CHARACTERIZATION OF GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR / INTERLEUKIN-2 FUSION cDNA AND THE USE OF MARROW STROMAL CELLS FOR CANCER IMMUNOTHERAPY

By

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ABSTRACT

Immunotherapy of cancer aims at achieving systemic anticancer responses capable of eradicating disseminated malignant cells. The disappointing outcomes associated with several immune-based clinical trials have highlighted the need to improve upon existing therapeutic strategies. The main objective of my thesis was to develop novel means in order to improve current cytokine-based anticancer strategies. The delivery of cytokines, or their encoding cDNA sequences, can induce antitumor immune responses. Interleukin (IL)-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are among the most potent cytokines able to induce tumor-specific systemic immunity, both in experimental models and clinical trials. Paradoxically, the combination of GM-CSF and IL-2 has been reported to downregulate certain immune functions, highlighting the unpredictability of dual cytokine use. In the first section of my thesis, I hypothesized that a GM-CSF and IL-2 fusion transgene (GIFT) could circumvent the limitations associated with dual cytokine expression yet preserve synergistic features. B16 mouse melanoma cells were gene modified to express GIFT (B16GIFT) and GIFT gene product was characterized *in vitro*. When injected into syngeneic mice, B16GIFT cells were unable to form tumors. When used as a whole cell tumor vaccine, irradiated B16GIFT could induce absolute protective immunity against wild type B16 tumors. In mice with established melanoma, B16GIFT therapeutic cellular vaccine significantly improved tumor-free survival when compared to B16 expressing both IL-2 and GM-CSF. Mechanistically, GIFT induced a significantly greater tumor site recruitment of macrophages and NK cells than combined GM-CSF and IL-2. I thus demonstrated that a fusion between GM-CSF and IL-2 can invoke greater antitumor effect than both cytokines in combination and that novel immunobiological properties can arise from such chimeric constructs.

Another means to improve current cytokine-based strategies is to limit the severe side-effects associated with their systemic administration. In view of that, I tested the hypothesis that primary marrow stromal cells (MSCs) can be used as a cellular vehicle for the tumor-localized delivery of immunostimulatory cytokines. Specifically, I investigated whether IL-2 gene-modified MSCs can be used to mount an effective immune response against the poorly immunogenic B16 melanoma model. My research

demonstrated that primary mouse MSCs can be efficiently gene-modified to secrete IL-2. Remarkably, IL-2 secreting MSCs embedded in a collagen-like matrix and injected in the vicinity of pre-established B16 tumors led to absence of tumor growth in 90% of treated mice. Injection of IL-2 secreting MSCs induced CD8 mediated tumor specific immunity and was dependent upon CD8 and NK cells, but not CD4 cells.

Therefore, despite their previously reported immunosuppressive effects on allogeneic immune responses, I provided evidence that primary MSCs can be used as transgenic delivery vehicles to enhance immune responses in syngeneic hosts. In order to further characterize the effect of MSCs on autologous immunity, I investigated the immunomodulatory properties of MSCs during syngeneic antigen-specific immune responses. I provide experimental evidence that syngeneic MSCs behave as conditional antigen-presenting cells. My research demonstrated that IFNγ can induce mouse MSCs to process and present antigenic peptides derived from a soluble xenoprotein (i.e. ovalbumin) and activate *in vitro* antigen-specific T cells. When injected *in vivo* into syngeneic mice, ovalbumin-pulsed IFNγ-treated MSCs induced potent ovalbumin-specific cellular immune responses and protected mice against ovalbumin-expressing tumors. My studies further showed that human MSCs can also acquire antigen-presenting functions upon IFNγ stimulation, thereby activating antigen-specific T cell hybridomas. Taken together, my results strongly suggest that in syngeneic conditions, IFNγ-stimulated MSCs behave as conditional antigen presenting cells able to activate antigen-specific immune responses.

Overall, my research opens the door for the development of new immunotherapeutic strategies based on (i) the improvement of cytokine potency by molecular fusion and (ii) the improvement of cytokine delivery by the use of gene modified somatic MSCs, and may reveal MSCs as previously unrecognized cellular regulators of physiological immune responses.

RÉSUMÉ

L'immunothérapie du cancer a pour but de générer une réponse immunitaire efficace et systémique capable d'éradiquer les cellules cancéreuses disséminées dans l'organisme. Les résultats décevants de récentes études cliniques visant à tester l'efficacité de différents traitements immunothérapeutiques nous amènent à envisager le développement de nouvelles stratégies. L'objectif général de ma thèse fut de développer de nouvelles méthodes en vue d'améliorer les stratégies actuelles d'immunothérapie du cancer basées sur l'administration de cytokines. L'administration de cytokines immunostimulantes, ou l'expression du cDNA de ces dernières, peut induire des réponses anticancéreuses systémiques thérapeutiquement relevantes. L'IL-2 (*interleukin-2*) et le GM-CSF (*granulocyte-macrophage colony-stimulating factor*) sont deux des plus puissantes cytokines démontrées comme étant capables de générer de telles réponses, et ce à la fois chez les modèles animaux et chez les patients. Cependant, la combinaison de l'IL-2 et du GM-CSF peut, dans certains cas, induire des effets immunitaires paradoxaux, limitant par le fait même leur utilisation combinée. En première partie de ma thèse, j'ai testé l'hypothèse que l'expression d'une protéine chimérique née de la fusion entre GM-CSF et IL-2 – dénommée GIFT – peut induire un effet antitumoral supérieur à celui obtenu suite à l'expression de GM-CSF et IL-2, exprimés seuls ou en combinaison. À cette fin, des cellules B16 de mélanomes de souris furent génétiquement modifiées pour exprimer GIFT (B16GIFT). Lorsque des souris syngéniques immunocompétentes furent injectées avec des cellules B16GIFT, aucune des souris ne développa de tumeur. De même, l'administration de cellules B16GIFT irradiées, dans le cadre d'une vaccination antitumorale prophylactique, protégea l'ensemble des souris contre le développement de tumeurs B16 non-modifiées. Remarquablement, chez des souris ayant une tumeur B16 préétablie, l'injection de cellules B16GIFT irradiées induisit une réponses antitumorale supérieure à celle observée suite à l'injection de B16 exprimant en combinaison GM-CSF et IL-2. L'analyse de ces réponses immunitaires nous a indiqué que l'expression de GIFT provoque une infiltration tumorale significativement supérieure de macrophages et de cellules NK (*natural killer*) comparativement à l'expression de GM-CSF et IL-2 en combinaison.

Une autre façon d'améliorer les stratégies actuelles d'immunothérapie du cancer consiste à restreindre les effets secondaires associés à l'administration systémique de cytokines en localisant à la tumeur l'expression de leurs gènes. En deuxième partie de thèse, j'ai donc testé l'hypothèse que les cellules stromales de la moelle osseuse peuvent être utilisées afin de délivrer de façon localisée des cytokines antitumorales, spécifiquement l'IL-2. Mes recherches ont démontré que les cellules stromales peuvent être génétiquement modifiées pour sécréter de l'IL-2 et qu'elles peuvent être utilisées afin d'induire une réponse immunitaire antitumorale significative. À l'aide de souris immunodéficientes, j'ai démontré que cette réponse immunitaire fut requiert la présence de lymphocytes CD8 et NK, mais est indépendante de la présence de lymphocytes CD4.

Mes recherches suggèrent donc que malgré les études antérieures démontrant les effets immunosuppressifs des cellules stromales contre les réponses immunitaires allogéniques, celles-ci peuvent être efficacement utilisées afin de générer des réponses immunitaires syngéniques. De façon à mieux caractériser le rôle immuno-modulatoire des cellules stromales, j'ai étudié l'effet de ces cellules lors d'une réponse immunitaire syngénique définie. Mes recherches ont démontré que les cellules stromales de la moelle osseuse agissent comme cellules présentatrice d'antigènes suite à une stimulation à l'interféron-γ (IFNγ). Lorsque l'on injecte des cellules stromales stimulées à l'IFNγ et pulsées à l'ovalbumine à des souris syngéniques, elles induisent une réponse immunitaire substantielle spécifique à l'ovalbumine. De plus, cette réponse est suffisante pour protéger la totalité des souris contre le développement de tumeurs exprimant l'ovalbumine. Enfin, mes recherches suggèrent que les cellules stromales humaines de la moelle osseuse agissent également comme cellules présentatrices d'antigènes.

En conclusion, mes travaux de recherches ont permis de démontrer que les stratégies actuelles d'immunothérapie du cancer, basée sur l'effet antitumoral de cytokines immunostimulantes, peuvent être améliorées (i) par l'utilisation de protéines chimériques nées d'une fusion entre deux cytokines antitumorales et (ii) par l'administration *in situ* de cellules stromales de la moelles osseuse génétiquement modifiées pour exprimer un transgène thérapeutique. Finalement, mes travaux suggèrent que les cellules stromales de la moelle osseuse peuvent agir comme cellules présentatrices d'antigènes et possiblement jouer un rôle préalablement insoupçonné lors de réponses immunitaire endogènes.

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PREFACE

Cancer is one of the main causes of death in the country, killing approximately 1 out of 4 Canadians. In its 2005 annual report, the National Cancer Institute of Canada estimated that on the basis of current incidence rates, 38% of Canadian women and 44% of Canadian men will develop cancer during their lifetimes (http://www.ncic.cancer.ca).

One of the most promising fields in cancer research is the development of therapeutic strategies based on immune recognition and destruction of cancer cells. The ultimate goal of cancer immunotherapy being to achieve induction of a tumor-specific immune response capable of eradicating disseminated malignant cells. Recent advances in the fields of molecular immunology have permitted unambiguous demonstrations that clinically relevant tumor-specific immunity is achievable. It is in that context of new hopes that I proudly present my Ph. D. thesis to the committee members.

In the course of my doctoral studies, I have tested three original hypotheses that have led to the development of novel immune-based therapeutic strategies. My work has been published in three first-author original peer-reviewed papers, which are presented in their integrality as distinct chapters of this thesis in accordance with the *McGill guidelines concerning thesis preparation*:

- Chapter 2: **Stagg J**, Wu JH, Bouganim N, Galipeau J. Granulocyte-macrophage colony-stimulating factor and interleukin-2 fusion cDNA for cancer gene immunotherapy. Cancer Res. 2004 Dec 15;64(24):8795-9.

- Chapter 3: **Stagg J**, Lejeune L, Paquin A, Galipeau J. Marrow stromal cells for interleukin-2 delivery in cancer immunotherapy. Hum Gene Ther. 2004 Jun;15(6):597-608.

- Chapter 4: **Stagg J**, Pommey S, Eliopoulos N, Galipeau J. Interferon-γ-Stimulated Marrow Stromal Cells: A New Type of Non-Hematopoietic Antigen Presenting Cell. Blood. 2005 (in press).

In addition to the work presented in this thesis, I was involved in collaborative studies with other members of the Lady Davis Institute, which led to the following publications:

- Eliopoulos N, **Stagg J**, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I and II mismatched recipient mice. Blood. 2005 (in press).

- Duguay D, Mercier F, **Stagg J**, Martineau D, Bramson J, Servant M, Lin R, Galipeau J, Hiscott J. In vivo IRF-3 Tumor Suppressor Activity in B16 Melanoma Tumors. Cancer Research 2002 Sep 15;62(18):5148-52.

Finally, I was author of the following review:

- **Stagg J**, Galipeau J. Pseudotyped retrovectors for tumor-specific delivery of toxic suicide genes. 2001. IDrugs, 4(8): 928-934.

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GENERAL INTRODUCTION

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1.1. CANCER IMMUNOSURVEILLANCE

The importance of the immune system at controlling cancer has been debated ever since the late 19th century when a surgeon by the name of William Coley reported sporadic cancer regressions after administration of bacterial extracts in order to induce inflammation[1]. In 1967, Burnett outlined the first concepts of cancer immune surveillance[2], hypothesizing that tumor-specific determinants could induce immune responses able to control tumor growth[3]. In his "cancer immunosurveillance" theory, cells that failed to repair DNA mutations but survived programmed cell death are detected by the immune system. His hypothesis has been until recently difficult to validate, the two major obstacles being: (i) the lack of experimental models, and (ii) the fact that human immune deficiencies, although informative, often impact on different biological pathways independent of the immune system. In addition, while cancers do appear at increased frequency in long-term immunosuppressed patients, they are often limited to cancers with a strong association with viral infections, mainly Epstein-Barr and herpes virus associated malignancies[4]. However, the development of gene-targeted animal models has allowed scientists to define for the first time the importance of the immune system at controlling the oncogenic process. An overview of these recent studies in the field of tumor immunology is described in the following section.

1.1.1 Cancer immunosurveillance effectors

i) Adaptive immunity

One of the most important studies demonstrating the presence of cancer immunosurveillance mechanisms was reported by Robert Schreiber's group[5]. To test the hypothesis that an intact immune system can protect against nascent tumors, they generated knock-out mice deficient in the recombinase activating gene (RAG)-2 unable

to rearrange lymphocyte receptors. These mice thus fail to produce αβ T cells, B cells, natural killer (NK) T cells, and γδ T cells. The reported studies demonstrated that lymphocytes indeed play a key role at controlling nascent tumors. They observed that $Rag2^{-/-}$ mice were three times more susceptible to 3'-methylcholanthrene (MCA)-induced sarcomas than their wild type counterparts. In addition, $Rag2^{-/-}$ mice developed significantly more spontaneous epithelial tumors (predominantly gastrointestinal carcinomas) than did wild type mice. Others have since confirmed an essential role of the mouse adaptive immune system in cancer immunosurveillance and better defined the importance of specific immune subsets to this phenomenon[6-7]. Girardi and colleagues, using T cell receptor (TCR) $\beta^{-/-}$ or $TCR\delta^{-/-}$ gene-targeted mice, provided additional support to Shreiber's studies by demonstrating that αβ and γδ T cells make critical but distinct contributions to the surveillance of carcinogen-induced tumors[8-9].

ii) Innate immunity

By studying mice deficient in NK and NKT cells – using the monoclonal antibody (mAb) anti-NK1.1 and using $J\alpha281^{-/-}$ mice, respectively – Smyth and colleagues demonstrated that in addition to adaptive lymphocytes, innate immune cells also participate in the control of neoplasia in mice[10-11]. Other studies have reported that macrophages can also participate in cancer immunosurveillance[12-13]. Macrophages possess both the direct ability to kill tumor cells in an antigen-independent manner, and to act as professional antigen-presenting cells (APCs) stimulating the generation of cytotoxic T-cells[14-15]. Other innate immune effectors such as neutrophils and eosinophils may also play an important role at controlling tumor growth[16-19]. The serendipitous finding of a transmissible trait that confers immune-mediated cancer resistance to wild type and, most remarkably, T cell deficient mice recently reinforced the importance of the innate immune system in cancer immunosurveillance[20-21]. Taken together, independent studies using gene-targeted immunodeficient mice highlighted the importance of both the innate and adaptive arms of the immune system in the surveillance of cancer.

1.1.2. Essential functions of immune effectors

The production of IFNγ and the capacity of immune effectors to kill cancer cells are the two most critical functions presently identified as essential for cancer immuno-surveillance[6].

i) IFNγ production

Studies using gene-targeted mice with distinct defects in IFNγ signaling have demonstrated the importance of this cytokine in the protection of mice against carcinogen-induced and spontaneous tumors. For instance, mice deficient in the receptor for IFNγ (IFNγ-R1), the signal transducer and activator of transcription (STAT)-1 (which is the transcription factor mediating signaling by IFNγ[22]) or IFNγ itself were found to be much more sensitive to MCA-induced carcinogenesis[23-24] and to spontaneously developing lymphomas[25]. IFNγ is a secreted cytokine with pleiotropic effects, including: (i) promoting antigen-specific $CD4^+$ helper type 1 and cytotoxic CD8+ T cells activation; (ii) orchestrating leukocyte interactions with the endothelium; (iii) activating macrophages tumoricidal activity; (iv) upregulating antigen-presentation via major histocompatibility complex (MHC) class I and II molecules; (v) suppressing tumor cell proliferation; (vi) sensitizing tumor cells to apoptosis; and (vii) blocking neo-angiogenesis associated with cancer growth[26-27].

ii) The perforin pathway

The second most critical function of immune effectors is their ability to kill tumor cells. One of the killing mechanisms of effector cells is through the release of cytoplasmic granules containing various proteins such as perforin (pfp) and granzymes[28]. In this pathway, perforin is first released from the granules and then enables the entry of cytotoxic proteins such as granzymes[29]. The development of $pfp^{-/-}$ mice demonstrated that perforin is essential in the protection against primary and metastatic tumors[30-31]. Indeed, it was shown that 50% of aging $pfp^{-/-}$ mice developed spontaneous lymphomas and that this development of lymphomas was accelerated when the mice were crossed on a p53-deficient background[32]. Granzymes, on the other hand, although they play a key

role in target cell death, were shown not to be essential for cancer immunosurveillance[33]. The crucial importance of the IFNγ and perforin pathways was recently reinforced by Street SE *et al.*[24] who reported that perforin and IFNγ pathways can act independently in order to control nascent tumors. Moreover, they demonstrated that perforin and IFNγ pathways account for all the natural antitumor activity mediated by the innate NK and NKT cells.

iii) Death ligands

Another mechanism that effector cells use to kill cancer cells is the expression apoptosis-inducing tumor necrosis factor (TNF)-family members[34]. At least three members have been shown to be involved in cancer immunosurveillance: Fas ligand (FasL), TNFα and TNF-related apoptosis inducing ligand (TRAIL). These cell-membrane-bound and secreted proteins have been shown to induce tumor apoptosis in numerous cancer models. Poehlein CH *et al.*[35] observed that the adoptive transfer of tumor-specific T cells deficient in perforin and IFNγ could still mediate tumor regression in a TNFα-dependent manner, suggesting a role for TNFα in the endogenous anticancer response. FasL deficient mice (*gld* mice) were similarly shown to be more prone to develop spontaneous cancers, especially in the B cell compartment[36]. TRAIL, which preferentially induces apoptosis in a wide range of tumor cells but not in normal cells, has also been shown to play a role in host protection from tumor initiation and metastasis[37]. Interestingly, the tumor-protection effect of TRAIL is impaired in IFNγ-deficient or NK cell-depleted mice[38].

1.1.3 Mechanisms of immune recognition of cancer cells

Two main mechanisms are involved in the immune recognition of cancer cells. Firstly, components of the innate immune system use pattern-recognition receptors and stress-induced surface molecules to directly recognize tumor cells. Secondly, adaptive immune cells are activated by APCs to recognize tumor-associated antigens (TAA). Professional APCs such as dendritic cells (DCs) thereby capture dying tumor cells or tumor debris, migrate to regional lymph nodes and process the tumor antigens for presentation to the adaptive arm of the immune system[39].

i) The "danger" model

The immune system has evolved to detect danger signals and the main sentinels of such danger signals are the professional APCs, namely macrophages, B cells and DCs. At present, DCs are identified as the most important APCs in the induction of innate and adaptive immune responses[40]. DCs constantly sample their environment in order to educate the immune system. In a non-inflammatory environment, DCs capture self-antigens and tolerize the immune system to these antigens, thus avoiding self-destruction. In an inflammatory environment triggered by danger signals, DCs get activated into mature DCs and initiate antigen-specific immune responses and memory immunity[41-43]. Since cancer immunosurveillance can induce adaptive immune responses, it was hypothesized that transformed cells can produce danger signals. In a 2003 issue of *Nature*, Kenneth Rock's group reported that uric acid produced from nascent tumors acts as an important danger signal to the immune system[44]. It was also demonstrated that tumors can release heat shock proteins able to activate APCs via Toll-like receptors, and to release extracellular matrix derivates such as hyaluronic acid and heparan sulfates, thereby inducing inflammatory responses akin to microbial-mediated inflammation[45-47].

ii) Direct recognition of tumor cells

In addition to alerting the immune system, danger signals modulate protein expression on tumor cells themselves. Heat-shock proteins, for instance, induce the expression of stress ligands that bind to the lectin-like receptor NKG2D expressed on several immune effectors such as NK cells, macrophages and CD8+ T cells[48-50]. Ligation of NKG2D activates these effector cells in an antigen-independent manner and triggers degranulation and perforin-mediated apoptosis of tumor cells. NKG2D interacts with a number of distinct families of stress-induced MHC class I chain-related (MIC) ligands. These MIC proteins are exclusively expressed on the gastrointestinal epithelium in normal tissue, but are found highly expressed on primary lung, kidney, prostate, ovary, colon and melanoma cancer cells[51]. In mice, NKGD2 interacts with the H-60 minor histocompatibility antigen and the retinoic acid early inducible (Rae-1) family of membrane-bound proteins[52-53]. NK cells can also target cancer cells that have downregulated or lost MHC class I

molecules[54]. While the selection of MHC class I-low tumor cells is a powerful strategy to avoid killing by antigen-specific lymphocytes, NK cells can recognize MHC class I-low tumor cells via inhibitory receptors specific for MHC class I molecules. Binding of these receptors to MHC class I prevents killing of normal cells while allowing killing of MHC class I-low tumor cells or virus-infected cells.

iii) Adaptive immune recognition of tumor cells

Once DCs have captured tumor antigens and have become mature DCs because of their inflammatory environment, they migrate to regional draining lymph nodes in order to induce the adaptive arm of the immune system. Mature DCs process the antigens into small peptides and load them onto MHC class I molecules to activate CD8+ T cells, and onto MHC class II molecules to activate CD4+ T cells[42]. T cell activation requires, in addition to antigen presentation, costimulatory signals provided by mature DCs[55]. Ultimately, properly activated CD8+ T cells will differentiate into cytotoxic T lymphocytes (CTLs) and memory CD8+ T cells, while CD4+ T cells will differentiate into "helper" T cells and memory CD4+ T cells. CD4+ helper T cells are the major cells orchestrating immune responses[56-58]. Once activated, CD4+ helper T cells differentiate into type 1 (Th1) or type 2 (Th2) helper cells, specialized in secreting distinct cytokines in order to help cellular and humoral immune responses, respectively. Th1 cells make IFNγ and IL-2 in order to enhance cytotoxic CD8+ T cells responses, while Th2 cells produce IL-4, IL-5, IL-10 and IL-13 in order to enhance antibody responses[59-61]. CD4+ Th1 cells further facilitate CD8+ T cell activation *via* binding of their CD40 ligand to CD40 on the DCs, which enhances MHC class I presentation by the DCs[62].

When DCs do not get properly matured by their environment, they fail to upregulate costimulatory molecules – such as CD80 (B7-1) and CD86 (B7-2) – essential for T cell activation[42]. It is currently believed that immature DCs continuously sample living and dying cells and present the captured antigens without costimulation in order to induce peripheral tolerance. In that context, self-reactive T cells become antigen-unresponsive, a phenomenon known as anergy, identified by an absence of both proliferation and interleukin (IL)-2 production after re-exposure to the antigen[63].

1.2 IMMUNOEDITING

Because of the genetic instability of cancer and the induction of peripheral tolerance to tumor antigens, the immune system is often incapable of preventing the growth of tumors. After having exerted pressure to remove tumor cells, the immune system remodels the initial tumor phenotype enabling surviving tumor cells to escape immune control mechanisms. This phenomenon, known as "immunoediting", thus selects for tumor cells better able to grow in immunocompetent hosts, continuously shaping the tumor phenotype through immunological pressure[64].

Similarly to the selective pressure induced by chemotherapy and hormone therapy, immunoediting is based on the heterogeneity and molecular instability of tumors. It has been hypothesized that immunoediting results from three distinct phases: (i) the elimination phase, which includes immunosurveillance mechanisms, (ii) the equilibrium phase, which is a period of tumor latency where the immune system exerts enough pressure to contain the tumor but is insufficient to fully destroy it, and (iii) the escape phase, in which surviving tumor cells begin to expand uncontrolled because of genetic instability[65]. The equilibrium phase hypothesis is based on clinical observations of cancer transmission following organ transplantation. For example, two patients were reported to have developed melanoma 2 years after each receiving kidneys from the same donor treated for melanoma 16 years before the transplantations but considered tumor-free since[66]. This and other case reports suggest that undetectable tumors can be kept in latency by an intact immune system, but become subsequently able to grow aggressively when the immune system is compromised such as following transplantation immunosuppression[67-68].

Having evaded immunosurveillance, tumors can progress using their genetic instability to their advantage. Genetic instability thus creates tumors that are favored by natural selection through immunological and molecular pressure afforded by their host. Tumors enter then a growth phase. This phenomenon is often referred to as tumor "immune escape" or "immune evasion", although escape and evasion imply an active process

rather than the differential survival of tumor subclones as it is the case. The next section presents an overview of the major tumor "immune escape" mechanisms.

