (TFS, 33.3 *versus* 0%; $P < 0.0001$; Fig. 2*A*). By contrast, 0% of the vaccinated recipients of allogeneic BMT survived challenge. In addition, vaccination failed to alter the kinetics of tumor development in recipients of allogeneic BMT, which demonstrated a lack of primary antitumor activity. These results demonstrate that immunization with irradiated, GM-CSF-secreting B16 cells fail to stimulate antitumor immunity after allogeneic BMT.

Similar results were obtained in a second BMT strain combination, LP→B6, in which the strains differ only in MiHAs. B6 recipients were transplanted after 11 Gy TBI with 5×10^6 BM and 1×10^6 splenic T cells from syngeneic B6 or allogeneic LP donors. After wild-type tumor challenge, none of the control animals survived without tumor beyond day 30, and no allogeneic graft-*versus*-tumor activity was evident after allogeneic BMT (Fig. 2*B*). Although vaccination stimulated protective antitumor immunity in 50% of nontransplanted animals ($P < 0.05$) and 25% of syngeneic BMT recipients ($P < 0.05$), 0% of allogeneic BMT recipients rejected the tumor challenge ($P < 0.05$ *versus* syngeneic BMT).

GVHD-associated Immunodeficiency Limits Vaccine Efficacy after Allogeneic BMT. GVHD is known to cause significant delays in immunological reconstitution after BMT (21–23), and we hypothesized that poor immunological reconstitution in the context of GVHD impaired antitumor activity. The effect of vaccination on tumor-specific T-cell responses was analyzed *in vitro* 1 week after vaccination (Table 2). The phenotype of lymphocytes in the spleen was not affected by the vaccination. Immunophenotyping of splenocytes 7 weeks post-BMT revealed severely reduced T- and B-lymphocyte numbers in recipients of allogeneic BMT with significant GVHD as described previously (36, 37), whereas numbers of CD4$^+$

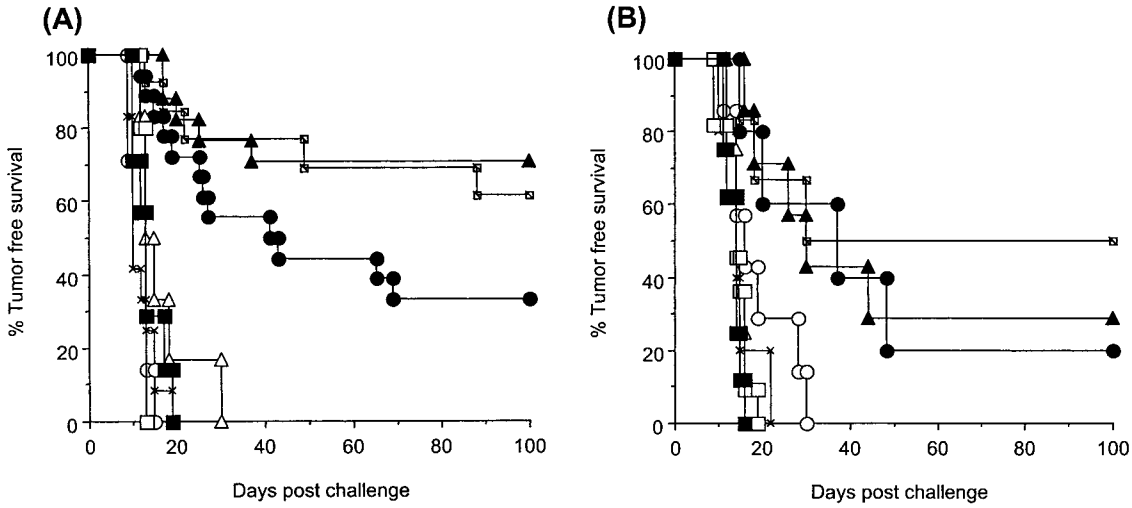

Fig. 2. Vaccination confers significant antitumor immunity after allogeneic TCD BMT but not allogeneic BMT. Lethally irradiated BMT recipients were transplanted with BM and splenic T cells from syngeneic or allogeneic donor mice. Each group of animals was divided into a vaccination group and a control group. Animals were vaccinated 6 weeks after BMT with 5×10^5 irradiated, GM-CSF-secreting B16 cells and challenged with 1×10^6 live B16 1–4 weeks later. *A*, SJL→B6SJLF1. Percentage of TFS after tumor challenge in recipients of syngeneic BMT (○, control, Syn BMT, − vax, $n = 7$; ●, vaccinated, Syn BMT, + vax, $n = 18$), allogeneic BMT (□, control, Allo BMT, − vax, $n = 5$; ■, vaccinated, Allo BMT, + vax, $n = 7$), allogeneic TCD BMT (△, control, AlloTCD BMT, − vax, $n = 6$; ▲, vaccinated, AlloTCD BMT, + vax, $n = 17$), and naive B6SJLF1 (×, control, − BMT, − vax, $n = 5$; ⧅ vaccinated, − BMT, + vax, $n = 7$). *B*, LP→B6. Percentage of TFS after tumor-challenge recipients of syngeneic BMT (○, control, $n = 7$; ●, vaccinated $n = 5$), allogeneic BMT (□, control, $n = 8$; ■, vaccinated $n = 11$), allogeneic TCD BMT (△, control, $n = 4$; ▲, vaccinated, $n = 7$), and naive B6 (×, control, $n = 8$; ⧅ vaccinated, $n = 11$). Data derive from three similar experiments.

T cells, natural killer cells, B cells, and myeloid cells, but not $CD8^+$ cells, were normal 7 weeks after syngeneic BMT. Culture of splenocytes harvested 1 week after immunization showed marked T-cell proliferative responses to B16 cells in vaccinated but not in control animals. In addition, vaccination did not prime T cells to respond to B6SJLF1 peritoneal cells or anti-CD3 cross-linking, which indicated vaccination-specific induction of antitumor reactivity. Whereas T cells from vaccinated recipients of syngeneic BMT proliferated as potently as cells from naive animals, recipients of allogeneic BMT showed little detectable B16-specific T-cell proliferation, even when T-cell numbers were normalized prior to culture. These results demonstrate that functional immune reconstitution of T-cell responses to B16 is associated with tumor eradication *in vivo* and that vaccine efficacy is abolished by the immunodeficiency associated with GVHD.

Vaccination after Allogeneic TCD BMT Generates Potent Antitumor Immunity. TCD of the donor inoculum is able to prevent the immunosuppression associated with GVHD after allogeneic BMT, but it also impairs immune reconstitution in clinical BMT (38). We, therefore, asked whether TCD of semiallogeneic BM could also provide for sufficient immune reconstitution to provide antitumor immunity in this allogeneic BMT model. B6SJLF1 recipients were transplanted after 11 Gy TBI with 5×10^6 TCD BM from SJL donors. Vaccination stimulated the development of striking antitumor activity (Fig. 2*A*; TFS, 70.6 *versus* 0%; $P < 0.001$), equivalent to that observed in nontransplanted vaccinated animals (TFS, 61.5%). Similar effects were found after BMT across MiHA differences (LP→B6), in which vaccination after TCD BMT also resulted in substantial levels of antitumor activity (TFS, 28.6%; Fig. 2*B*).

The effect of vaccination on tumor-specific T-cell responses was analyzed *in vitro* 1 week after vaccination (Table 2). Allogeneic TCD BMT recipients showed normal numbers of all cell phenotypes except $CD8^+$ cells by 6 weeks after BMT. T-cell proliferation to B16 stimulators in these animals was restored to normal levels. A recent study demonstrated that GM-CSF-based B16 cell vaccine require both Th1 and Th2 cytokine responses for the induction of maximal antitumor immunity (39). We, therefore, examined T-cell cytokine responses to vaccination after BMT. Analysis of the conditioned media obtained from cocultures of splenocytes from vaccinated animals and B16 stimulators revealed substantial levels of GM-CSF, IL-4, IL-5, IL-10, IFN-γ, and IL-2, similar to the profile observed in tumor-infiltrating lymphocytes stimulated by GM-CSF-based tumor vaccines in human melanoma patients (17). Cytokine responses in vaccinated TCD BMT recipients were never less than responses after syngeneic BMT and often equivalent to that seen in vaccinated naive animals. The development of proliferation and cytokine production to B16 *in vitro* correlated closely with the efficacy of the vaccine and tumor destruction *in vivo*. Comparable results were obtained in the LP→B6 system (data not shown). These results demonstrate that dual Th1 and Th2 cytokine responses that are closely associated with the development of antitumor immunity against B16 tumor can be induced by vaccination after BMT, including allogeneic TCD BMT.

Vaccination with a GM-CSF Whole Tumor Cell Vaccine Does Not Break Tolerance to Host Antigens after Allogeneic TCD BMT. Theoretically, whole tumor cell vaccines could present a significant risk of exacerbating GVHD by focusing increased reactivity to histocompatibility antigens shared by the tumor and host. To determine the effect of vaccination on GVHD severity, we monitored the survival and clinical GVHD score (range, 0–10) of immunized allogeneic BMT recipients, as described previously (32). GVHD was severe in the SJL→B6SJLF1 BMT model, with 36% mortality from GVHD by the time of vaccination (Fig. 3*A*). Clinical scores of GVHD severity in surviving allogeneic animals ranged from 5 to 7 by 4 weeks after allogeneic BMT, but it was mild or absent in recipients of syngeneic or TCD BMT (Fig. 3*B*). Importantly, vaccination did not exacerbate GVHD in any group, and, in particular, it did not cause increased skin disease or, depigmentation, as has been reported in other strategies to eliminate B16 tumors (40). Similar results were observed in the LP→B6 BMT model across MiHA differences, in which GVHD was relatively mild, and only 15% of the animals died by the time of vaccination (Fig. 3*C*). As expected, clinical GVHD scores were low, but even mild GVHD was not intensified by vaccination (Fig. 3*D*). Together, these findings demonstrate that GM-CSF-

Table 2 *Immunophenotypic and functional T-cell responses to vaccination*

Data represent mean ± SD of three animals from one of two similar experiments.

BMT	No	No	Syn	Syn	Allo	Allo	Allo TCD	Allo TCD
GVHD	−[a]	−	−	−	+	+	−	−
vaccination	−	+	−	+	−	+	−	+
Immunophenotype (× 10^6/spleen)[b]								
CD4+	21.9 ± 1.6	25.6 ± 5.5	21.5 ± 3.9	26.8 ± 7.1	0.1 ± 0.1[c]	0.6 ± 1.1[c]	28.6 ± 5.7	32.2 ± 6.9
CD8+	16.0 ± 0.8	17.0 ± 2.2	8.9 ± 1.8[c]	10.8 ± 2.9[c]	0.1 ± 0.2[c]	0.3 ± 0.3[c]	5.9 ± 1.3[c]	6.7 ± 1.6[c]
DX5+	3.4 ± 0.4	3.8 ± 1.1	1.8 ± 0.4	2.8 ± 0.6	0.0 ± 0.0[c]	0.2 ± 0.5[c]	1.1 ± 0.2[c]	2.8 ± 0.6
B220+	54.5 ± 2.7	54.7 ± 12.3	59.7 ± 5.7	61.7 ± 14.7	0.0 ± 0.0[c]	1.4 ± 3.3[c]	61.3 ± 9.7	65.0 ± 8.4
Gr-1+	2.8 ± 0.7	2.8 ± 1.2	2.2 ± 1.0	3.3 ± 1.0	0.5 ± 0.7[c]	0.5 ± 0.5[c]	2.0 ± 0.6	3.0 ± 1.9
CD11b+	2.2 ± 0.3	2.5 ± 0.6	2.3 ± 0.8	2.9 ± 1.1	0.5 ± 0.7[c]	0.7 ± 0.7[c]	3.3 ± 0.6	4.6 ± 2.0
T-cell proliferation (× 10^3 cpm)[d]								
+B16	2.1 ± 0.5	99.2 ± 12.6[c]	3.5 ± 0.8	100.7 ± 15.2[c]	0.7 ± 0.5	1.6 ± 0.7	3.1 ± 0.6	97.3 ± 13.0[c]
+B6SJLF1	6.9 ± 1.4	5.9 ± 1.8	6.4 ± 1.6	5.0 ± 0.1	ND	ND	3.0 ± 0.5	3.2 ± 0.3
+αCD3	392.2 ± 31.9	471.0 ± 50.1	382.0 ± 20.6	405.2 ± 14.4	ND	142.5 ± 21.1[c]	380.0 ± 41.9	387.0 ± 37.0
T-cell cytokine response[e]								
GM-CSF (pg/ml)	79.1 ± 15.1	1283 ± 42[c]	105.8 ± 26	771 ± 36[c]	ND	ND	57.0 ± 6.6	1574 ± 123[c]
IFN-γ (units/ml)	UD	33.8 ± 3.3[c]	UD	16.1 ± 0.8[c]	ND	ND	UD	17.2 ± 0.5[c]
IL-2 (units/ml)	UD	0.5 ± 0.1[c]	UD	1.0 ± 0.1[c]	ND	ND	UD	3.9 ± 0.5[c]
IL-4 (pg/ml)	UD	421 ± 30[c]	UD	362 ± 18[c]	ND	ND	UD	383 ± 31[c]
IL-5 (pg/ml)	UD	2678 ± 12[c]	UD	1801 ± 192[c]	ND	ND	UD	1545 ± 201[c]
IL-10 (pg/ml)	UD	2583 ± 296[c]	UD	1027 ± 195[c]	ND	ND	UD	1823 ± 116[c]

[a] −, no; +, yes; Syn, syngeneic; Allo, allogeneic; UD, undetectable; ND, not determined.

[b] Recipients were vaccinated 6 weeks post-BMT and splenocytes were harvested 1 week later. Control mice were not immunized (three mice/group).

[c] $P < 0.01$ compared with naive.

[d] Spleens from three to four animals per group were combined. (2×10^5) T cells were cultured for 5 days with 2×10^4 irradiated, IFN-γ-treated B16 cells, 10^5 irradiated B6SJLF1 peritoneal cells, or with anti-CD3 MσAbs for 3 days in quadruplicate. Proliferation was determined by incubation of cells with [^{3}H]thymidine (1 μCi) for the last 16 hours of culture.

[e] Cytokine levels in supernatants harvested from 4-day cultures with B16 stimulators. Splenocytes from control animals did not produce detectable levels of cytokines.

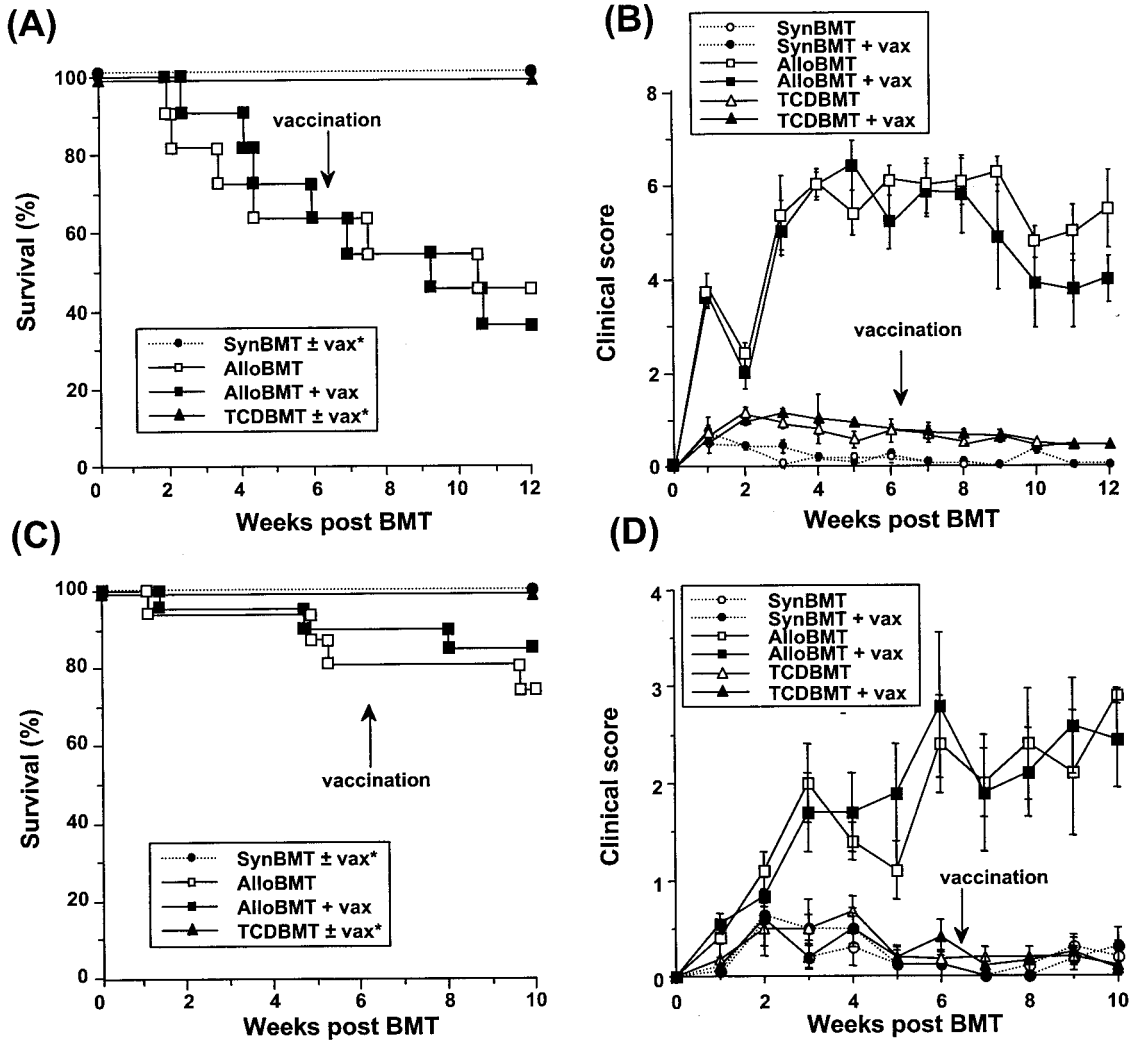

Fig. 3. Clinical course of GVHD after vaccination. Animals were vaccinated 6 weeks after BMT. Survival (*A* and *C*) and GVHD scores (*B* and *D*) were determined. *A* and *B*, SJL→B6SJLF1. *C* and *D*, LP→B6. Clinical GVHD scores were assessed weekly after BMT by a scoring system that sums changes in five clinical parameters (maximum index, 10). Clinical scores represent mean ± SE. SynBMT, syngeneic BMT; AlloBMT, allogeneic BMT; vax, vaccination. *A* and *C*, ●, SynBMT with and without vax; □, AlloBMT; ■, AlloBMT + vax; ▲, TCDBMT with and without vax. *B* and *D*, ○, SynBMT; ●, SynBMT + vax; □, AlloBMT; ■, AlloBMT + vax; △, TCDBMT; ▲, TCDBMT + vax.

based tumor cell vaccines do not exacerbate GVHD when administered after allogeneic BMT.