1.2.1 Altering tumor antigen expression

Since MHC molecules and antigen processing are critical to immunosurveillance, many human tumors display losses of MHC molecules or proteins essential to the antigen presentation pathway such as the transporter associated with antigen processing-1 (TAP1) protein and proteasome subunits[69]. Other signaling deficiencies have been identified that indirectly lead to antigen presentation defects, such as mutations in the IFNγ pathway[70]. However, correlative observations between MHC class I expression and cancer progression have yet to be confirmed in controlled experiments as playing a role in tumor immune evasion. For instance, gene-targeted loss of TAP-1 or proteasome subunits in knock-out mice does not increase the incidence of spontaneous tumors[71]. In addition, loss of tumor MHC sometimes predicts better clinical survival in cancer patients[72] and high MHC class I levels have been correlated with metastatic spread and poor prognosis[73]. It thus still needs to be determined whether MHC downregulation in malignant cells is coincidental or functionally relevant in the generation of tumors' phenotype.

1.2.2 Immunosuppressive molecules

At later stages, an impressive variety of immunosuppressive molecules can be found secreted by tumor cells or by the immune system in response to cancer[74]. The best-known tumor-mediated immunosuppressors are IL-10, transforming growth factor (TGF)-β, prostaglandin E2 (PGE2) and vascular endothelial growth factor (VEGF).

IL-10 plays a central role at inducing immune dysregulation in cancer patients[75]. IL-10 has been shown to inhibit a number of immune functions, including lymphocyte proliferation, type 1 cytokine production, antigen presentation and cytotoxicity *in vivo*[76-77]. Increased concentrations of IL-10 are often detected in the serum of patients with

cancer. In antigen presentation, IL-10 compromises maturation of DCs and inhibits IL-12 production[76-77]. IL-10 was also shown to enhance DCs apoptosis[78] and to downregulate MHC class I expression[79]. TGF-β is also frequently found in cancer patients and is associated with a poor prognostic[80-82]. The sources of TGF-β are both living tumor cells and apoptotic cells. The major effect of TGF-β is to inhibit the activation, proliferation and activity of lymphocytes[83-84]. PGE2, on the other hand, is expressed by tumor cells as a result of upregulated expression of cyclooxygenase 2, and increases the production of IL-10 by lymphocytes and macrophages[85-86]. Finally VEGF, in addition to its angiogenic properties, inhibits DCs differentiation and maturation[87-88]. In patients with lung, head and neck, and breast cancers, increased VEGF levels correlates with a decrease in the function and number of DCs[89].

1.2.3 Death receptor signaling

As outlined above, death receptors play a major role in cancer immunosurveillance. Accordingly, defects in death receptor signaling are a major mechanism that contributes to the survival of tumor cells. Mutations and loss of the Fas receptor on tumor cells, as well as components essential for its signaling, have been identified in numerous malignancies, such as myeloma, non-Hodgkin's lymphoma and melanoma[90-91]. The loss of signaling through the TRAIL receptors in human cancer cells has also been documented[92]. Tumors can further block the perforin death pathway by overexpressing the serine protease inhibitor PI-9, which inactivates granzyme proteins[93].

Conversely, tumor cells can "defend" themselves by expressing death receptor ligands such as FasL, inducing apoptosis of Fas expressing immune effector cells. This has been observed in a variety of human cancers, including lung carcinoma[94], melanoma[95], colon carcinoma[96] and hepatocellular carcinoma[97]. However, independent studies have shown that injection of FasL-transfected tumor cells actually accelerated their immune rejection compared to wild type counterparts[98-100]. This paradox suggests that FasL may be pro-inflammatory in some circumstances. On the other hand, the observations that FasL expression in tumor samples correlates with poor prognostic may simply reflect the role

of Fas-FasL interactions in the activation-induced cell death (AICD) of antitumor T cells, either by "suicide" or by "fratricide"[101].

1.2.4 Regulatory T cells

Another important mechanism by which tumor cells become "invisible" to immune effectors is through the regulatory process that restricts the induction of autoimmunity. Because antitumor immunity is in essence an autoimmune response, it is confronted to the potent mechanisms that prevent self-destruction. Regulatory T cells (Tregs) are thought to be the main players governing peripheral immune tolerance[102-104]. It is in 1995 that Sakaguchi *et al.* provided clear evidence of the existence of Tregs[105]. They demonstrated that when CD4+ T cells from normal mice were depleted of the fraction expressing CD25 (the α chain of the IL-2 receptor) and injected into nude mice, all the mice developed autoimmune diseases. Most importantly, autoimmune responses could be prevented by the coadministration of CD4+CD25+ T cells. Since this study, independent groups have confirmed the existence of Tregs, both in rodents and in humans, and identified their major role in maintaining peripheral self-tolerance[102-104]. Naturally occurring Tregs constitutively express the transcription factor FoxP3 that can be induced by TGF-β, the glucocorticoid-induced TNF receptor (GITR) and most of them the CD25 receptor. They are selected by the thymus (at least in part) and their generation is dependent upon IL-2. In mice, they represent 5-10% of the peripheral CD4+ T cells, divide upon antigen encounter *in vivo*[106] and their suppressive effect is dependent upon TGF-β signaling in effector cells[107]. Recent studies suggested that Tregs belong to a class of "nonclassical" lymphocytes expressing Toll-like receptors (TLR), which activation enhances their suppressive properties[108]. Tregs have been implicated in the downregulation of antitumor immune responses at both the priming phase and effector phase[109-110]. Treg depletion with anti-CD25 antibody or low dose cyclophosphamide has been shown to improve T cell-based tumor clearance in several types of mouse cancers[110]. Using the B16 mouse melanoma model, Turk *et al.*[111] demonstrated that endogenous anticancer immunity could induce tumor rejection only in the absence of CD4+ Tregs. Furthermore, it has been shown that human cancer patients

have increased numbers of peripheral and tumor-infiltrating Treg that functionally inhibit tumor-specific T cells[112].

1.3 IMMUNOTHERAPY: ENHANCING CANCER IMMUNOGENICITY

I have presented thus far evidences that strongly suggest that the immune system endogenously controls nascent tumors. I also described how the immune system can specifically target malignant cells, and how it shapes through immunological pressure the tumor phenotype allowing selection of a range of subversive methods by tumor cells. In view of these evidences, the question then becomes what can be done *therapeutically* to enhance cancer immunogenicity and induce clinically relevant immune responses?

As aforementioned, an important aspect that affects tumor immunogenicity is the microenvironment surrounding a given tumor, more specifically the cytokines and chemokines induced in response to danger signals.

Is it possible then to harness effective antitumor immune responses by manipulating the tumor microenvironment? Forni and colleagues were the first to demonstrate this was indeed possible[113]. They showed that manipulating the cytokines present is a tumor's environment can provoke dramatic changes in the host response to cancer. In the following sections, I describe the physiological role of two cytokines identified as playing a major role in modulating tumor immunity: interleukin (IL)-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF).

1.3.1 Interleukin-2 (IL-2)

i) IL-2 signaling

IL-2 is a typical four α-helix cytokine identified as a growth factor for T cells. The IL-2 receptor (IL-2R) is a member of the type I cytokine receptor superfamily and is composed of three distinct subunits: IL-2Rα (CD25), IL-2Rβ (CD122) and the common γ

chain (γc; CD132)[114-115]. The IL-2Rβ and the γc are both essential and sufficient for signaling upon IL-2 binding. The IL-2Rα, on the other hand, confers high affinity binding to IL-2 without participating in the signaling events. In fact, IL-2Rα enhances the affinity of the receptor complex for IL-2[114]. Interestingly, it was shown that mice genetically deficient in IL-2Rα display an equivalent phenotype than mice deficient in IL-2, suggesting that physiological concentrations of IL-2 are not sufficient to induce signaling of the heterodimeric IL-2β/γc receptor[116]. Since the IL-2Rα is considered the key regulator in controlling the physiological responses to IL-2, it is not surprising that it is tightly regulated. IL-2Rα can be induced by the cytokines IL-2, IL-4, IL-5 and IL-10, and by viral infections[114]. Zurawski *et al.* reported that 13 solvent-accessible residues of mouse IL-2 are critical for its interaction with IL-2Rα[117].

In contrast to the IL-2Rα chain that is specific for IL-2, the IL-2Rβ and γ chains also make up for the IL-15 receptor (together with the IL-15Rα chain) and are expressed on monocytes/macrophages, eosinophils, neutrophils, NK cells, NKT cells and CD8+ memory T cells[114]. The γc subunit, in addition, is found on most cells of hematopoietic origin and contributes to the receptors for IL-4, IL-7, IL-9 and IL-21[118].

The IL-2Rβ is considered the most important signaling subunit of the IL-2R. Like other members of the cytokine receptor superfamily, IL-2Rβ contains conserved motifs in its membrane-proximal region[119]. Two of these motifs, termed Box1 and Box2, confer to IL-2Rβ constitutive binding sites for the Janus kinase (JAK)-1. For the γc, these motifs are constitutively associated with JAK-3[118]. Upon ligand binding, the receptor complex dimerizes and JAK-1 and JAK-3 get tyrosine transphosphorylated, which increases their catalytic activity, inducing tyrosine phosphorylation of the cytoplasmic tail of IL-2Rβ. The phosphotyrosine residues of IL-2β then serve as docking sites for molecules with phosphotyrosine-binding (PTB) or src-homology (SH2) domains, which are themselves targets of JAKs, triggering diverse downstream signaling events[114]. One of the six potential sites of tyrosine phosphorylation on IL-2Rβ, i.e. Tyr-338, is central to IL-2 signaling. This tyrosine links to the main IL-2 signaling events, i.e. induction of the

mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI-3K) pathway and the signal transducer and activator of transcription (STAT) pathway[120].

After tyrosine phosphorylation, the adapter molecule Shc, through its PTB domain, associates with IL-2Rβ. Shc further recruits the adaptor Grb2 and the nucleotide exchange factor son-of-sevenless (SOS), engaging the MAPK pathway[121]. Functional consequences of MAPK activation include c-fos mediated cell proliferation and Bcl-2/Bcl-x_L mediated cell survival. PI-3K is another adaptor molecule recruited by Shc[122]. PI-3K is a lipid kinase implicated in proliferation and survival. It regulates the transcription factor E2F, which controls cell cycle progression[123], and activates Akt, which in turn regulates Bcl-2 family proteins[122]. Activation of the STAT pathway is another important event in IL-2 signaling. Following tyrosine phosphorylation, STAT-5A and STAT-5B are recruited to the IL-2Rβ via their SH2 domains and are themselves phosphorylated by JAK-1 and JAK-3[124]. This leads to their dimerization and subsequent migration to the nucleus where they activate transcription of target genes[125]. An important role of STAT-5 is the induction of IL-2Rα transcription, the induction of FasL transcription as well as enhancement of cell growth and survival[126].

Like IL-2Rβ, the common γ chain is a member of the cytokine receptor superfamily. However, it directly activates only a very limited number of signals[118]. In fact, tyrosine-deficient γc still mediates proper IL-2 signaling and wild type γc cannot compensate for the lack of IL-2Rβ tyrosines[127]. The major role of γc actually lies in its association with JAK-3[128]. Unlike other JAKs, JAK-3 is uniquely associated with γc, as demonstrated by the fact that JAK-3 deficiency causes an identical phenotype to that of γc deficiency[129]. Since JAK-3 can be replaced with another JAK and still mediate γc signaling, it has been suggested that the major function of γc is to import a functional tyrosine kinase to the IL-2R complex[127]. The other function of γc appears to be the regulation of IL-2 receptor expression on cell surface. Indeed, it has been shown that γc mutant receptors lacking the

cytoplasmic tail display enhanced surface expression. In addition, three cytoplasmic domains within γc have been demonstrated to control IL-2 receptor internalization[130].

ii) Physiological role of IL-2

IL-2 is primarily produced by activated CD4+ T cells, but is also produced at lower levels by naïve CD8+ T cells, DCs and thymocytes[131-133]. IL-2 production is highly regulated by signals from the TCR and CD28. Without costimulation through CD28, T cells fail to stabilize IL-2 mRNA and do not produce IL-2 upon activation[134-135]. Two of the main functions of IL-2 are to activate innate immune effectors such as NK and NKT cells, and to promote the proliferation of activated T cells[136-137]. However, the idea that IL-2 is exclusively a proinflammatory cytokine was first questioned when it was demonstrated that IL-2-deficient mice died of autoimmune disorders[138]. It was shown that these mice were actually deficient in regulatory T cells (Tregs) and that their severe autoimmune responses could be prevented with the adoptive transfer of Tregs. This suggested that IL-2 was central in the production of Tregs, thus controlling immune homeostasis. Further evidence supports this model. For instance, the administration of IL-2 to IL-2-deficient mice restored the production of Tregs[139]. IL-2 was also shown to be essential for Tregs growth and suppressor functions[140-141]. In addition, most Tregs constitutively express the high affinity subunit of the IL-2R, i.e. the α chain (CD25) [141]. However, Tregs do not produce IL-2 but require IL-2[141]. A possible source of IL-2 for Tregs may come from autoreactive T cells. In this context, IL-2 produced by self-reactive T cells could interact with Tregs thus promoting their clonal expansion and/or survival in order to suppress the autoreactive T cells[142].

Notwithstanding the fact that IL-2 is crucial in the development of Tregs, it is also a potent activator of NK cells[143] and an important growth factor of activated T cells[144]. IL-2 is indeed sufficient *in vitro* to induce more than 1000-fold clonal expansion of T cells. Other cytokines have been shown to stimulate clonal expansion of T cells, such as IL-7 and IL-15, but it is IL-2 that induces the most efficient expansion[144]. The role of IL-2 in T cell activation is exemplified by the fact that it is the main γc cytokine secreted when T cells are initially activated *in vitro*. Indeed, the gene that encodes IL-2 is amongst the

immediate-early genes that are activated in T cells after activation through the TCR[145]. Furthermore, blockade of either IL-2 or IL-2R with monoclonal antibodies have been shown to inhibit T-cell proliferation and function *in vitro*[146]. Taken together, these studies suggested that IL-2/IL-2R interactions are responsible for the clonal expansion of activated T cells. However, with the recent development of IL-2 and IL-2R-deficient mice, it was demonstrated that some T cell proliferation occurs *in vitro* independently of IL-2[147-148]. Interestingly, although these T cells were able to expand to a certain extent, they were unable to perform full effector functions[149]. These studies suggested that TCR and costimulation are sufficient to induce some T cell proliferation, but that further expansion and most importantly effector functions are dependent upon IL-2. This crucial role for IL-2 in T-cell differentiation was however not observed *in vivo*. Surprisingly, IL-2 or IL-2R-deficient mice have relatively normal immune responses, including the induction of protective CTLs, functional helper T cells and production of antibodies[150-152]. IL-2 might be important, however, for the trafficking of the T cells to the infection site and proper NK activity[153-154]. Other studies support the notion that IL-2 may affect immune effector responses at a later stage. Indeed, the administration of IL-2 during the contraction phase of an immune response to viral infection resulted in an increase proliferation of antigen-specific T cells and to a larger pool of memory T cells[155]. This is supported by the fact that *in vivo* production of IL-2 by activated T cells only occurs when these T cells have divided more than five times[156]. Other *in vitro* studies have suggested a central role for IL-2 in controlling the contraction phase of T cell responses[157-158]. *In vitro*, IL-2 has been shown to sensitize activated T cells to apoptosis, a phenomenon known as activation-induced cell death (AICD). Upon re-encounter of the antigen, expanded T cells in the presence of IL-2 undergo AICD through Fas and TNF-dependent pathways[158]. However, IL-2 appears not to be mandatory in order to induce immune contraction, as other cytokines such as IL-4 and IL-7 can induce AICD[159-160] and that IL-2 or IL-2R-deficient T cells display a normal contraction phase *in vivo*[150].

Taken together, these studies demonstrated that: (i) IL-2 is essential in the development of Tregs; (ii) exogenous IL-2 can promote NK cell activation; (iii) exogenous IL-2 can promote antigen-specific T cell expansion; and (iv) the endogenous host immune

response has sufficient redundancy to allow effective T-cell immunity in the absence of IL-2. Malek and Bayer[141] recently proposed a "3 signals" model for T-cell responses. In their model, TCR signaling (signal 1) and costimulation (signal 2) are sufficient to induce limited T cell expansion, but necessitate signal 3 in order to induce effective T-cell responses. *In vitro*, IL-2 appears to be the major source of signal 3, while *in vivo*, considerable redundancy can compensate for the lack of IL-2. Signal 3 is thus suggested to be a "crucial checkpoint" in the immune response, inducing conditional development of effector cells, prevention of autoimmunity through its action on Tregs, and maintenance of immune homeostasis through AICD sensitization and promotion of antigenic memory.

1.3.2 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

i) GM-CSF signaling

GM-CSF (CSF-2) is a four α-helix colony stimulating factor identified based on its ability to stimulate the survival, proliferation and differentiation of myeloid precursors into granulocytes and macrophages[161-162]. The GM-CSF receptor (GM-CSFR) is a member of the type I cytokine superfamily and is composed of two subunits, the α subunit (CD116) which binds GM-CSF with low affinity, and the common β subunit (βc, CD131) which is shared by IL-3 and IL-5 receptors and has no binding affinity on its own for GM-CSF but is necessary for high affinity binding[163].

GM-CSFR is expressed on most myeloid precursors and CD34+ stem cells[164]. It is also express on mature monocytes, neutrophils, eosinophils, basophils, macrophages, dendritic cells, B and T fetal lymphocytes, endothelial cells, fibroblasts, osteoblasts and uterine cells. In addition to its physiological distribution, GM-CSFR has been shown to be expressed and to confer survival advantage to several types of tumor cells. It can be expressed in multiple myeloma, osteogenic sarcoma, breast carcinoma, lung carcinoma, prostate carcinoma, melanoma and on acute and chronic myeloid leukemia[165-166].

GM-CSFR activation occurs in a stepwise manner where GM-CSF first binds to the α subunit receptor, a complex that recruits the βc for high affinity binding[162]. Mutation studies demonstrated that there are two binding interfaces on GM-CSF important for its high affinity binding. First, residues on the fourth α helix of GM-CSF involving Asp112 are important for binding to the α subunit. Secondly, a conserved glutamate residue (E21) in the first α helix of GM-CSF is essential for high affinity binding to the βc[167-168].

In contrast to other cytokine receptors that exist as monomers, the βc subunit of the GM-CSF, IL-3 and IL-5 receptors exists as a homodimer[169]. The βc contains Box1 and Box2 conserved motifs in its membrane-proximal region, which confer constitutive binding sites for JAK-2[170]. Following ligand binding to the α subunit, the receptor complex dimerizes and JAK-2 is transphosphorylated increasing its catalytic activity and inducing phosphorylation of tyrosine residues on the cytoplasmic tail of the βc[162]. The phosphotyrosine residues then serve as docking sites for molecules with phosphotyrosine-binding (PTB) or src-homology (SH2) domains, which are themselves targets of JAKs, triggering downstream signaling events. The major GM-CSF mediated signaling pathways are the MAPK pathway[171], the PI-3K pathway[171-172] and the STAT-1 and STAT-5 pathways[173]. In contrast to IL-2 signaling, no single tyrosine residue has been found to be crucial for mediating any of the effects of GM-CSF[174]. Studies using dominant-negative JAK-2 indicated that JAK-2 activation is necessary and sufficient to induce proliferation in response to GM-CSF[175], while JAK-2 is not sufficient to promote GM-CSF mediated cell survival[176]. Although the βc is essential for GM-CSF, IL-3 and IL-5 signaling, it cannot account for cytokine specificity. Studies have demonstrated that distinct α receptor domains can confer such cytokine-specific cellular responses[177].

ii) Physiological role of GM-CSF

GM-CSF is a cytokine produced mainly by activated T cells and macrophages, but also by eosinophils, mast cells, basophils, fibroblasts and endothelial cells[162]. GM-CSF plays an important role at enhancing several immune functions in addition to promoting proliferation and differentiation of myeloid precursors. GM-CSF enhances DCs and

monocytes maturation, proliferation and migration[178], upregulates MHC class II expression on DCs and macrophages[179], enhances survival[180-181], induces degranulation, release of nitric oxyde radicals and phagocytosis of macrophages and neutrophils[182-186] and induces the expression of chemokine receptors on neutrophils[187]. The generation of GM-CSF-deficient gene-targeted mice revealed a crucial role for GM-CSF in lung immune responses[188]. The lungs of GM-CSF-deficient mice have defective macrophages, leading to an increased susceptibility to bacterial infections[189]. Interestingly, GM-CSF-deficient mice develop a pathology known as pulmonary alveolar proteinosis (PAP), characterized by an accumulation of phospholipids and surfactants in alveolar spaces. GM-CSF may thus be an important cytokine for the ability of macrophages in the lungs to phagocytose and degrade pathogens in the alveoli.

Another important role of GM-CSF is to act as a chemoattractant for monocytes, and neutrophils[190-191]. Since GM-CSF is produced by inflammatory leukocytes and activated endothelial cells, the chemotactic effects of GM-CSF may serve to recruit APCs and effector cells to "danger" zones. Other studies have shown that GM-CSF is also a chemoattractant for endothelial cells[192] and mesenchymal cells[193], suggesting that GM-CSF may be involved in tissue remodelling in addition to inflammation.

Chemotaxis of immune cells in response to "danger" signals is typically mediated by pertussis toxin-sensitive G protein-linked receptors[194]. In the case of GM-CSF, chemotaxis of neutrophil is only minimally affected by pertussis toxin suggesting a G-protein independent pathway[191]. Instead, the chemoattractant function of GM-CSF (as well as IL-8) has been linked to the activity of the ribosomal p70 S6 kinase (mTOR/p70S6K)[191]. The chemotactic potency of GM-CSF was shown to be as high as IL-8, but with a bell-shape dose-response with a maximal chemotactic effect at 10nM and the absence of any effect at doses higher than 20nM.

1.3.4. Recombinant cytokine therapy

The findings by Forni and colleagues that manipulating the cytokines present in a tumor's microenvironment could induce anticancer effects and protective immunity prompted clinical studies to test the anticancer potential of cytokines. Rosenberg and colleagues were the first to report successful treatment of cancer patients with recombinant IL-2 (recIL-2). They demonstrated that high-dose recIL-2 can induce clinical regression in 16% of patients with stage IV metastatic melanoma or renal cell carcinoma. Of the treated patients, 7% had sustained complete remission 10 years after treatment[195].

Administration of supraphysiological doses of recIL-2 causes activation of NK cells and clonal expansion of activated T cells[196]. IL-2 therapy combined with adoptive transfer of tumor-specific T lymphocytes has been shown to greatly enhance the response rate in melanoma patients (34% responders compared to 17% responders with IL-2 alone)[196-197]. One of the limiting factors of successful IL-2 therapy thus seems to be the frequency of tumor-specific T cells.

One approach to improve response rates to recIL-2 is to combine it with a tumor vaccine targeting a tumor antigen in order to enhance the frequency of tumor-specific T cells. Studies performed at the National Cancer Institute (USA) demonstrated that vaccination against an epitope of gp-100 (a melanocyte protein) was able to induce melanoma-specific T cells and, most importantly, enhanced clinical responses in 13 out of 31 patients when combined with high-dose recIL-2 compared to recIL-2 alone[198].

As for recombinant GM-CSF, it is mainly used clinically for neutropenic patients following autologous bone marrow transplants for mobilization of blood progenitor cells[161, 199-201]. Recombinant GM-CSF can also be administered to patients with acute myelogenous leukemia following induction chemotherapy. Placebo-controlled trials have shown that treatment with GM-CSF resulted in significant improvement in survival[202].

1.3.4 Cytokine gene therapy

Because of tumor heterogeneity, single TAA-directed vaccination is inevitably confronted to the loss of antigen expression by the tumor. In addition, we have little idea about the nature of dominant tumor rejection antigens for many cancers. Multi-TAAs directed vaccination is thus preferable to enhance the anticancer immunological pressure[203]. Vaccine formulations using tumor cells themselves as a source of antigens could theoretically offer the ability to prime immune responses to a broad spectrum of TAAs present on tumor cells. Since cytokines are potent modulators of immune responses, it is hypothesized that irradiated cytokine gene-modified tumor cells could be used as a cellular vaccine to induce therapeutic anticancer immune responses[204]. The development of high-efficiency gene transfer technologies has allowed investigators to test whether injection of irradiated cytokine gene-modified tumor cells could induce tumor-specific immunity.

i) IL-2 cancer gene therapy

Cytokine gene-modified tumor cells, because of tumor-localized cytokine expression, could circumvent the severe toxicity associated with systemic injection of recombinant cytokine while inducing tumor-specific systemic immunity. The first study comparing the relative anticancer potential of cytokines was performed by Dranoff and colleagues who gene-modified B16 mouse melanoma cells to express IL-2, IL-4, IL-5, IL-6, GM-CSF, IFNγ, IL-1 or TNF-α, and assessed whether cytokine expression could alter tumor immunogenicity[205]. They observed a modest delay in tumor formation when live B16 cells were gene modify to secrete IL-4, IL-6, IFNγ and TNF-α. When B16 tumors were gene-modified to secrete IL-2, however, they observed complete tumor rejection. In order to assess if IL-2 could induce protective antitumor immunity (against non gene-modified B16 cells), the mice that had rejected the IL-2 secreting B16 cells were re-injected with wild type B16 cells. A modest delay in wild type B16 tumor growth was observed. Dranoff's work set the stage for several pre-clinical and clinical studies evaluating the antitumor effects of cytokine cDNA gene transfer.

A. *In vivo* tumor-targeted IL-2 gene therapy

One of the first cancer gene therapy approaches consisted of *in vivo* delivery of the IL-2 cDNA. Using direct intratumoral administration of an adenoviral vector encoding for IL-2, Haddada *et al.*[206] followed by Addison *et al.*[207] were the first to demonstrate, in animal models of cancer, that intratumoral delivery of IL-2 cDNA can induce local as well as systemic antitumor effects. Several phase I clinical trials have since established that adenoviral vector-mediated tumor-targeted delivery of IL-2 is well tolerated in cancer patients, inducing minor side-effects such as fever and anorexia[208-209]. Although anticancer responses are not the primary objective of phase I trials, only few cases of clinical responses were reported, waning down the interest in pursuing phase II/III trials. In Canada, two phase I clinical trials testing adenoviral vector-delivery of IL-2 cDNA have been documented. In the first trial, 23 patients with melanoma or breast cancer were injected with increasing doses of adenoviral vectors ranging from 10^7 to 10^{10} plaque-forming-units (PFU)[208]. As expected, tumor-derived IL-2 expression was transient and became undetectable 7 days after injection. Of the 23 treated patients, two had signs of tumor regression at the injection site only, while all patients had systemic progression of their disease. In the second trial[209], 12 patients with localized prostate cancer were injected prior to prostatectomy with increasing doses of adenoviral vectors ranging from 10^9 to 10^{10} PFU. Similarly to the first trial, no toxicity was observed. Interestingly, the prostate-specific antigen (PSA) levels declined (mean 33%) in five of five evaluable patients injected at the lowest dose. However, at higher doses, PSA levels increased significantly during the first two weeks after injection to subsequently decline to levels observed prior to injection. After an 18 months follow-up, all patients had trace of PSA and there was no indication of disease relapse.

B. *Ex vivo* IL-2-expressing tumor vaccines

The major limitations of *in vivo* tumor-targeted gene-transfer are the transient transgene expression and anti-vector immune responses in the case of adenoviral vectors, and in the case of retroviral vectors, the insufficient expression of the therapeutic transgene. An alternative approach consists of gene modifying *ex vivo* cultured autologous tumor cells to express the desired transgene, and using them as an irradiated cellular vaccine to

induce therapeutic anticancer immune responses. After the demonstration in preclinical experiments that animal treated with *ex vivo* gene-modified IL-2 expressing tumor vaccine could develop antitumor CTLs[210], human clinical trials were initiated. In one trial, 12 patients with malignant melanoma were vaccinated once, twice or three times with 10 million irradiated autologous IL-2 secreting tumor cells[211]. Injection of *ex vivo* gene-modified IL-2 expressing tumor cells was associated with little toxicity. One of the limitations of the trial was the fact that a high proportion of patients could not be vaccinated because of cancer progression between tumor harvest and the completion of the vaccine preparation, highlighting the difficulty in generating autologous gene-modified tumor cultures. In that trial, no clinical responses were observed, but three patients had stable disease for 7-15 months and seven patients had detectable CTLs in the peripheral blood.

Because the generation of autologous tumor cell vaccines is time consuming and establishing tumor cell lines not always possible for every patient, allogeneic cellular tumor vaccines have been proposed as an alternative. It has been demonstrated that CTLs can specifically recognize and lyse both autologous and allogeneic melanoma cells based on shared tumor antigens. Furthermore, it has been suggested that the allogeneic response to a vaccine may enhance cross-priming of tumor antigens by professional APCs[212-213]. In a phase I clinical trial testing allogeneic tumor vaccines, 33 patients with metastatic melanoma were injected three times at weekly intervals with 60 million irradiated melanoma cells secreting 120ng of IL-2/10^6cells/24hr[214]. The treatment induced T infiltration in distant metastases in three patients, complete or partial metastatic regression in two patients and disease stabilization in seven patients including one who developed vitiligo.

ii) GM-CSF cancer gene therapy

As mentioned above, Dranoff and colleagues reported that IL-2 was the most effective cytokine at inducing rejection of live gene-modified tumors[205]. However, they also observed that it was GM-CSF expression that was the most efficient for inducing systemic protective immunity against non gene-modified wild type tumor cells[205]. In

their experiments, injection of irradiated GM-CSF expressing tumor cells could prevent both the growth of a subsequent challenge of wild type tumor cells and the growth of a three days pre-established tumor.

Histological examination of the inflammatory response mediated by irradiated GM-CSF secreting tumor cells revealed an intense recruitment of DCs, macrophages and granulocytes[215-216]. This suggested that one important function of GM-CSF in a tumor vaccine is its ability to recruit APCs to the vaccine site and thus enhance tumor antigen presentation. GM-CSF was also shown to upregulate costimulatory molecules and to induce high levels of CD1d expression on APCs[216]. CD1d is an MHC class I-like molecule which is known to present lipids antigens to NKT cells, especially the Vα14Jα281 invariant NKT cells[217]. The importance of invariant NKT cells to the adjuvant effect of GM-CSF was subsequently assessed. Invariant NKT cell were revealed to be essential, as immunization of Jα281-deficient mice with GM-CSF secreting tumor cells failed to protect the mice against a wild type tumor challenge[218]. Interestingly, T cell mediated cytotoxicity against B16 cells was comparable between the two groups. However, there was a downregulation of Th2 cytokine production (IL-4, IL-5 and IL-6) by T cells from vaccinated Jα281-deficient mice compared to control mice. This observation is consistent with the suggestion that antibodies may play an important role in GM-CSF stimulated immunity[219].

Simultaneously, other studies assessed the dose-response of GM-CSF antitumor effect. Jaffee and colleagues[220] demonstrated that effective GM-CSF cellular vaccination requires a minimal secretion level of 36ng/10^6 cells/24 hours, and that levels up to 10 fold higher did not result in increased antitumor effect. They also observed that the vaccination effect was significantly enhanced when GM-CSF expressing cells were administered in separate locations compared to a single location, suggesting that achieving multiple lymph node priming enhances the effect of GM-CSF expressing tumor vaccines.

Although GM-CSF can act as a potent adjuvant to tumor vaccines, several studies have demonstrated that several types of tumor cells use GM-CSF as a growth factor[165-166]. Furthermore, GM-CSF has been shown, in some circumstances, to suppress certain immune functions. For instance, it has been reported that GM-CSF can downregulate the activity of NK cells[221-222]. In addition, GM-CSF has been shown to suppress autoreactive T cells through the generation of CD4+CD25+ Tregs[223]. Paradoxical effects of GM-CSF on tumor growth were also reported by Serafini and colleagues[224] who demonstrated that GM-CSF producing tumor vaccines can either stimulate or suppress tumor-specific immunity depending on the dose administered. Importantly, they observed that the immunosuppressive effects of GM-CSF were observed when the systemic levels, independently of the local levels, exceeded a certain threshold. They identified this upper "vaccine-secretion limit" to be between 300 and 1500ng of GM-CSF/10^6 cells/24 hours. However, they cautioned about extrapolating this limit to human cancers as it can vary depending of the cytokine efficacy in distinct species and tumor heterogeneity. Notwithstanding this, it is clear that the development of GM-CSF-mediated tumor vaccines needs to take into account that above a certain dose, GM-CSF can induce immune suppression.

Three different types of GM-CSF-mediated tumor vaccines have been thus far tested in clinical trials: autologous GM-CSF-expressing tumor cell vaccines, allogeneic GM-CSF-expressing tumor cells vaccines and autologous tumor cells admixed with universal GM-CSF-expressing bystander cells vaccines.

In all clinical trials using autologous GM-CSF-producing cell vaccines, the major limitation was the ability to obtain the desired number of viable cells for gene transfer. In the first trial, renal cell carcinoma patients were injected with 4, 40 or 400 million GM-CSF-producing cells (42-149ng/10^6cells/24 hours)[225]. The number and intervals of vaccination were highly variable due to the difficulty of obtaining viable tumor cells, which made any dose-response difficult to assess. Importantly, there were no clinical toxicities reported. A clinical response, characterized by regression of multiple pulmonary metastases, was observed in one patient injected at the highest GM-CSF dose

(40 million cells secreting 149ng/10^6cells/24 hours). In the second trial, 33 patients with advanced metastatic melanoma were injected with 10 million GM-CSF-producing cells (84-965ng/10^6cells/24 hours) at 7, 14 or 28-day intervals for a total of 3, 6 or 12 vaccinations[226]. As in the first trial, no systemic toxicities were observed. Histological analyses of the vaccination sites revealed a significant infiltration of both CD4 and CD8 T cells and, surprisingly, a significant infiltration of plasma cells comprising nearly 50% of the infiltrate. Using standard clinical criteria, the authors observed one partial response, one mixed response and three minor responses.

More recently, Nemunaitis *et al.* published the results of a phase I/II clinical trial of autologous GM-CSF-secreting vaccination for Non-Small-Cell Lung Cancer (NSCLC)[227]. In this trial, 83 patients with early or late stage NSCLC were treated with intradermal injections of irradiated GM-CSF expressing tumor cells every two weeks. Remarkably, three advanced-stage patients who had previously failed chemotherapy achieved durable, complete tumor regressions lasting 6 months, 18 months and more than 22 months. Additionally, one minor response and two mixed responses were reported, while seven patients had stable disease. As predicted by preclinical studies, the antitumor effect was GM-CSF dose-dependent with a 1-year survival of 0% for patients receiving doses lower than 40ng of GM-CSF/10^6cells/24 hours, compared to a 1-year survival of 56% for patients receiving doses higher than 40ng of GM-CSF/10^6cells/24 hours. Although delayed-type hypersensitivity and immune cell infiltration could be observed, immunological responses were not associated with either tumor regression or survival. Similar clinical results were obtained from the Dranoff group using autologous GM-CSF-producing tumor cells for the treatment of NSCLC[228] and metastatic melanoma[229].

The second approach reported is the use of allogeneic GM-CSF producing tumor vaccines. As previously noted, the major limitation of autologous tumor vaccines is the difficulty to culture sufficient tumor cells and thus the difficulty to increase the vaccine dose or to continue vaccination. Because rejection antigens of specific cancers are often shared among different cancer patients, the use of allogenic cellular vaccines constitutes an alternative approach. In the first trial testing "off-the-shelf" allogeneic GM-CSF-

producing vaccines[230], 14 non-metastatic pancreatic cancer patients were injected with a mixture of two human pancreatic tumor cell lines expressing a *k-ras* mutation and gene-modified to produce GM-CSF (120-250ng/10^6cells/24 hours). The trial was a dose-escalation study with doses of 10, 50, 100 or 500 million cells per vaccine. Of interest, one patient vaccinated at the highest dose developed an acantholytic dermatosis (Grover's disease). In another clinical trial testing allogeneic GM-CSF-secreting tumor vaccines, 80 patients with hormone-refractory prostate cancer were enrolled in a phase II trial. Of the treated patients, 62% had improvement or stabilization of bone metastatic activity, with 2 patients with complete normalization[231]. Based on these results, a randomized phase III trial was initiated in 2004 on approximatively 600 patients in order to compare allogeneic GM-CSF-expressing prostate cancer vaccination with standard chemotherapy.

Finally, an alternative approach that has been reported combines the advantages of both autologous and allogeneic vaccines. The strategy consists of retrieving patients' tumor cells with minimal tumor cell processing and coupling them to an allogeneic cell preparation previously modified to express high and stable amounts of GM-CSF. Such "bystander" vaccine has been reported by Borello *et al.* who have generated a cell line that expresses high levels of GM-CSF and lacks expression of MHC class I and class II molecules[232]. They hypothesized that the absence of MHC molecules will limit the chances of generating allogeneic responses to the bystander cells, thus biasing the response towards the patients' tumor antigens.

iii) Other anticancer cytokines

IL-12 is another recently identified anticancer cytokine. IL-12 is a type I cytokine produced by dendritic cells, macrophages, B cells and possibly other phagocytic cells upon encounter with pathological agents[233]. The main effect of IL-12 is to induce the production of IFNγ by T cells and NK cells. IL-12 also promotes the differentiation of CD4+ T cells into Th1 cells, and has been shown to inhibit cancer-induced neoangiogenesis[234]. The antiangiogenic properties of IL-12 seem to be mediated by IFNγ as neutralizing anti-IFNγ antibodies prevented its effect[235]. Although IL-12 has been

shown to enhance the rejection of a variety of murine tumors, clinical studies have revealed that IL-12 can induce severe toxicity. In the first phase II clinical trials[236-237], the administration of recombinant IL-12 at a dose of 500ng/kg resulted in the death of 2 out of 17 patients and hospitalization of 12 patients. In a subsequent trial, ovarian cancer patients were injected with 250ng/kg recombinant IL-12 as a single dose followed by a 2-week rest period, followed by cycles of 5 daily injections and 16-day rest until disease progression[238]. It was determined that a single injection 2 weeks before consecutive dosing had a major effect in decreasing IFNγ production and associated toxicity. In that trial, one patient was a partial responder and 13 had stable disease.

In order to minimize the toxic side-effects associated with recombinant IL-12, many studies have explored gene therapy approaches. Overall, tumor-mediated IL-12 gene expression leads to the induction of potent cellular anticancer immunity dependent on CD8+ T cells and NK cells. In the only reported clinical trials assessing IL-12 gene therapy approaches, the investigators tested the antitumoral effect of intratumorally injected allogeneic[239] or autologous fibroblasts[240] transfected with the IL-12 cDNA. With the injection of IL-12-producing autologous fibroblasts, tumor regressions were observed in 4 of 9 patients while one patient had regression of a distant non-injected tumor[240]. Finally, in animal models of cancer, synergistic antitumor effects have been observed when combining IL-12 cDNA with other cytokines (GM-CSF, IL-15, IL-2 and IL-18), chemokines (lymphotactin, IP-10) or adoptive cell therapy[241].

The immune system is characterized by a high degree of redundancy exemplified by the sharing of many cytokine receptor subunits. IL-2 and IL-15 for instance, share two of their three-receptor subunits. It is thus not surprising that IL-15 has also been reported to possess potent anticancer properties[242]. These two cytokines also share many physiological functions. Shared functions between IL-2 and IL-15 include initial stimulation of activated lymphocytes and the activation of NK cells[242]. However, IL-2 and IL-15 provide opposing signals to the adaptive immune response. For instance, while IL-2 is an inducer of lymphocyte activation-induced cell death (AICD), IL-15 inhibits this AICD and stimulates the maintenance of CD8+ memory T cells[243-244].

It has been demonstrated that IL-15 can induce potent anticancer responses in a wide spectrum of animal models of cancer[242]. In some cases, IL-15 mediated anticancer effects were found to be superior to IL-2[245]. Following adoptive transfer of tumor-specific lymphocytes, recombinant IL-15 has been shown to be superior to recombinant IL-2 enhancing anticancer responses against established tumors. IL-15 was also shown to be a potent adjuvant to DNA vaccination strategies[246]. However, because it antagonises AICD and thus self-tolerance, IL-15 carries the additional risk compared to IL-2 of inducing survival of autoreactive T cells, which would otherwise be eliminated[242]. This could potentially lead the development of autoimmune diseases. In addition, IL-15 has been shown to induce the release of high levels of TNFα and IL-1β, thus limiting the therapeutic dose that can be safely administered to patients[243].

1.4 GENE DELIVERY VECTORS

Gene delivery vectors can be defined as technical vehicles facilitating the transfer of genetic material into target cells. These vectors can be derived either from a viral system or from a non-viral system. The most commonly used viral vectors are derived from retroviruses, adenoviruses and adeno-associated viruses (AAV). Non-viral vectors are either plasmid DNA or chemically synthesized compounds. The major factors influencing the type of vector to use are: (1) the durability of expression, (2) the "transducibility" of the target cell, and (3) the size of the gene to be transferred[247]. I hereafter briefly describe the major gene transfer vectors.

1.4.1 Retroviral vectors

Retroviruses are composed of two positive single RNA strands, copied upon infection by the viral reverse transcriptase into double-stranded DNA that then migrates to the nucleus where it can stably integrate into the host genome[248]. One of the most widely used retroviruses in gene therapy is the Moloney Murine Leukemia Virus (MMLV). MMLV

is a retrovirus with four viral genes: *gag, pol, pro* and *env*. In order to generate a retroviral vector, coding sequences from the MMLV genome are removed and replaced with a therapeutic gene[249-250]. The deletion of the viral sequences, however, makes it necessary to express these genes in *trans* in what are known as "packaging" cell lines. Packaging cell lines thus express the *gag, pol, pro* and *env* genes, however without their packaging sequence. The subsequent transfer into these cells of a plasmid encoding a therapeutic gene with minimal viral sequences results in the production of replication incompetent retroviral particles capable of transferring the therapeutic gene into target cells. The minimal viral sequences required are: (1) the long terminal repeats (LTRs), (2) the primer binding site (PBS), (3) the polypurine tract (PPT) and (4) the packaging sequence[250]. For MMLV-derived vectors, two different glycoproteins of the envelope can be used to induce gene transfer in target cells: the ecotropic glycoprotein, which binds an amino acid transporter present only on murine cells[251], and the amphotropic glycoprotein, which binds a phosphate transporter that is present on most cell types including rodents and humans[252].

The major advantages of using retroviral vectors lies in their ability to selectively gene-modify dividing cells (with the exception of lentiviruses) and to induce stable expression of a therapeutic transgene by integrating it into the genome. Most retroviral vectors offer the possibility to introduce therapeutic transgenes up to 8-10 kb in size[253]. The titers obtained are usually between 10^5 and 10^6 infectious particles per millilitre and can be concentrated to more than 10^{12} infectious particles per millilitre when the viral particles incorporate the G-glycoprotein from the vesicular stomatitis virus envelope[254]. The two major disadvantages with the use of retroviral vectors are the risks of insertional mutagenesis and the possible generation of replication competent retroviruses (RCR). Insertional mutagenesis has been shown to occur as a consequence of retroviral gene transfer in human hematopoietic cells resulting in the development of leukemia in patients injected with these cells[255]. The other major concern is the risk of generating RCR[256]. Although there has thus far been no such report from clinical trials, it is possible that a replication-incompetent retroviral vector administered *in vivo* could recombine with endogenous viruses and generate a new RCR virus.

1.4.2 Adenoviral vectors

Adenoviruses are double stranded DNA viruses and replicate within the nucleus outside of the chromosome[257]. In humans, wild type adenoviruses cause self-limiting acute respiratory infections. Initial attachment of adenoviruses to target cells is mediated by a fiber protein that binds to the Coxakie adenovirus receptor (CAR)[257]. In contrast to retroviral vectors, adenoviral vectors are used to achieve gene transfer of non-dividing cells. Because of their size (30-35kb), they can be used as vectors for the delivery of large sequences[258]. Furthermore, the expression level of the therapeutic gene is generally greater than what can be obtained with retroviral vectors. However, because of immune responses against the vector and its non-integrating nature, the gene expression is only transient. This is a serious limitation to the use of adenoviral vectors, but is less of a problem for gene therapy approaches that require short-term expression. There are three major concerns with the use of adenoviral vectors: (1) organ inflammation due to immune reactions against the vector, (2) the development of tolerance to the vector that could result in disease upon infection with wild type adenoviruses, and (3) the generation of replication competent adenoviruses[259]. However, for the latter concern, malignancies are less likely to be induced since adenoviruses do not stably integrate into the host genome.

1.4.3 Adenovirus-associated vectors (AAV)

AAV are single-stranded DNA viruses of approximatively 4.5 kb that replicate and integrate in the nucleus in the presence of a helper virus[260]. Therefore, AAV require co-infection with another virus, usually an adenovirus or a herpes simplex virus. AAV have not been associated with human disease, but 90% of humans show evidence of prior infection with AAV[260-261]. Wild type AAV integrate at a specific region on chromosome 19, but AAV vectors integrate randomly since they lack the Rep protein necessary for site-directed integration[261]. Similarly to retroviral vectors, AAV vectors are deleted of all coding sequences, avoiding immune responses to viral proteins. The most common

method of packaging AAV vectors consist of transfecting an inverted terminal repeat (ITR)-flanked plasmid encoding the therapeutic gene and a *rep-cap* expression plasmid (deleted of ITR) into adenoviral-infected 293 cells. The major advantage of AAV vectors is their ability to stably gene modify non-dividing cells, and their major disadvantage is their small packaging limit of 4.5 kb. The potential risks of using AAV vectors are: (1) the presence of contaminating adenovirus which can cause adverse side-effects, (2) the potential for insertional mutagenesis, and (3) the generation of wild-type AAV as the result of recombination between the vector and the packaging plasmid[262].

1.4.4 Non-viral vectors

Non-viral vectors are gene transfer techniques that do not involve a viral particle. They include naked DNA plamids amplified in bacteria or eukaryotic cells, and chemically synthesized oligodeoxynucleotides[263]. Plasmid DNA can contain up to 15 kb of genetic material and require cationic liposomes or receptor-mediated targeting in order to efficiently enter a target cell. Synthesized oligodeoxynucleotides contain between 10 and 25 bases and are generally used to alter processing, translation or stability of targeted RNA. Alternatively, oligodeoxynucleotides can serve as inhibitors of DNA transcription or decoys to transcription factors. The major advantage of non-viral vectors is the absence of risk of generating competent viruses, while the major disadvantage is the generally transient expression of the plasmid-encoded genes. The potential risks of non-viral vectors are the risks of insertional mutagenesis if the plasmid integrates and the toxicities associated with the compounds that are used to facilitate cell entry[264].

1.5 BONE-MARROW STROMAL CELLS (MSCs)

1.5.1 Definition of MSCs

The term stroma, derived from the Greek "stromos" meaning "mattress", is used to identify the supportive connective tissue associated with a given organ[265]. In the bone

marrow, the stroma applies to the non-hematopoietic tissues that support hematopoiesis and lymphopoiesis[266]. This is achieved through structural support on the one hand, and by supplying growth factors and cell-cell interactions on the other. Bone marrow stroma consists of a heterogeneous population of cells, including osteogenic cells, adipocytes, reticular cells, macrophages, vascular endothelial cells and smooth muscle cells surrounding blood vessels, as well as marrow stromal cells[266].

The first report of a population of stem cells in the bone marrow stroma is attributed to Friedenstein[267], who described the growth of colony forming unit fibroblasts (CFU-F) from cell suspensions of bone marrow aspirates. He observed that a small fraction of adherent cells formed foci of two to four cells, remained dormant for 2 to 4 days and then began to multiply rapidly. Remarkably, these marrow stromal cells (MSCs) had the ability to differentiate into bone- and cartilage-like colonies. These initial observations have been since confirmed by several investigators who demonstrated that the cells isolated following Friedenstein's technique were able to differentiate into osteoblasts, chondroblasts, adipocytes and myoblasts[268-271]. Because of their mesenchymal plasticity, adherent cells from MSCs cultures are thus often referred to as mesenchymal stem cells. When plated at low cell density (1-10 cells/cm^2), adherent MSCs can be cloned as single-cell-derived colonies[272-274]. Individual MSCs colonies, however, have been shown to possess variable degree of plasticity[268]. MSCs cultures are thus heterogeneous, containing subpopulations of early progenitors and more committed progenitors. Morphologically, two distinctive types of cells are found within MSCs cultures: small rapidely self-renewing multipotent cells (RS cells) and more mature slowly replicating larger cells[274-276]. If maintained at low cell densities, MSCs cultures remain rich in multipotent RS cells until approximatively 50 population doublings, where cultures are then dominated by the larger cells[275,277]. However, RS cells are quickly lost during expansion when MSCs are maintained at high density[278]. Whenever plated at low density, MSCs display a stationary phase of 2-4 days, followed by an exponential growth phase, and by another stationary phase[275-277]. Notably, if conditioned media from early log phase cultures is added to new MSCs cultures, the expansion potential of MSCs is significantly increased. This was recently attributed to Dickkopf-1 (Dkk-1) protein, an

inhibitor of the Wnt signaling pathway, secreted by MSCs in early log phase[279]. It was demonstrated that when MSCs approach their stationary phase, Dkk-1 and its receptor LRP6 are downregulated, while the gene for *Wnt5a* is upregulated. It is thus hypothesized that in the bone marrow, density-dependent expression of Wnt5a and Dkk-1 prevents MSCs from overpopulating the marrow.

Recently, studies by Verfaillie and colleagues suggested that multipotent RS cells are not the earliest progenitors withing MSCs cultures. They reported the isolation of another marrow stem cell referred to as multipotential adult progenitor cells (MAPCs) that possess greater plasticity than MSCs cultures[280]. The main difference between MAPCs and MSCs is that MAPCs require medium containing a mixture of growth factors that are not required for MSCs cultures.