In light of this absence of GVHD after vaccination, we evaluated T-cell responses to host antigens *in vitro* in these recipients of allogeneic TCD BMT. Analysis of donor engraftment at 4 and 6 weeks after BMT in peripheral blood disclosed mixed donor/host chimerism of $CD3^+$ T cells in TCD BMT recipients (29.1 ± 4.1% and 67.5 ± 8.1% donor type at 4 and 6 weeks, respectively), although myeloid cells were completely of donor origin by 4 weeks post-BMT. Overall donor engraftment was 74 ± 5% donor at 4 weeks and 84 ± 4% donor at 6 weeks after BMT. Splenocytes were harvested 1 week after vaccination, and T-cell proliferative and cytotoxic responses were analyzed (Fig. 4). T cells from unvaccinated naive SJL proliferated in response to B6SJLF1 peritoneal cells, but T cells from TCD BMT recipients did not (Fig. 4*A*), which confirmed the acquisition of tolerance to host antigens after allogeneic TCD BMT. After vaccination, TCD BMT recipients proliferated to B16 stimulators *in vitro* without proliferating to B6SJLF1 antigens, which demonstrated that vaccination induced B16-antigen specific T-cell responses (Fig. 4*A*). Similar results were obtained in CTL assays (Fig. 4, *B* and *C*). Vaccination produced equivalent cytotoxic responses to B16 tumors after allogeneic TCD BMT and syngeneic BMT (data not shown). As expected, T cells from vaccinated SJL mice lysed B6 ConA blasts but did not lyse SJL ConA blasts. Although unvaccinated SJL mice possessed little detectable cytotoxicity against B16, vaccination significantly enhanced this cytotoxicity, similar to observations in immunized melanoma patients (17). Vaccination did not augment cytolytic activity against B6 ConA blasts, confirming tolerance to host antigens *in vitro*. Thus, despite mixed donor/host chimerism after allogeneic TCD BMT, GM-CSF-based tumor cell vaccines were able to stimulate effective antitumor immunity and did not elicit immune responses to host alloantigens either *in vitro* or *in vivo*.

In these experiments, immune reconstitution occurred in the absence of tumor, perhaps preventing the acquisition of tolerance to tumor antigens. To examine this possibility, we repeated this experiment with tumor present during immune reconstitution. After allogeneic TCD BMT (SJL→B6SJLF1), recipients were given s.c. injections with 10^5 irradiated B16 cells on days 0, 7, 14, and 21 of BMT. Mice were subsequently vaccinated with irradiated, GM-CSF-secreting B16 cells at 6 weeks post-BMT and were challenged at 7 weeks post-BMT and monitored for survival, clinical scores, and tumor development (Table 3). TFS of BMT recipients receiving both repeated injections of irradiated B16 cells (Group E) and vaccination was equivalent to that of recipients receiving vaccination without such

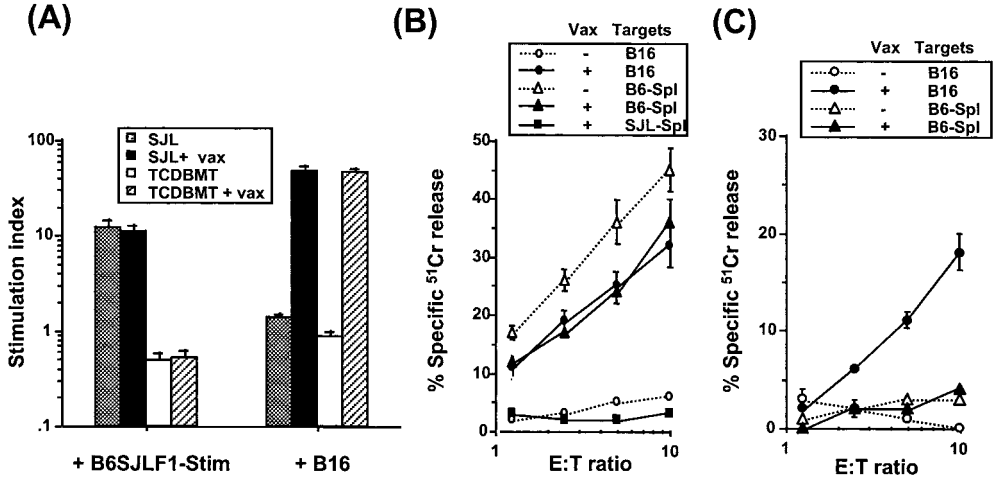

Fig. 4. Induction of B16-specific T-cell responses and antihost tolerance after TCD BMT. *A*, naive SJL and recipients of TCD BMT were vaccinated. Splenocytes were harvested one week after vaccination and spleens from three animals per group were combined. T cells (2×10^5) were cultured for 5 days with 2×10^4 irradiated, IFN-γ-treated B16 cells or with 10^5 irradiated SJL or B6SJLF1 peritoneal cells (*Stim*). Proliferation was determined by incubation of cells with [^{3}H]thymidine (1 μCi) for the last 16 h of culture. Data are shown as mean ± SD of stimulation index (cpm in culture with B16 or B6SJLF1/cpm in culture with SJL) from quadruplicate culture. ▩, SJL; ■, SJL+ vax; □, TCDBMT; ▨, TCDBMT + vax. *B* and *C*, cytotoxicity of splenic T cells from naive SJL (*B*) and from TCD BMT recipients (*C*) cultured for 5 days with B16 stimulators as determined in a standard 4-h ^{51}Cr release assay against B16 targets, B6 ConA blasts (B6-Spl), or SJL ConA blasts (SJL-Spl). Data represent results from two similar experiments. ○, − Vax, Target B16; ●, + Vax, Target B16; △, − Vax, Target B6-Spl; ▲, + Vax, Target B6-Spl; ■, + Vax, Target SJL-Spl.

injections (group D). Thus, the presence of tumor antigens during immune reconstitution did not prevent the development of antitumor immunity stimulated by this vaccine protocol.

Antitumor Activity Induced by Vaccination Post-BMT Is Long-Lasting. To determine whether vaccination stimulated the development of long-lasting antitumor immunity, we challenged mice that had rejected an initial tumor inoculum of 10^6 wild-type B16 cells at 5 months after immunization. We found that 67–75% of syngeneic BMT and 100% of allogeneic TCD BMT recipients eliminated the second tumor challenge, which demonstrated the induction of immunological memory by this vaccination strategy (Table 4).

Table 4 *Antitumor immunity induced by vaccination is long-lasting in transplant recipients*

Vaccinated mice that rejected initial tumor challenges were rechallenged with 1×10^6 live B16 cells 5 months after vaccination, and the numbers of surviving animals were reported.

Recipients	BMT	Tumor-free survivors at day 100 of rechallenge
Experiment 1		
B6	No	5/5 (100%)
B6	Syngeneic	8/12 (67%)
Experiment 2		
B6SJLF1	No	3/4 (75 %)
B6SJLF1	Syngeneic	5/5 (100%)
B6SJLF1	Allogeneic TCD	7/7 (100%)

DISCUSSION

The ability of cancer vaccines to enhance antitumor immunity after BMT are influenced by the toxicities of the conditioning regimen, the requirement for immunological reconstitution, and the immunosuppression associated with allogeneic BMT. To study the complex interactions of these variables, we have examined the vaccination properties of irradiated, GM-CSF-secreting B16 melanoma cells after BMT. GM-CSF-based vaccines require both $CD4^+$ and $CD8^+$ T cells for successful immunization (7), and, thus, present a stringent test of immunological function post-BMT.

In preliminary studies, we examined the relationship between immunological reconstitution and responsiveness to vaccination by performing a time course analysis of immunization with irradiated, GM-CSF-secreting B16 cells after syngeneic BMT (B6→B6). Vaccination generated substantial levels of antitumor immunity by 4 weeks and full levels by 6 weeks post-BMT, demonstrating a rapid recovery from the toxicities of the conditioning regimens. Splenic $CD4^+$ T cells recovered in significant numbers by 4 weeks and reached normal levels by 6 weeks, whereas $CD8^+$ T cells achieved only 50% of normal levels by 8 weeks post-BMT. These findings confirm recent observations in a different BMT model (41), demonstrate that immune reconstitution is critical for effective vaccination, and underscore the correlation between T-cell recovery and vaccination efficacy. Although elimination of B16 tumor has been reported to occur independently of $CD4^+$ cells (40), our results confirm that vaccination with GM-CSF-secreting B16 cells results in both $CD4^+$ and $CD8^+$ T-cell sensitization to tumor.

We then examined the ability of vaccination to generate antitumor immunity after allogeneic BMT. Immunization 6 weeks after syngeneic BMT with GM-CSF-secreting B16 cells generated potent antitumor immunity, as measured by both tumor protection and by B16-specific T-cell responses *in vitro*. However, when allogeneic BMT recipients were vaccinated, no antitumor activity was induced in two different BMT models. The absence of antitumor activity correlated with the immunosuppression associated with GVHD; spleens obtained from allogeneic BMT recipients showed marked lymphoid hypoplasia and functional T-cell defects that are typical of GVHD-associated immune deficiency (21, 22, 36, 37, 42). It, therefore, seemed likely that GVHD-associated immunodeficiency limits vac-

Table 3 *Presence of tumor antigens during immune reconstitution did not induce tolerance to tumor*

Naive mice or BMT recipients of allogeneic TCD BM received injections of 10^5 irradiated B16 cells at days 0, 7, 14, and 21 post-BMT. Subsequently mice were vaccinated at 6 weeks and challenged at 7 weeks after BMT.

Group	BMT	Irradiated tumor injection	Vaccination	Tumor-free survivors at day 30 of challenge
A	−[a]	−	−	0/3 (0%)
B	−	−	+	2/5 (40%)
C	+	+	−	0/4 (0%)
D	+	−	+	4/8 (50%)
E	+	+	+	4/10 (40%)

[a] −, no; +, yes.

cine efficacy after allogeneic BMT; this experimental result is consistent with clinical studies evaluating posttransplant immunization against tetanus and poliovirus, in which impaired responses to vaccination were associated with chronic GVHD (43, 44). Thus, although GVHD has a known beneficial antitumor effects against hematological malignancies and certain solid tumors (45), its associated immunodeficiency may inhibit efforts to enhance tumor eradication through this type of vaccination strategy after allogeneic BMT.

Remarkably, this vaccination strategy was extremely effective after allogeneic BMT when the donor inoculum was depleted of T cells to prevent GVHD and resulted in mixed chimerism. This efficacy was manifest in terms of both tumor protection and the development of T-cell responses specific for B16 melanoma antigens. The induction of tumor-specific cytokine production, proliferation, and cytotoxicity after vaccination was closely associated with efficacy of vaccination evident after both allogeneic TCD BMT and syngeneic BMT. Reconstitution to normal levels of $CD4^+$ T cells (but not $CD8^+$ T cells) was observed by 6 weeks after TCD BMT as well as after syngeneic BMT. These findings demonstrate that TCD that prevents the development of GVHD, allows sufficient reconstitution of T cells from donor stem cells and can thereby restore the efficacy of vaccination. In this case, a functional thymus is critical for repopulation of the periphery with competent T cells because expansion of donor T cells is not an option after TCD BMT. Unfortunately, such rapid reconstitution is unlikely to occur in adult humans, in which the age-related reductions in thymic regenerative capacity often result in incomplete restoration of T-cell homeostasis after TCD BMT (46). Novel approaches to stimulate immune reconstitution will be required in older patients with poor thymic function.

The tumor-specific T-cell production of GM-CSF, IFN-γ, IL-2, IL-4, IL-5, and IL-10 does not fit a classic Th1 or Th2 cytokine pattern and suggests that multiple immunological effector mechanisms are induced by GM-CSF-based vaccines. Pathological studies of the skin at vaccination sites and challenge sites in mice and humans receiving GM-CSF-secreting tumor cell vaccine have revealed an extensive local influx of T cells, B cells, macrophages, dendritic cells, and eosinophils (7, 17, 39). It has recently been demonstrated that vaccination with GM-CSF-secreting B16 cells required both Th1 and Th2 cytokines from $CD4^+$ T cells for the induction of maximal antitumor immunity (39). This cytokine profile has also been observed in human Phase I clinical trials of vaccination with irradiated, GM-CSF-secreting melanoma cells (17). These observations strongly suggest a central role of $CD4^+$ T cells in the induction of antitumor immunity by GM-CSF-secreting whole tumor cell vaccine. Our studies demonstrate that transplanted mice can generate both Th1 and Th2 cytokine responses after BMT as well as nontransplanted mice. The efficacy of vaccination after syngeneic or allogeneic TCD BMT was also comparable with that seen in nontransplanted mice, which may be explained by the nearly normal quantitative and qualitative immune reconstitution in these animals.

Interestingly, the protective antitumor immunity induced by GM-CSF vaccination was long-lasting and displayed immunological memory, evidenced by the ability of vaccinated mice to reject a tumor challenge 5 months later. Clinical studies of BMT patients show a loss of donor-derived immunity (20, 27, 44), which suggests the need for antigenic stimulation to an immune system that is newly generated from donor BM cells, hence the recommendation of post-BMT vaccination against infectious agents (47).

To determine whether vaccination with GM-CSF-secreting B16 cells broke tolerance to host antigens, we evaluated a group of immunized mice for progression of GVHD. The SJL→B6SJLF1 model presents a highly stringent test for GVHD exacerbation, because the donor and recipient differ at MHC I and II loci in addition to MiHAs. Although immunization was performed in mice that had already developed significant GVHD, this cellular-based vaccine caused no exacerbation of GVHD. Although GVHD in the LP→B6 BMT model (disparate MiHAs only) was less intense than in the other model system, again vaccination had no significant influence on the course of GVHD. Vaccination also did not induce GVHD after TCD BMT in either strain combination. Lastly, our experiments determined that the presence of tumor cells during immune reconstitution, as might occur during clinical BMT when some malignant cells survive high-dose conditioning, does not induce tolerance to tumor antigens and does not prevent the efficacy of vaccine. However it should be noted that administration of irradiated B16 cells may not be immunologically equivalent to viable tumor cells because irradiated B16 cells are known to have low MHC expression and are poor immunogens.

Our studies confirm and extend recent observations in a different allogeneic BMT model when the use of a cellular-based vaccine provided tumor-specific immunity *in vivo* without exacerbation of GVHD (48). The mechanisms underlying the dissociation of antitumor activity and GVHD in recipients of TCD BMT involve the establishment of tolerance to host antigens. Tumor challenge demonstrated that most naive SJL (MHC- and MiHA-discordant) and LP (MiHA-discordant) mice, but not B6 (syngeneic) donor mice rejected a lethal inoculum of B16 melanoma. This observation shows that B16 cells express a sufficient amount of MiHAs or MHC to stimulate the immune system, although B16, a well-known tumor, has little detectable MHC I and MHC II molecules (35). Studies of T-cell proliferative and cytotoxic responses to allogeneic targets and B16 tumors demonstrated that: (*a*) vaccination induced SJL T-cell responses directed against B16-associated antigens; (*b*) donor T cells derived from SJL TCD BMT were tolerant of host B6 antigens; and (*c*) vaccination with B16 GM-CSF cells did not break tolerance of host antigens by donor T cells. Tolerance of host antigens was associated with the presence of mixed chimerism in TCD BMT recipients, and induction of mixed chimerism has now become a major strategy to induce tolerance after allogeneic BMT (49). These results show that vaccination is capable of stimulating donor T cells to generate antitumor immunity despite their acquisition of tolerance to host antigens in the recipient thymus, which prevents GVHD after vaccination. However, our data regarding B16 may not be representative of all tumors because of its low MHC expression and the profound role of natural killer cells in its rejection (50).

In other systems, antitumor effects are closely associated with GVHD. A recent study in which allogeneic BMT donors were immunized with IL-2-secreting tumor cells demonstrated a concomitant increase in both antitumor activity and GVHD (51). By contrast, our experiments clearly show that vaccination of recipients with GM-CSF-secreting tumor cells after TCD BMT generates antitumor activity that is separable from GVHD. Immunization of recipients rather than donors may have several advantages; vaccinations can: (*a*) be administered after the acquisition of tolerance to host antigens by donor cells; (*b*) stimulate the newly developing immune system, resulting in long-lasting immunity; and (*c*) avoid unnecessary exposure of healthy donors to tumor cells and foreign proteins such as alloantigens. Because TCD is associated with a marked reduction in the frequency and intensity of GVHD and antitumor activity (52), the ability of tumor vaccination to increase antitumor immunity without GVHD in this setting has important clinical implications. If substantive immune reconstitution can be achieved in patients after BMT, this approach may be able to overcome the multiple immunological defects associated with progressive cancer and, in so doing, enhance the overall potency of tumor vaccines. The work presented here provides a framework for crafting clinical trials aimed at evaluating the effi-

cacy of this strategy, perhaps in combination with other approaches such as donor lymphocyte infusions.

ACKNOWLEDGMENTS

We are grateful to Yani S. Brinson for her technical assistance and Dr. Kenneth R. Cooke for helpful discussions.

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Overlapping roles for granulocyte-macrophage colony-stimulating factor and interleukin-3 in eosinophil homeostasis and contact hypersensitivity

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Studies of mice rendered deficient in granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) have established unique roles for these cytokines in pulmonary homeostasis, resistance to infection, and antigen-specific T- and B-cell responses. In addition to these distinctive properties, however, GM-CSF and IL-3 also stimulate the development and activation of hematopoietic cells in many similar ways, raising the possibility that each factor might partially compensate for the other's absence in singly deficient mice. To test whether endogenous GM-CSF and IL-3 mediate redundant functions in vivo, we generated mice lacking both cytokines through sequential gene targeting experiments in embryonic stem (ES) cells. Surprisingly, doubly deficient animals, but not single knockouts, showed increased numbers of circulating eosinophils. Doubly deficient mice, moreover, developed weaker contact hypersensitivity reactions to haptens applied epicutaneously than mice deficient in either factor alone. Together, these findings delineate overlapping roles for GM-CSF and IL-3 in hematopoiesis and immunity. (Blood. 2001;97:922-928)

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) stimulate the proliferation, differentiation, and activation of hematopoietic cells in vitro in many similar ways.[1] These overlapping functions reflect, at least in part, the shared use of the βc subunit for receptor signaling.[2] The proximity of GM-CSF and IL-3 genomic sequences on mouse chromosome 11 and human chromosome 5 further underscores their close relationship and suggests that these cytokines may have evolved from an ancient gene duplication.[3]

Notwithstanding these similarities, mice rendered singly deficient in GM-CSF or IL-3 manifest distinct phenotypes. Animals deficient in GM-CSF display normal steady-state hematopoiesis, but develop a lung disease resembling pulmonary alveolar proteinosis (PAP).[4,5] The pathogenesis of PAP involves a reduction in surfactant clearance[6] by defective alveolar macrophages.[7,8] The βc knockout mice acquire comparable lung abnormalities[9,10] due to closely related perturbations in surfactant metabolism[11] and mount abrogated eosinophil responses as a consequence of the loss of βc-mediated interleukin-5 (IL-5) signaling.[12,13] Intriguingly, humans with PAP harbor high titers of neutralizing anti–GM-CSF antibodies[14] or, less commonly, mutations in βc.[15] GM-CSF–deficient mice also show compromised antigen-specific IgG and cytotoxic T-cell responses, interferon-γ (IFN-γ) production, and phagocyte function.[16-19] Together, these immune defects confer an increased susceptibility to *Listeria monocytogenes*, group B streptococcus, and *Pneumocystis carinii*,[20-22] but partial protection against endotoxin challenge[23] and collagen-induced arthritis.[24]

Although IL-3–deficient mice similarly display intact steady-state hematopoiesis, unlike GM-CSF–deficient animals, they maintain normal pulmonary homeostasis.[25] Mice deficient in IL-3 mount attenuated mast cell and basophil responses to parasite infection that result in compromised worm expulsion.[26] They also show partial reductions in contact hypersensitivity reactions to haptens applied epicutaneously.[25] Mice rendered deficient in βIL-3, a second signaling chain for this cytokine, develop blunted IL-3 responses, but show no perturbations of steady-state hematopoiesis.[9,27]

Mice deficient in GM-CSF or IL-3 have been interbred with other hematopoietic growth factor knockouts to uncover possible redundancies of cytokine function in vivo.[28] Mice lacking both GM-CSF and granulocyte colony-stimulating factor (G-CSF), unlike either single mutant, develop neutropenia early in life, resulting in increased mortality.[29] Mice deficient in both GM-CSF and macrophage colony-stimulating factor (M-CSF) show more extensive pulmonary pathology and a higher incidence of fatal bacterial pneumonia than GM-CSF single knockouts.[30] Mice deficient in both IL-3 and c-*kit* signaling display more severe defects in mast cell expansion and parasite resistance than either single knockout.[26] On the other hand, mice lacking both IL-3 and *mpl* fail to develop further compromises in thrombopoiesis when compared with single *mpl* knockouts.[31]

Because IL-3 signals through both βc and βIL-3, IL-3–deficient mice have been crossed with βc knockouts to generate mice with disrupted GM-CSF, IL-3, and IL-5 function.[32] Although these animals mount reduced eosinophil responses due to the loss of IL-5 signaling and develop PAP due to the absence of GM-CSF signaling, no additional abnormalities have been described. In an effort to study more thoroughly the impact of dual GM-CSF and

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Submitted August 1, 2000; accepted October 24, 2000.

Supported by the Swiss National Science Foundation (N.M., S.G.), the Swiss Cancer League (S.G.), the Cancer Research Institute/Partridge Foundation, and CA74886 (G.D.). G.D. is a Clinical Scholar of the Leukemia and Lymphoma Society. S.G. and N.M. should be considered equal first authors.

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IL-3 ablation, without a concurrent loss in IL-5 signaling, we generated mice lacking both GM-CSF and IL-3 through sequential gene targeting experiments in embryonic stem (ES) cells. Surprisingly, doubly deficient animals have increased numbers of circulating eosinophils and are markedly compromised in contact hypersensitivity reactions.

Materials and methods

Generation of GM-CSF/IL-3–deficient mice

Genomic sequences spanning the GM-CSF locus were excised from pPNT-GM-CSF and introduced into pPHT,[33] a targeting vector designed for hygromycin and ganciclovir double selection. pPHT-GM-CSF was electroporated into an IL-3 heterozygous-deficient D3 ES cell clone selected for high germline transmission.[25] ES cells were propagated on G418/hygromycin resistant feeders (derived from C57Bl/6J-TgN[pPWL512hyg]1Ems JR2354 mice),[34] and clones resistant to hygromycin, G418, and ganciclovir were characterized by Southern analysis. A full-length complementary DNA (cDNA) probe was used to analyze the IL-3 locus and the previously reported probe[4] shown in Figure 1 was used to analyze the GM-CSF locus. Targeted clones were injected into C57Bl/6 blastocysts to generate chimeras that transmitted the doubly mutant allele through the germline. Animals inheriting the targeted allele were interbred to obtain homozygous, doubly deficient animals. The mutant allele was then back-crossed 9 generations onto both C57Bl/6 and Balb/c backgrounds. The βc-deficient mice[10] were similarly bred onto both C57Bl/6 and Balb/c backgrounds for a total of 9 generations. The βc-deficient mice were then crossed with GM-CSF/IL-3–deficient mice to obtain homozygous, triply deficient animals.

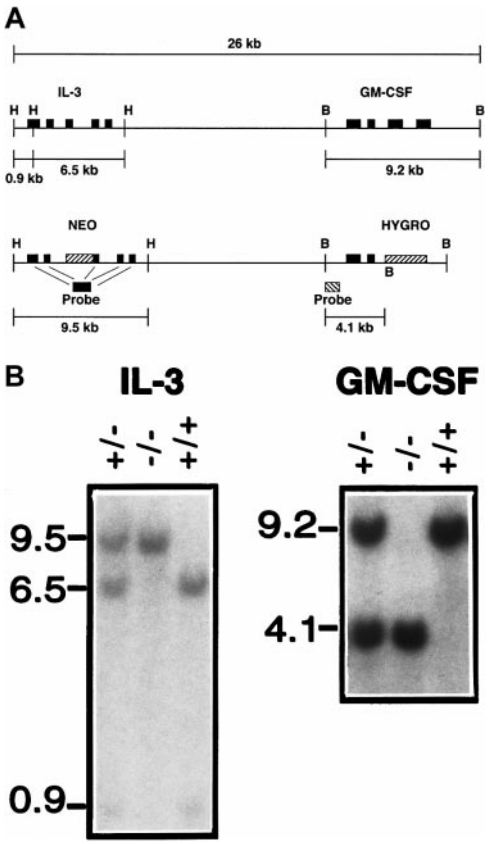

Figure 1. Generation of GM-CSF/IL3–deficient mice. (A) Structure of the wild-type and targeted GM-CSF/IL-3 loci. (B). Genotyping of wild-type, heterozygous, and homozygous doubly deficient animals. Tail DNA was digested with either *Hind*III or *Bam*HI and characterized by Southern analysis using the indicated probes.

Hematologic evaluation

Hematocrits, total and differential white blood cell and platelet counts, and bone marrow colony forming units-granulocyte, -macrophage, -granulocyte-macrophage, and -granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-G, -M, -GM, and –GEMM, respectively) were determined as previously described.[4] CFU-eosinophils (CFU-Eo) were determined in triplicate by culturing 7.5×10^5 bone marrow cells in MethoCult (StemCell Technologies, Vancouver, BC, Canada) supplemented with either 10 or 100 ng/mL recombinant IL-5. Bone marrow eosinophil numbers were determined on stained marrow sections using a micrometer and counting 300 cells along a linear millimeter in randomly chosen fields and on stained cytospins of bone marrow aspirates counting a total of 300 nucleated cells. For bone marrow transplantation experiments, 5×10^6 nucleated blood cells were harvested from donor femurs and injected into lethally irradiated (1100 rads in 2 doses) recipients. Peripheral blood counts were determined at days 9, 23, 64, and 100 after transplantation. GM-CSF, IL-3, and IL-5 were measured by enzyme-linked immunosorbent assay (ELISA) with the appropriate monoclonal antibodies (Endogen, Woburn, MA; Pharmingen, San Diego, CA).

Dendritic cells

Live B16-F10 melanoma cells (5×10^5) secreting murine flt3-ligand were injected subcutaneously into C57Bl/6 mutant and control animals to increase dendritic cell numbers, as previously described.[35] Splenocytes and thymocytes were harvested 14 days later and stained with fluorescein isothiocyanate (FITC)- or phycoerythtin (PE)-conjugated monoclonal antibodies to CD11c, CD11b, I-A^b, CD8α, CD1d, CD3ϵ, CD4, NK1.1, B7-1, B7-2, and CD40 in the presence of blocking antibodies against FcγIII/II receptors (Pharmingen).

Contact hypersensitivity

Mice at least 7 weeks of age were sensitized epicutaneously on day 0 with 70 μL 4% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, Sigma, St Louis, MO) in acetone/olive oil (4:1) and challenged 5 days later on the ear with 20 μL 0.5% oxazolone or carrier only. To assess responsiveness to FITC (Sigma), mice were sensitized on day 0 with 400 μL 2.5% FITC in acetone/dibutyl phthalate (1:1) and challenged on day 6 with 40 μL 1.5% FITC. The hapten-specific increase in ear thickness at 24 hours was determined with a micrometer. Draining lymph nodes were harvested 24 to 48 hours after FITC application, processed to single cell suspension, and stained for major histocompatibility complex (MHC) II, B7-1, CD1d, and Ox40-ligand.[36] For correction experiments, mice were injected intraperitoneally and subcutaneously with a total of 4700 ng GM-CSF and 810 ng IL-3, beginning 2 days before and finishing 2 days after sensitization (−48 hours, −24 hours, −18 hours, −4 hours, 0 hours, +4 hours, +18 hours, +24 hours, +48 hours). This regimen involved more intensive dosing than previously examined in studies of IL-3 singly deficient animals[25] and was undertaken based on pilot experiments indicating an important dose-response effect. Cytokines were harvested from B16 cells engineered to secrete GM-CSF and IL-3.[37,38] Control supernatants were from wild-type B16 cells.

Histology

Tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. They were then sectioned at 4 μm thickness and stained with hematoxylin and eosin. A semiquantitative scoring scheme for the intensity of contact hypersensitivity reactions was established as follows: trace, minimal edema, rare infiltrating lymphocytes or granulocytes, no epidermal changes; 1+, mild edema, focal infiltration of lymphocytes or neutrophils, no epidermal changes; 2+, easily visible edema, diffuse but scattered infiltration of lymphocytes, neutrophils, and eosinophils, foci of intraepidermal neutrophils; 3+, marked edema with numerous lymphocytes, many neutrophils and eosinophils, few intraepidermal abscesses; 4+, marked edema with numerous lymphocytes, neutrophils and eosinophils, many subcorneal and intraepidermal abscesses, focal keratinocyte necrosis.

Statistics

A one-way analysis of variance was used for statistical analysis. When significant differences were observed ($P < .05$), pairwise t tests were performed, using the Bonferroni correction for the multiple comparisons examined.

Results

Generation of GM-CSF/IL-3–deficient mice

Because GM-CSF and IL-3 are separated by only 14 kb on chromosome 11,[3] doubly deficient mice could not be obtained by interbreeding single knockout animals. Thus, mice lacking both cytokines were generated through sequential gene targeting experiments in ES cells. A hygromycin cassette replacing exons 3 and 4 of the GM-CSF locus was introduced by homologous recombination into IL-3 heterozygous deficient ES cells[25] (Figure 1A). Two correctly targeted clones gave rise to germline transmission following injection into C57Bl/6 blastocysts. Genotyping of progeny mice revealed that GM-CSF and IL-3 were disrupted on the same allele (Figure 1B). Heterozygous mutant mice were interbred to generate homozygous GM-CSF/IL-3–deficient animals. Mutant mice were obtained at the expected frequencies, remained clinically healthy throughout 18 months of observation, and were fertile. Supernatants of concanavalin A-stimulated splenocytes from mutant animals showed no immune-reactive GM-CSF or IL-3 protein as determined by ELISA (not shown), confirming the generation of a null allele. The mutant allele was back-crossed 9 generations onto Balb/c and C57Bl/6 backgrounds for detailed analysis. Additional studies are required to delineate whether the modest decrease in fertility of GM-CSF–deficient animals[29,39] is influenced by the simultaneous ablation of IL-3.

Pathology

Complete pathologic examination of GM-CSF/IL-3–deficient mice revealed abnormalities restricted to the lungs. A progressive accumulation of surfactant in the intra-alveolar spaces and an extensive lymphoid hyperplasia around both airways and veins was observed. Alveolar macrophages demonstrated a marked increase in intracellular surfactant. These features were similar to those previously found in GM-CSF–deficient mice,[4,5] and morphologic analysis did not reveal an exacerbation by the concurrent loss of IL-3. Tissue hematopoietic populations and lymphoid organs failed to disclose additional alterations.

Hematopoiesis

The hematocrits and total circulating white blood cell and platelet counts were normal in GM-CSF/IL-3–deficient mice. Unexpectedly, examination of stained blood smears revealed that circulating eosinophils were increased in doubly deficient mice, as compared to single knockouts and wild-type controls (Table 1). In contrast, circulating neutrophils, lymphocytes, and monocytes were not affected. Bone marrow-derived CFU-G, -M, -GM, and -GEMM were not altered in GM-CSF/IL-3–deficient animals, and bone marrow precursors did not show enhanced sensitivity to IL-5 in vitro (CFU-Eo in response to 10 ng IL-5 for +/+ mice: 15, 7, 15, 16.7; for −/− mice: 14, 6.3, 16.7, 13.7. Colony sizes were equivalent between +/+ and −/−mice). Enumeration of bone marrow eosinophils by examination of both fixed core sections and cytospins of marrow aspirates did not reveal differences between wild-type and doubly deficient animals (percent eosinophils for +/+ mice: 2.3, 1.3, 2, 1.3; for −/− mice: 1.7, 1.3, 1.3, 1.7). Although no IL-5 was consistently measurable in the blood (the sensitivity of ELISA was 25 pg/mL), interbreeding of GM-CSF/IL-3–deficient and βc-deficient mice resulted in abrogation of the eosinophilia (Table 1), strongly suggesting the participation of IL-5 in this response. Triply deficient mice also demonstrated an unexpected reduction in circulating lymphocytes.

To characterize hematopoiesis in GM-CSF/IL-3–deficient mice further, we lethally irradiated mutant animals and transplanted them with doubly deficient marrow. GM-CSF/IL-3–deficient mice achieved reconstitution that was comparable to wild-type controls, although there was a modest delay in the kinetics of leukocyte recovery (not shown), similar to that previously observed for GM-CSF–deficient animals.[40]

Dendritic cell development

Recent studies have underscored the striking abilities of GM-CSF and IL-3 to stimulate the growth and differentiation of dendritic cells from hematopoietic precursors.[41] However, we and others previously reported that mice deficient in GM-CSF or IL-3 maintained normal numbers of spleen and lymph node dendritic cells.[4,25,42] Analysis of the spleens, thymi, and lymph nodes of GM-CSF/IL-3–deficient mice similarly revealed normal numbers of both myeloid- and lymphoid-type dendritic cells (not shown).

In an effort to identify other factors that might contribute to dendritic cell development in these animals, we implanted syngeneic tumor cells engineered to secrete high levels of flt3-ligand.[35] These cells serve as an efficient vehicle for the systemic administration of flt3-ligand, a cytokine that dramatically augments dendritic cell numbers in wild-type mice.[43] By 14 days after injection, there was a marked increase in splenocytes staining positive for CD11c and MHC II in both mutant and wild-type animals, with an average of 25% positive cells per spleen (Figure 2A,B). Because injection of flt3-ligand–expressing tumor cells produced a 3- to 4-fold increase in total spleen cellularity, a nearly 100-fold expansion of

Table 1. Peripheral blood counts in GM−/−IL3−/− mice

Genotype	RBC ($\times 10^6$)	Platelets ($\times 10^3$)	WBC ($\times 10^3$)	Eosinophils ($\times 10^3$)	Lymphocytes ($\times 10^3$)	Neutrophils ($\times 10^3$)	Monocytes ($\times 10^3$)
+/+	9.5 ± 1.2	1054 ± 281	6.3 ± 2.1	0.08 ± 0.03	5.1 ± 1.6	0.98 ± 0.41	0.14 ± 0.04
GM−/−IL3−/−	9.6 ± 0.4	1074 ± 209	8.1 ± 1.9	0.32* ± 0.10	6.3 ± 1.5	1.26 ± 0.39	0.19 ± 0.08
GM−/−	9.2 ± 0.2	1008 ± 363	6.9 ± 2.2	0.09 ± 0.04	5.5 ± 1.7	1.14 ± 0.52	0.15 ± 0.08
IL3−/−	9.9 ± 0.4	1113 ± 126	8.0 ± 1.1	0.12 ± 0.04	6.5 ± 0.8	1.20 ± 0.32	0.19 ± 0.06
βc−/−GM−/−IL3−/−	9.7 ± 0.4	1197 ± 135	5.1†† ± 1.4	0.02† ± 0.02	4.1§ ± 1.2	0.89 ± 0.21	0.12 ± 0.05

Results (mean ± SD) of a 300 cell count differential for 10 mice (ages 2-5 months) per group.
*GM−/−IL3−/− versus +/+, GM−/−, IL3−/−, and βc−/−GM−/−IL3−/−, P = .0001, .0001, .0002, and .0001.
†βc−/−GM−/−IL3−/− versus GM−/−IL3−/− and +/+, P = .0001.
††βc−/−GM−/−IL3−/− versus GM−/−IL3−/−, P = .0017.
§βc−/−GM−/−IL3−/− versus GM−/−IL3−/−, P = .0025.

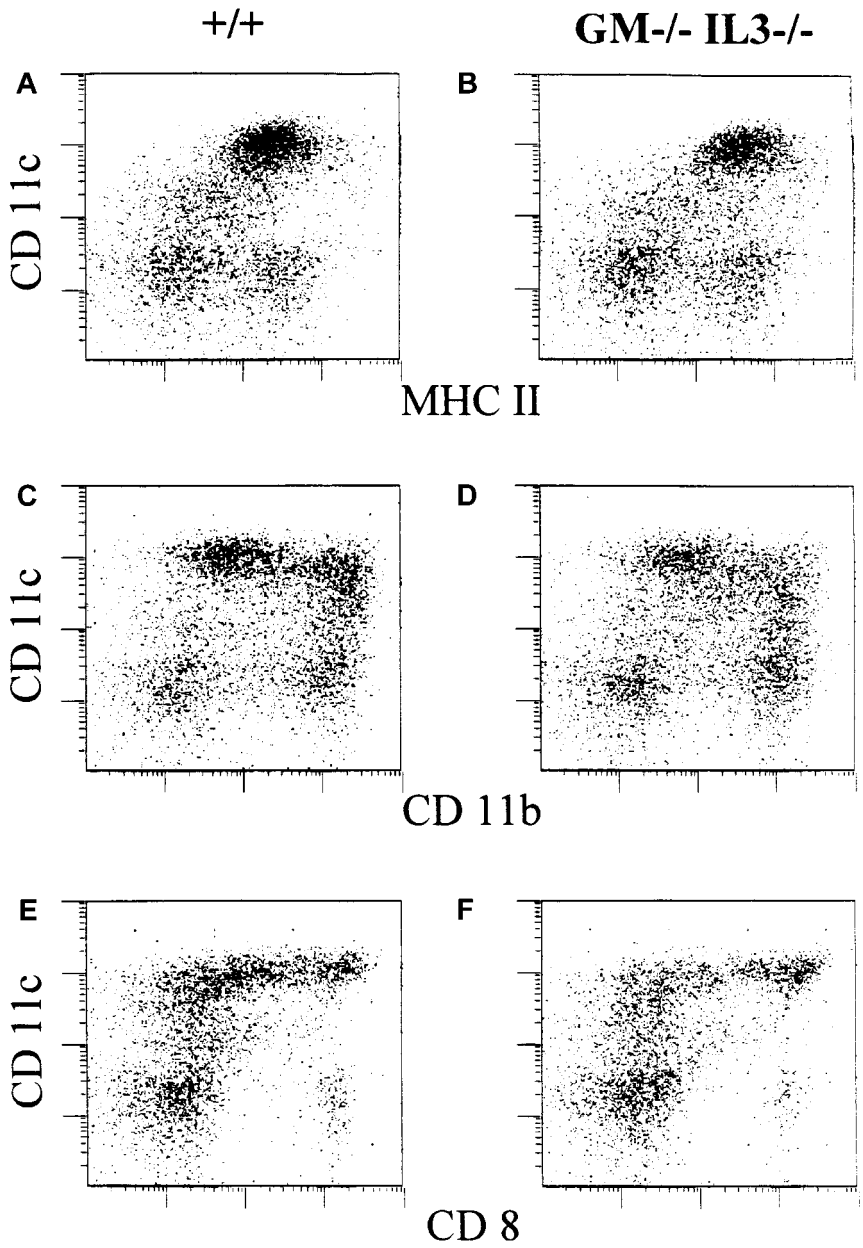

Figure 2. GM-CSF/IL-3–deficient and wild-type mice mount comparable dendritic cell responses to Flt3-ligand. Splenocytes from mutant and wild-type animals were harvested 14 days after injection of Flt3-ligand–secreting B16 cells and stained for CD11c, MHC II, CD11b, and CD8. (A,C,E) Wild-type mice. (B,D,F) GM-CSF/IL-3–deficient mice.

dendritic cell numbers was accomplished in the absence of GM-CSF and IL-3. Flt3-ligand–secreting tumor cells stimulated the generation of both myeloid-type ($CD8\alpha^-$, $CD11b^+$) and lymphoid-type ($CD8\alpha^+$, $CD11b^-$) dendritic cells (Figure 2C-F).[43,44] No differences in B7-1, B7-2, CD40, or CD1d expression were observed between doubly deficient and wild-type animals (not shown). Taken together, these results suggest that flt3-ligand may be a critical regulator of dendritic cell development in vivo.