1.5.2 Phenotypic characterization of MSCs

At present, the only method of defining MSCs is by assessing the expression of membrane-bound surface antigens. Several antibodies have been described to identify human and rodent MSCs. However, all of them recognize antigens expressed on a variety of other cell types. In human, the SH-2 antibody was the first to be used in immunoselection methods to isolate MSCs[268]. The SH-2 antibody reacts with the TGF-β receptor endoglin (CD105) expressed on MSCs and endothelial cells[281]. The antibodies SH-3 and SH-4, also generated to identify MSCs, recognize distinct epitopes of the membrane-bound ecto-5'-nucleotidase (CD73)[282]. Both human and mouse MSCs express CD105 and CD73. Additionally, human and mouse MSCs have been reported to consistently express the hyaluronate receptor CD44. In order to rule out any contamination of MSCs preparations by hematopoietic or endothelial cells, MSCs are routinely tested for the expression of CD45 (common leukocyte antigen) and CD31 (PECAM-1; highly expressed on endothelial cells) antigens. The expression of the hematopoietic stem cell (HSC) marker CD34 is also commonly used to assess the presence of contaminating HSC in human MSCs preparations. While human and

BALB/c MSCs are consistently negative for CD34, it as been reported that C57BL/6 mouse MSCs express CD34 in a heterologous fashion (10-20% positivity)[283]. Hence, MSCs can be defined as being positive for the expression of CD105, CD73 and CD44, negative for the expression of CD45 and CD31, and negative for the expression CD34 with the exception of C57BL/6 mice.

1.5.2 Biology of MSCs

It has been shown that long-term cultures of hematopoietic stem cells (HSCs) require the presence of MSCs[284]. One of the main role of MSCs is thus to provide the microenvironment necessary for hematopoiesis, including the secretion of cytokines and growth factors such as macrophage-CSF (M-CSF), Flt-3L, stem-cell factor (SCF), IL-6, IL-7, IL-8, IL-11, IL-12, IL-14 and IL-15. Upon IL-1α stimulation, MSCs can further produce IL-1α, LIF, G-CSF and GM-CSF[285]. In addition to their role in hematopoiesis, MSCs are also implicated in lymphopoiesis[286]. Maturation of B cells, for instance, occurs in the bone marrow and involves interactions with MSCs, which provides them with SCF and IL-7[287]. It has also been suggested that the bone marrow stroma, through T cell-MSCs interactions, might be involved in extrathymic T cell lymphopoiesis[288]. Indeed it was shown that when thymocytes are seeded on MSCs, immature double negative (CD4-CD8-) and double positive (CD4+CD8+) T cells preferentially adhere to MSCs and proliferate[288]. In addition, studies suggest that the bone marrow plays an important role in endogenous immune responses by hosting and regulating adaptive immunity. For instance, Mazo *et al.*[289] demonstrated that central memory CD8+ T cells are preferentially recruited to the bone marrow. Intriguingly, homing of memory CD8+ T cell was mediated by CXCL12, a chemokine abundantly produced by MSCs. Furthermore, it has been demonstrated that naïve T cells can home to the bone marrow where they are activated in an antigen-dependent manner to blood-circulating tumor antigens[290]. Taken together, the bone marrow is being revealed as a unique lymphoid organ able to activate naïve T cells and to recruit memory T cells and thus MSCs may represent a previously unrecognized player of physiological immune responses.

1.5.3 MSCs in regenerative medicine

The plasticity of MSCs makes them ideal candidates for regenerative medicine and tissue engineering. Clinical studies based on transplantation of MSCs are generating promising results for the treatment of several diseases. I will hereafter review recent developments in the use of MSCs for the treatment of osteogenesis imperfecta, cardiovascular diseases and neuronal diseases. Osteogenesis imperfecta is a genetic disorder in which osteoblasts produce defective type I collagen, thereby inducing numerous fractures, skeletal deformities and retarded bone growth[291]. There is currently no cure for osteogenesis imperfecta. Horwitz and colleagues were the first to demonstrate that allogeneic bone marrow transplantation can significantly improve the condition of osteogenesis imperfecta patients via engraftment of mesenchymal progenitor cells[292]. Recently, they demonstrated that infusion of purified allogeneic MSCs enhances the clinical benefits of allogeneic marrow transplantation[293]. Remarkably, they provided evidence that transplanted allogeneic MSCs engraft in the bone and differentiate into osteoblasts without the requirement for preparative chemotherapy.

Another field of interest in regenerative medicine is the development of MSC-based therapy for the treatment of cardiovascular diseases. Since adult cardiomyocytes have limited regenerative capacity, implantation of progenitor cells with cardiac plasticity has been suggested for the regeneration of damaged cardiac cells after myocardial infarction[294]. Various cell types have been studied for this purpose, including fetal cardiomyocytes[295-297], skeletal myoblasts[298], endothelial progenitor cells[299] and MSCs[300-302]. Bone marrow is, at present, the most frequent source of cells used for clinical cardiac repair[303-304]. In the case of MSCs, studies have suggested they have the ability to home to sites of tissue injury including the infarcted myocardium[305-306]. The factors responsible for inducing MSCs migration have however yet to be identified. Since the therapeutic benefit of MSCs transplantation following myocardial infarction decreases over time and appears to occur in less than 72 hours[307], it has been proposed that paracrine factors secreted by MSCs are mainly responsible for the observed effects. In a recent issue of

Nature Medicine, Victor Dzau's group provided the first evidence in support of this hypothesis[308]. They demonstrated that supernatants from hypoxia-exposed MSCs, and to a greater extent Akt gene-modified MSCs, can significantly prevent apoptosis of cardiomyocytes after myocardial infarction in rats.

Another aspect of MSCs is their reported ability to cross the blood–brain barrier and to migrate preferentially to an ischemic cortex where they have been shown to differentiate into microglia and astroglia[309]. Li *et al.* reported that intravenous injection of human MSCs 1 day after stroke can improve functional outcome in rats after as early as 7 days[310]. They observed, however, that less than 2% of injected MSCs express neuronal differentiation markers, leading them to hypothesize that paracrine factors possibly secreted by MSCs, and not differentiation of MSCs, may be responsible for the observed therapeutic benefits. The interaction of MSCs with the host brain may furthermore lead MSCs and parenchymal cells to produce abundant growth factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), both of which were detected in MSC-injected regions.

In summary, the characterization of the mechanisms responsible for MSCs-induced tissue repair, whether in the bones, brain or heart, should allow scientists to develop new therapeutic strategies for the treatment of catastrophic diseases.

1.5.4 Immune properties of MSCs

The interest in MSCs for regenerative medicine was invigorated with reports that MSCs display immunosuppressive properties when transplanted in allogeneic hosts, suggesting that "universal donor" MSCs may be used for "off-the-shelf" cell therapy. It has been shown that MSCs are able to: (i) suppress the proliferation of allogeneic T cells in response to mitogen or allogeneic cells[311-313, 316, 317]; (ii) inhibit the production of IFNγ and tumor-necrosis factor (TNF)-α and increase the production of IL-10[318]; (iii) induce T cell division arrest anergy[319]; (iv) inhibit the maturation and function of antigen presenting cells such as monocytes and dendritic cells[320-321]; (v) decrease alloantigen-

specific cytotoxicity of CD8 T cells and natural killer (NK) cells[322]; and (vi) favor the differentiation of CD4 T cells with presumed regulatory activity[322]. In a non-human primate animal model, *in vivo* injection of MSCs led to a modest yet significant prolongation of skin graft survival comparable to immunosuppressive agents[323]. The immunosuppressive effects of MSCs on allogeneic immune responses has also been shown to increase the tumorigenicity of B16 mouse melanoma cells when injected in allogeneic hosts[317]. In humans, the clinical potential of the immunosuppressive properties of MSCs has been exemplified by LeBlanc and colleagues[324] who reported in a case study that administration of haploidentical human MSCs following allogeneic stem cell transplantation could reverse the severe grade IV acute graft-versus-host disease (GVHD) of a patient.

At present, the exact mechanism responsible for MSC-mediated immunosuppression remains imprecise. Djouad *et al.* demonstrated that the immunosuppressive effects of MSCs are mediated by soluble factors secreted when MSCs are cocultured with allogeneic splenocytes[317]. Likewise, other studies reported that soluble factors from MSCs/splenocytes cocultures, independently of cell-contact, are responsible for inhibiting T-cell proliferation[311, 313]. Djouad *et al.* further provided evidence that the immunosuppressive effects were actually mediated through the generation of CD8+ regulatory T cells[317]. The identification of the soluble factors responsible for MSC-mediated immunosuppression is still controversial. Hepatocyte growth factor (HGF), transforming growth factor (TGF)-β1[31], indoleamine 2,3-dioxygenase (IDO)[325], IL-10[320] and unidentified factors[312, 317, 326] have been implicated. Other studies suggested instead that contact-dependent mechanisms are required[316, 320].

Since MSCs are known to express low levels of MHC class II molecules and to upregulate these molecules together with adhesion molecules upon stimulation with IFNγ, MSCs may behave as antigen presenting cells. To assess whether MSCs could stimulate T cells, two studies[311-312] used irradiated human MSCs as stimulators for allogeneic peripheral blood mononuclear cells (PBMC) in one-way mixed cell cultures. While control allogeneic PBMC were efficient stimulators, allogeneic MSCs did not

induce any proliferation of PBMC. In addition, the pretreatment of MSCs with IFNγ did not improve the stimulating capacity of MSCs despite MHC class II upregulation. In fact, IFNγ-treated MSCs were more immunosuppressive than non-treated MSCs when added to third-party allogeneic mixed lymphocyte reactions (MLR)[311]. However in another report[314], one-way MLR were not inhibited by irradiated MSCs although third-party MLR were, contradictimg the two aforementioned studies. Notably, the human MSCs used in the latter experiments expressed significant levels of MHC class II molecules without IFNγ stimulation, suggesting that different subsets of MSCs may induce distinct immunological effects. One study suggested that MSCs may behave differently in autologous/syngeneic conditions: Beyth *et al.* reported that human MSCs cocultured with autologous purified human CD4+ T cells and SEB superantigen can activate CD4+ T cells[320]. Notwithstanding that antigen processing was not required in these experiments to induce T cell activation, it suggested that human MSCs were able to provide sufficient MHC class II and costimulation signaling to induce CD4+ T cell activation. In summary, if the immunosuppressive effects of MSCs on allogeneic or third party immune responses have been well described, the effect of MSCs on autologous/syngeneic immune responses has been largely overlooked.

1.5.5 Spontaneous transformation of human MSCs

It is important to mention that a recent study published by Rubio *et al.* reported that human adult MSCs derived from adipose tissue can spontaneously transform after long-term *in vitro* culture[327]. This study is the first report of spontaneous transformation of adult human stem cells. The authors demonstrated upon *in vitro* isolation, all human MSCs preparations (10 out of 10) were able to bypass senescence phase. Remarkably, 30% of MSCs samples displayed trisomy of chromosome 8 after senescence bypass. Normally, when cells bypass senescence, they continue to grow until telomeres become too short and then enter a crisis phase, characterized by chromosome instability and mass apoptosis. In their study, 50% of MSCs preparations further bypassed the crisis phase and were able to grow in soft agar 4-5 months after isolation. When these MSCs were injected into immunodeficient mice, all mice developed tumors, suggesting that 50% of

MSCs preparations had spontaneously transformed. Karyotype analysis of transformed MSCs revealed nonrandom chromosome rearrangements. Notably, telomerase activity was detected in all transformed MSCs samples while presenescence and post-senescence MSCs showed no detectable levels of telomerase. This study highlights the importance of better defining the biology of MSCs in order to establish safe criteria for their use in cell therapy protocols. The characterization of the effects of culture expansion on the genetic stability of MSCs will thus be critical to their clinical use.

1.6 SPECIFIC RESEARCH AIMS

The main objective of the research presented in this thesis was to develop novel approaches in order to improve cytokine-based immunotherapeutic strategies against cancer. The specific research aims were:

1. To test the hypothesis that a fusion between two cytokines cDNA, specifically GM-CSF and IL-2, would circumvent the limitations associated with the combinatorial use of cytokines and may induce novel anticancer effects;

2. To test the hypothesis that primary marrow stromal cells can be used as a cellular vehicle for the tumor-localized delivery of anticancer cytokines, specifically IL-2, thereby limiting the severe toxicity associated with systemic administration of recombinant cytokines;

3. To test the hypothesis that primary marrow stromal cells are important immune-modulatory cells and can be exploited in order to induce therapeutic antitumor immunity.

CHAPTER 2

Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-2 Fusion cDNA for Cancer Gene Immunotherapy.

Reference: Stagg J, Wu JH, Bouganim N, Galipeau J. Granulocyte-macrophage colony-stimulating factor and interleukin-2 fusion cDNA for cancer gene immunotherapy. Cancer Res. 2004 Dec 15;64(24):8795-9.

CHAPTER 2: Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-2 Fusion cDNA for Cancer Gene Immunotherapy.

2.1 ABSTRACT

Genetic engineering of tumor cells to express both GM-CSF and IL-2 can induce synergistic immune antitumor effects. Paradoxically, the combination has also been reported to downregulate certain immune functions, highlighting the unpredictability of dual cytokine use. We hypothesized that a GM-CSF and IL-2 fusion transgene (GIFT) could circumvent such limitations yet preserve synergistic features. We designed a fusion cDNA of murine GM-CSF and IL-2. Protein structure computer modelling of GIFT protein predicted for intact ligand binding domains for both cytokines. B16 mouse melanoma cells were gene modified to express GIFT (B16GIFT) and these cells were unable to form tumors in C57bl/6 mice. Irradiated B16GIFT whole cell tumor vaccine could also induce absolute protective immunity against challenge by live B16 cells. In mice with established melanoma, B16GIFT therapeutic cellular vaccine significantly improved tumor-free survival when compared to B16 expressing both IL-2 and GM-CSF. We show that GIFT induced a significantly greater tumor site recruitment of macrophages than combined GM-CSF and IL-2 and that macrophage recruitment arises from novel chemotactic feature of GIFT. In contrast to GM-CSF's suppression of NK cell recruitment despite co-expression of IL-2, GIFT leads to significant functional NK cell infiltration as confirmed in NK-defective *beige* mice. In conclusion, we demonstrated that a fusion between GM-CSF and IL-2 can invoke greater antitumor effect than both cytokines in combination and novel immunobiological properties can arise from such chimeric constructs.

2.2 INTRODUCTION

The delivery of cytokines, or their encoding cDNA sequences, has been broadly explored in order to increase tumor cell immunogenicity. Interleukin (IL)-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are among the most potent cytokines able to induce tumor-specific systemic immunity, both in experimental models and clinical trials[196, 328]. By comparing the antitumor effect of different cytokines in the B16 mouse melanoma model, Dranoff *et al.* reported that GM-CSF was the most effective in generating systemic immunity protecting mice against a distant tumor while IL-2 was the most effective at inducing loco-regional tumor rejection[205]. Given the complementing nature of their actions, several groups have since demonstrated powerful antitumor synergy between GM-CSF and IL-2[329-330]. However, other studies reported that the combination of GM-CSF and IL-2 could induce inhibitory signals downregulating the functions of certain immune effectors[331-332]. These conflicting results highlight the importance - and the difficulty - of optimizing the activity between two agents with different pharmacological properties. Alternatively, bi-functional proteins generated from the fusion of two distinct cytokines have been shown to recapitulate synergistic effects while eliminating the need for dual delivery[333]. Moreover, a fusion protein may possess unheralded biopharmaceutical properties, which may trigger novel beneficial responses. We here report the first engineering of a GM-CSF and IL-2 fusion transgene (GIFT). We provide evidence that this GM-CSF and IL-2 fusion displays novel antitumor properties greater than those of combined GM-CSF and IL-2 for cancer immunotherapy.

2.3 MATERIALS AND METHODS

2.3.1 Animals and cell lines

The C57bl/6 derived B16F0 (B16) mouse melanoma cells were generously given by MA Alaoui-Jamali (Lady Davis Institute, Montreal, QC, Canada) and maintained in DMEM

(Wisent technologies) 10% FBS (Wisent technologies) and 50 U/ml Pen/Strep (Wisent technologies). CTLL-2 and JAWSII cells were purchased from ATCC (American Type Culture Collection) and maintained as per ATCC recommendations. C57bl/6 wild type female mice were obtained from Charles River (Laprairie Co., QC, Canada). Immunodeficient $CD8^{-/-}$, $CD4^{-/-}$ and *beige* mice were obtained from Jackson Laboratory. All mice were used for experimentation at 4-8 weeks of age.

2.3.2 Vector construct and virtual protein modeling

Mouse IL-2 and GM-CSF cDNAs were obtained from the National Gene Vector Laboratories (The University of Michigan), excised by restriction digest and inserted into bicistronic retroviral plasmids allowing co-expression of GFP[334]. The nucleotide sequence of the fusion product of GM-CSF and IL-2 cDNAs was confirmed by DNA sequencing at the Guelph Molecular Supercentre (University of Guelph, Ontario, Canada). Based on the templates 2gmf, 1m47 and 4hb1 from PSI-BLAST[335] searches, a 3-dimensional model of the GIFT gene product was built using Modeller 6.2[336]. Here, 50 structure models were generated and the one with lowest objective function was selected for analysis using the Procheck3.5 software[337].

2.3.3 Transgene expression

The retroviral plasmids were introduced into GP+AM12 packaging cells (ATCC) and supernatant used to gene modify B16 cells. Single B16 clones were isolated by cell sorting and further expanded. Supernatant from clonal populations was tested by ELISA for cytokine expression (BioSource, San Diego, CA), or immunoblotted using anti-mouse IL-2 or anti-mouse GM-CSF antibodies (BD Biosciences).

2.3.4 Cytokine-dependent proliferation assays

CTLL-2 or JAWSII cells were plated at 10^4 cells/well of a 96-well plate with increasing concentrations of cytokines from gene modified B16 cells. The cells were incubated for

48hrs, and 20µl of 5mg/ml MTT solution was incorporated for the last 4hrs of incubation. The reaction was stopped by adding 200µl DMSO and absorbance read at 570nm.

2.3.5 Murine B16 tumor implantation and therapeutic modeling

One million cytokine-secreting B16 cells were injected subcutaneously (n=14 per group) in C57bl/6 mice and tumor growth monitored over time. For prophylactic B16 vaccinations, one million irradiated (50Gy) cytokine-secreting B16 cells were injected subcutaneously and challenged 14 days later on the contralateral flank with $5x10^4$ wild type B16 cells. For therapeutic B16 vaccination experiments, $2x10^4$ wild type B16 cells were injected subcutaneously into wild type, $CD8^{-/-}$, $CD4^{-/-}$ or *beige* mice and treated at days 1 and 7 with peritumoral injection of 10^6 irradiated (50Gy) B16-GIFT, B16-GMCSF, B16-IL2, or 10^6 B16-GMCSF plus 10^6 B16-IL2 cells (n=10 per group). This experiment was repeated (n=10 per group) in wild type mice and the results combined for statistical analysis. All implanted B16 clones produced similar and comparable molar quantities of the cytokine(s) analyzed (0.7±0.2 pmol/10^6cells/24hrs).

2.3.6 Immune effector infiltration analysis

One million cytokine-secreting B16 cells (in 50ul PBS) were mixed to 500µl Matrigel™ (BD Biosciences, CA, USA) at 4°C and injected subcutaneously in C57bl/6 mice (n=4 per group). After 2 days, implants were surgically removed and incubated 90min with a solution of collagenase type IV 1.6mg/ml (Sigma-Aldrich, Oakville, ON, Canada) and DNAseI 200µg/ml (Sigma-Aldrich) in PBS (Mediatech). After incubation with anti-Fcγ III/II mAb (clone 2.4G2, BD Pharmingen) for 1h, cells were incubated for 1h at 4°C with anti-mouse PE-Mac3 and biotin-Ly6G6C, PE-NK1.1, or proper isotypic controls, followed by streptavidin-APC for 15min. Labeled cells were subsequently analyzed by flow cytometry with a Becton-Dickinson FACScan.

2.3.7 Macrophage migration assay

Murine peritoneal macrophages were isolated from C57bl/6 mice by lavage of the abdominal cavity with RPMI solution and were consistently >65% Mac-1 positive by flow cytometric analysis. Immediately after isolation, 10^5 cells/well were plated in the upper chamber of a 0.15% gelatin coated 5μm Transwell plate. The lower chambers were filled in duplicates with 600μl of RPMI 10% FBS with or without 6nM of GIFT, GM-CSF, IL-2 or 6nM of GM-CSF and 6nM of IL-2 obtained from the supernatant of cytokine-secreting B16 cells. After 5hrs at 37°C, the upper chamber was removed, thoroughly washed, removed from cells on the upper filter with a cotton swab, fixed in methanol and stained with violet blue dye. The cells on the bottom filter of 10 high power fields (400x) were counted for each well and the results depicted on a histogram. The experiment was performed twice will similar results.

2.4 RESULTS AND DISCUSSION

Part of the synergy between GM-CSF and IL-2 comes from the fact that GM-CSF can promote proliferation and differentiation of antigen-presenting cells (APCs), which may initiate a tumor-specific immune response that can be subsequently amplified by IL-2[328]. For tumor-infiltrating lymphocytes (TILs), addition of GM-CSF to IL-2 has been reported to result in faster proliferation and enhanced tumor cytotoxicity[338]. In addition, GM-CSF and IL-2 have been shown to enhance monocyte activation and cytotoxicity against melanoma cells in vitro[339-340], and to prolong polymorphonuclear neutrophil survival[341-342]. However, GM-CSF and IL-2 used in combination can sometimes induce paradoxical effects. Skog *et al.* reported that combined GM-CSF and IL-2 therapy could induce inhibitory signals in colorectal carcinoma patients, downregulating the functions of monocytes, NK cells and B cells compared to therapy with GM-CSF alone[331]. Lee *et al.* reported that although GM-CSF and IL-2 expression was synergistic at inhibiting primary mouse colon adenocarcinoma growth, it abrogated the protective effect against wild type tumor challenge compared to single cytokine expression[332].

The difficulty in predicting the outcome of GM-CSF and IL-2 combined therapy may come from their distinct pharmacokinetic and biologic properties. The half-life of IL-2 in the circulation is extremely short (approximately 10min) while the half-life of GM-CSF can extend to 50-85 min[343]. Furthermore, GM-CSF is a potent initiator of an adaptive immune response, whereas IL-2 promotes innate antitumor activity. Soliciting these two functional immune pathways contemporaneously may lead to unheralded antagonism. Indeed, GM-CSF has been shown to downregulate certain aspects of the innate immune response such as NK cytotoxicity[344] and these may in part explain the observations of others[331-332] especially if tumor production of GM-CSF and IL-2 varies in space and time. We hypothesize that a single bi-functional fusion protein, through constant equimolar availability of both subunits, could limit paradoxical effects. Granted, such a fusion protein would be bereft of a true physiological role and may trigger novel responses. In this study, we report the engineering of a GM-CSF and IL-2 fusion transgene. We provide evidence that a fusion between two cytokines can invoke greater antitumor effect than both cytokines in combination.

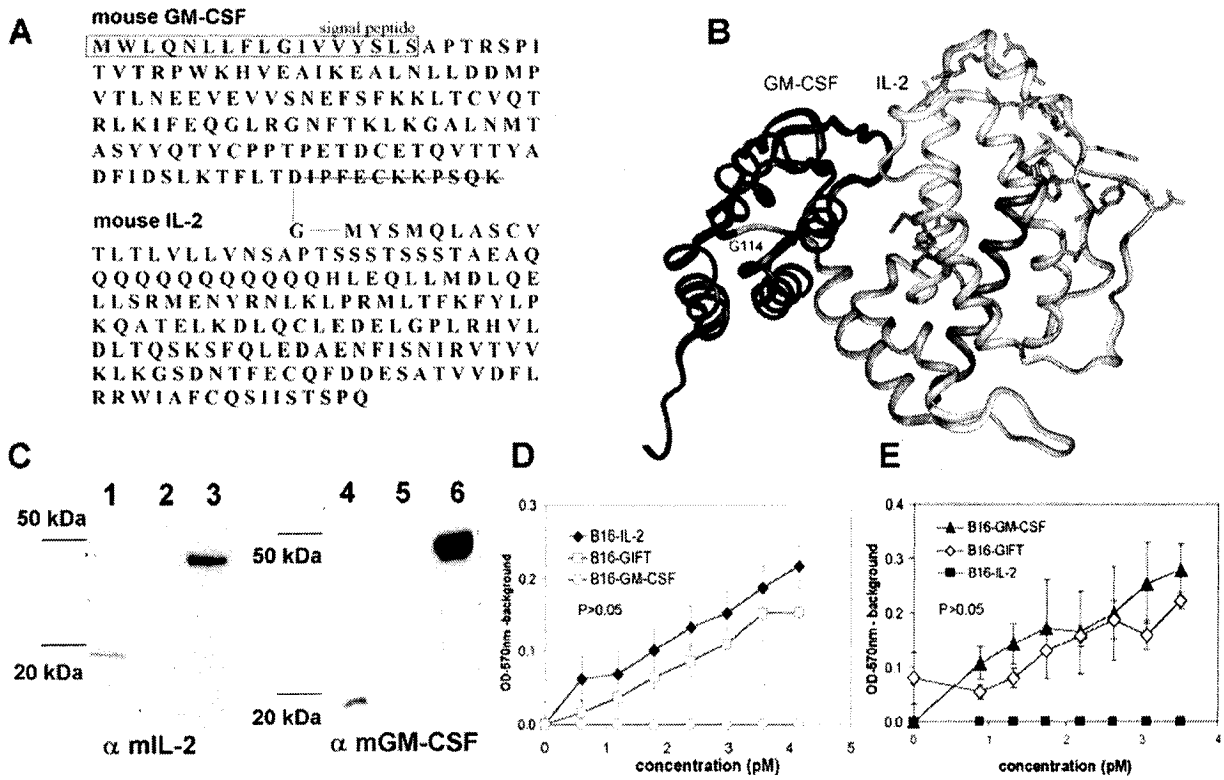
The cDNAs for mouse GM-CSF and mouse IL-2 were cloned in framed after a 33bp deletion at the 3' end of the GM-CSF cDNA. We utilized the IL-2 signal peptide sequence as an intercytokine bridge. The resulting fusion transgene, named GIFT, was confirmed by sequencing analysis. Figure 1A illustrates the predicted amino acid sequence of the gene product encoded by GIFT. Computer-based analysis of the GIFT gene product predicted that the signal peptide (orange ribbon) and glutamic tract (pink ribbon) of the mouse IL-2 precursor would form α-helix structure linking the mature GM-CSF to the mature IL-2 (Figure 1B), allowing proper folding of both subunits and availability of crucial receptor binding residues.