Contact hypersensitivity

To evaluate dendritic cell function in GM-CSF/IL-3–deficient mice, we compared the abilities of mutant and wild-type animals to develop contact hypersensitivity reactions to epicutaneously applied haptens. Contact hypersensitivity is a form of delayed-type hypersensitivity in which hapten-protein conjugates are presented by cutaneous dendritic cells, following their migration to regional lymph nodes, to hapten-specific $CD4^+$ and $CD8^+$ T lymphocytes.[45-48] On secondary hapten challenge, sensitized T cells initiate a local inflammatory response.

Although GM-CSF/IL-3–deficient mice were indistinguishable from wild-type littermates in the initial reaction to oxazolone challenge (data not shown), they exhibited a dramatically reduced response on secondary challenge, as measured by ear swelling (Figure 3A). The degree of compromise was significantly greater than that previously reported for IL-3–deficient mice.[25] Similar results were observed on both C57Bl/6 and Balb/c backgrounds and when FITC was used as the hapten (not shown).

Although no pathologic differences between GM-CSF/IL-3–deficient and wild-type mice were noted in untreated skin or skin at the sensitization site, marked differences were apparent in skin at the challenge site (Figure 4A-C). In wild-type animals, the inflammatory response was characterized by an intense cellular infiltrate consisting primarily of neutrophils, lymphocytes, and eosinophils, which was associated with substantial dermal edema,

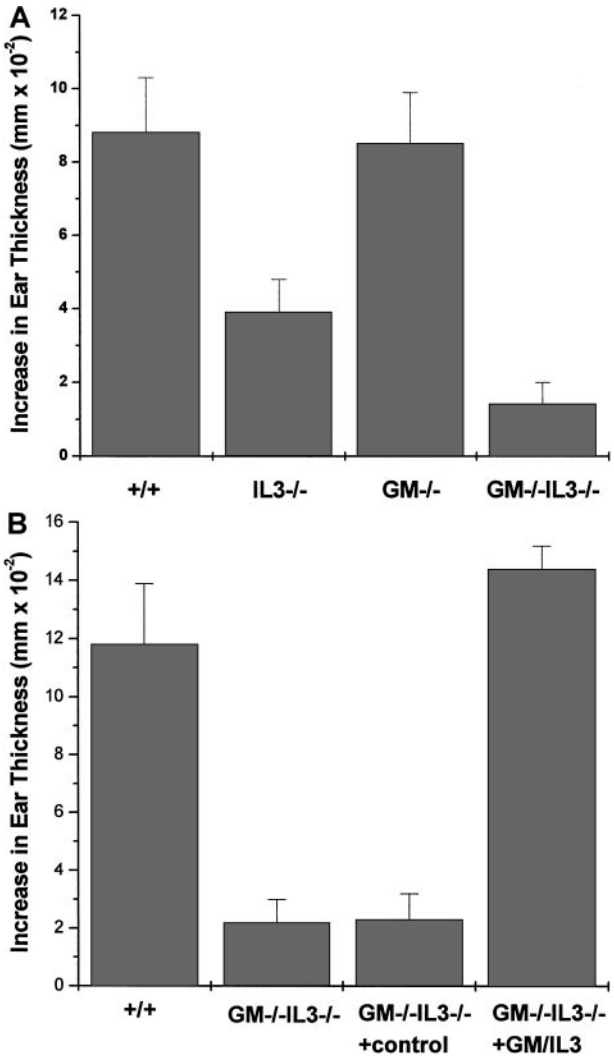

Figure 3. Contact hypersensitivity reactions are compromised in GM-CSF/IL-3–deficient mice. Mice were sensitized with oxazolone on the abdomen and foot pads on day 0 and challenged on the ear on day 5. Ear thickness was measured with a micrometer. (A) Wild-type versus IL-3–deficient, $P < .0001$. GM-CSF/IL-3 deficient versus IL-3 deficient, $P < .0001$. Similar results were observed in 8 independent experiments on both the C57Bl/6 and Balb/c backgrounds. (B) Defective contact hypersensitivity reactions can be reversed by the administration of GM-CSF and IL-3 protein during initial sensitization. Wild-type versus GM-CSF/IL-3 deficient, $P < .0001$. GM-CSF/IL-3 deficient versus control treatment, not significant. GM-CSF/IL-3 deficient versus GM-CSF/IL-3 treatment, $P < .0001$. Wild-type versus GM-CSF/IL-3 treatment, not significant. Similar results were observed in 5 independent experiments.

hyperkeratosis, and focal intraepidermal abscesses (4+, see "Materials and methods" for description of semiquantitative scoring scheme). GM-CSF/IL-3–deficient animals, in contrast, generated a dramatically less intense cellular infiltrate with much less edema and little keratinocyte activation (trace to 1+). IL-3–deficient mice displayed intermediate reactions (2+) and GM-CSF–deficient animals developed strong reactions (3+), but these were reduced compared to wild-type controls (not shown).

To delineate whether the compromise in contact hypersensitivity reflected a defect during the priming phase of the response, we injected doubly deficient mice with GM-CSF and IL-3 protein at the time of initial hapten application. Remarkably, the administration of these factors resulted in complete reconstitution of the attenuated secondary reaction, as measured both by ear swelling (Figure 3B) and pathologic analysis, where the intensity and character of the corrected response were indistinguishable from wild-type levels (Figure 4D,E). These findings formally establish a dual requirement for GM-CSF and IL-3 during hapten sensitization.

To explore this requirement further, we analyzed the dendritic cells that migrated to the draining lymph node following FITC application in doubly deficient and wild-type animals. Similar numbers of FITC-positive cells were found in both groups, and these cells showed comparable staining for CD11c, MHC II, B7-1, and CD1d (Ox40-ligand was not detected). Additional studies are required to identify which features of dendritic cells are compromised by the absence of GM-CSF and IL-3.

Discussion

The generation of GM-CSF/IL-3–deficient mice has provided a system to test the hypothesis that these molecules mediate redundant functions in vivo. The experiments presented here definitively establish overlapping roles for these cytokines in both hematopoiesis and immunity.

Previous studies demonstrated that IL-3/βc-deficient mice have reduced numbers of circulating eosinophils, likely due to the abrogation of IL-5 signaling.[32] It was thus surprising to find increased numbers of circulating eosinophils in GM-CSF/IL-3–deficient animals. IL-5 likely contributed to this eosinophilia, however, because mice lacking GM-CSF, IL-3, and βc showed decreased numbers of eosinophils. The mechanism underlying the eosinophilia is currently unclear. The numbers of mature eosinophils and their progenitors were not increased in the bone marrow of GM-CSF/IL-3–deficient mice, suggesting that the steady-state production of this lineage is probably not altered. It is possible that the egress of eosinophils from the circulation is compromised, and future investigations aimed at quantifying eosinophil numbers in a variety of tissue populations will help clarify this idea further. A prolongation of eosinophil life span is an additional consideration that needs to be explored. In either case, it is tempting to speculate that IL-5 normally competes with GM-CSF and IL-3 in signaling through βc.

Because recent investigations have highlighted the abilities of GM-CSF and IL-3 to stimulate dendritic cell development,[41] we quantified these cells in the spleen, thymus, and lymph nodes of GM-CSF/IL-3–deficient animals. However, as in our previous studies of mice singly deficient in GM-CSF[4] or IL-3,[25] dendritic cell numbers were not altered in the doubly mutant mice. These results suggest that GM-CSF and IL-3 are either not involved in steady-state dendritic cell development or are components of a larger network of redundant cytokines. In this context, flt3-ligand is likely to play a decisive role, based on its ability to increase dendritic cells in GM-CSF/IL-3–deficient mice by nearly 2 logs. Indeed, a recent report of flt3-ligand knockout mice demonstrated a substantial reduction of dendritic cell numbers in the spleen, thymus, and lymph nodes.[49]

Although GM-CSF/IL-3–deficient mice maintained normal dendritic cell numbers, these animals were markedly compromised in priming contact hypersensitivity reactions. The degree of impairment significantly exceeded that observed in GM-CSF or IL-3 single knockouts, establishing a dual requirement for both cytokines in this response. Although the administration of GM-CSF and IL-3 protein at the time of hapten sensitization reversed the defect, we have not yet identified the specific pathway compromised in GM-CSF/IL-3–deficient mice. Unlike CCR7 knockout mice,[50] which fail to mount contact hypersensitivity

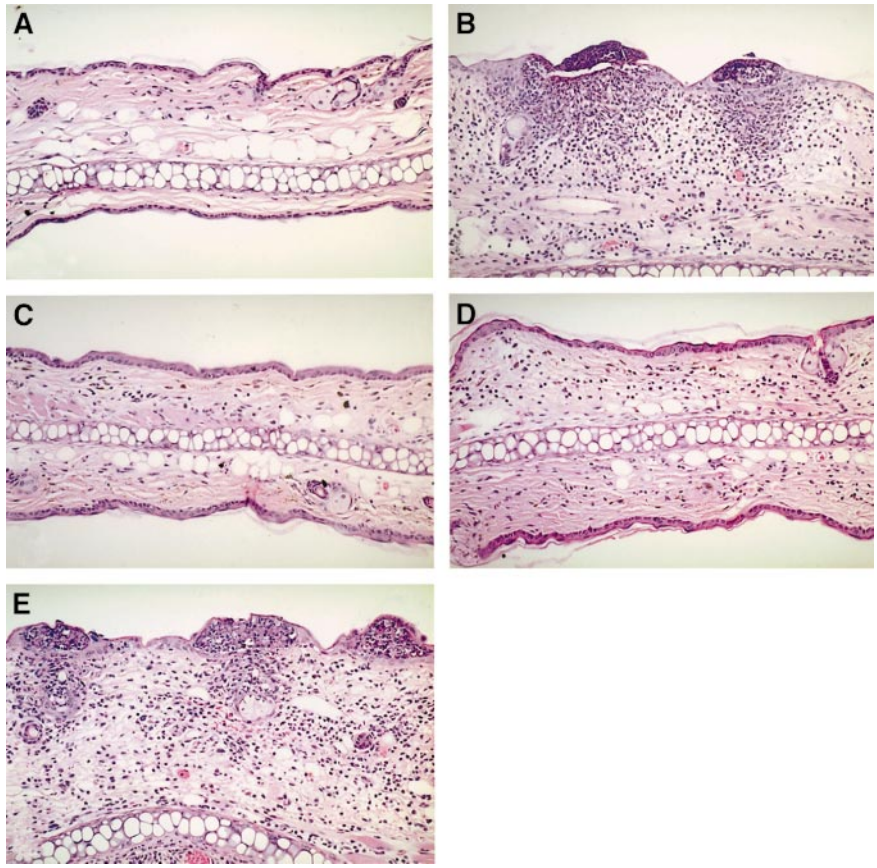

Figure 4. Pathologic analysis of contact hypersensitivity. Ears were harvested 24 hours after secondary oxazolone challenge. (A) Wild-type mouse, diluent only. (B) Sensitized wild-type mouse, hapten challenge. (C) Sensitized GM-CSF/IL-3–deficient mouse, hapten challenge. (D) Sensitized GM-CSF/IL-3–deficient mouse plus control treatment, hapten challenge. (E) Sensitized GM-CSF/IL-3-deficient mouse plus GM-CSF/IL-3 treatment, hapten challenge. All panels at ×200.

reactions due to the lack of dendritic cell migration from the skin to the draining lymph nodes, GM-CSF/IL-3–deficient animals display normal numbers of hapten-loaded dendritic cells in the draining nodes. Moreover, although B7 family members[51,52] and Ox40-ligand[36] also contribute to hapten-specific priming, we were unable to detect differences in these molecules between doubly deficient animals and wild-type controls. Additional experiments are required to elucidate the mechanisms underlying defective hapten sensitization in the absence of GM-CSF and IL-3.

Our own studies have revealed that vaccination with irradiated tumor cells engineered to secrete GM-CSF and, to a lesser extent IL-3, stimulate potent, specific, and long-lasting antitumor immunity.[37,38,53] Although we have not yet examined the susceptibility of GM-CSF/IL-3–deficient mice to tumor induction, recent investigations have established a striking inverse correlation between the ability to generate contact hypersensitivity reactions to polycyclic hydrocarbons and susceptibility to the carcinogenic effects of these agents.[54] These results raise the intriguing possibility that GM-CSF and IL-3 may, like IFN-γ,[55] contribute to cancer immunosurveillance.

Acknowledgments

We thank Glenn Begley (WAIMR) and Lorraine Robb (WEHI) for providing βc-deficient mice and Andy Chen and Arlene Sharpe (Brigham and Womens' Hospital) for providing antibodies against OX40-ligand. We thank Christine Sheehan and Esther Brisson (Albany Medical College) for excellent help with the histologic specimens, Susan Lazo-Kallanian and John Daley for excellent help with the fluorescence-activated cell sorting studies, Patricia Bernardo for excellent help with the statistics, Kurt Edelman for excellent technical help, and the staff of the Redstone Animal Facility for excellent help with maintenance of the mouse colony. We thank Jim Griffin for critical review of the manuscript.

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blood

2009 113: 6658-6668
Prepublished online Mar 12, 2009;
doi:10.1182/blood-2008-06-161075

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

Role of GM-CSF signaling in cell-based tumor immunization

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a potent adjuvant in cancer vaccination; however, the specific role of endogenous GM-CSF remains unknown. We performed cell-based vaccination in 2 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$^{-/-}$, whereas all βc$^{-/-}$ animals died. Altogether, our results strongly suggest that although 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. (Blood. 2009;113:6658-6668)

Introduction

Recent insights into the cellular and molecular mechanisms underlying the host antitumor response have led to the development of several strategies for enhancing antitumor immunity.[1-3] 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.[4-6] 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 antitumor immunity in multiple murine tumor models.[7-10] 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,[12,13] pancreatic,[14] prostate[15] and renal cell carcinoma.[16] Despite the data from animal models and phase 1 clinical trials, the critical role of GM-CSF is not well characterized and several reports have raised concern about potential detrimental effects 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 et al, 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$^+$ regulatory T cells (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 dendritic cells (DCs).[21] Although the critical role of DCs in priming antigen-specific responses is well established,[22] several studies have identified specific DC characteristics that are critical in the induction of a potent antitumor vaccination activity.[23] For example, although both GM-CSF and Flt3-ligand induce the marked expansion of DCs,[24,25] we have shown that GM-CSF–secreting tumor cells promoted higher levels of protective immunity than vaccination with FLT3-L–secreting tumor cells.[10] 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 (which evokes the involvement of natural killer T [NKT] cells) on DCs.[10] We have also shown that tumor protection induced by GM-CSF–secreting tumor cell vaccine was abrogated in CD1d-deficient mice, whereas vaccinated wild-type (WT) mice mount protective tumor immunity.[26] 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 IFN-γ secretion and tumor-specific cytotoxicity remained unchanged.[26]

Previous mouse studies exploiting gene-targeting techniques or neutralizing antibodies have established that both CD4$^+$ and CD8$^+$ T cells are required for efficient vaccination.[7,9] Other investigations

Submitted June 4, 2008; accepted February 19, 2009. Prepublished online as *Blood* First Edition paper, March 12, 2009; DOI 10.1182/blood-2008-06-161075.

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have revealed a central role for $CD4^+$ T cells in the production of IFN-γ and IL-4 and the activation of eosinophils and macrophages to produce nitric oxide, and reactive oxygen species in GM-CSF–secreting tumor vaccination.[9] 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.[27]

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 antitumor efficacy.[2,28] 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.[29] Together, these studies reveal important roles for GM-CSF in both the priming and effector phases of antitumor 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 granulocyte and macrophage expansion and it also induces dendritic cell recruitment and maturation. Most of GM-CSF's 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.[30] GM-CSF–deficient mice showed modestly reduced numbers of DC populations in hematopoietic organs and tissue.[31] Study of GM-CSF–deficient mice revealed abnormal alveolar macrophage function with decreased surfactant clearance, leading to alveolar proteinosis.[30] Spontaneous tumors were not described but increase in mortality to several pathogens, mainly encapsulated organisms, has been reported.[32,33]

In an effort to further clarify the requirement for endogenous GM-CSF in antitumor immunity, we used 2 distinct antitumor vaccination models in 2 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 ($GM\text{-}CSF^{-/-}$), IL-5 ($IL\text{-}5^{-/-}$), or βc ($\beta c^{-/-}$) 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, whereas IL-3 activity is maintained because of an additional β subunit, specific for IL-3 ($\beta\text{-}_{IL\text{-}3}$).[34] 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 $GM\text{-}CSF^{-/-}$, $IL\text{-}3^{-/-}$, GM-CSF + $IL\text{-}3^{-/-}$, and $\beta c^{-/-}$ in BALB/c background as well as WT littermates.

Methods

Animals

Mice deficient in GM-CSF,[30] βc,[35] IL-3,[36] and both IL-3 and GM-CSF[37] were backcrossed at least 9 generations onto the C57BL/6 and BALB/c strains. IL-5–deficient mice[38] were generated in a pure C57BL/6 background. Animals studied were at least 10 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 (mAbs) and isotype controls were purchased from BD Pharmingen (San Diego, CA) unless stated: phycoerythrin (PE)–conjugated anti-CD11c (hamster IgG, clone HL3) and isotype control (hamster IgG, clone A19-3); FITC-labeled mAbs 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); AlexaFluor488–conjugated anti-F4/80 (rat IgG2a, clone BM8; Caltag Laboratories, Burlingame, CA), and isotype controls (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.[10] GM-CSF secretion was approximately 150 ng/10^6 cells per 24 hours, as determined by enzyme-linked immunosorbent assay (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, Mannheim, Germany).

Tumor models

For tumorogenicity experiments, C57BL/6 and BALB/c mice were injected subcutaneously in the interscapular region with 1×10^5 live B16 and 2×10^6 live RENCA cells, respectively. Animals were killed when tumors reached 10 mm in diameter or became ulcerated. For vaccination experiments, C57BL/6 mice were injected subcutaneously in the abdomen with 1×10^6 irradiated (35 gray) B16-GM cells and challenged 7 days later with 5×10^5 live B16 cells injected subcutaneously in the upper back; BALB/c mice were injected subcutaneously in the abdomen with 1×10^6 irradiated (35 gray) RENCA cells and challenged 7 days later with 5×10^6 live RENCA cells injected subcutaneously in the upper back. Mice were followed for 3 months after tumor challenge. In all experiments, confirmation of genotype was performed by polymerase chain reaction (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 (100 gray) B16 cells (2×10^4) in 2 mL complete medium supplemented with 10 units/mL IL-2. Supernatants were harvested after 5 days and assayed for GM-CSF, IL-5, IL-10, IL-13, and IFN-γ by ELISA using the appropriate monoclonal antibodies (Endogen, Woburn, MA and BD Biosciences, Erembodegem, Belgium).

GM-CSF ELISA

The GM-CSF secretion from RENCA cells was determined by ELISA Kit (OptEIA from BD Biosciences, Erembodegem, Belgium), as indicated by the manufacturer. Briefly, the RENCA cells were irradiated at 35 gray and seeded at a density of 10^6 cells per well. The GM-CSF release in the supernatant was measured after 24 hours.

Cytokine measurement using 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 cytometric bead assay (CBA) system (BD PharMingen). Standard curves were determined for

each cytokine from a range of 20 to 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 4% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone; Sigma-Aldrich, St Louis, MO) in acetone–olive oil (4:1). Mice were challenged 5 days later on the right ear with 20 μL 0.5% oxazolone in acetone–olive oil and with carrier acetone–olive oil alone on the left ear. The ear thickness was measured on both ears with a micrometer at 24 hours after challenge. Results are presented as the increased thickness of the hapten-treated ear minus the nonspecific swelling (carrier-treated ear). Data represent mean values of 5 mice per group. Similar results were obtained in 3 independent experiments.

Histopathology

Vaccination sites were removed at day 5 and processed for pathologic examination. Tissues were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin, cut to 5-micrometer thickness, and stained with hematoxylin and eosin (H&E). Images were viewed with an Axioskop 2 plus microscope (Göttingen, Germany) and taken with an AxioCam HRc serie1.6 camera (Carl Zeiss, Jena, Germany). Software used was AxioVision 3.1 (Carl Zeiss).

Flow cytometric analysis of cells at the vaccination site

Mice were immunized subcutaneously with 10^6 irradiated tumor cells (B16 or B16-GM). Four days later, mice were killed, 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 collagenase I (Sigma-Aldrich) and 2 mg/mL hyaluronidase I (Sigma-Aldrich) at 37°C during 30 minutes. 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 FACSCalibur cytometer (Becton Dickinson, Mountain View, CA). Living cells were identified by the nonpermeant DNA dye 7-amino-actinomycin D (Sigma-Aldrich). Data were analyzed with WINMDI software written by J. Trotter (Scripps, La Jolla, CA) and Cell-Quest software (Becton Dickinson). Fluorescence-activated cell sorting (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 2-tailed Student *t* test was used to evaluate *P* values between experimental groups. A *P* value less than .05 was considered statistically significant.

Results

Comparison of tumor immunity among WT, GM-CSF$^{-/-}$, IL-5$^{-/-}$, and βc$^{-/-}$ mice

Because 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 antitumor immunity. In these experiments, adult female C57BL/6 GM-CSF$^{-/-}$ animals and littermate controls were vaccinated with 10^6 irradiated B16-GM and challenged 1 week later with 5×10^5 live B16 cells. Consistent with previous reports, vaccinated WT mice efficiently rejected tumor challenge (long-term protection of 75% of animals), whereas nonimmunized WT animals uniformly developed progressive tumors (Figure 1A). Interestingly, vaccinated GM-CSF$^{-/-}$ mice showed tumor protection that was equivalent to immunized WT controls, with 75% of animals rejecting tumor challenge (Figure 1A).

Studies have shown that although βc mediates most GM-CSF signaling, some evidence suggests that the α chain alone may transduce signals.[40] 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.[41] To learn more about the pathways stimulated by GM-CSF–secreting tumor cells, we have evaluated the ability of βc$^{-/-}$ mice to generate tumor immunity. Although the tumorigenicity of B16 cells in WT, GM-CSF$^{-/-}$, βc$^{-/-}$, and GM-CSF + IL-3 double knockout mice appeared the same (Figure 1C), vaccination with irradiated B16-GM cells failed to induce any tumor protection in βc$^{-/-}$ mice (Figure 1A). All mutant animals rapidly developed growing tumors at the challenge site, with kinetics comparable with unvaccinated WT animals (Figure 1A). Because βc is also implicated in IL-5 signaling,[35,42] we therefore evaluated the role of endogenous IL-5 to generate tumor immunity. IL-5$^{-/-}$ mice vaccinated with irradiated B16-GM cells showed a 75% survival rate, in comparison with an 80% survival rate in WT mice (Figure 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 5×10^6 live RENCA cell challenge (Figure 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. Whereas all vaccinated βc$^{-/-}$ animals succumb to tumor challenge, all vaccinated WT and GM-CSF$^{-/-}$ mice showed protective antitumor response (Figure 2A). We then evaluated the tumorogenicity of RENCA cells in different knockout mice and found out that this remains identical among WT, GM-CSF$^{-/-}$, βc$^{-/-}$, and GM-CSF + IL-3$^{-/-}$ double knockout mice (Figure 2B).

As vaccination with RENCA cells induces 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 antitumor 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 (Figure 2C). In contrast, unmodified B16-F10 melanoma cells do not release any GM-CSF (Figure 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 (Figure 2A).

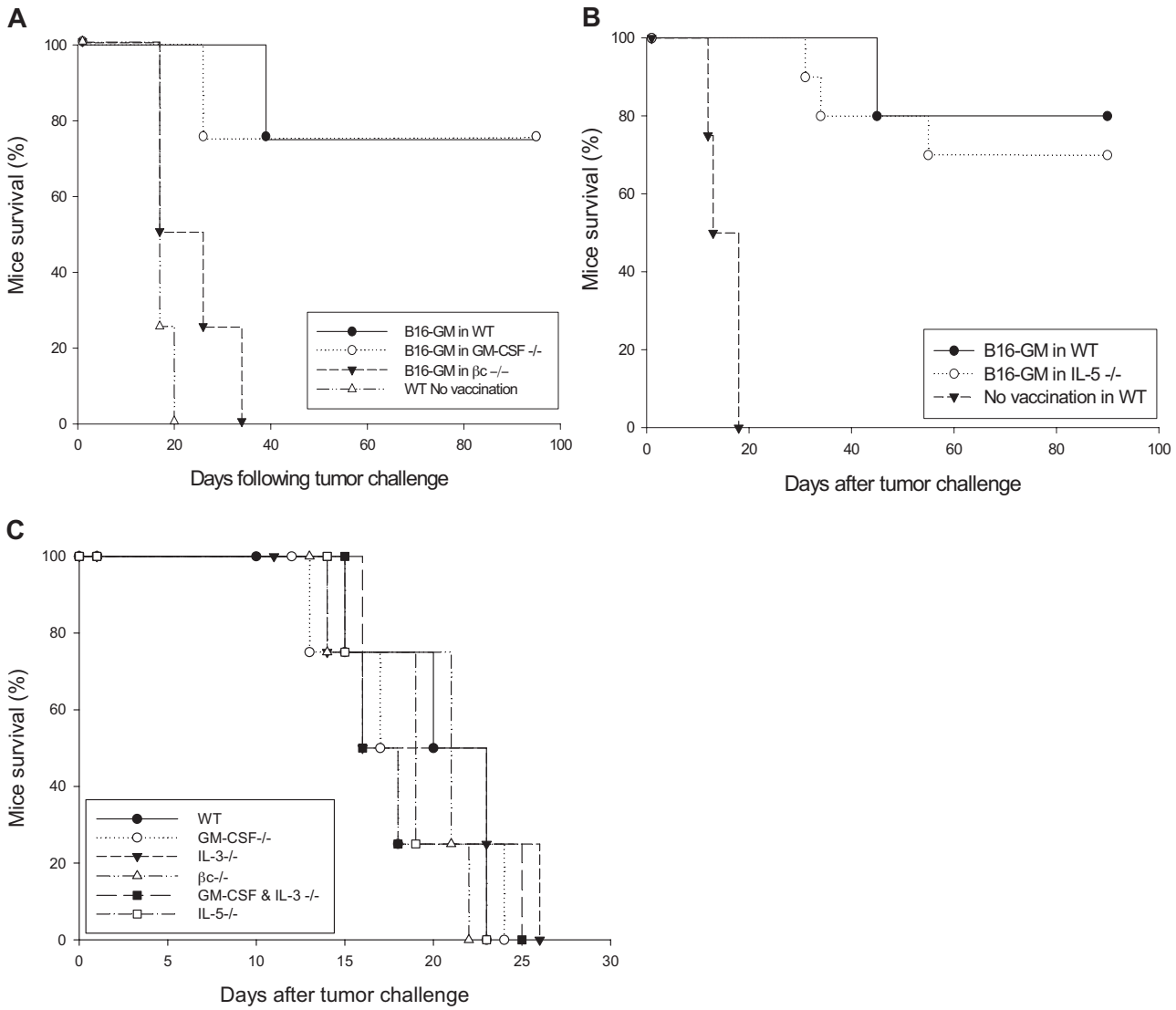

Figure 1. GM-CSF–secreting tumor cell vaccination in WT, GM-CSF, IL-5, or βc knockout C57BL/6 mice. (A) GM-CSF$^{-/-}$, βc$^{-/-}$, WT, or (B) IL-5$^{-/-}$ and WT C57BL/6 mice were immunized subcutaneously on the abdomen with 10^6 irradiated, B16-GM cells (5 per group). One week later, the vaccinated mice as well as the unvaccinated WT controls, were challenged subcutaneously on the back with 5×10^5 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 3 independent experiments. (C) Tumorogenicity of B16-F10 cells in different knockout mice. Survival of WT and indicated knockout C57BL/6 mice inoculated with 5×10^5 B16-F10 cells. This experiment is representative of 3 independent experiments.

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 and B16-FL induces 40% and 25% (respectively) of survival upon tumor challenge compared with 75% tumor protection after B16-GM vaccination (Figure 3A). This is consistent with the previously published data where GM-CSF remains the most potent cytokine in the induction of tumor protection.[7] On the contrary, none of these cytokine-producing cells was able to protect βc$^{-/-}$ mice, with all mice showing progressive tumor growth upon challenge (Figure 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, IFN-γ, TNF-α, and IL12p70 using CBA system. Irradiated B16 WT, B16-FL, B16-IL3, and B16-GM cells (10^6) 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 (Figure 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 after GM-CSF–based tumor vaccination in different knockout mice

Vaccination with irradiated B16-GM cells stimulated comparable antitumor 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 (Figure 4A). To delineate the basis for the loss of antitumor immunity in βc$^{-/-}$ mice, we characterized the generation

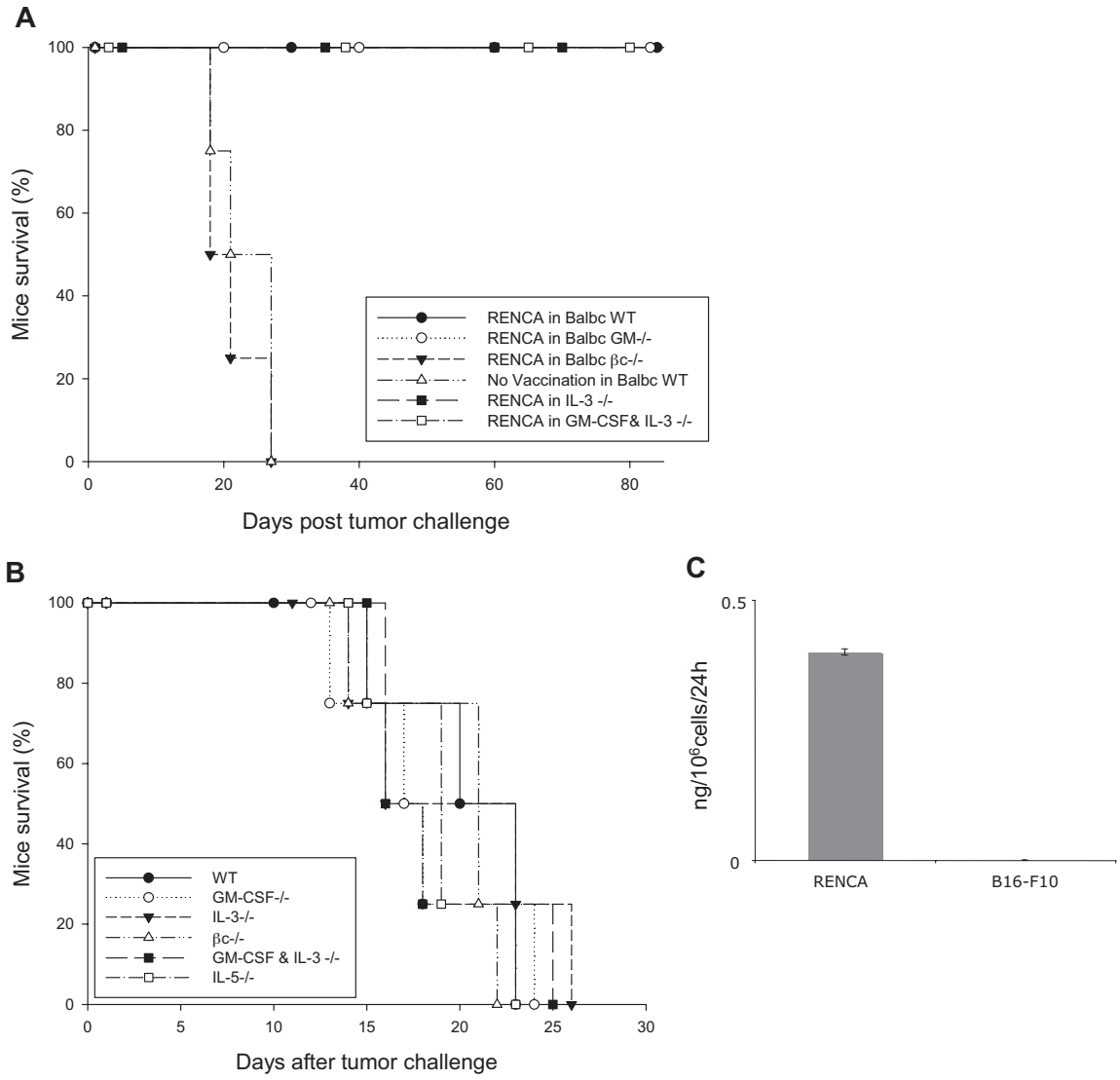

Figure 2. Tumor protection of RENCA cells in WT and different knockout BALB/c mice. (A) WT and indicated knockout BABL/c mice were vaccinated with 10^6 irradiated RENCA cells and challenged 1 week later with 5×10^6 RENCA tumor cells. The survival curve indicates percentage of mice that survived the challenge. The graphic represents data of 4 independent experiments (n = 4 mice/group). (B) Tumorogenicity of RENCA cells in different knockout mice. WT and indicated knockout BALB/c mice were inoculated on the back with 2×10^6 RENCA tumor cells. Mice were killed when tumor size reached 15 mm in diameter or was ulcerated. This experiment is representative of 3 independent experiments. (C) Spontaneous production of GM-CSF by RENCA cells. Irradiated RENCA or B16-F10 cells (10^6) were seeded in a 10-mm plate and GM-CSF release was detected by enzyme-linked immunosorbent assay (ELISA) from their supernatant after 24 hours. The graph is representative of 3 independent experiments.

of antitumor 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 (Figure 4B). Furthermore, consistent with the results of tumor immunity (Figure 1B), the development of antitumor effector response in immunized IL-5$^{-/-}$ mice was comparable with WT animals (Figure 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 (Figure 4C).

Histopathologic analysis of the immunization site in different knockout mice vaccinated with B16-GM

To understand the lack of vaccination efficacy in βc$^{-/-}$ mice, we performed histopathologic analyses of the site of tumor antigen capture 5 days after B16-GM immunization. This enabled us to investigate potential defects in the early phase of the response. In WT (Figure 5A) and GM-CSF$^{-/-}$ (Figure 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 (Figure 5C) except for reduced eosinophils. In contrast, the vaccination site in βc$^{-/-}$ mice showed minimal infiltrates and no inflammation (Figure 5D). Interestingly, this reaction was comparable with the response evoked in WT mice by vaccination with irradiated unmodified B16 cells (Figure 5E).

βc$^{-/-}$ mice immunized with GM-CSF–secreting tumor cells failed to recruit myeloid DCs at the site of vaccination

It has been shown that GM-CSF–secreting tumor cells induce the generation of potent antitumor immunity by increasing the local recruitment and maturation of myeloid-derived DCs.[10] We therefore evaluated the capacity of immunized GM-CSF$^{-/-}$, IL-5$^{-/-}$, and βc$^{-/-}$ mice to recruit DCs at the vaccination site.