Bicistronic retrovectors allowing coexpression of the GFP reporter were then generated for GIFT, GM-CSF, IL-2 and used to gene-modify B16 mouse melanoma cells. Immunoblotting of cultured cell supernatant confirmed that GIFT gene product was secreted and consisted of a protein of expected molecular weight (Figure 1C).

Figure 1:

Cloning of a bi-functional mouse GM-CSF and IL-2 fusion transgene (GIFT). (A) Predicted amino acid sequence of the fusion transgene. **(B)** Computer model of GIFT gene product built by comparative modeling. The region of the fusion that corresponds to the signal peptide (orange ribbon) and glutamic tract (pink ribbon) of mouse IL-2 precursor was predicted to form α-helix. There are 98.9% of residues in the most favored and allowed regions of the Ramachandran plot, which indicates that stereochemical quality of the model is excellent. Residue G114 (in green) connects GM-CSF (purple ribbon) to IL-2 (the orange, cyan, pink and blue ribbons). The C-terminal α-helix is shown in blue. The side chains of solvent accessible residues that are important for mouse IL-2 to interact with IL-2Rα are in red sticks, whereas the 4 residues that are important for interaction with other subunits of IL-2R are in black sticks. **(C)** Immunoblotting of conditioned supernatant from GIFT gene-modified B16 cells with anti-mouse IL-2 and anti-mouse GM-CSF monoclonal antibodies (1 and 5: recombinant mouse IL-2, 2 and 4: recombinant mouse GM-CSF, 3 and 6: supernatant from B16-GIFT). **(D)** CTLL-2 and **(E)** JAWSII cell proliferation assays as determined by MTT incorporation after 48hrs incubation with increasing concentrations of cytokines from conditioned supernatant of gene-modified B16 cells (CTLL-2: $P>0.05$ between B16-GIFT and B16-IL2; JAWSII: $P>0.05$ between B16-GIFT and B16-GMCSF). Mean of triplicates are shown ± s.e.m. of one representative experiment of three.

Figure 1


A
mouse GM-CSF
signal peptide
MWLQNLLFLGIVVYSLSAPTRSPI
TVTRPWKHVEAIKEALNLLDDMP
VTLNEEVEVVSNEFSFKKLTCVQT
RLKIFEQGLRGNFTKLKGALNMT
ASYYQTYCPPTPETDCETQVTTYA
DFIDSLKTFLTDIPFECKKPSQK
mouse IL-2
G MYSMQLASCV
TLTLVLLVNSAPTSSSTSSSTAEAQ
QQQQQQQQQQQQHLEQLLMDLQE
LLSRMENYRNLKLPRMLTFKFYLP
KQATELKDLQCLEDELGPLRHVL
DLTQSKSFQLEDAENFISNIRVTVV
KLKGSDNTFECQFDDESATVVDFL
RRWIAFCQSIISTSPQ
B
GM-CSF
IL-2
G114
C
1 2 3 4 5 6
50 kDa
20 kDa
50 kDa
20 kDa
α mIL-2
α mGM-CSF
D
B16-IL-2
B16-GIFT
B16-GM-CSF
P>0.05
OD-570nm -background
concentration (pM)
E
B16-GM-CSF
B16-GIFT
B16-IL-2
P>0.05
OD-570nm - background
concentration (pM)

Clonal populations of cytokine-secreting B16 cells were then isolated by FACS and their conditioned media tested by ELISA for cytokine secretion. For comparison purposes, we selected clonal populations secreting comparable molar quantities of GIFT, GM-CSF or IL-2 (0.7 ± 0.2 pmol /10^6cells/ 24hrs).

In order to test the bi-functionality of GIFT, cytokine conditioned supernatant was tested at different concentrations for its ability to stimulate proliferation of IL-2 dependent CTLL-2 cells (Figure 1D) and GM-CSF dependent JAWSII cells (Figure 1E). As demonstrated by 3 distinct MTT incorporation experiments, GIFT was able to stimulate CTLL-2 cells at a similar level to IL-2 ($P>0.05$ by T-test), and to stimulate JAWSII cells at a similar level to GM-CSF ($P>0.05$ by T-test). Our results thus confirmed the *in vitro* bifunctionality of GIFT gene product.

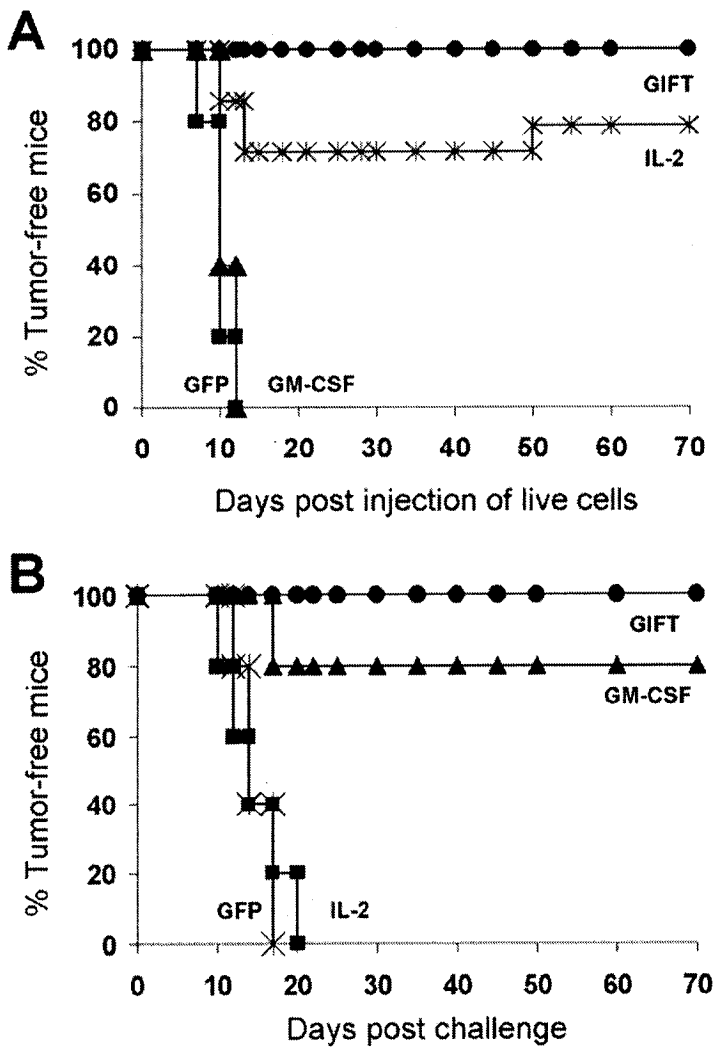
To assess GIFT *in vivo* antitumor effect, we first proceeded with a set of experiments where 10^6 live cytokine-secreting B16 cells were injected subcutaneously into cohorts of immunocompetent syngeneic C57bl/6 mice (n=14). Consistent with previous studies[205], we observed that IL-2 expression but not GM-CSF expression by live B16 cells could prevent tumor growth (respectively 78% and 0% of mice rejected the implant). In comparison, all mice injected with GIFT expressing B16 cells rejected the tumor implant ($P<0.05$ by T-test with IL-2; Figure 2A). Importantly, the observed absence of tumor growth was not the result of clone-specific cell proliferation rates, as determined by MTT incorporation assays in vitro ($P>0.05$; data not shown). Neither was it due to an idiosyncratic property of this clone, as polyclonal B16-GIFT tumors were also rejected (data not shown).

We then tested whether GIFT engineered B16 cells could induce protective immunity against a wild type challenge of B16 cells – in essence a prophylactic tumor cell vaccine. C57bl/6 mice were injected subcutaneously into the right flank with 10^6 irradiated (50Gy) cytokine-secreting B16 cells and challenged 14 days later with $5x10^4$ wild type B16 cells into the contralateral flank. Consistent with previous studies[205], we observed that GM-CSF expression but not IL-2 expression could induce systemic protective immunity when

Figure 2:

Loco-regional antitumor effects and systemic protective antitumor immunity induced by GIFT. **(A)** Immunocompetent C57bl/6 mice were injected subcutaneously with 10^6 live cytokine-secreting B16 cells and tumor growth monitored over time ($P<0.05$ between B16-GIFT and B16-IL2 by Log-rank). **(B)** For prophylactic vaccinations, immunocompetent C57bl/6 mice were first injected subcutaneously with 10^6 irradiated (50Gy) cytokine-secreting B16 cells and then challenged 14 days later on the contralateral flank with a subcutaneous injection of $5x10^4$ wild type B16 cells ($P>0.05$ between GIFT and GM-CSF by Log-rank). B16-GIFT (black circles), B16-IL2 (stars), B16-GMCSF (black triangles) and B16-GFP (black squares).

Figure 2


A
100
80
60
40
20
0
% Tumor-free mice
GIFT
IL-2
GFP
GM-CSF
0
10
20
30
40
50
60
70
Days post injection of live cells
B
100
80
60
40
20
0
% Tumor-free mice
GIFT
GM-CSF
GFP
IL-2
0
10
20
30
40
50
60
70
Days post challenge

given as an irradiated cellular tumor vaccine (respectively 80% and 0% of mice rejected the challenge). In comparison, all mice vaccinated with irradiated B16-GIFT rejected the subsequent challenge ($P>0.05$ with GM-CSF; Figure 2B). Taken together, our results demonstrate that in addition to its potent loco-regional effect against live tumor cells, GIFT is able to induce systemic antitumor immunity, protecting mice against a distant injection of wild type B16 cells, thereby combining the innate immune effects of IL-2 and the adaptive immune effects of GM-CSF, without any observable mutual interference.

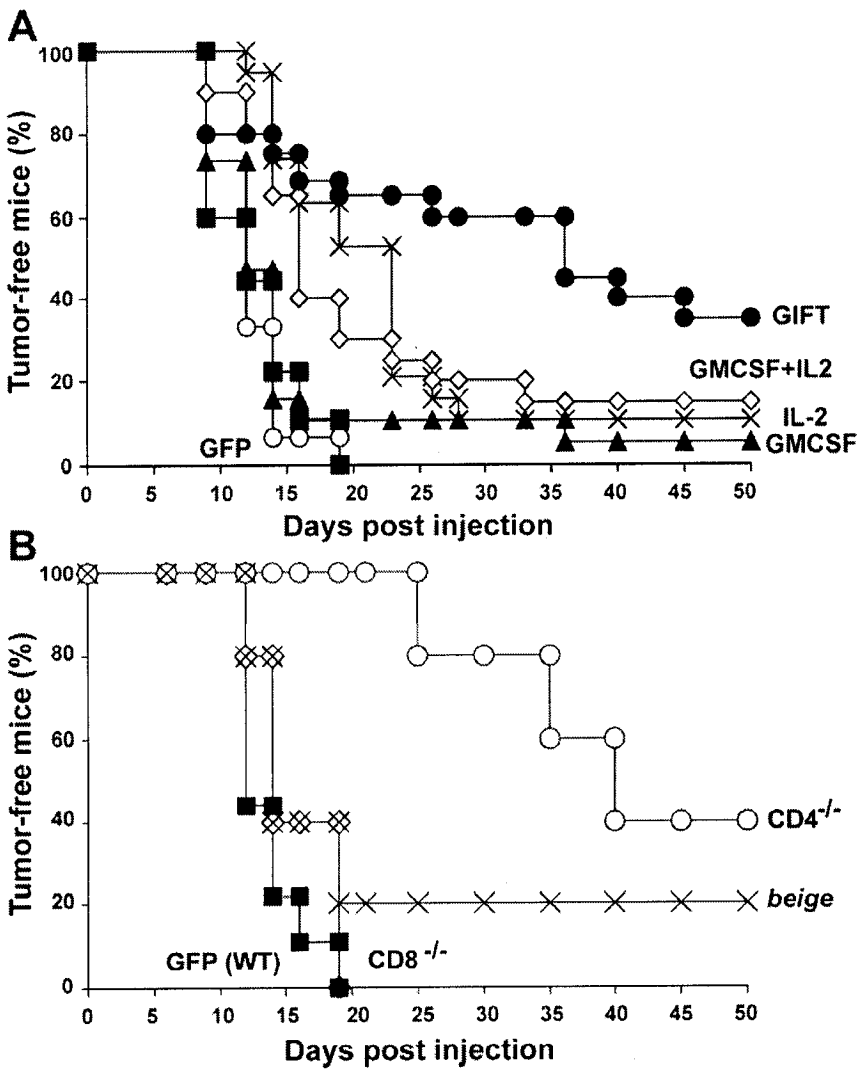
We also compared GIFT's antitumor action to a combination of IL-2 and GM-CSF as assessed in a therapeutic cancer cell vaccine strategy. First, $2x10^4$ B16 cells were injected subcutaneously into C57bl/6 mice. Then on days 1 and 7, the same mice with pre-established live B16 tumors were injected peritumoraly with 10^6 irradiated B16-GIFT cells, or a mixture of 10^6 B16-GMCSF and 10^6 B16-IL2 cells (Figure 3A). At equimolar cytokine secretion rates, the treatment with a GIFT expressing cellular vaccine was significantly greater than a vaccine expressing both IL-2 and GM-CSF ($P=0.0407$ by Log rank), IL-2 alone ($P=0.035$ by Log rank) or GM-CSF alone ($P=0.0003$ by Log rank). Treatment of $CD4^{-/-}$ tumor-bearing mice with GIFT was indistinguishable from treatment of wild type tumor-bearing mice, indicative of a T helper independent immune response (Figure 3B). In contradistinction, $CD8^{-/-}$ mice treated with GIFT failed to develop antitumor immune response ($P<0.05$ by Log rank compared to wild type). NK cells were also implicated, as treatment of NK-defective *beige* tumor-bearing mice was significantly reduced, but not completely abolished, compared to treatment of wild type mice ($P<0.05$ by Log rank compared to wild type).

Our observation that GIFT tumor cell vaccines were more effective than a combination of both GM-CSF and IL-2 at equimolar concentration suggested that GIFT may possess supplementary and novel immunopharmacological properties when compared to the combination of GM-CSF and IL-2. We hypothesized that immune cells expressing both the GM-CSF and the low-affinity IL-2 receptors could mediate such distinct properties in response to GIFT. Macrophages and neutrophils are known to express both the GM-CSF

Figure 3:

GIFT is more potent than combined GM-CSF and IL-2 and requires CD8 and NK cells for antitumor effects. (A) Immunocompetent C57bl/6 mice were injected subcutaneously with $2x10^4$ wild type B16 cells. Then on days 1 and 7, the same mice were injected peritumoraly with 10^6 irradiated (50Gy) B16-GIFT (black circles), B16-IL2 (stars), B16-GMCSF (black triangles) or B16-GFP cells (black squares), or a mixture of 10^6 B16-GMCSF and 10^6 B16-IL2 cells (white diamonds) and tumor growth monitored over time. **(B)** Immunodeficient $CD4^{-/-}$ (white circles), $CD8^{-/-}$ (white diamonds), *beige* (stars) or immunocompetent (black squares) C57bl/6 mice were injected subcutaneously with $2x10^4$ wild type B16 cells. Then on days 1 and 7, the same mice were injected peritumoraly with 10^6 irradiated (50Gy) B16-GIFT or B16-GFP cells and tumor growth monitored over time.

Figure 3


A
Tumor-free mice (%)
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45 50
Days post injection
GIFT
GMCSF+IL2
IL-2
GMCSF
GFP
B
Tumor-free mice (%)
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45 50
Days post injection
CD4-/-
beige
GFP (WT)
CD8 -/-

and the low affinity IL-2 receptors and have been reported to play a role in the antitumor effect induced by GM-CSF and IL-2. We thus compared the level of macrophage and neutrophil infiltration of early cytokine-secreting B16 tumors. As shown in Figure 4, GIFT induced a significantly more robust infiltration of macrophages than GM-CSF, IL-2, or even a combination of both GM-CSF and IL-2 ($P<0.05$ by T-test). On the other hand, the number of neutrophils was significantly greater in response to GIFT compared to IL-2 or GM-CSF alone ($P<0.05$ by T-test), but similar to the number of neutrophils in response to combined GM-CSF and IL-2 ($P>0.05$ by T-test). In order to determine if the enhanced macrophage infiltration was the result of a direct chemotactic effect of GIFT, migration assays were performed with mouse peritoneal macrophages. As shown in Figure 4C, GIFT was able to induce migration of significantly more macrophages than equimolar concentration of combined GM-CSF and IL-2 ($P<0.05$ by T-test).

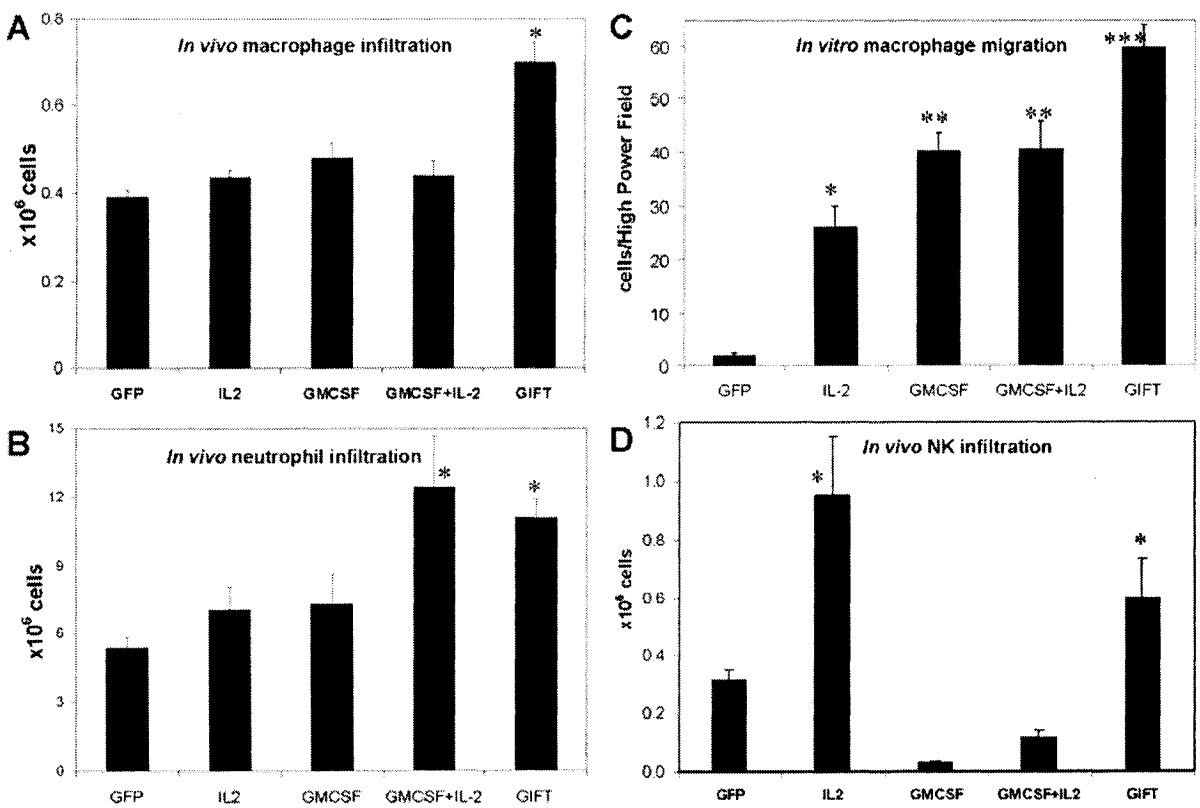
An intriguing observation was the significant suppression of NK infiltration by GM-CSF when compared to controls ($P<0.05$ between GM-CSF and GFP by T-test). The effect was not rescued by co-expression of IL-2 ($P>0.05$ between GM-CSF and GM-CSF+IL2 by T-test). However, GIFT retained the ability to recruit NK cells as did IL-2 alone (Figure 4D). Recombinant human GM-CSF has been shown to suppress NK cell formation *in vitro*[345] and NK cytotoxicity *in vivo*[344]. This may explain in part the inability of GM-CSF alone to reject live tumor cells as we and others[205] have observed. GM-CSF's dominant negative effect on NK cells may also help explain in part the apparent inferiority of GM-CSF and IL-2 combination to GIFT as part of a therapeutic vaccine.

In conclusion, we have demonstrated that the nucleotide sequence encoding for the fusion of GM-CSF and IL-2 cDNA can be utilized as a therapeutic transgene for gene therapy of cancer, recapitulating the potent antitumor effects of both GM-CSF and IL-2. Furthermore, this fusion gene product appears to have immunopharmacological properties distinct of GM-CSF and IL-2 used alone or in combination. This is the first report that a fusion between two cytokines can invoke greater antitumor effect than both

Figure 4:

GIFT mediated recruitment of innate immune cells. Immunocompetent C57bl/6 mice were injected subcutaneously with 10^6 cytokine-secreting B16 cells mixed in Matrigel. Implants were then surgically removed after 2 days, dissolved to single cell suspensions and analyzed by flow cytometry for the presence of macrophages **(A)**, neutrophils **(B)** and NK cells **(D)** and depicted in histograms of mean cell number per implant ± s.e.m. (n=4 per group). **(C)** *In vitro* macrophage migration assay. Fresh peritoneal macrophages were plated for 5hrs in Transwell plates with lower chambers filled in duplicates with or without cytokine(s). The cells on the bottom filters of 10 high power fields (400x) were counted for each well and the results depicted as mean cell number per high power field ± s.e.m. Error bars smaller than icons do not appear. For T-tests, *: $P<0.05$ compared to GFP; **: $P<0.05$ compared to *; ***: $P<0.05$ compared to **).

Figure 4


A
In vivo macrophage infiltration
x10^6 cells
0.8
0.6
0.4
0.2
0
GFP
IL2
GMCSF
GMCSF+IL-2
GIFT
*
B
In vivo neutrophil infiltration
x10^6 cells
15
12
9
6
3
0
GFP
IL2
GMCSF
GMCSF+IL-2
GIFT
*
*
C
In vitro macrophage migration
cells/High Power Field
60
50
40
30
20
10
0
GFP
IL-2
GMCSF
GMCSF+IL2
GIFT
*
**
**
***
D
In vivo NK infiltration
x10^6 cells
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GFP
IL2
GMCSF
GMCSF+IL2
GIFT
*
*

cytokines in combination and suggest that chimeric fusion cytokine transgenes may serve as novel genetic biopharmaceuticals for cancer immunotherapy.

2.5 ACKNOWLEDGMENTS

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CHAPTER 3

Marrow Stromal Cells for Interleukin-2 Delivery in Cancer Immunotherapy.

Reference: Stagg J, Lejeune L, Paquin A, Galipeau J. Marrow stromal cells for interleukin-2 delivery in cancer immunotherapy. Hum Gene Ther. 2004 Jun;15(6):597-608.

Preface to Chapter 3:

We showed in Chapter 2 that the expression of a chimeric fusion protein between IL-2 and GM-CSF drastically enhanced the antitumoral effect of each cytokine either expressed alone or in combination as part of a therapeutic tumor vaccine. This demonstrated that fusion protein bioengineering can greatly enhance the potency of a given cytokine. Another approach to improve cytokine-based cancer therapies and limit severe side effects is to constrain the release of cytokine to the tumor site. In the next chapter, we tested the hypothesis that primary bone marrow stromal cells can be used as an efficient tumor-localized delivery vehicle of anticancer cytokines.

CHAPTER 3: Marrow Stromal Cells for Interleukin-2 Delivery in Cancer Immunotherapy.