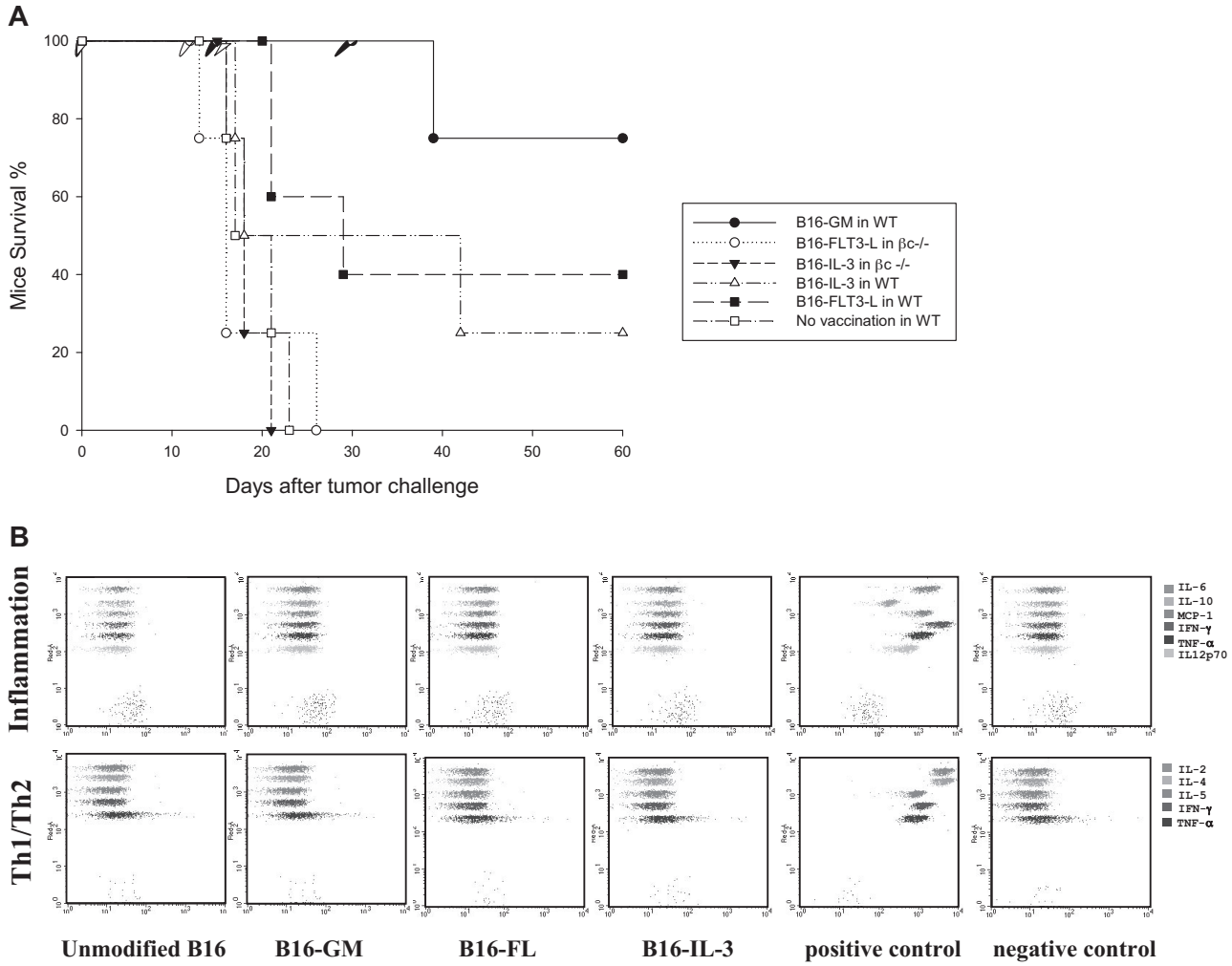

Figure 3. Tumor protection in WT and βc knockout mice vaccinated with different cytokine-producing tumor cells. (A) Survival of C57BL/6 mice vaccinated subcutaneously with irradiated cytokine-producing B16 cells. Mice were vaccinated with 10^6 B16 cells secreting GM-CSF, FLT3-L, or IL-3 and challenged 1 week later with 5×10^5 viable unmodified B16 cells. The graph is representative of 3 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 was collected after 24 hours and IL-2, IL-4, IL-5, IL-6, IL-10, IL12p70, MCP-1, IFN-γ, and TNF-α were measured using inflammation and Th1/Th2 CBA kits. The positive and negative controls represent the DMEM complete medium and the 1250 pg/mL CBA standard curve, respectively. Each plot is representative of 1 single cell supernatant and illustrates 1 of the 2 independent experiments.

WT mice vaccinated with irradiated B16-GM tumor cells have significantly increased numbers of $CD11c^+CD11b^+$ DCs (36% ± 3.4%; SEM of 6 independent experiments) compared with WT animals vaccinated with irradiated unmodified B16 tumor cells (3.4% ± 1.9%; SEM of 5 independent experiments; Figure 6A). Furthermore, all recruited $CD11b^+CD11c^+$ DCs were $CD8\alpha^-$ (data not shown).

Similarly to WT animals, DC recruitment was increased after B16-GM immunization in GM-$CSF^{-/-}$ (43% ± 3.3%; SEM of 6 independent experiments; Figure 6B) and IL-$5^{-/-}$ (39% ± 3.9%; SEM of 6 independent experiments) mice (Figure 6C). GM-$CSF^{-/-}$ (5% ± 0.5%; SEM of 6 independent experiments; Figure 6B) and IL-$5^{-/-}$ mice (3% ± 0.6%; SEM of 5 independent experiments; Figure 6C) vaccinated with unmodified B16 tumor cells showed a low percentage of DC recruitment that was similar to WT mice. In contrast, $\beta c^{-/-}$ mice fail to recruit DCs after B16-GM vaccination (4% ± 0.9%; SEM of 3 independent experiments; Figure 6D). This recruitment was similar to the vaccination of $\beta c^{-/-}$ mice with B16 WT tumor cells (4.25% ± 0.6%; SEM of 3 independent experiments; Figure 6D). Together, these observations suggest that the loss of antitumor immunity in $\beta c^{-/-}$ 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 $\beta c^{-/-}$ mice

To address whether $\beta c^{-/-}$ 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 DCs, after their migration to regional lymph nodes, to hapten-specific $CD4^+$ and $CD8^+$ T lymphocytes.[43] Upon secondary hapten challenge, sensitized T cells initiate a local inflammatory response. As shown in Figure 7, $\beta c^{-/-}$ 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

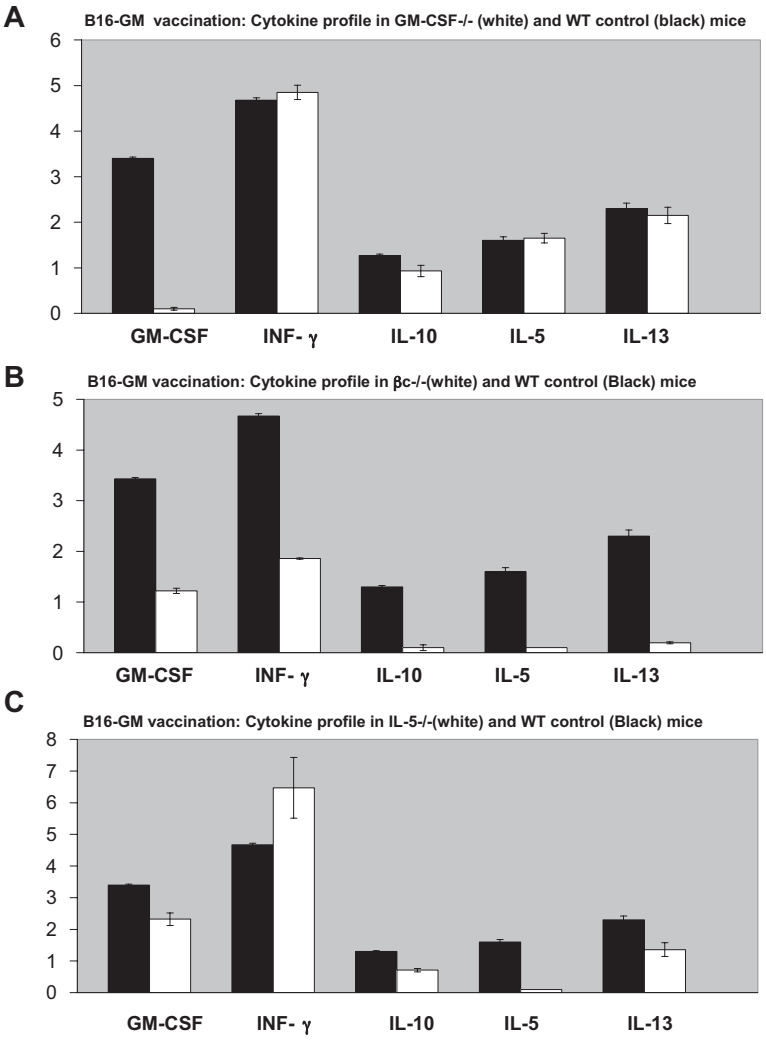

Figure 4. Comparison of tumor-induced cytokine profile in B16-GM vaccinated mice. Quantification of cytokines release by ELISA in cell suspension supernatants from spleen of B16-GM immunized C57BL/6 mice (■) or knockouts (A) GM-CSF$^{-/-}$ (B) βc$^{-/-}$ and (C) IL-5$^{-/-}$ mice (□), cocultured with irradiated B16 cells during 5 days, in the presence of IL-2. Values are in nanograms per milliliter. Error bars represent the standard deviation from triplicate samples of 1 single experiment. Similar results were obtained on 3 independent experiments.

effector phases of antitumor 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.[17] In animal studies, GM-CSF has proven active when delivered or produced at the vaccination site. The adjuvant or antitumor immunization effect is thought 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 to 300 ng/10^6 cells per 24 hours.[7,44] In contrast, local release of high-dose GM-CSF at the vaccination site or injected intraperitoneally has been shown to block the immune response leading to the down-regulation of immune defense in antitumor reactions.[18] In addition, high doses of GM-CSF injected subcutaneously have shown to be detrimental, resulting in the expansion of myeloid suppressor cells.[19]

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 antitumor 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 2 different antitumor 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 antitumor immunization in at least 2 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 both GM-CSF–secreting B16 cells and the unmodified RENCA cells was completely abrogated in βc$^{-/-}$ mice. This was associated with a failure

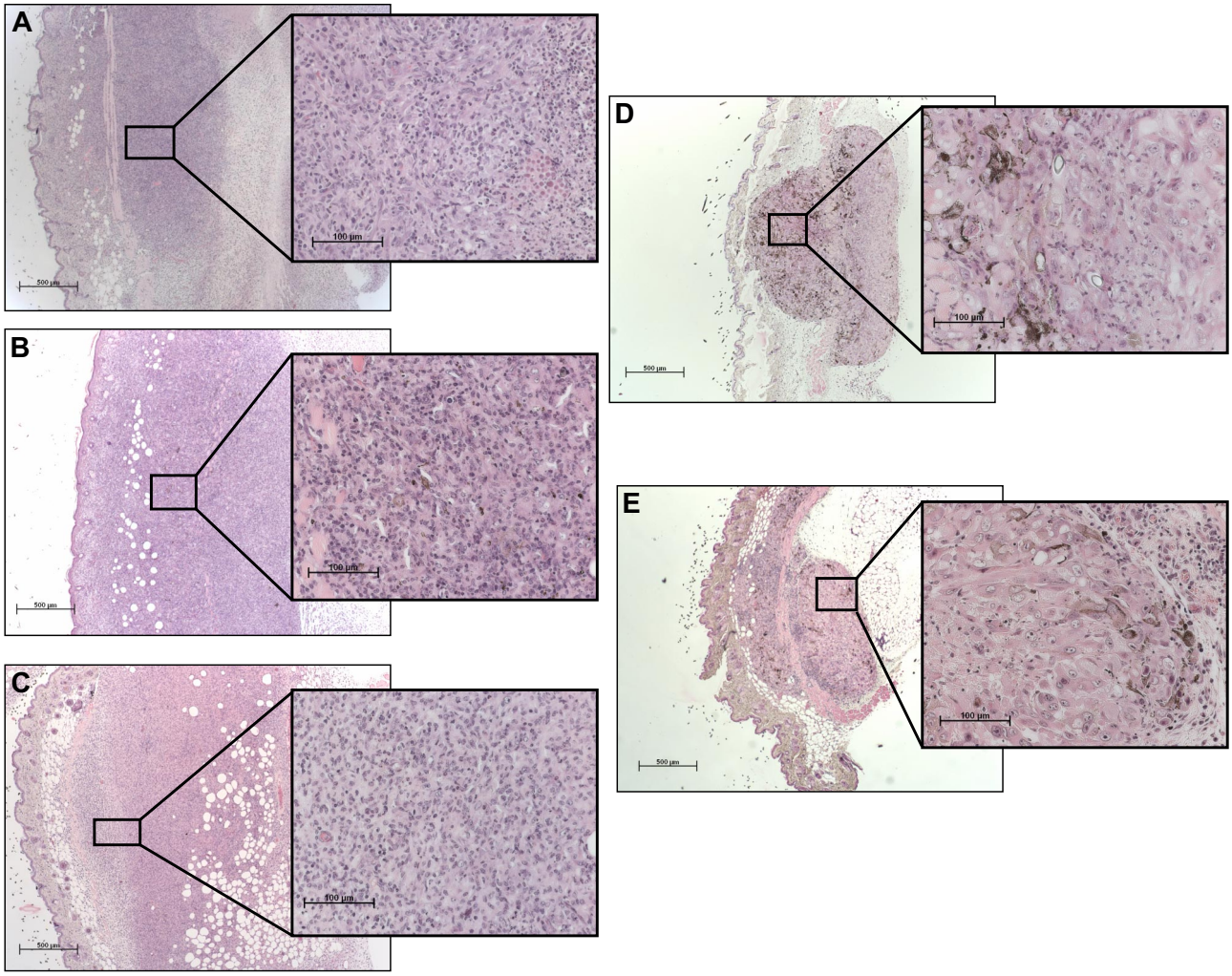

Figure 5. Histopathologic analysis of the vaccination site. (A) WT, (B) GM-CSF$^{-/-}$, (C) IL-5$^{-/-}$, and (D) βc$^{-/-}$ mice were immunized subcutaneously on the abdomen with 10^6 irradiated B16-GM cells. As control, WT mice were immunized with 10^6 irradiated B16 cells (E). Five days later, tissue samples were collected and fixed in formalin before paraffin embedding. Samples were then stained with hematoxylin and eosin. Magnification: 10 × 2.5 and 10 × 20.

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 ($\beta_{IL\text{-}3}$), 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 antitumor 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.

Because βc 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 2 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 βc$^{-/-}$ 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 antitumor effectors in immunized GM-CSF$^{-/-}$ animals was also similar to WT littermates. Although 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-γ. The inability to uptake apoptotic cells by GM-CSF$^{-/-}$ antigen-presenting cells (APCs) led to autoimmunity

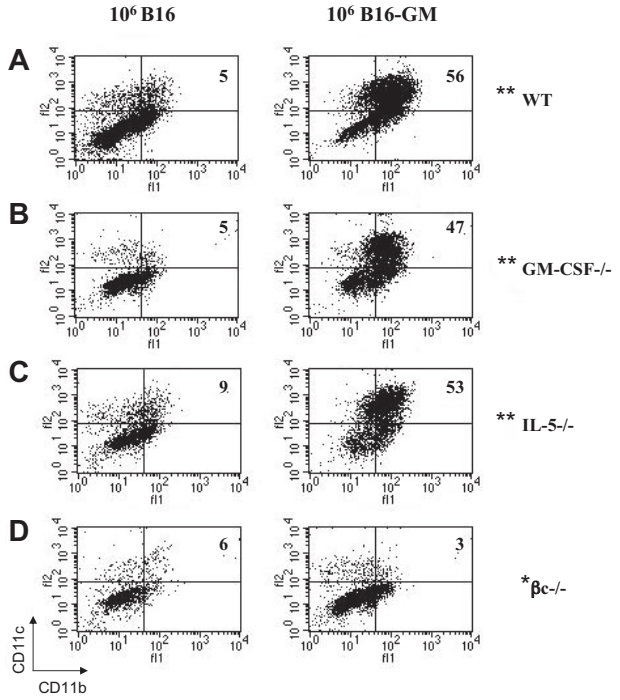

Figure 6. Flow cytometric analysis of DC recruitment at the site of vaccination after B16 or B16-GM immunization. C57BL/6 WT (A), GM-CSF$^{-/-}$ (B), IL-5$^{-/-}$ (C), and βc$^{-/-}$ (D) mice were immunized with irradiated B16 or B16-GM (10^6 cells). Four days later cells at the vaccination sites were isolated and subsequently analyzed by flow cytometry for the presence of CD11c$^+$, CD11b$^+$, and CD8α$^+$ DCs. Results are percentages and are representative of 1 of at least 3 animals per group. The difference observed between B16 and B16-GM in each group was highly significant (**$P \leq .005$) except for the βc$^{-/-}$ group (*$P = .45$).

via decreased numbers of regulatory T cells (Tregs), whereas double knockout mice lacking both GM-CSF and IFN-γ showed a marked increase in the incidence of both solid and hematologic tumors.[20,47]

Finally, tumor vaccination in βc$^{-/-}$ mice was completely inefficient and this was associated with a marked decrease in IL-5, IL-10, and IL-13 production and lack of inflammatory cells influx at the vaccination site (Figures 4B, 5D). This is probably due to the lack of DC recruitment at the site of vaccination in βc$^{-/-}$ mice (Figure 6D) that is mandatory in the initiation of effective antitumor 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,[48] 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 a 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 Th2 responses.[49] 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 antitumor immunization. Working hypotheses include the lack of recruitment and/or differentiation of a subclass of DCs 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.[50] 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 antitumor immunity observed in βc$^{-/-}$ mice and may bring further understanding to improve cell-based antitumor immunization schemes.

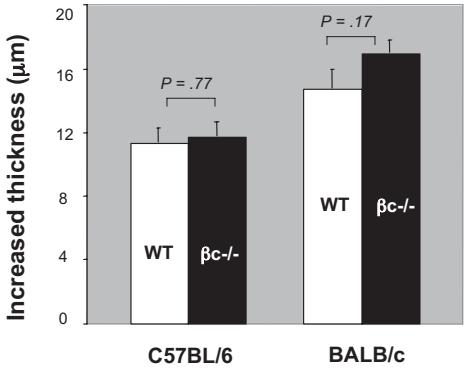

Figure 7. Contact hypersensitivity reactions in βc$^{-/-}$ and WT mice. C57BL/6 and BALB/c mice were sensitized with oxazolone on the abdomen and foot pads on day zero. Five days later, mice were challenged on the right ear with 0.5% oxazolone in acetone–olive oil. The left ear was treated with the carrier (acetone–olive oil) alone. The ear thickness was measured with a micrometer at 24 hours after challenge. Results are presented as the increased thickness of the hapten-treated ear minus the nonspecific swelling (carrier-treated ear). Data represent mean values of 5 mice per group. Similar results were obtained in 3 independent experiments.

Acknowledgments

The authors thank Glenn Begley for providing the βc$^{-/-}$ mice and for his comments and helpful discussions and Paul Walker for carefully reading the paper.

This work was supported by the Swiss National Science Foundation (PNR 37) (Berne, Switzerland), La Ligue genevoise contre le cancer (Geneva, Switzerland), la Fondation pour la lutte contre le cancer (Zurich, Switzerland; N.M.), the Cancer Research Institute/Partridge Foundation (New York, NY), and CA 74886 (G.D). G.D. is a Clinical Scholar of the Leukemia & Lymphoma Society (White Plains, NY).