3.1 ABSTRACT

Marrow stromal cells (MSCs) can be easily gene-modified and clonally expanded making them ideal candidates for transgenic cell therapy. However, recent reports suggest that MSCs possess immunosuppressive effects, which may limit their clinical applications. We investigated whether interleukin(IL)-2 gene-modified MSCs can be used to mount an effective immune response against the poorly immunogenic B16 melanoma model. We first show that primary MSCs mixed with B16 cells and injected subcutaneously in syngeneic recipients does not affect tumor growth. On the other hand, IL-2 producing MSCs mixed with B16 cells significantly delayed tumor growth in an IL-2 dose-dependent manner. Furthermore, we observed that matrix-embedded IL-2 producing MSCs injected in the vicinity of pre-established B16 tumors led to absence of tumor growth in 90% of treated mice ($P<0.001$). We demonstrated that tumor-bearing mice treated with IL-2 producing MSCs developed CD8 mediated tumor specific immunity and significantly delayed tumor growth of a B16 cell challenge ($P<0.05$). In addition, treatment of cd8-/-, cd4-/- and *beige* mice revealed that CD8+ and NK cells, but not CD4+ cells, were required to achieve antitumor effect. In conclusion, MSCs can be exploited to deliver IL-2 and generate effective immune responses against melanoma in mice with normal immune systems.

3.2 OVERVIEW SUMMARY

Marrow stromal cells (MSCs) are appealing as a cellular vehicle for delivery of anti-cancer gene products because they can be readily harvested, easily gene-modified and clonally expanded to clinically relevant numbers. We report here that primary MSCs can be used as efficient IL-2 delivery vehicles. This is the first description in immunocompetent animals that gene-modified MSCs can be used for cancer

immunotherapy. We observed that after co-injection of B16 melanoma cells and IL-2 producing MSCs, a significant dose-dependent delay in tumor growth occurred. We further demonstrate that embedding IL-2 producing MSCs in a matrix-scaffolding enhances their value as a biopharmaceutical. Indeed, peritumoral injection of Matrigel-embedded MSCs-IL2 eradicated pre-established melanoma. The immune response induced by such treatment included CD8-mediated tumor-specific cytotoxicity and was dependent upon CD8 T cells and NK cells. This novel biopharmaceutical approach could be utilized for the treatment of cancer patients with local minimal residual disease.

3.3 INTRODUCTION

Bone marrow derived marrow stromal cells (MSCs) are pluripotent cells that can be easily expanded *ex vivo* and differentiated into various cell lineages[268, 346]. Isolated from simple bone-marrow aspirates by their adherence properties, MSCs are phenotypically identified by the absence of CD45 and CD31 cell surface markers, and by the presence of CD44, CD105, SH2 and SH3 markers[285]. Since MSCs are present in humans of all ages, that they can be harvested in the absence of prior mobilization and that they maintain their precursor phenotype following gene modification, MSCs are attractive as autologous cellular vehicles for the delivery of therapeutic gene products.

Several pre-clinical studies have shown that gene-modified MSCs can be used to efficiently deliver *in vivo* various therapeutic proteins[347-350]. We have recently developed a method by which gene-modified MSCs can deliver therapeutic levels of erythropoietin in nonmyeloablated, immunocompentent animals by embedding them in a collagen matrix scaffolding prior to injection[351]. This method allows for stable transgene expression and permits removal when desired of the transgenic cells and therefore control over the release of the therapeutic protein.

We here report a novel application of this method for *in vivo* delivery of antitumoral cytokines in the context of cancer immunotherapy. Currently, cytokine therapy is used in

the clinic to treat certain malignancies but is limited by the severe toxicity associated with systemic administration of the recombinant proteins, therefore limiting its use to selected few patients[196, 352]. An alternative to the current treatment involves cancer-localized cytokine gene expression. Various cytokine genes, including interleukin-2 (IL-2), when expressed by or at the vicinity of tumor cells, can successfully generate potent systemic anticancer responses[208, 353-354]. A complementary approach to tumor-targeted gene delivery involves the use of normal cell as vehicles for paracrine delivery of cytokines to the tumor environment[355-357]. However, there are drawbacks associated with the use of terminally differentiated somatic cells such as fibroblasts. First, skin fibroblasts have been shown to inactivate introduced vector sequences[358-359]. Second, pre-programmed replicative-senescence would make it difficult, especially in the aged cancer patients, to culture expand *ex vivo* large amounts of gene-modified somatic cells and to isolate clonal populations[360]. We hypothesized that the use of postnatal adult stem cells, such as MSCs, may address this issue.

Primary MSCs possess, however, properties of their own which may influence the desired therapeutic effect when used as transgenic cellular vehicles. Recently, several studies have demonstrated that primary mouse, baboon and human MSCs exhibit immunosuppressive properties in mixed lymphocyte reactions induced by allogeneic cells[311-320]. This phenomenon was reported to be mediated by soluble factors such as hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGF-β1), or other unidentified factors secreted by MSCs. Furthermore, mouse MSCs were reported to favor tumor growth of B16 melanoma cells in allogeneic mice[317] possibly due to the fact that MSCs express receptors for several tumor stroma-derived growth factors. Conversely, these intrinsic properties of MSCs can be exploited to target tumor environment as shown by Studeny *et al.*[361]. They demonstrated that injection of gene-modified IFN-β producing MSCs targeted the tumor's environment and significantly inhibited proliferation of IFN-β sensitive melanoma cells. However, because this latter work was performed in immunodeficient mice, it is unknown if the immunosuppressive properties of MSCs would impede any antitumoral effect generated by a transgene product in fully immunocompetent recipients. Although MSCs are attractive cellular

vehicles for the delivery of therapeutic proteins, it remained unclear whether they could be used in the context of immunostimulation.

The aim of this study was thus to investigate whether primary cytokine gene-modified MSCs could be used to mount an effective immune response against cancer. To test this hypothesis, we retrovirally engineered primary mouse MSCs to secrete IL-2, a well characterized anticancer cytokine, and tested their ability to prevent tumor growth of B16 mouse melanoma cells. Recombinant IL-2 is currently approved for the treatment of metastatic melanoma and renal cell carcinoma[196], and IL-2 has also been investigated as adjuvant therapy in other malignancies such as lymphoma[362-363]. B16 melanoma cells do not express MHC class II and express only very low levels of MHC class I molecules, rendering them poorly immunogeneic[364]. We demonstrated that primary mouse MSCs engineered to secrete IL-2 can efficiently induce a potent, long-lasting and tumor-specific anticancer response in fully immunocompetent animals. The immune response generated with IL-2 producing MSCs was dependent upon CD8+ and NK cells, consistent with previous work on IL-2 anticancer effects. These results demonstrated for the first time that cytokine gene-modified MSCs can be used as an autologous cellular vehicle for immunostimulation in the context of cancer immunotherapy.

3.4 MATERIALS AND METHODS

3.4.1 Cell isolation and culture

Primary mouse MSCs were isolated from C57bl/6 mice as previously described[351]. Briefly, whole marrow from the femurs and tibias of 4-8 weeks old female mice (Charles River, Laprairie Co., QC, Canada) was flushed in DMEM (Wisent technologies, St-Bruno, QC, Canada) 10% fetal bovine serum (FBS) (Wisent technologies) and 50 U/ml Pen/Strep (Wisent technologies), plated for 5 days, washed and fresh media added to the adherent cells every 3-4 days. MSC cultures were then gene-modified between PD5 and PD10 and kept frozen in liquid nitrogen until further use.

The B16 F0 (B16) cell line is a non-metastatic melanoma model derived from C57bl/6 mice and was generously given by MA Alaoui-Jamali (Lady Davis Institute, Montreal, QC, Canada). Cells were maintained in DMEM (Wisent technologies) 10% FBS (Wisent technologies) and 50 U/ml Pen/Strep (Wisent technologies).

3.4.2 Retroviral vectors and MSC transduction

Culture-expanded MSCs were gene-modified using retrovectors generated from GP+E86 packaging-cells (American Type Culture Collection) transfected with pIRES-EGFP[334] or pIL2-IRES-EGFP plasmids. The cDNA for mouse IL-2 was obtained from the National Gene Vector Laboratories (University of Pennsylvania, PA, USA), excised by *Xho*I-*Hpa*I digest and cloned into pIRES-GFP after *Bam*HI-*Xho*I digest in order to generate pIL2-IRES-EGFP. The retroviral plasmids were transfected into GP+E86 cells utilizing lipofectamine reagent following manufacturer's instructions (GIBCO-BRL, Gaithesburg, MD, USA). Transduction of primary MSCs was performed as described (Eliopoulos *et al.* 2003). Six days after transduction, GFP expressing MSCs were sorted and plated at 1 cell per well in 96-well plates. Clonal populations were expanded and tested for the presence of IL-2 in the culture media by enzyme-linked immunosorbent assay (ELISA) specific for mouse IL-2 (BioSource International, Camarillo, CA). For Southern blot analysis, 10 μg of genomic DNA isolated from stably engineered MSCs or unmodified MSCs using the QIAamp DNA mini kit (Qiagen, Mississauga, ON, Canada) was digested with *Nhe*I, separated by electrophoresis in 1% agarose, and transferred to a Hybond-N nylon membrane (Amersham, Oakville, ON, Canada). The probe was prepared by ^{32}P radiolabeling of the EGFP complete cDNA utilizing a Random Primed DNA Labeling Kit (Roche Diagnostics, Indianapolis, IN, USA) and was hybridized with the membrane. The blot was exposed to a Kodak X-Omat film.

3.4.3 Flow cytometry analysis of MSCs

Culture-expanded engineered MSCs or unmodified MSCs were detached using 0.05%Trypsin-0.53mM EDTA (Wisent technologies) and incubated with the following mAbs: PE-labeled rat anti mouse CD45 (clone 30-F11), CD44 (clone IM7) or Flk1 (clone Avas12α1), PE-labeled mouse anti-mouse H-2Db or H-2Kb, biotin-conjugated rat anti-mouse CD31 (clone 390) or CD34 (clone RAM34), PE-labeled rat IgG2a or IgG2b, PE-labeled mouse IgG2a, or biotin-conjugated rat IgG2a Abs (all from BD Pharmingen, San Diego, CA, USA). Biotinylated Abs were revealed by PE-streptavidin (BD Pharmingen). Cells were washed and events acquired using a Coulter EPICS flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed by means of Win MDI 2.7 software.

3.4.4 Transplantation of engineered MSCs admixed with melanoma cells

Culture-expanded engineered MSCs and B16 cells were detached using 0.05%Trypsin-0.53mM EDTA (Wisent technologies), concentrated by centrifugation and resuspended to the desired concentration in phosphate buffered solution (PBS) (Mediatech, Herndon, VA, USA). A volume of 100µl/mouse containing the desired cell number of engineered MSCs mixed to B16 cells was injected subcutaneously in the right lateral flank of 4-8 weeks old female C57bl/6 mice (Charles River).

3.4.5 Transplantation of matrix-embedded engineered MSCs

B16 cells were detached using 0.05%Trypsin-0.53mM EDTA (Wisent technologies), concentrated by centrifugation, resuspended to the desired concentration in PBS (Mediatech), and injected subcutaneously in the right lateral flank of 4-8 weeks old female C57bl/6 normal, cd8-/-, cd4-/- or *beige* mice (Jackson Laboratories) in a volume of 100µl/mouse. Twenty-four hours later, culture-expanded engineered MSCs were detached using 0.05%Trypsin-0.53mM EDTA (Wisent technologies) concentrated by centrifugation and resuspended to a final concentration of $2x10^7$ cells/ml in PBS (Mediatech). For each mouse treated, one million engineered MSCs (50µl) were mixed

to 500µl Matrigel™ (BD Biosciences, CA, USA) at 4°C and injected subcutaneously at the site of previously injected B16 or in the contralateral flank. All groups consisted of cohorts of 10 mice.

3.4.6 Tumor-specific apoptosis assays

Apoptosis assays were performed on the direct quantitative flow cytometry analysis of annexin-V expression as previously described[365]. Splenocytes from MSC-IL2 treated or untreated mice were isolated and CD8+ cells purified using Sin Sep™ cell separation antibody cocktail (Stem Cell Technologies, Vancouver, Canada) and used fresh as effectors in annexin-V assays. B16 cells or EL4 cells were labelled with 2uM PKH26 (Sigma-Aldrich, Oakville, ON, Canada) following manufacturer's instructions and used as target cells. Labelled target cells were seeded into 96 well U-bottom plates (10^4 cells/well) and incubated with the effector cells at different effector/target ratios for 4 hours in the presence of 20U of rIL-2/ml. After coincubation, the cells were washed twice in PBS and resuspended in 100ul annexin-binding buffer (BD Pharmingen). Annexin-V-FITC and propidium iodine staining was performed following manufacturer's instructions (BD Pharmingen) and flow cytometry analysis was carried out on a FACScan cytometer (BD, San Jose, CA). The specific effector mediated apoptosis was determined by gating of PKH26-positive cells and by the formula: %specific apoptosis = [%ann-positive cells in sample - %ann-positive cells in control]/[1-%ann-positive cells in control].

3.4.7 Removal and processing of the implants

At defined time points after subcutaneous injection of matrix-embedded MSCs, implants were surgically removed and placed in 12-well plates in 1ml/well of a solution of collagenase type IV 1.6mg/ml (Sigma-Aldrich, Oakville, ON, Canada) and DNAseI 200µg/ml (Sigma-Aldrich) in PBS (Mediatech). The explants were then cut in small pieces with scissors and incubated at 37°C for 90 minutes. The cell suspensions were further dissociated by repeated pipetting, transferred to a 15ml Falcon tube (BD

Biosciences) and washed by adding 5ml of PBS (Mediatech) and subsequently centrifuged at 1,500 rpm for 5 minutes. Supernatant was removed and cell pellet resuspended in 1ml of 2 vol 0.8% ammonium chloride for red blood cells lysis, immediately centrifuged and resuspended in PBS (Mediatech) with 3% FBS (Wisent technologies). To determine the fate of matrix-embedded MSCs *in vivo*, cell suspensions were counted using an hemacytometer and analyzed by flow cytometry after incubation with propidium iodine at 1mg/ml (Sigma-Aldrich) to allow exclusion of dead cells, or analyzed at 10 days after incubation with PE-labeled rat anti-mouse Flk1 (clone Avas12α1), biotin-conjugated rat anti-mouse CD34 (clone RAM34) followed by PE-streptavidin, PE-labeled rat IgG2a, or biotin-conjugated rat IgG2a followed by PE-streptavidin (all Abs from BD Pharmingen).

3.4.8 Immunofluorescence microscopy of engineered MSCs

At 10 days post-injection, the implants were excised, placed in OCT compound (Sakura Finetek, Torrance, CA, USA) and snap frozen in liquid nitrogen. The tissue was stored at -80°C, sectioned (8-12 μm) and processed for immunofluorescence microscopy. Nonspecific binding was blocked by incubation with 1% goat serum (Jackson ImmunoResearch Laboratories) in PBS for 1h at room temperature. In order to reveal GFP expression, the sections were incubated with a rabbit anti-GFP Ab (1:1000) at +4°C overnight, followed by incubation with a FITC-conjugated goat anti-rabbit secondary Ab for 30 min (Abs generously given by Stéphane Richard, Lady Davis Institute, Montréal, Canada). For endothelial differentiation assessment, frozen sections were incubated with biotin-conjugated rat anti-mouse CD31 (BD Pharmingen clone 390) at +4°C overnight or control isotype, followed by incubation with PE-streptavidin (BD Pharmingen) for 30 min.

3.4.9 Analysis of lymphoid infiltrate

Culture-expanded engineered MSCs and B16 cells were detached using 0.05%Trypsin-0.53mM EDTA (Wisent technologies), concentrated by centrifugation and resuspended

in PBS (Mediatech). B16 cells were mixed with engineered MSCs to a final concentration of $2x10^6$ and $2x10^7$ cells/ml respectively. For each mouse, 50µl of the mixture was mixed to 500µl Matrigel at 4°C and injected subcutaneously in the right lateral flank of 4-8 weeks old female C57bl/6 mice. At 5 and 10 days after subcutaneous injection, mice were sacrificed and the implants were surgically removed and processed as described above to obtain single cell suspensions. After incubation with anti-Fcγ III/II mAb (clone 2.4G2), three-color staining was performed by incubating 10^6 cells for 30 min at +4°C with one of the following combinations: biotin-conjugated rat anti-mouse CD3ε (clone 2C11), PE-labeled rat anti-mouse CD4 (clone RM4-5) and FITC-labeled rat anti-mouse CD8α (clone 53-6.7); biotin-conjugated rat anti-mouse CD3ε (clone 2C11) and PE-labeled rat anti-mouse NK1.1 (clone PK136); biotin-conjugated rat anti-mouse CD3ε (clone 2C11), PE-labeled rat anti-mouse CD4 (clone RM4-5) and FITC-labeled rat anti-mouse CD25 (clone7D4). Biotinylated Abs were revealed by PE-CyChrome (all Abs from BD Pharmingen). Cells were washed and events acquired using a Coulter EPICS flow cytometer (Beckman Coulter) and analyzed by means of Win MDI 2.7 software.

3.5 RESULTS

3.5.1 Primary MSCs can be retrovirally engineered to secrete IL-2.

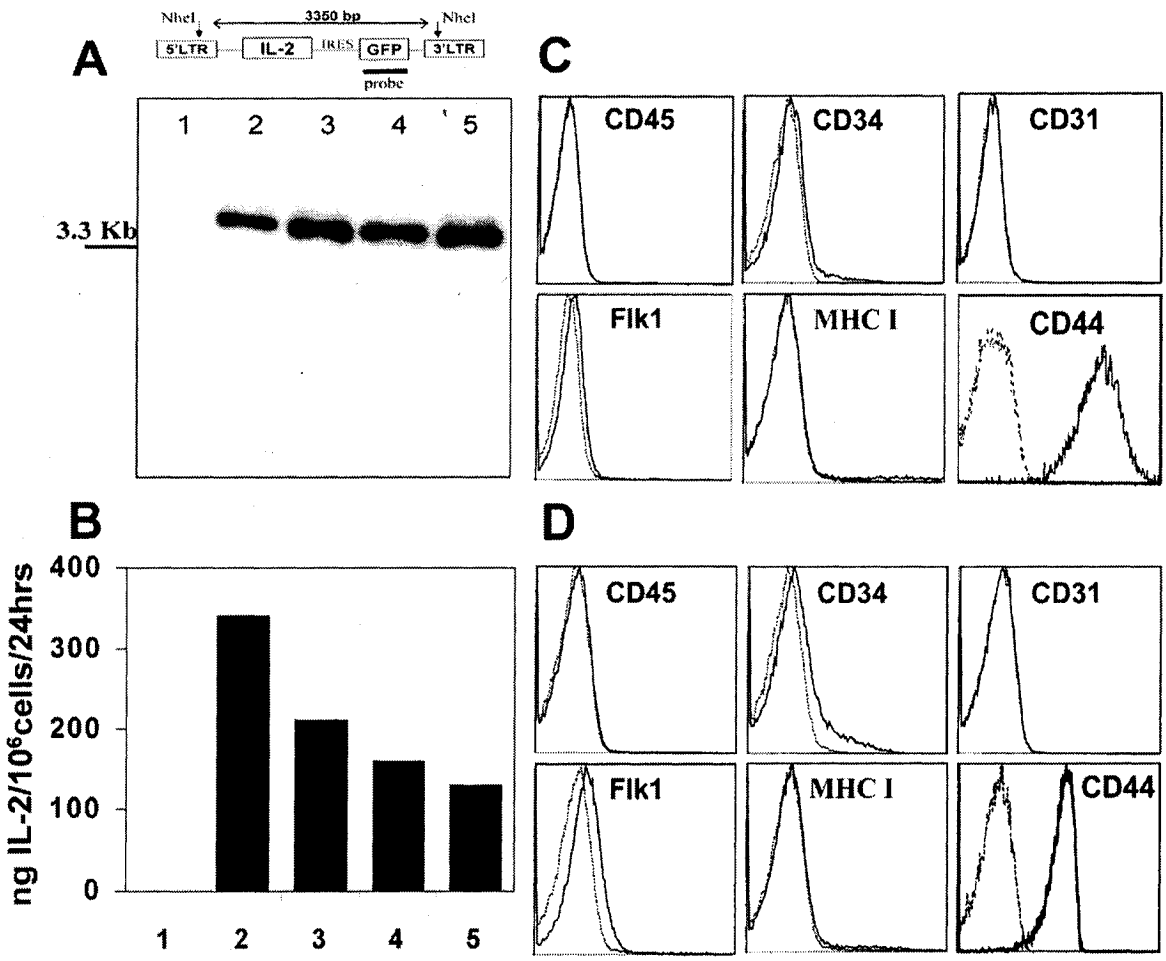
Primary mouse MSCs were gene-modified using recombinant retrovectors to express a bicistronic construct encoding the mouse IL-2 cDNA and the reporter GFP (MSC-IL2). As control, primary MSCs were gene-modified to express only GFP (MSC-GFP). Gene modified MSC clones were isolated and stable transgene integration was confirmed by Southern blot analysis. (Figure 5A).

Four MSC-IL2 clones were selected and were shown to secrete respectively 340ng (MSC-IL2-high), 211ng (MSC-IL2-moderate), 160ng (MSC-IL2-low) and 130ng (MSC-IL2-lowest) of IL-2/10^6cells/24hrs as determined by ELISA (Figure 5B). We next

Figure 5: Characterization of culture-expanded MSCs and IL-2 transgene expression.

Primary MSCs were isolated from C57bl/6 female mice, cultured in DMEM 10% FBS and gene modified using recombinant retrovectors to express a bicistronic construct encoding the mouse IL-2 cDNA and the reporter GFP, or GFP only. (A) Genomic DNA from unmodified MSCs (lane 1), or clonal populations of IL-2 gene-modified MSCs (lanes 2-5) was analyzed by Southern blot for retrovector integration after enzymatic digestion with *Nhe*I and probed with a ^{32}P-labeled GFP probe. (B) IL-2 producing MSC clones were shown to secrete respectively 340ng (MSC-IL2-high; lane 2), 211ng (MSC-IL2-moderate; lane 3), 160ng (MSC-IL2-low; lane 4) and 130ng (MSC-IL2-lowest; lane 5) of IL-2/10^6cells/24hrs as determined by ELISA. (C) Unmodified or (D) IL-2 gene-modified primary mouse MSCs recovered by trypsization after 30-40 population doublings were stained with mAbs against CD45, CD34, MHC-I, CD31, Flk-1, CD44, or control IgG Abs, and analyzed by flow cytometry. Plots show isotype control IgG staining profile (doted line) versus specific Ab staining profile (thick line).

Figure 5


A
NheI
3350 bp
NheI
5'LTR
IL-2
IRES
GFP
3'LTR
probe
1 2 3 4 5
3.3 Kb
C
CD45
CD34
CD31
Flk1
MHC I
CD44
B
ng IL-2/10^6cells/24hrs
400
300
200
100
0
1 2 3 4 5
D
CD45
CD34
CD31
Flk1
MHC I
CD44

performed cell surface antigen analysis of culture-expanded MSCs by flow cytometry. The phenotype of culture-expanded MSCs isolated from C57bl/6 mice was $CD45^-$, $CD34^{low}$, $CD31^-$, $Flk1^{low}$, MHC I^- and $CD44^+$ (Figure 5C). We then determined the effect of IL-2 expression on engineered MSCs. As shown in figure 5D, IL-2 transgene expression does not alter the phenotype of primary mouse MSCs.