Authorship

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

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Cancer Gene Therapy (2011), 1–10

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ORIGINAL ARTICLE

Cell encapsulation technology as a novel strategy for human anti-tumor immunotherapy

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant in autologous cell-based anti-tumor immunotherapy has recently been approved for clinical application. To avoid the need for individualized processing of autologous cells, we developed a novel strategy based on the encapsulation of GM-CSF-secreting human allogeneic cells. GM-CSF-producing K562 cells showed high, stable and reproducible cytokine secretion when enclosed into macrocapsules. For clinical development, the cryopreservation of these devices is critical. Thawing of capsules frozen at different time points displayed differences in GM-CSF release shortly after thawing. However, similar secretion values to those of non-frozen control capsules were obtained 8 days after thawing at a rate of >1000 ng GM-CSF per capsule every 24 h. For future human application, longer and reinforced capsules were designed. After irradiation and cryopreservation, these capsules produced >300 ng GM-CSF per capsule every 24 h 1 week after thawing. The *in vivo* implantation of encapsulated K562 cells was evaluated in mice and showed preserved cell survival. Finally, as a proof of principle of biological activity, capsules containing B16-GM-CSF allogeneic cells implanted in mice induced a prompt inflammatory reaction. The ability to reliably achieve high adjuvant release using a standardized procedure may lead to a new clinical application of GM-CSF in cell-based cancer immunization.
Cancer Gene Therapy (2011) **0,** 000–000. doi:10.1038/cgt.2011.22

Keywords: cell encapsulation; cytokine; drug release; genetic engineering; immunostimulation

Introduction

Considerable advances in the field of anti-tumor immunotherapy have resulted in promising strategies for the treatment of malignancies. More recently, it has led to the first Food and Drug Administration approved clinical application that uses an autologous dendritic cell-based strategy. Various approaches have been developed, such as the administration of tumor peptides or epitope-enhanced peptides,[1,2] naked DNA expression plasmids,[3] pulsed dendritic cells[4,5] and the use of whole tumor cells.[6,7] Autologous tumor cells contain all the tumor-associated antigens, rendering their application interesting in avoiding the isolation of one specific epitope and allowing a broader anti-tumor immune response. Allogeneic tumor cells have also been extensively studied, but their survival in the recipient is short as they are quickly eliminated by the host immune response (human leukocyte antigen-dependent and innate natural killer-dependent mechanisms). Furthermore, allogeneic tumor cells may not have the necessary antigens to stimulate a specific immune response against the host's own tumor.

To enhance the host immune response, autologous or allogeneic tumor cells have been genetically engineered to produce various cytokines.[8] This strategy was applied in several cell-based vaccination trials in animal models and was evaluated for the immunomodulating activities or adjuvant effects of the cytokines on tumor rejection.[9–14] Granulocyte-macrophage colony-stimulating factor (GM-CSF) was revealed to have a potent stimulation effect on cells at the immunization site, enhancing the tumor-associated antigens' processing and presentation to CD4+ and CD8+ T cells.[15] This approach has been shown to induce potent, specific and long-lasting anti-tumor immunity in mice previously vaccinated with GM-CSF-producing irradiated tumor cells, leading to the rejection of small tumor burden of melanoma cells and lung, colon and renal cancer cells.[10] The use of *ex vivo*

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Received 4 October 2010; revised 12 January 2011; accepted 10 March 2011

genetically modified tumor cells producing GM-CSF as an immunization method has been extended to a variety of animal tumor models, such as leukemia,[16] melanoma[17] and glioma.[18] Furthermore, preclinical and clinical trials, including patients suffering from renal cell carcinoma,[19] melanoma,[20,21] prostate cancer,[22] pancreatic cancer[23] or non-small-cell lung cancer,[24,25] have also been performed. These promising studies have demonstrated the ability of GM-CSF to induce an efficient anti-tumor immune response when produced locally at the tumor cell injection site, and displaying minimal toxicity and no systemic adverse reaction. The autologous approach, however, imposes a requirement for individualized genetic engineering of autologous tumor cells leading to unpredictable levels of GM-CSF secretion.

To circumvent these limitations, a strategy for genetically modifying an allogeneic cell line that can act as a bystander cytokine producer at the vaccination site, was developed. The immunization is then performed by co-injection of the bystander cells and unmodified autologous tumor cells. In a mouse model, a major histocompatibility complex-negative allogeneic cell line genetically engineered to produce GM-CSF was shown to afford an anti-tumor immune response that was equivalent to or better than those achieved using genetically modified autologous tumor cells.[26] Furthermore, the investigators reported the generation of the modified K562 human erythroleukemia cell line, which produces large quantities of GM-CSF, for use in clinical applications. However, using allogeneic tumor cells engineered to produce a stable amount of the cytokine leads to the rapid destruction of the cells, and it does not allow for a sustained delivery of the adjuvant over several days. Recently, a phase III clinical trial in prostate cancer was prematurely terminated after analysis of the early data. In addition, the immune response against the newly exposed allogeneic antigens of the bystander cell line rather than the desired tumor-associated antigens potentially skews the immune reaction. From a clinical point of view, an ideal immunotherapy approach should combine the benefits outlined above, and a prolonged and predictable release of the immunmodulatory molecule at the injection site, and the ability to remove the source of cytokine production in case of side effects. Cell encapsulation technology may satisfy these criteria by allowing cell transplantation across an immunological barrier and preventing the contact between the encapsulated cells and the host immune cells. In addition, the properties of the permeable membrane of the capsule ensures a selective influx of molecules essential for cell survival, and a selective outflow of metabolic byproducts and the cytokine of interest. This strategy has been studied extensively as a sustained delivery system to provide biologically active molecules like erythropoietin,[26–29] coagulation factors[30] and neurotrophic factors.[31,32] Clinical trials have been conducted in diabetic patients[33] and patients suffering from amyotrophic lateral sclerosis.[34] Further studies of the biomaterial compatibility[35] and the host immune reaction, to obtain long-term encapsulated cells survival,[29,36] have been carried out.

Here, we report the development of a novel strategy for clinical anti-tumor immunotherapy using a physically immuno-isolated GM-CSF-producing bystander cell line enclosed into hollow fiber macrocapsules.

Materials and methods

Cell lines

The K562 cell line is a human erythroleukemia cell line[37] supplied by the American Type Culture Collection (ATCC, Rockville, MD). K562 cells were cultured in RPMI 1640 medium (GIBCO-BRL, Life Technologies, Baltimore, MD), supplemented with 10% fetal calf serum, 50 U ml^{-1} penicillin–streptomycin, 2 mM L-glutamine and grown in suspension culture at 37 °C in a 5% CO_2 humidified environment. K562-hGM-CSF cells were obtained by transfecting K562 cells using the standard calcium phosphate precipitation method with the pUCMD-hGM-CSF plasmid and the puromycin resistance gene-containing pJ6Omega-puro plasmid. K562-hGM-CSF cells were selected in culture medium containing 2 mg ml^{-1} puromycin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). A master cell bank and clinical production lots were tested for sterility (Harvard Gene Therapy Initiative, Harvard Medical School, Boston, MA).

The B16 mouse melanoma cell line was obtained from ATCC and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 U ml^{-1} penicillin–streptomycin. B16 cells were genetically modified for the expression of mGM-CSF using retroviral-mediated gene transfer, as previously described.[38]

Capsule design

One-centimeter long macrocapsules were obtained by cutting hollow fibers of polyethersulfone (Azko Nobel Faser, Wupperthal, Germany) with a molecular weight cut-off of 280 kDa, an external diameter of 720 mm and an internal diameter of 524 mm. Before cell loading, syringe hub adaptors were glued to one extremity with a photopolymerizable acrylate-based glue (Luxtrack LCM 23, Notestik, Electronic Materials and Adhesives, Rancho Dominguez, CA) illuminated for 30 s at a wavelength of 460 nm. For encapsulation of adherent cells, such as B16 cells, scaffold matrices were inserted into capsules. The matrices were obtained from a polyvinyl alcohol sponge provided by Rippey Corporation (El Dorado Hills, CA) using a hollow drill with an internal diameter corresponding to the inner dimensions of the capsule. The polyvinylalcohol rods were washed in sterile ultra-pure water, dried at room temperature and inserted into the polyether sulfone capsules before sealing the extremity with the acrylate-based glue. The fibers were finally sterilized with ethylene oxide at 55 °C and kept 10 days at room temperature to eliminate any traces of gas. Capsules designed for human application were cut at a length of 2 cm, and were reinforced by insertion of a titanium coil grade 2 (0.2 × 0.1 mm) (Heraeus, Cossonay, Switzerland) into the capsule.

Cell encapsulation

Cells were diluted with culture medium to obtain a suspension of 10^4 cells ml^{-1}. Using a 50 ml syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) fitting the adaptor hub, 10 ml or 20 ml of cell suspensions (10^5 or 2×10^5 cells) were injected into a 1-cm or 2-cm long microporous membrane, respectively. The hub adaptor was cut-off, and the extremity of the capsule sealed as described above. The loaded capsules were kept in culture medium at 37 °C in a 5% CO_2 humidified environment for the duration of the experiment. The culture medium was replaced three times a week.

Capsules freezing and thawing

After loading of the K562-hGM-CSF cells, the capsules were maintained in culture medium at 37 °C in a 5% CO_2 humidified environment for 1 or 3 days before freezing. At that time, capsules were placed in small silicone tubes (Sococchim SA, Lausanne, Switzerland) measuring 2.5 cm in length with an internal diameter of 3 mm and an outer diameter of 3.8 mm. These tubes had previously been sealed at one end with the acrylate-based glue as described above and sterilized with ethylene oxide at 55 °C. Tubes were filled with approximately 300 µl of culture medium supplemented with 10% dimethylsulfoxide (Axon Lab AG, Baden-Daettwil, Switzerland) and placed vertically in conventional tubes for cell storage without culture medium. The capsules were then frozen using an automated freezer with a program cooling 1 °C min^{-1} until −80 °C. The thawing procedure consisted of placing the silicone tubes in six-well culture dishes containing complete culture medium at 37 °C. After extraction of the capsules from the silicone tubes, they were rinsed with culture medium and placed in 12-well dishes at 37 °C in a 5% CO_2 humidified environment.

Cells and capsules release of GM-CSF

K562-hGM-CSF or B16-mGM-CSF cells were plated at a density of 10^5 cells per well in six-well dishes containing 1 ml of culture medium. After 2 h, culture medium was harvested and centrifuged (4 min, 1400 revolutions per min). Supernatant was filtered through 0.22 mm filters and stored frozen at −20 °C. K562-hGM-CSF- or B16-mGM-CSF-containing capsules were periodically tested for their ability to secrete GM-CSF by placing them in 12-well dishes with 1 ml culture medium. After 2 h, culture medium was harvested and stored frozen at −20 °C. The same procedure was repeated with cells and capsules after irradiation (10 000 rads from a ^{137}Cs source) to evaluate the GM-CSF production and the cell survival. GM-CSF was quantified using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Capsule implantation in mice

Adult female BALB/c mice (Charles River Laboratories, Saint-Germain sur l'Abresle, France), were anesthetized by inhalation of isoflurane (Forene, Abbott Ireland, Sligo, Republic of Ireland). Animals were placed in a back position for surgery. The capsules were ventrally implanted in a subcutaneous and extra-peritoneal location through a 14G Abbocath catheter (Abbott). The entry site in the skin was closed using a non-resorbable suture (Prolene 6/0, Johnson and Johnson Intl, European Logistics Center, Brussels, Belgium). On recovery, the animals were returned to the animal care facility where they had access to food and water *ad libitum*. The experiments were performed according to a protocol approved by our local Committee on Animal Experimentation, in agreement with Swiss Federal Law.

Capsule histology

Capsules intended for histological analysis were fixed in 4% paraformaldehyde with 1% glutaraldehyde for a minimum of 3 h. They were then dehydrated through grading alcohol bath cycles and embedded in glycolmethacrylate (Leica Instruments, Nussloch, Germany). Capsules were cut at a thickness of 4 µm and stained with hematoxylin and eosin (Papanicolaou, Merck KgaA, Darnstadt, Germany).

Statistical analysis

Results are shown as the mean ± s.e.m. The results obtained were analyzed for statistical significance between the various groups using a Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

Results

Generation of the K562-hGM-CSF and the B16-mGM-CSF cell lines

The human erythroleukemia cell line K562 was used for the generation of an hGM-CSF-producing bystander cell line. GM-CSF secretion from these cells was quantified and repeated three times. A secretion of 2992 ± 49 ng GM-CSF per 10^6 cells every 24 h was obtained. In terms of safety regarding the use in clinical applications, it is recommended that cell replication be limited by irradiation. Following irradiation with 10 000 rads, daily GM-CSF release studies and assessments of the viable cell count using the Trypan blue vital coloration assay, were performed. The results indicated a decrease in GM-CSF production from 3216 ± 235 ng GM-CSF per 10^6 cells every 24 h 1 day after cell irradiation to 2 ± 0 ng GM-CSF per 10^6 cells every 24 h after 14 days ($n = 4$) (data not shown). The cell viability also showed a decrease in approximately 95% over the same 14-day period (data not shown). The benefit of the development of a bystander cell line is that the modified cells can be used for many applications. In contrast, immunotherapeutic approaches based on individualized genetic engineering of autologous tumor cells can only be used for one application. Therefore, a K562-hGM-CSF cell line was engineered and tested in the experiments described below.

GM-CSF secretion from capsules containing K562-hGM-CSF cells

K562-hGM-CSF cells were enclosed into 1-cm long hollow fiber macrocapsules at a density of 10^5 cells per capsule. As the sustained release of GM-CSF over several days is essential for use in potential clinical applications,

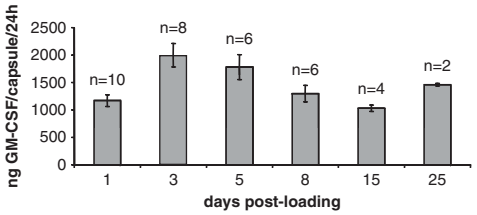

Figure 1 Time course of GM-CSF secretion from capsules containing K562-hGM-CSF cells loaded at a density of 10^5 cells per capsule.

the ability of encapsulated cells to produce the cytokine was evaluated over time, measuring the GM-CSF release at day 1, 3, 5, 8, 15 and 25 after capsule loading. The results indicated GM-CSF secretion values higher than 1000 ng GM-CSF per capsule every 24 h over a 25-day period. A slight decrease in cytokine production was observed after 8 days (Figure 1).

GM-CSF secretion from K562-hGM-CSF capsules after freezing and thawing

For the use in clinical applications, it is crucial to be able to produce large quantities of loaded capsules and to store them safely. For these reasons, we tested whether or not the encapsulated cells could be frozen and stored. We evaluated capsule freezing at two post encapsulation times. One group of capsules was frozen 1 day (group A) and another group 3 days (group B) after K562-hGM-CSF cells encapsulation (10^5 cells per capsule). Both groups were compared with a control group described above consisting of capsules loaded with K562-hGM-CSF cells but not submitted to the freezing/thawing procedure (group C). Groups A and B were thawed after several days at $-80\,^{\circ}C$ and maintained in culture under the same conditions as group C. GM-CSF release measurements were performed at day 1, 3, 5, 8, 15 and 25 after capsule thawing. At each time point, one to three capsules were fixed for histological analysis after the GM-CSF release procedure. The results showed secretion of GM-CSF through day 25 with 553 ± 251 ng GM-CSF per capsule every 24 h for group A and 703 ± 82 ng GM-CSF per capsule every 24 h for group B (Figure 2). Frozen capsules had a lower secretion capacity immediately after thawing, but recovered to comparable values with the control group after 8 days in culture (group A 1174 ± 145, group B 1137 ± 115 and group C 1298 ± 149 ng GM-CSF per capsule every 24 h). Comparing the results from groups A and B, we observed lower values for group A than for group B that were statistically significant until day 8. After day 8, both groups reached a similar secretion capacity. Furthermore, group B capsules had a higher GM-CSF secretion before the freezing procedure than capsules from group A. Histological analysis showed cell survival in the capsules, although some necrotic debris appeared over time (Figure 3).

GM-CSF secretion from K562-hGM-CSF capsules after irradiation

Capsules containing K562-hGM-CSF cells were tested for their secretion ability after an irradiation procedure of 10 000 rads ($n = 4$) in a similar manner to the non-

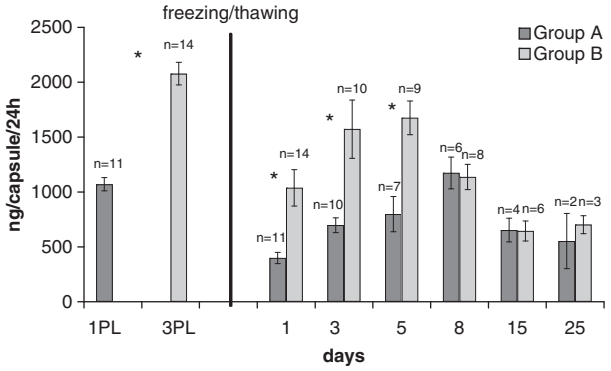

Figure 2 Time course of GM-CSF secretion from K562-hGM-CSF capsules after freezing and thawing. Capsules were frozen 1 day (group A) or 3 days (group B) after cell loading. 1PL = 1 day post-loading: GM-CSF secretion from group A before capsule freezing. 3PL = 3 days post-loading: GM-CSF secretion from group B before capsule freezing. *The statistically significant ($P < 0.05$) differences between the groups.

encapsulated cells. The results indicated a decrease in GM-CSF production over a period of 14 days with a GM-CSF secretion value of 895 ± 33 ng per capsule every 24 h 2 days after the irradiation procedure, and reaching 13 ± 1 ng per capsule every 24 h after 14 days *in vitro* (data not shown).

GM-CSF secretion from K562-hGM-CSF capsules after irradiation and freezing/thawing

K562-hGM-CSF capsules were submitted to irradiation and freezing/thawing procedures. Then their ability to secrete GM-CSF over time was evaluated according to the sequence of the procedures. Capsules containing 10^5 K562-hGM-CSF cells were either irradiated and immediately frozen (group A, Figure 4a), or frozen first, then thawed and maintained in culture medium for 1 day followed by irradiation (group B, Figure 4b). Before the procedures, groups A and B showed similar secretion values of 1007 ± 99 and 1120 ± 70 ng GM-CSF per capsule every 24 h, respectively. After the procedures, groups A and B again showed similar results at 24 h, with secretion values of 487 ± 52 ng GM-CSF per capsule every 24 h and 520 ± 19 ng GM-CSF per capsule every 24 h, respectively. A higher decrease in GM-CSF secretion after 7 days was observed for group A (188 ± 45) than for group B (355 ± 22) ($P = 0.006$).

GM-CSF secretion from K562-hGM-CSF capsules designed for human application

For future human application, we tested the K562-hGM-CSF cells with the clinical grade capsules. These capsules were designed to have a length of 2 cm and were reinforced with a titanium coil (Figure 5a). They were loaded with 2×10^5 K562-hGM-CSF cells and maintained in culture for 3 days before irradiation and freezing procedures ($n = 4$) were performed. The GM-CSF secretion was quantified over a 7-day period after thawing. The results showed a GM-CSF production of 3860 ± 460 ng

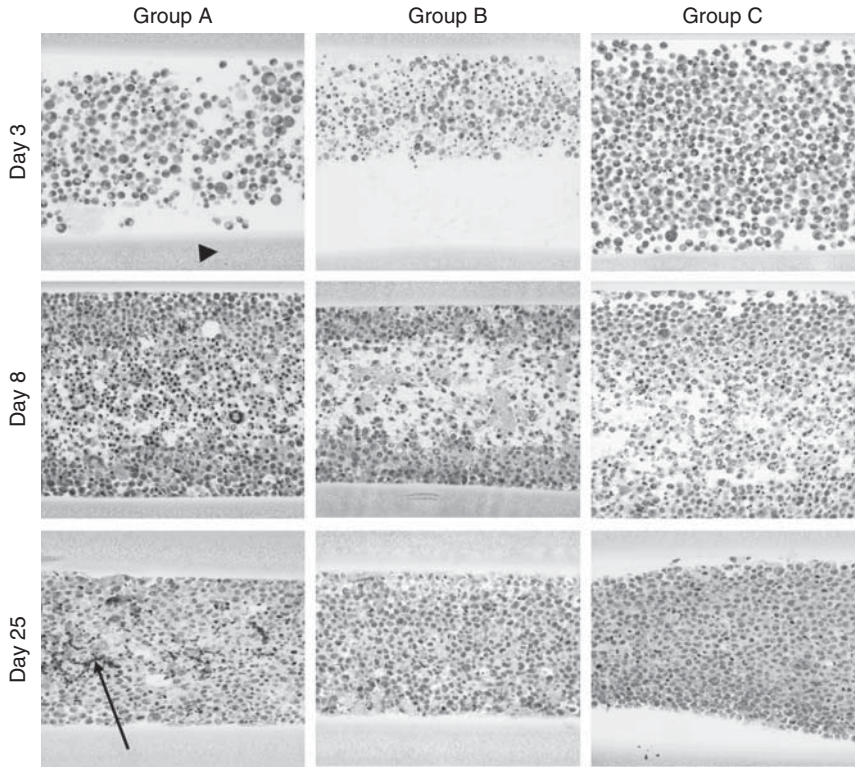

Figure 3 Histologies of capsules cultured *in vitro* for 3, 8 and 25 days after thawing. Capsules were frozen 1 day (group A) or 3 days (group B) after cell loading. Group C represents capsules that were not frozen. Arrowhead: capsule membrane. Arrow: necrotic debris.

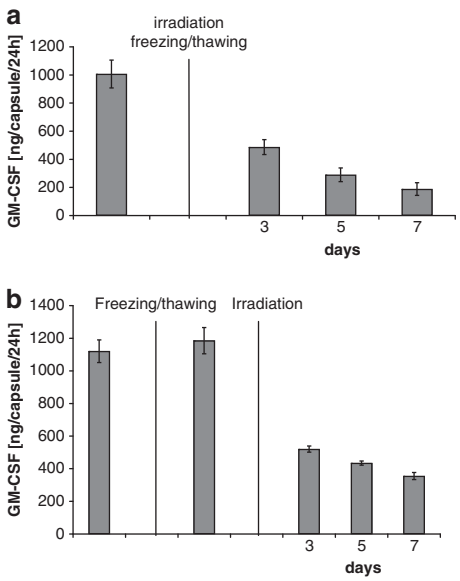

Figure 4 Time course of GM-CSF secretion from K562-hGM-CSF capsules following irradiation with 10 000 rads and freezing and thawing (**a**) ($n=3$), or freezing and thawing followed by irradiation (**b**) ($n=6$).

per capsule every 24 h after 3 days in culture. Following the irradiation, freezing and thawing procedures, this production decreased over time, reaching 308 ± 49 ng per capsule every 24 h, 7 days after thawing (Figure 5b).

In vivo *survival of encapsulated K562 cells*

K562 cells were loaded into capsules at the same cell density as previously described. After 3 days *in vitro*, capsules were implanted in mice in a ventral subcutaneous extra-peritoneal location. After 5 days, capsules were retrieved and histological analysis was performed. Results showed that encapsulated K562 cells are able to survive for 5 days under xenogeneic conditions (Figure 6).

In vivo *implantation of encapsulated B16-mGM-CSF cells*

As a preliminary test for the *in vivo* application of the cell encapsulation strategy for anti-tumor immunotherapy, the biological activity of encapsulated GM-CSF-secreting cells was evaluated under allogeneic conditions by assessing the local inflammatory reaction at the implantation site. The B16-mGM-CSF cells used in the experiments secreted 2644 ± 197 ng GM-CSF per 10^6 cells every 24 h ($n=4$). Capsules were loaded with B16-mGM-CSF or unmodified B16 cells as a control. *In vitro* secretion tests showed stable cytokine production over 25 days (data not shown). The pre-implantation cytokine secretion values were 153 ± 11 ng GM-CSF per capsule every 24 h ($n=8$). Capsules were retrieved after 3 days *in vivo* and the site of implantation, and the capsules with the surrounding tissue, were evaluated. Mice implanted with B16-mGM-CSF capsules showed a pericapsular inflammatory reaction that was not observed in mice implanted with non-secreting capsules (Figure 7).

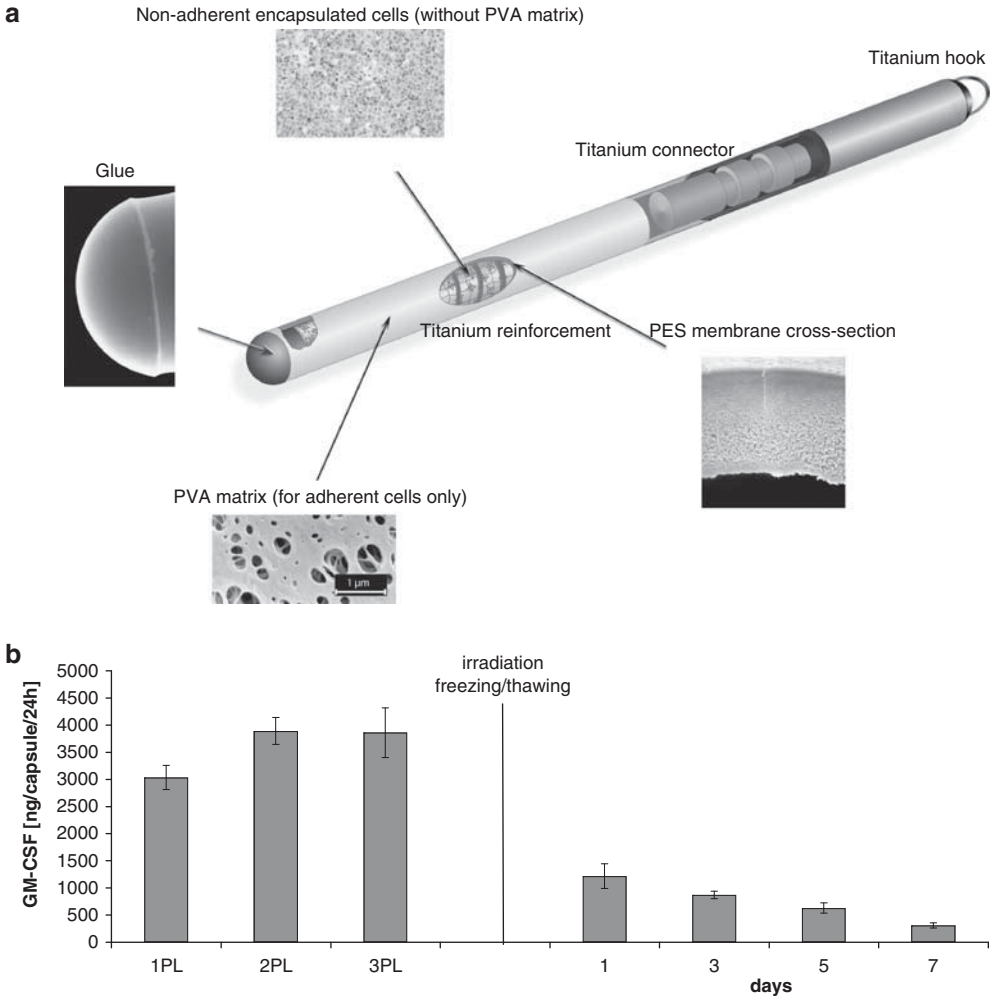

Figure 5 (**a**) Design of the capsule developed for human application showing the titanium coil inserted into the hollow fiber. (**b**) Time course of GM-CSF secretion from 2-cm-long capsules reinforced with a titanium coil and containing K562-hGM-CSF loaded at a density of 2×10^5 cells per capsule. Capsules were irradiated with 10 000 rads before freezing and thawing ($n = 4$).

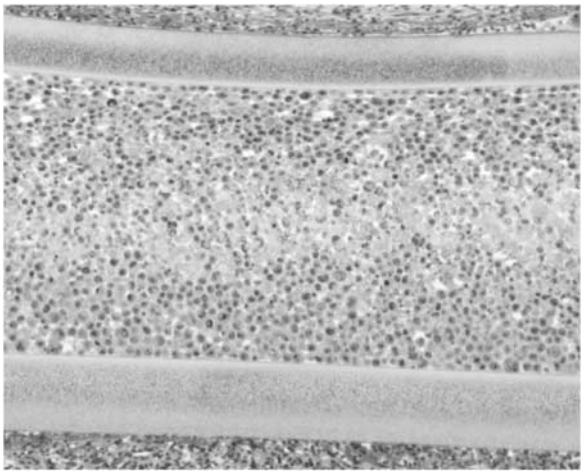

Figure 6 Histology of a capsule containing K562 cells implanted after 5 days in a BALB/c mouse.

Discussion

In this study, we report the development of a novel strategy for anti-tumor immunotherapy using human GM-CSF-producing cells enclosed into hollow fiber capsules. This device was designed for implantation in close proximity to autologous tumor cells that act as the richest source of tumor antigens. Such co-implantation should enhance dendritic cell recruitment and activation of a potent stimulation of the anti-tumor immune response.

Cell encapsulation of several proteins such as erythropoietin and neurotrophic factors has been extensively reported in *in vitro, in vivo* and clinical studies.[29,33,36] This technology was developed as a replacement strategy for long-term protein release. In such applications, a critical requirement was to achieve the lowest inflammatory response around the capsule to avoid deleterious effects for encapsulated cells and allow for a long-term release of

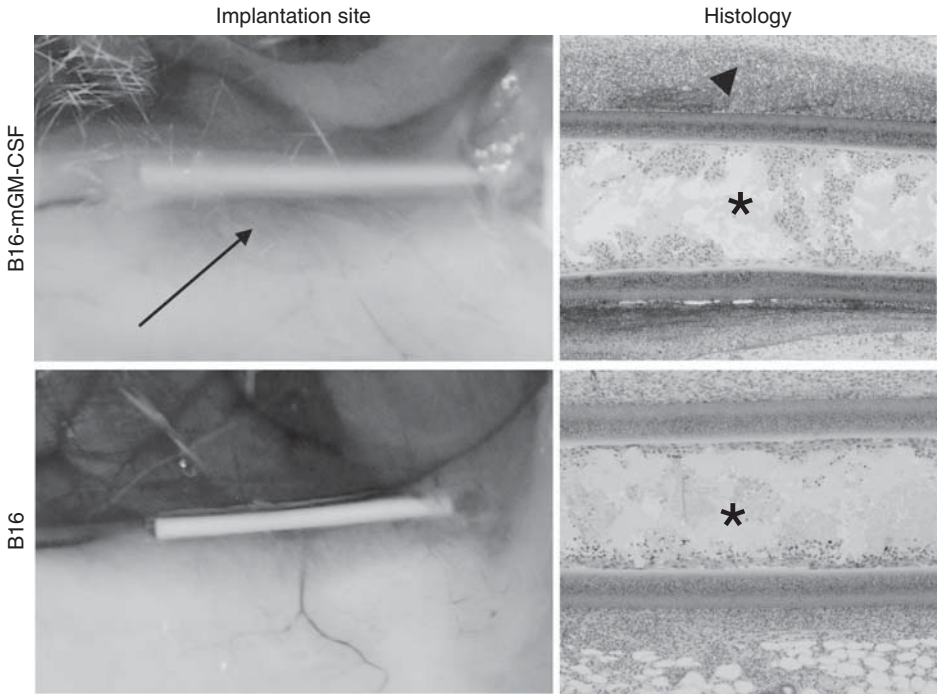

Figure 7 Implantation site and histologies of capsules containing B16-mGM-CSF or unmodified B16 cells after 3 days in BALB/c mice. Arrow: macroscopic peri-capsular inflammatory reaction. Arrowhead: microscopic peri-capsular inflammatory reaction. Asterisk: polyvinyl alcohol (PVA) matrix as a scaffold for the encapsulated cells.

the therapeutic protein. Even with the immune protection of the device, shed antigens crossing the membrane can stimulate a host immune response against the encapsulated cells. Immunoglobulins as well as smaller inflammatory and cytotoxic agents may diffuse through the membrane and cause immune toxicity,[39,40] which can be an issue for long-term application of the encapsulation technology for therapeutic proteins production. The device developed in this study was aimed at inducing a strong local inflammatory reaction for a limited period of time and results indicated a preserved cell survival over a 25-day period, which is more than expected for anti-tumor immunotherapy application.

The secretion of GM-CSF should result in an ideal milieu for triggering an anti-tumor immune response. This immunostimulatory strategy has been widely studied in murine models and phase I clinical trials using irradiated tumor cells genetically engineered to produce GM-CSF.[10,15,19,21–23,41–43] The inability to obtain reproducible levels of the cytokine, and the labor-intensive requirements for individualized genetic engineering, led to the development of allogeneic bystander cells for the local delivery of GM-CSF, such as the K562 human erythroleukemia cell line.[44–46] However, despite their undetectable expression of human leukocyte antigen class I and II, the high sensitivity of allogeneic K562 cells to natural killer cytotoxicity prevented continuous release of the cytokine for several days. In this context, the encapsulation technology has the advantage of allowing a sustained release of the cytokine at the implantation site by the allogeneic cells being physically isolated from the host immune response.

The K562-hGM-CSF cell line was obtained after transfection using the standard calcium phosphate method of the GM-CSF gene. Owing to biosafety concerns, this genetic modification method is preferable over a retroviral infection approach because retroviral infection involves risks of development of competent recombinant retroviral particles *in vivo*. K562-hGM-CSF cells secreted approximately 3000 ng per 10^6 cells every 24 h, which is higher than previously reported values that range from 42 to 1403 ng per 10^6 cells every 24 h.[19,22,41,42,44,45,47] Although the GM-CSF threshold for an effective immunostimulation effect has been evaluated to be 36 ng per 10^6 cells every 24 h,[48] there are no clearly defined values reported for the site of cell injection. On the other hand, Serafini *et al*.[49] reported that a high-dose of GM-CSF could impair the immune response through the recruitment of myeloid suppressor cells. This inhibitory effect was attributed mainly to systemic rather than local secretion of GM-CSF. These experiments were performed in mouse models that do not give rise to a simplified calculation for the identification of the upper therapeutic limit in humans. The systemic inhibitory effect of GM-CSF may imply that the plasmatic dosage increases after injection of the GM-CSF-producing cells. Thus, the effective immunostimulatory range falls between the lower threshold described above and the value leading to a plasmatic change in GM-CSF dosage. Further studies to evaluate this range by measuring other

variables, such as the quantification of myeloid suppressor cells, are needed.

For potent application of anti-tumor immunotherapy, there is a need to obtain a secretion of GM-CSF *in vivo* over a 3- to 7-day period, allowing time for dendritic cell recruitment and activation.[10,48] Combining this requirement with safety concerns, we evaluated the secretion time profile of irradiated K562-hGM-CSF cells that either were in suspension or encapsulated. Irradiation provides a supplementary safety level over the enclosure of allogeneic cells that would be rejected by the host immune system in case of capsule breakage. Secretion results indicated a progressive decrease in GM-CSF secretion over a 14-day period that corresponds with the dying of irradiated cells. As the capsule cryopreservation is essential for clinical development, the secretion capacity of encapsulated cells frozen at day 1 or 3 after capsule loading was compared with that of the non-frozen control group. Until day 8, capsules frozen at day 1 showed lower GM-CSF secretion than capsules frozen at day 3 and the non-frozen control. These observations can be explained by the proliferation potential of the cells inside the capsule. With a cell density loading of 10^5 cells per capsule, there is sufficient volume for cell growth until the inner content of the capsule is occupied. This hypothesis was confirmed by histological analysis at day 1 and 3. After thawing, however, both groups reached comparable maximum secretion values, despite a lower value the day after thawing. This is probably due to partial cell death induced by the freezing/thawing procedures. Finally, histological analysis showed the development of necrotic debris inside the capsules appearing over time. This necrotic core is a typical feature associated with cell confinement within the device[50] and reflects the impaired transfer of oxygen and nutrients to the central part of the capsule. This phenomenon is undoubtedly increased by the malignant properties of the cells, having lost their contact proliferation inhibition. As the capsule irradiation before freezing/thawing seems a safer and more practical approach for human application, experiments with capsules intended for human application evaluated the GM-CSF secretion from capsules that had been irradiated before freezing. The results showed a secretion of >300 ng GM-CSF per capsule every 24 h after 7 days in culture, which might be in the desired clinical range of secretion.

Preliminary testing of this approach was performed in mice. Encapsulated K562 cells showed a preserved cell survival after 5 days under such xenogeneic conditions, which is encouraging regarding future allogeneic applications. Mimicking potential human applications, the biological properties of GM-CSF were tested in an allogeneic model using encapsulated B16 mouse melanoma cells secreting mGM-CSF. Encapsulated B16-mGM-CSF cells released approximately 150 ng per capsule every 24 h. The difference in both secretion and cell density between encapsulated B16-mGM-CSF and K562-hGM-CSF cells may be explained by the presence of the polyvinyl alcohol matrix in the capsules containing the B16-mGM-CSF cells, decreasing the inner space for cell growth. Mice implanted with B16-mGM-CSF capsules showed a prompt inflammatory response around the capsules as early as 3 days post-implantation, confirming the potent and rapid effect of GM-CSF secretion. This inflammatory reaction undoubtedly impeded the efficient nutrient supply through the membrane, and might explain the lower density of enclosed cells after capsule retrieval. As previously mentioned, the aim of this strategy is to obtain an inflammatory reaction rather than long-term cell survival in the capsules. Further experiments will evaluate the ability of the encapsulation strategy for GM-CSF secretion to induce protective anti-tumor immunity. According to the efficiency of this process, it will be possible to modify the initial cytokine secretion values by changing the cell density loading and/or the capsule design.

This novel strategy affords greater control of the GM-CSF production rate as compared with previously described individualized gene transfer of autologous tumor cells. It will permit the separation of the source of GM-CSF and targeted antigens, and the conservation of the whole pattern of tumor-associated antigens by avoiding any genetic modification of these cells. The ability to freeze the GM-CSF-producing devices allows a simplification and standardization of the anti-tumor immunotherapy concept, which would consist of the combined implantation of a thawed capsule and irradiated autologous tumor cells.

Conflict of interest

N Mach is stocks owners of MaxiVAX SA. The other authors declare no conflict of interest.

Acknowledgements

This work was supported in part by the Swiss National Science Foundation (PNR 37), the Swiss Confederation's innovation promotion agency in collaboration with MaxiVAX SA, la Ligue genevoise contre le cancer and la Fondation pour la lutte contre le cancer.

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