3.5.2 IL-2-producing MSCs delay B16 melanoma tumor growth *in vivo*.

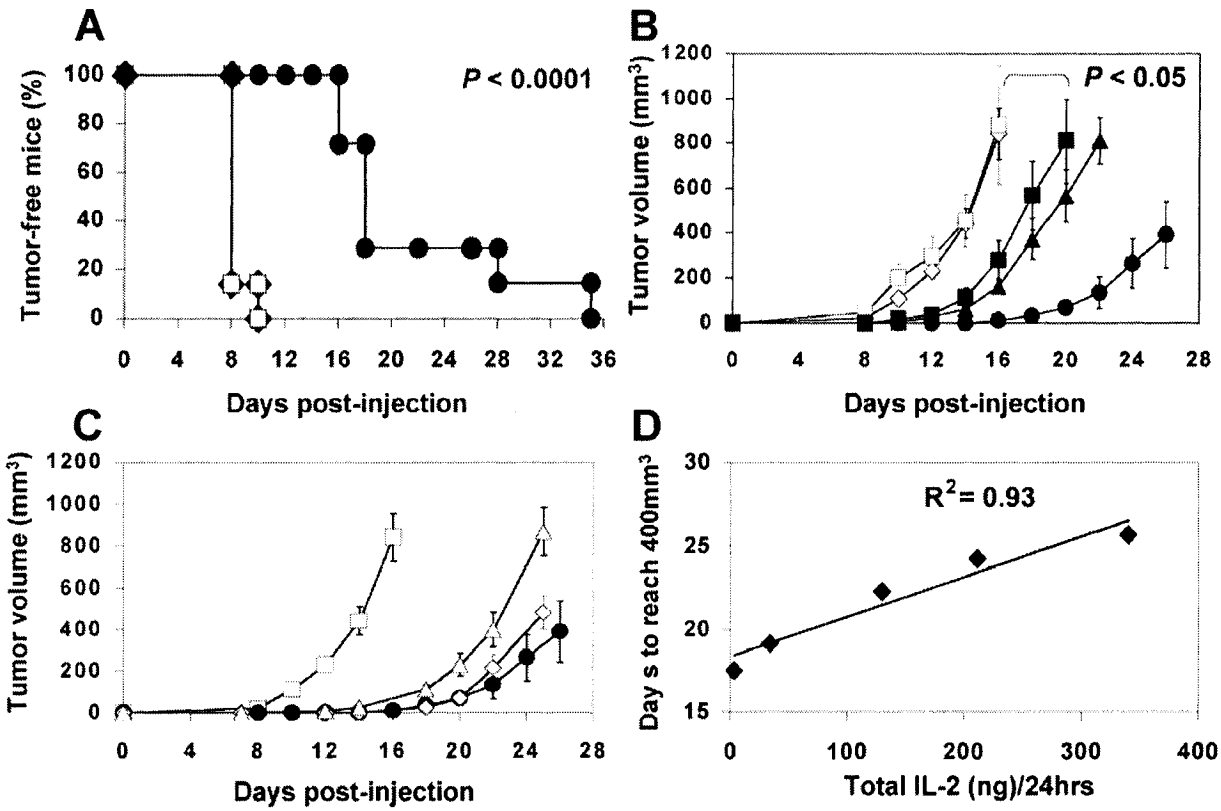
We determined whether primary MSCs had an effect on the growth of B16 melanoma cells when transplanted together in normal C57/bl6 mice. All mice injected subcutaneously with 10^5 B16 cells, or 10^5 B16 cells admixed with 10^6 MSC-GFP, developed palpable tumors by 10 days post-injection. Importantly, B16 tumor growth kinetics was unaltered by the presence of MSC-GFP. In contrast, when IL-2 producing MSCs were transplanted with B16 cells, a significant delay in tumor growth was observed as it took 35 days before all mice injected developed palpable tumors (Figure 6A; $P<0.0001$). Systemic IL-2 plasma concentration measured using a commercial ELISA kit was below detectable levels (<13pg/ml) 24 hours or 10 days post-injection in all mice injected (data not shown). We then evaluated dose-effect by mixing 10^5 B16 cells with a range of MSC-IL2-high, i.e. 10^4, 10^5 or 10^6 cells (Figure 6B). A significant antitumor effect was observed when as low as 10^4 MSC-IL2-high were mixed to 10^5 B16 cells ($P<0.05$). To ensure that the antitumor effect observed with MSC-IL2-high was not an idiosyncratic property of this clone, the dose-effect was assessed using a panel of distinct MSC-IL2 clones (Figure 5C) secreting different levels of IL-2. All the MSC-IL2 clonal populations tested induced an IL-2 dose-dependent antitumor effect ($R^2= 0.93$; data not shown).

3.5.3 Matrix embedding of IL-2-producing MSCs improves antitumor activity.

Based on our previous work[351], we speculated that providing a matrix support to injected IL-2 producing MSCs would improve their therapeutic value. We therefore compared

Figure 6: Antitumor effect of IL-2 gene-engineered MSCs when mixed to B16 cells. (A) Cohorts of syngeneic mice were injected subcutaneously with 10^5 B16 cells (white squares), a mixture of 10^6 MSC-IL2-high with 10^5 B16 cells (black circles) or a mixture of 10^6 MSC-GFP with 10^5 B16 cells (black diamonds) (n=10 per group). (B) We assessed the anti-tumor dose-effect by coinjecting subcutaneously a mixture of 10^5 B16 cells and a range of MSC-IL2-high, i.e. 10^4 (black squares), 10^5 (black triangles) or 10^6 cells (black circles). Control mice were injected with 10^5 B16 cells only (white squares) or 10^5 B16 cells mixed to 10^6 MSC-GFP (white diamonds). Mean are shown (n=5) ± s.e.m. of one representative experiment of two.

Figure 6


A
Tumor-free mice (%)
100
80
60
40
20
0
P < 0.0001
0 4 8 12 16 20 24 28 32 36
Days post-injection
B
Tumor volume (mm3)
1200
1000
800
600
400
200
0
P < 0.05
0 4 8 12 16 20 24 28
Days post-injection
C
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0 4 8 12 16 20 24 28
Days post-injection
D
Day s to reach 400mm3
30
25
20
15
R2= 0.93
0 100 200 300 400
Total IL-2 (ng)/24hrs

the antitumor effect of matrix-embedded MSC-IL2 to that of free MSC-IL2 cell injection. In the first set of experiments, B16 tumors were pre-established subcutaneously and treated 24 hours later by peritumoral injection of 10^6 matrix-embedded MSCs. When a tumor burden of 10^5 B16 cells was pre-established subcutaneously, all mice treated with matrix-embedded MSC-GFP developed palpable tumors by 15 days. Importantly, tumor growth kinetic was unaltered by the presence of matrix-embedded MSC-GFP. In contrast, 40% of mice treated with 10^6 matrix-embedded MSC-IL2-high were still tumor-free more than 100 days after treatment ($P<0.01$; Figure 7A). This antitumor effect was even more pronounced when the tumor burden was lowered to 2 x 10^4 B16 cells, as 90% of treated mice were still tumor-free more than 100 days after treatment ($P<0.001$; Figure 7B). To assess whether antitumor effects could have been mediated solely by elevated systemic levels of IL-2, we injected mice with 10^6 matrix-embedded MSC-IL2-high in the opposite flank to the pre-established tumor ($2x10^4$ B16 cells). We found there was no significant antitumor effect, confirming that systemic delivery of IL2 by embedded cells is not sufficient for antitumor effect ($P>0.05$; Figure 7C). The enhanced effectiveness of matrix-embedded MSCs is further exemplified by the fact that peritumoral injection of 10^6 "free" MSC-IL2-high into a low-burden pre-established tumor did not procure any advantage over MSC-GFP injection or no treatment ($P>0.05$; Figure 7D).

3.5.4 Fate of matrix-embedded MSC-IL2

We investigated the fate of IL-2-producing MSCs when embedded in the matrix and injected subcutaneously into syngeneic mice. After subcutaneous injection of 10^6 matrix-embedded MSC-IL2 or control MSCs, embedded cells were retrieved at defined time points from experimental mice and the explants dissolved in a collagenase solution. Single cell suspensions were generated and analyzed for GFP and cell surface markers expression. Flow cytometry revealed that 40% of MSC-IL2 expressed *de novo* CD34 and Flk1 (VEGF receptor-2) (Figure 8A,B). As further revealed by immunofluorescence microscopy on frozen sections 10 days after subcutaneous injection (Figure 8C), IL-2

Figure 7: **Matrix-embedding of IL-2 producing MSCs improves antitumor activity.** Cohorts of syngeneic C57bl/6 mice (n=10) were injected subcutaneously with (A) 10^5 or (B) $2x10^4$ B16 melanoma cells only (white squares) or treated twenty-four hours later with peritumoral injection of 10^6 matrix-embedded MSC-IL2-high (black circles) or MSC-GFP (black diamonds). (C) 10^6 matrix-embedded MSC-IL2-high (black circles) or MSC-GFP (black diamonds) were injected subcutaneously in the opposite flank of tumor-bearing mice injected with $2x10^4$ B16 cells. Untreated mice are represented by white squares. (D) Antitumor effect of peritumoral injection of 10^6 "free" (non-embedded) MSC-IL2-high (black circles) or MSC-GFP (black diamonds) into tumor-bearing mice injected with $2x10^4$ B16 cells. Untreated mice are represented by white squares.

Figure 7

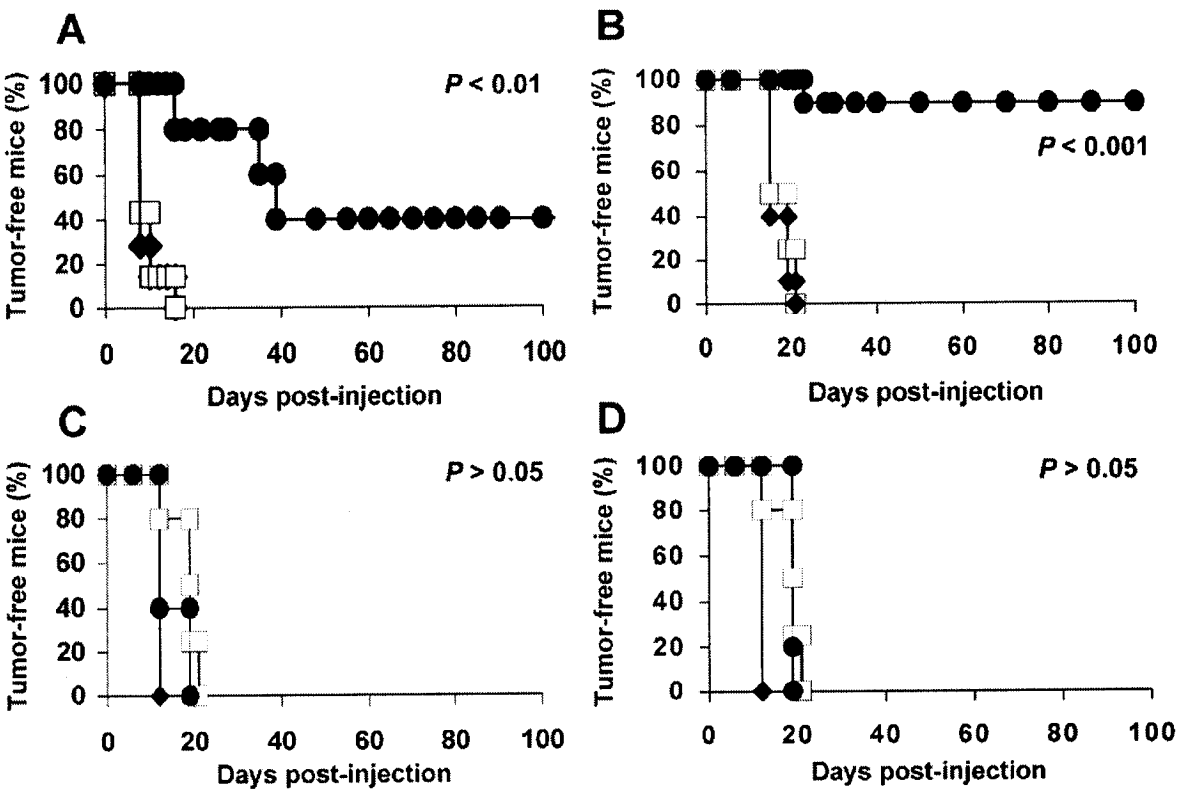
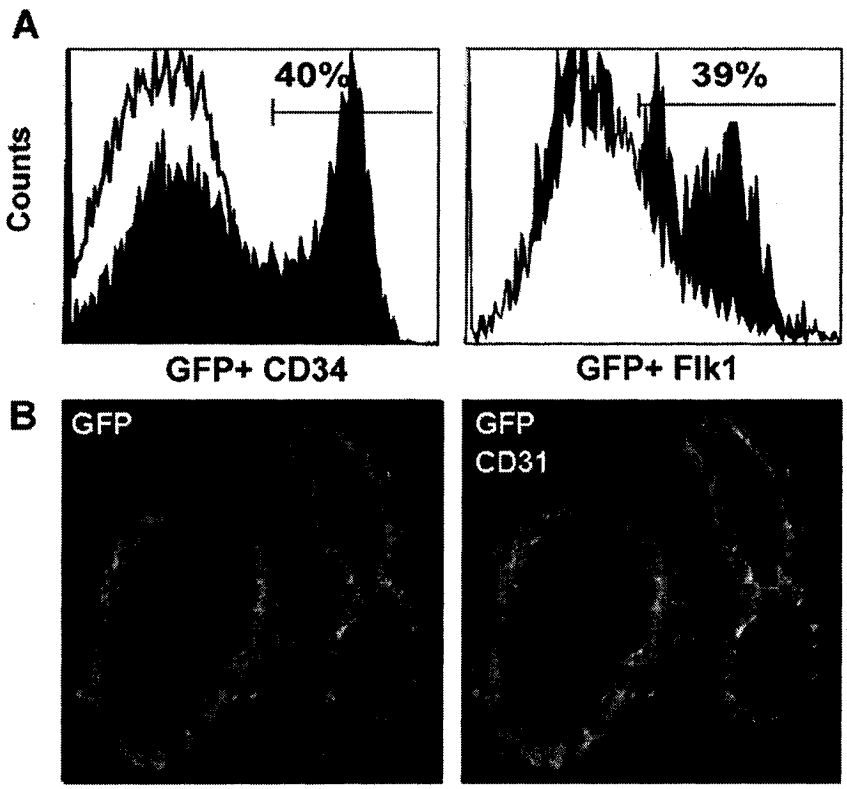

A
P < 0.01
Tumor-free mice (%)
100
80
60
40
20
0
0
20
40
60
80
100
Days post-injection
B
P < 0.001
Tumor-free mice (%)
100
80
60
40
20
0
0
20
40
60
80
100
Days post-injection
C
P > 0.05
Tumor-free mice (%)
100
80
60
40
20
0
0
20
40
60
80
100
Days post-injection
D
P > 0.05
Tumor-free mice (%)
100
80
60
40
20
0
0
20
40
60
80
100
Days post-injection

Figure 8: **Fate of matrix-embedded MSC-IL2**.

(A) Syngeneic C57bl/6 mice were injected subcutaneously with 10^6 matrix-embedded MSC-IL2-high. At 10 days post-injection, the implants were removed and processed to obtain single cell suspensions, and analyzed by flow cytometry for CD34 or Flk1 expression. Plots show isotype control IgG staining profile (in white) versus specific Ab staining profile (in black). (B) Immunofluorescence microscopy of matrix-embedded MSC-IL2-high at 10 days after injection reveals the expression of the endothelial marker CD31 on GFP positive MSC-IL2 (400x magnification; red: CD31-PE; green: MSC-IL2; yellow: expression of CD31 on MSC-IL2).

Figure 8



producing MSCs participated in the formation of blood vessel-like structures within the implant, and a fraction of them co-expressed the endothelial marker CD31, consistent with our previous studies[351, 366]. In order to determine if matrix-embedded MSC-IL2 secrete IL-2 after *in vivo* injection, day 10 MSC-IL2 cells were sorted out from the explants, placed in culture for 24 hours and their supernatant tested for the presence of IL-2 by ELISA. The recovered MSC-IL2, although partly transdifferentiated, secreted identical levels of IL-2 as they did prior to injection (data not shown).

3.5.5 Tumor-specific adaptive immune response in MSC-IL2 treated mice

We tested whether treatment of a pre-established tumor with matrix-embedded IL-2 producing MSCs could induce adaptive immunity. In order to assess adaptive immune response, mice that had previously rejected a subcutaneous tumor injection of $2x10^4$ B16 cells were challenged with a new implant of $2x10^4$ B16 cells on the opposite flank and monitored for tumor growth. We observed a significant delay in tumor occurrence in mice previously treated with matrix-embedded MSC-IL2-high versus naïve mice (Figure 9A; $P<0.05$). To test specificity of the induced response, CD8+ splenocytes of MSC-IL2 treated mice or control mice were isolated 30 days after challenge (75% ± 4% purity; data not shown) and used has effectors in an apoptotic assay against labelled B16 or EL4 target cells. Target cells (10^4cells/well) and CD8+ splenocytes were cocultured for 4hrs at different effector/target (E/T) ratios and apoptotic target cell fraction determined by flow cytometry analysis as described in materials and methods. Our results demonstrated that MSC-IL2 treated mice generated a CD8 mediated tumor-specific immune response against B16 melanoma cells (Figure 9B) compared to untreated mice (Figure 9C).

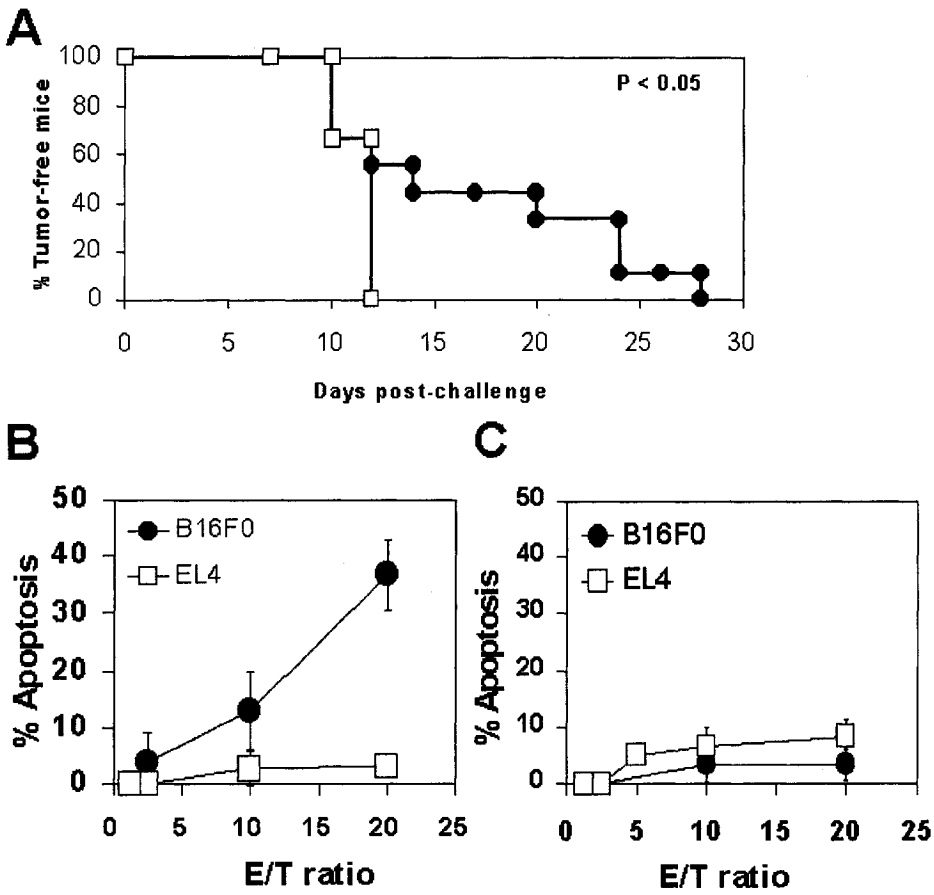
3.5.6 Cellular immune effector analysis

In order to determine which cellular immune effectors were required for anticancer immune response in our system, B16 melanoma were pre-established in cd4-/-, cd8-/- and *beige* mice, and treated with matrix-embedded IL-2 producing MSCs. All CD4 deficient

Figure 9: Tumor-specific immune response in MSC-IL2 treated mice.

(A) Syngeneic C57bl/6 mice (n=9) which rejected a subcutaneous tumor injection of $2x10^4$ B16 F0 melanoma cells after treatment with peritumoral injection of 10^6 MSC-IL2-high embedded in Matrigel were challenged on the opposite flank with $2x10^4$ B16 cells 14 days after treatment (black circles). Control group consisted of naïve mice (white squares). At day 30 post-challenge, $CD8^+$ splenocytes from MSC-IL2 treated mice (B) or control mouse (C) were isolated and used has effector cells in an apoptotic assay against labelled B16 or EL4 target cells (10^4 cells/well). Mean of triplicates are shown ± s.e.m. of a representative experiment of two. Error bars smaller than icons do not appear.

Figure 9


A
100
80
60
40
20
0
% Tumor-free mice
P < 0.05
0
5
10
15
20
25
30
Days post-challenge
B
C
50
40
30
20
10
0
% Apoptosis
B16F0
EL4
0
5
10
15
20
25
E/T ratio

mice effectively rejected the B16 tumor when treated with matrix-embedded IL-2 producing MSCs, indicative of a T helper independent immune response (Figure 10A). Conversely, 80% of CD8 deficient mice treated with matrix-embedded IL-2 producing MSCs developed palpable tumors, confirming an essential role for CD8+ T cells in the observed antitumor effect (Figure 10B). However, there was a significant difference in tumor occurrence between MSC-GFP treated wild type mice and MSC-IL2 treated cd8-/- mice (P =0.02), suggesting that other immune effectors were involved. We thus investigated the possible implication of NK cells by injecting *beige* mice with B16 cells and treated them with matrix-embedded MSC-IL2. As shown in figure 10C, all *beige* mice developed palpable tumors after MSC-IL2 treatment, suggesting that NK cells in addition to CD8+ T cells were required effectors in the MSC-IL2 treatment.

3.5.7 Lymphoid infiltrate associated with MSC implants

In order to further characterize the immune infiltration recruited by the paracrine delivery of IL-2 by engineered MSCs, tumor/MSCs implants were retrieved at 5 and 10 days post-injection, dissolved in a collagenase solution and analyzed by flow cytometry for the expression of CD3, CD4, CD8, NK1.1 and CD25. Compiled analyses demonstrated that after 5 days, IL-2 delivery by engineered MSCs resulted in an early recruitment of T cells (Figure 11A) and NK cells (Figure 11B) 6-fold greater than what was observed in control tumors (P<0.001). After 10 days, the number of infiltrating NK cells was still 3-fold higher (P<0.05), while the level of T cell infiltration was similar in the three groups (P>0.05). However, there was a significant difference in the type of CD3+ subsets recruited in association with IL-2-producing MSCs after 10 days. Relative to control, we observed a significant decrease of CD4+CD25- T cells recruited by IL-2 producing MSCs (P<0.01), while the infiltration of CD4+CD25+ T cells was not significantly different. On the other hand, the number of CD8+ T cells was significantly decreased while a significant fraction of CD3+ T cells coexpressed the NK marker NK1.

Figure 10: Cellular immune effector analysis.

Cohorts of syngeneic immunodeficient mice (n=5), i.e. (A) cd4-/- (black diamonds), (B) cd8-/- (black sqares) or (C) NK-defective *beige* (black triangles) mice, were injected subcutaneously with $2x10^4$ B16 melanoma cells and treated twenty-four hours later with peritumoral injection of 10^6 matrix-embedded MSC-IL2-high. Control mice were injected with $2x10^4$ B16 cells only (n=11; white squares) or treated with 10^6 matrix-embedded MSC-GFP (n=10; white diamonds). Tumor occurrence of wild type mice treated with 10^6 matrix-embedded MSC-IL2-high is represented in black circles (n=10).

Figure 10

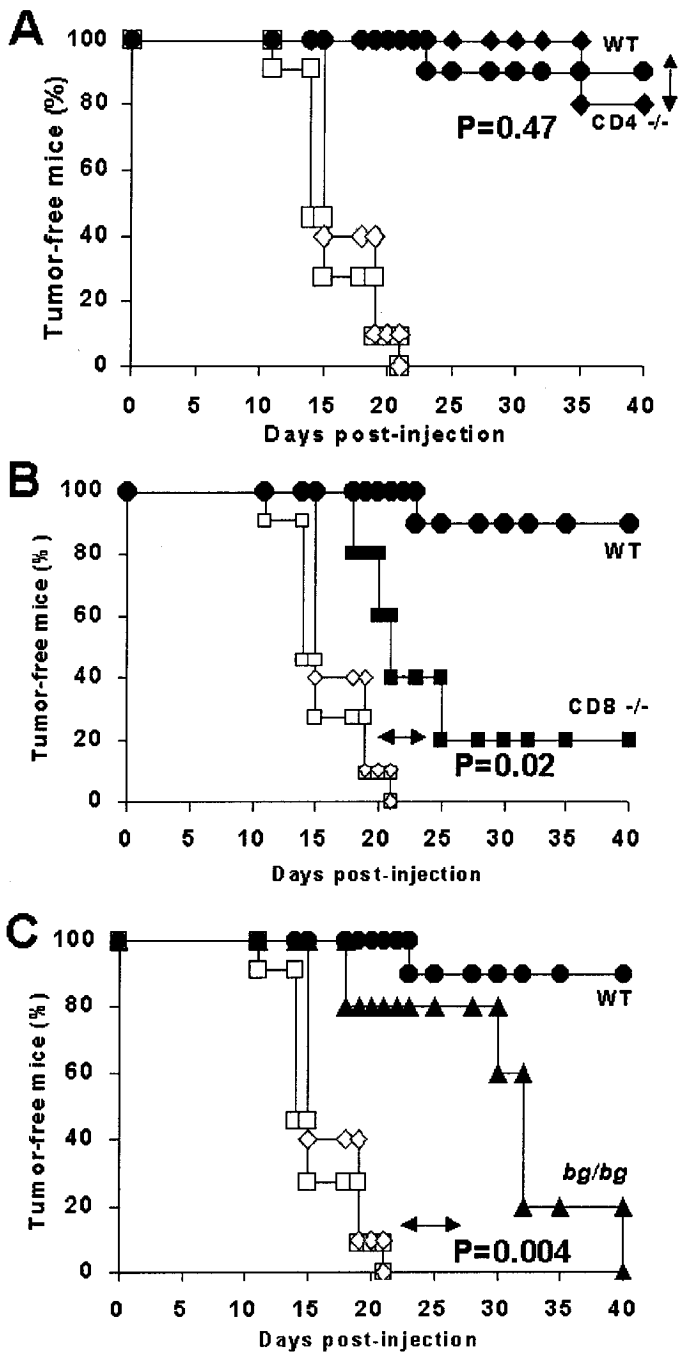
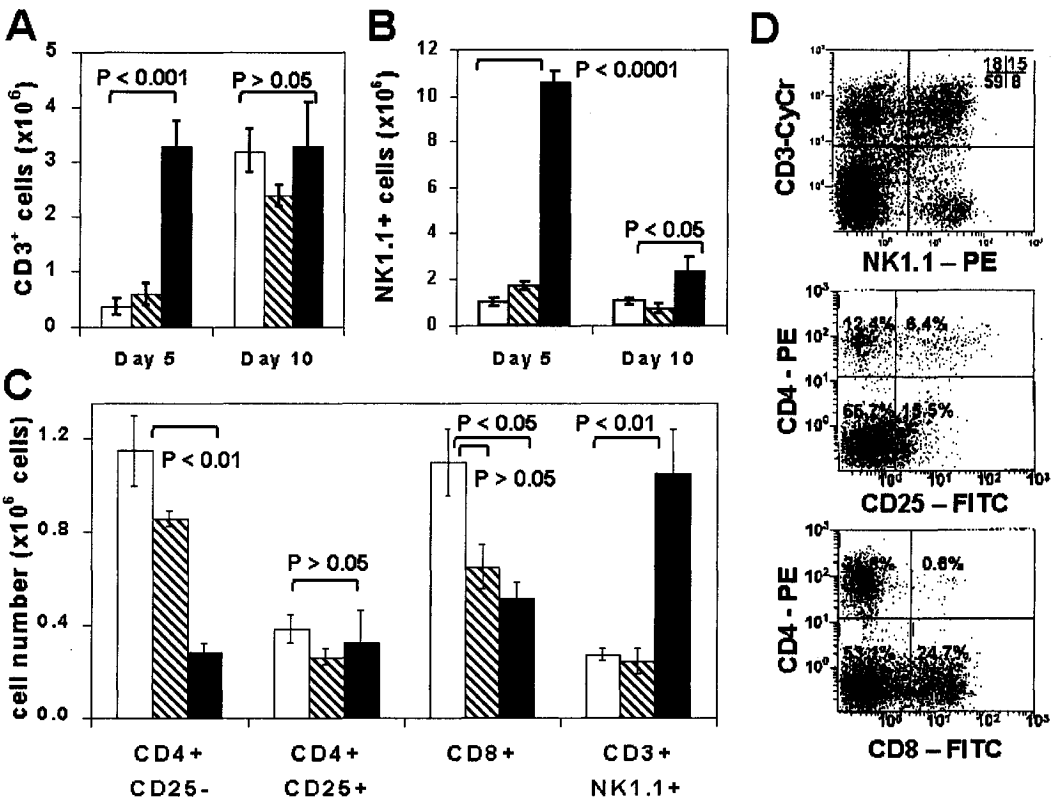

A
100
80
60
40
20
0
Tumor-free mice (%)
WT
CD4 -/-
P=0.47
0
5
10
15
20
25
30
35
40
Days post-injection
B
100
80
60
40
20
0
Tumor-free mice (%)
WT
CD8 -/-
P=0.02
0
5
10
15
20
25
30
35
40
Days post-injection
C
100
80
60
40
20
0
Tumor-free mice (%)
WT
bg/bg
P=0.004
0
5
10
15
20
25
30
35
40
Days post-injection

Figure 11: Lymphoid infiltrate associated with IL-2 producing MSCs.

Cohorts of syngeneic mice (n=10) were injected subcutaneously with 10^5 B16 (white bars), 10^5 B16 cells mixed to 10^6 MSC-IL2-GFP (diagonal bars) or 10^5 B16 cells mixed to 10^6 MSC-IL2-high (black bars) embedded in Matrigel. At 5 and 10 days after injection, the mice were sacrificed and the implants excised and processed to obtain single cell suspensions. Cells were counted and analyzed by flow cytometry after three-color staining with mAbs against CD3ε, CD4, CD25, CD8α and NK1.1 antigens as described in methods. (A) Absolute number of infiltrating $CD3^+$ cells at 5 and 10 days after injection. (B) Absolute number of infiltrating $NK1.1^+$ cells at 5 and 10 days after injection. (C) Infiltrating $CD3^+$ subset analysis at 10 days after injection. (D) Representative examples of flow cytometry analysis at 10 days after injection of B16 cells and MSC-IL2-high.

Figure 11


A
CD3+ cells (x10^6)
P < 0.001
P > 0.05
Day 5
Day 10
B
NK1.1+ cells (x10^6)
P < 0.0001
P < 0.05
Day 5
Day 10
C
cell number (x10^6 cells)
P < 0.01
P > 0.05
P < 0.05
P > 0.05
P < 0.01
CD4+ CD25-
CD4+ CD25+
CD8+
CD3+ NK1.1+
D
CD3-CyCr
NK1.1 – PE
18.5
59.8
CD4 - PE
CD25 – FITC
12.4%
6.4%
55.7%
25.5%
CD4 - PE
CD8 – FITC
0.6%
24.7%

3.6 DISCUSSION

Primary MSCs are pluripotent cells with a very robust *ex vivo* expansion capacity, which makes them ideal candidates for regenerative and transgenic cell medicine. A potential clinical application of MSCs is their use for the delivery of therapeutic proteins following *ex vivo* genetic engineering and autologous transplantation. Several studies have demonstrated, essentially in immunocompromised animals, successful *in vivo* delivery of various proteins by gene-modified MSCs. This includes delivery of factor VIII, factor IX, IL-3 and IFN-β[347-350]. We have shown, in addition, that gene-modified autologous MSCs can be used to generate a subcutaneous implant in order to deliver erythropoietin in unconditioned normal hosts[351]. This method allows for pharmacological delivery of secreted proteins while allowing removal of the transgenic cells if unforeseen complications were to arise.

Although MSCs have generated clinical interest in the delivery of various therapeutic gene products, their utility for delivery of immunostimulatory proteins was uncertain due to the recent observations that MSCs possess intrinsic immunosuppressive properties able to suppress allogeneic mixed lymphocyte reactions and to favour tumor growth in allogeneic recipients[311-320]. To address this, we gene-modified primary mouse MSCs to secrete IL-2 and tested whether these cells could be used to generate an effective antitumoral immune response against a poorly immunogenic tumor.

We first generated bicistronic retroviral vectors encoding the murine IL-2 cDNA and the GFP reporter gene, and gene-modified primary C57bl/6 MSCs. We analyzed the cell surface phenotype of IL-2 producing MSCs clonal populations and showed that IL-2 expression did not substantially alter the phenotype of primary mouse MSCs. Indeed, control and IL-2 producing MSCs were $CD45^-$, $CD34^{low}$, $CD31^-$, MHC I^{low}, $Flk1^{low}$ and $CD44^+$. Clonal variability of CD44 expression could explain the observed difference. This phenotype is identical to that we have previously reported[351, 366] and similar to that reported for MSCs derived from C57bl/6 mice[283].

We next tested the effect of MSCs on the growth of B16 melanoma cells in normal immunocompetent mice. It had been shown recently that C3H mouse MSCs, when coinjected or intravenously administered, allow proliferation of B16 tumor cells in allogeneic immunocompetent animals[317]. The authors suggested that soluble factors released from MSCs mediated allogeneic tumor growth. We thus performed an experiment where MSC-GFP were coinjected with B16 cells into syngeneic immunocompetent mice and found that MSCs did not alter tumor growth kinetics. This was true even when 10 fold more MSCs were mixed to B16 cells. Therefore, we conclude that autologous MSCs do not appear to have intrinsic properties facilitating B16 tumor progression in C57bl/6 mice.

While coinjection of control MSC-GFP did not affect tumor growth of B16 melanoma, coinjection of IL-2 producing MSCs significantly delayed B16 tumor growth in immunocompetent mice. This antitumor effect was directly dependent upon the dose of IL-2 delivered by the engineered MSCs. Interestingly, a significant delay in tumor growth was achieved when as low as 10^4 IL-2 producing MSCs were co-injected with 10^5 B16 cells. To rule out any idiosyncratic effect of a given clonal populations, distinct IL-2 producing MSCs population were coinjected with B16 cells and similar IL-2 dose-dependent antitumor effects were observed (data not shown).

Since we had previously shown that *in vivo* delivery of erythropoietin was greatly enhanced when primary gene-modified MSCs are embedded within a collagen matrix[351], we tested the hypothesis that matrix-embedded IL-2 producing MSCs could be used to treat established B16 melanoma in mice. We demonstrated that when matrix-embedded IL-2 producing MSCs were injected in the vicinity of pre-established low-burden B16 melanoma, up to 90% of mice failed to develop a tumor. As previously shown[351] with naïve MSCs and Epo gene-modified MSCs, we observed that IL-2 producing MSCs participate in neovascularization of the collagen implant. Although a fraction of the MSCs had undergone phenotypic conversion to endothelial cells, stable release of IL-2 was maintained after injection. At 18 days after implantation, we determined that 10% of the initial number of matrix-embedded MSCs remained, whether they were IL-2

producing MSCs or control MSC-GFP (data not shown). Our previous work using Epo-secreting MSCs[351] demonstrated that the surviving fraction of matrix-embedded MSCs is sufficient to induce long-term delivery (>200days) of the therapeutic gene product. Therefore, despite survival of only a small fraction of matrix-embedded MSCs, significant long-term therapeutic effect is achieved.

We assessed the immune response generated and showed that mice that had rejected a pre-established melanoma after treatment with matrix-embedded IL-2 producing MSCs acquired significant protection against a tumor challenge with B16 cells. Furthermore, CD8+ tumor-specific splenocytes were detectable more than 30 days post therapy. In order to determine which immune effectors were required for this anticancer immune response, B16 melanoma were pre-established in cd4-/-, cd8-/- or NK-deficient *beige* mice, and treated with matrix-embedded IL-2 producing MSCs. The combined results demonstrated that B16 treatment with IL-2 producing MSCs is dependent upon CD8+ and NK cells, but independent of CD4+ cells.

When tumor infiltrating lymphocytes were analyzed by flow cytometry, we observed that IL-2 delivery by engineered MSCs resulted in early recruitment of T cells and NK cells, 6-fold greater after 5 days compared to controls. When infiltrating T cells were analyzed 10 days after injection, we observed that IL-2 producing MSCs led to a robust infiltration of CD3+ cells that coexpressed NK1.1. It has been previously shown that upon stimulation with IL-2 or viral infection, purified NK1.1- T cells can rapidly acquire surface expression of NK1.1, consistent with our observation[367-368]. Interestingly, CD8+ expression was decreased when IL-2 producing MSCs were injected ($P<0.05$), but not when MSC-GFP were injected ($P>0.05$). We may speculate that this decrease could be the result of exposure to IL-2, since it has been shown that IL-2 can downregulate CD8α expression on T cells[369]. Another unheralded observation is the significant decrease of CD4+CD25- T cells recruited by IL-2 producing MSCs, whereas levels of classical immunoregulatory CD4+CD25+ T cells remained unchanged. We may speculate that cells within the CD4+CD25- subset may play a negative immunoregulatory role, and their depletion enhances anticancer cellular immunity. This is supported by the fact that

anergic CD4+CD25- T cells have been described, and possess nearly identical capacity to block T cell proliferation as CD4+CD25+ regulatory T cells[370-371].

Importantly, our analysis of the cellular immune infiltrate demonstrated that control MSC-GFP failed to modulate, either negatively or positively, the immune response when transplanted with B16 cells in syngeneic recipients. This is in contrast with the reported immunosuppressive effect of MSCs on B16 growth in allogeneic recipients[317]. On the other hand, our results with IL-2 producing MSCs are consistent with the literature on IL-2 anticancer effects[242], and demonstrate that MSCs can be effectively exploited as a cellular vehicle to stimulate an immune response against poorly immunogeneic tumors. Interestingly, the fact that MSC-IL2 treated mice could mount tumor-specific CD8-dependent systemic immunity is in contrast with what has been reported using IL-2 producing fibroblasts. Paracrine delivery of high doses of IL-2 by engineered fibroblasts, although able to prevent tumor implantation, was ineffective in generating systemic immunity[372]. We may speculate that MSCs in themselves provide unidentified cofactors that may act synergistically with IL-2 in generating a memory immune response. For example, it has been reported that bone marrow-derived MSCs provide cofactors essential for NK cell activity[373].

There have been numerous prior reports – including clinical trials - describing cancer targeted IL-2 gene delivery with replication-defective viral vectors[208, 211, 374]. Though local anticancer effects were noted, little to no systemic anticancer activity was noted. A distinguishing feature of our work is the unambiguous observation that IL-2 delivery by MSCs adjacent to a pre-established low-burden tumor implant leads to outright rejection but also leads to measurable and biologically relevant anticancer CD8+ T-cell mediated adaptive immunity. Our experiments reveal that this response did not occur as a result of non-specific systemic levels of IL-2 since an implantation of MSC-IL2 far removed from the tumor site did not lead to a measurable anti-cancer response *in vivo*. Therefore, it appears that the sustained paracrine delivery of IL-2 afforded by MSCs in physical proximity to the tumor site is required for the pleiotropic immune effects observed. We speculate that here lies the difference with previous cancer targeted IL-2 delivery

strategies. It would be expected that tumor-localized IL-2 levels would decline rapidly when IL2-engineered tumor cells are destroyed by immune-mediated responses, whereas our MSC-IL2 platform allows for a more sustained, pharmacologically relevant production of IL-2 at the tumor site. This proof-of-principle supporting the use of MSCs for sustained local delivery of an anticancer immunostimulatory interleukin could also be exploited for delivery of other interleukins, cytokines, chemokines and the like. The clinical utility of this strategy could be readily applied in the setting of debulked locally advanced disease – minimal residual disease – where local application of MSC-IL2 in the tumor bed site may lead to a local as well as a systemic anticancer effect and protect from local or systemic cancer relapse from low-burden residual or metastatic disease.

In conclusion, the results presented here add to the numerous potential applications of MSCs in cell medicine. Although it has been shown that MSCs possess immunosuppressive properties in allogeneic settings, we demonstrated that primary MSCs did not affect syngeneic B16 tumor growth and that IL-2 producing MSCs could generate CD8 and NK mediated systemic immunity against B16 cells. MSCs are particularly appealing as an autologous cellular vehicle for delivery or IL-2 – or any other secreted anti-cancer immunomodulator – for the following reasons: (i) *ex vivo* expansion of engineered clonal MSCs to clinically relevant numbers is readily feasible in human adults, (ii) MSCs display tumor tropism, (iii) engineered MSCs secreting IL-2 coupled to a synthetic matrix demonstrated vascular plasticity and may be exploited for pharmacological anticancer purposes as here demonstrated. Although the matrix used for this proof-of-concept, i.e. Matrigel, serves as a useful support vehicle in mice, it is incompatible to human use. Our previous work demonstrated that collagen might be an adequate minimal component in mediating MSCs survival in implants[351]. Using human compatible type I bovine collagen, we showed that matrix-embedded Epo-secreting MSCs were able to maintain high hematocrit levels ($P<0.001$) for more than 100 days in mice. This suggests that therapeutic gene product delivery could be achieved in patients using human compatible matrices. This strategy could be of significant utility in locally advanced solid tumors where high-dose systemic IL-2 therapy has shown some promise, but whose modest benefit is outweighed by unacceptable systemic toxicities.

3.7 AKNOWLEDGMENTS

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CHAPTER 4

Interferon-γ-Stimulated Marrow Stromal Cells: A New Type of Non-Hematopoietic Antigen Presenting Cell.

Reference: Stagg J, Pommey S, Eliopoulos N, Galipeau J. Interferon-γ-Stimulated Marrow Stromal Cells: A New Type of Non-Hematopoietic Antigen Presenting Cell. 2005. Blood (in press).

Preface to Chapter 4:We demonstrated in Chapter 3 that despite their previously reported immunosuppressive effects on allogeneic immune responses, primary MSCs can be used as transgenic delivery vehicles to enhance immune responses in syngeneic immunocompetent hosts. In order to further characterize the effect of MSCs on autologous immunity, we investigated the immunomodulatory properties of MSCs during syngeneic antigen-specific immune responses.

CHAPTER 4: Interferon-γ-Stimulated Marrow Stromal Cells: A New Type of Non-Hematopoietic Antigen Presenting Cell.

4.1 ABSTRACT

Several studies have demonstrated that marrow stromal cells (MSCs) can suppress allogeneic T cell responses. However, the effect of MSCs on syngeneic immune responses has been largely overlooked. We here describe that primary MSCs derived from C57BL/6 mice behave as conditional antigen-presenting cells (APCs) and can induce antigen-specific protective immunity. IFNγ-treated C57BL/6 MSCs, but not unstimulated MSCs, cocultured with ovalbumin-specific MHC class II restricted hybridomas in the presence of soluble ovalbumin induced significant production of IL-2 in an antigen dose-dependent manner ($P<0.005$). IFNγ-treated MSCs could further activate *in vitro* ovalbumin-specific primary OT-II-derived CD4+ T cells. C57BL/6 MSCs were however unable to induce antigen cross-presentation via MHC class I pathway. When syngeneic mice were immunized intraperitoneally with ovalbumin-pulsed IFNγ-treated MSCs, they developed antigen-specific cytotoxic CD8+ T cells and became fully protected (10 out of 10 mice) against ovalbumin-expressing E.G7 tumors. Human MSCs were also studied for antigen presenting functions. IFNγ-treated DR1-positive human MSCs, but not unstimulated human MSCs, cocultured with DR1-restricted influenza-specific humanized T cell hybridomas in the presence of purified influenza matrix protein 1 induced significant production of IL-2. Taken together, our data strongly suggest that MSCs behave as conditional APCs in syngeneic immune responses.

4.2 INTRODUCTION

Primary cultures of bone marrow stromal cells (MSCs) contain pluripotent cells with a robust *ex vivo* expansion capacity[268, 285]. Pre-clinical and clinical studies have demonstrated that MSCs can be used for tissue repair[307, 375], for delivery of therapeutic gene products[347-351] and for enhancing engraftment of autologous peripheral-blood stem cells[376]. MSCs can differentiate along multiple cell lineages, including adipocytes, chondrocytes, osteocytes, myocytes, astrocytes, neurons, endothelial cells and lung epithelial cells[268, 272, 366, 377-379]. Since no single surface marker has been described for purification, MSCs are generally isolated based on their adherence to tissue culture plates, resulting in a semi-homogenous population characterized by the absence of CD45 and CD31, and by the expression of CD105, CD73 and CD44[307]. MSCs express low levels of major histocompatibility complex (MHC) class I molecules while, as a general rule, they do not constitutively express MHC class II molecules[312-313, 325]. One study, however, reported constitutive MHC class II expression on MSCs[314]. Both MHC class I and class II molecules get upregulated following IFNγ treatment, with a more heterogeneous expression between individual cells for MHC class II molecules[315-316, 325]. Costimulatory molecules such as CD80, CD86, CD40 and CD40L are not known to be expressed nor induced on human MSCs, while mouse MSCs can be found to express CD80[316].

MSCs are also known to secrete a wide spectrum of growth factors and cytokines implicated in different aspects of hematopoiesis[285] and lymphopoiesis[286]. One important feature of MSCs is their recently identified *in vitro* immunosuppressive properties against allogeneic immune responses. It has been shown that MSCs are able: (i) to suppress the proliferation of allogeneic T cells in response to mitogen or allogeneic cells[311-313, 316, 317]; (ii) to inhibit the production of IFNγ and tumor-necrosis factor (TNF)-α and increase the production of IL-10[318]; (iii) to induce T cell division arrest anergy[319]; (iv) to inhibit the maturation and function of antigen presenting cells such as monocytes and dendritic cells[320-321]; (v) to decrease alloantigen-specific cytotoxicity of CD8 T cells and natural killer (NK) cells[322]; and (vi) to favor the differentiation of CD4 T cells with presumed

regulatory activity[322]. The clinical potential of the immunosuppressive properties of MSCs has been exemplified by LeBlanc and colleagues[324] who reported in a case study that administration of haploidentical human MSCs following allogeneic stem cell transplantation could reverse the severe grade IV acute graft-versus-host disease (GVHD) of a patient. At present, the exact mechanism responsible for MSC-mediated immunosuppression remains imprecise. Soluble factors such as hepatocyte growth factor (HGF), transforming growth factor (TGF)-β1[31], indoleamine 2,3-dioxygenase (IDO)[325], IL-10[320] and unidentified factors[312, 317, 326] have been implicated. Other studies suggested instead that contact-dependent mechanisms are required[316, 320].

If the immunosuppressive effects of MSCs on allogeneic or third party immune responses have been well described, the effect of MSCs on syngeneic immune responses has been largely overlooked. In order to further characterize the effect of MSCs on autologous immunity, we investigated the immunomodulatory properties of MSCs during a syngeneic antigen-specific immune response. Unexpectedly, we observed that syngeneic MSCs behave as conditional antigen-presenting cells. We demonstrated that IFNγ can induce mouse MSCs to process and present antigenic peptides derived from a soluble xenoprotein (ovalbumin) and activate *in vitro* antigen-specific T cells. When injected *in vivo* into syngeneic mice, ovalbumin-pulsed IFNγ-treated MSCs induced potent ovalbumin-specific cellular immune responses and protected mice against ovalbumin-expressing tumors. We further demonstrated that human MSCs can also acquire antigen-presenting functions upon IFNγ stimulation, thereby activating antigen-specific T cell hybridomas. Taken together, our results strongly suggest that in syngeneic conditions, IFNγ-stimulated MSCs behave as conditional antigen presenting cells able to activate antigen-specific immune responses.

4.3 RESULTS:

4.3.1 Phenotypic characterization of primary MSCs

Primary MSCs were isolated from C57BL/6 mice as previously described[380-381]. Cultured in differentiation media, MSCs were able to give rise to osteogenic and adipogenic cells (Figure 12A). Phenotypically, MSCs were negative for CD45, CD31 (data not shown), CD54, CD86 and CD40 expression and were positive for CD105, MHC class I (H-2Kb) and CD80 expression as determined by flow cytometry (Figure 12). When exposed to recombinant mouse IFNγ (50ng/ml) for 20 hours, MSCs upregulated MHC class I, MHC class II and CD54, but not CD80, while they remained negative for CD86, CD40, and CD45 expression (Figure 12B).

4.3.2 Immunosuppressive effects of MSCs

We evaluated the immunosuppressive effect of C57BL/6-dervied mouse MSCs on allogeneic mixed lymphocyte cultures. In accordance with previous studies[316], the addition of MSCs to allogeneic cocultures of C57BL/6 and BALB/c splenocytes significantly inhibited the activation levels of the cocultures ($P<0.05$ by T-test; Figure 13A). Also consistent with previous studies[325], pre-stimulation of MSCs with recombinant IFNγ did not hinder their allogeneic immunosuppressive effect ($P>0.05$ by T-test; Figure 13A).

In order to test whether MSCs could suppress syngeneic immune responses, we used a previously described ovalbumin-specific T-T hybridoma assay[382]. In this assay, immortalized dendritic cells (DC2.4 cells) are cocultured for 20 hours with syngeneic MHC class II restricted ovalbumin-specific T-T hybridomas (MF2.2D9 cells) in the presence of increasing doses of soluble ovalbumin. Twenty hours later, antigen-specific T cell activation is assessed by measuring the level of IL-2 released in the supernatant. When soluble ovalbumin was added to DC2.4 and MF2.2D9 cocultures, significant levels

Figure 12: *In vitro* characterization of C57BL/6-derived MSCs.

A) Primary MSCs were isolated from the femurs and tibias of C57BL/6 female mice, culture expanded in DMEM 10% FBS and differentiated into osteogenic and adipogenic lineage cells as described in the methods section. Alizarin Red S was used to stain calcium in the mineralized extracellular matrix and Oil Red O was used to stain adipocytic vesicles. Top panels show stained undifferentiated cells and bottom panels show stained differentiated cells. (B) C57BL/6 MSC were isolated from the femurs and tibias of C57BL/6 female mice, culture expanded in DMEM 10% FBS and analyzed by flow cytometry for CD45, CD105, MHC class I (H2-Kb), MHC class II (I-Ab), CD80, CD86, CD40 and CD54 cell surface expression. Where indicated, MSCs were first treated with recombinant mouse IFNγ (50ng/ml) for 20hrs prior to flow cytometry analysis. Plots show isotype control IgG staining profile (doted line) versus specific Ab staining profile (thick line).

Figure 12

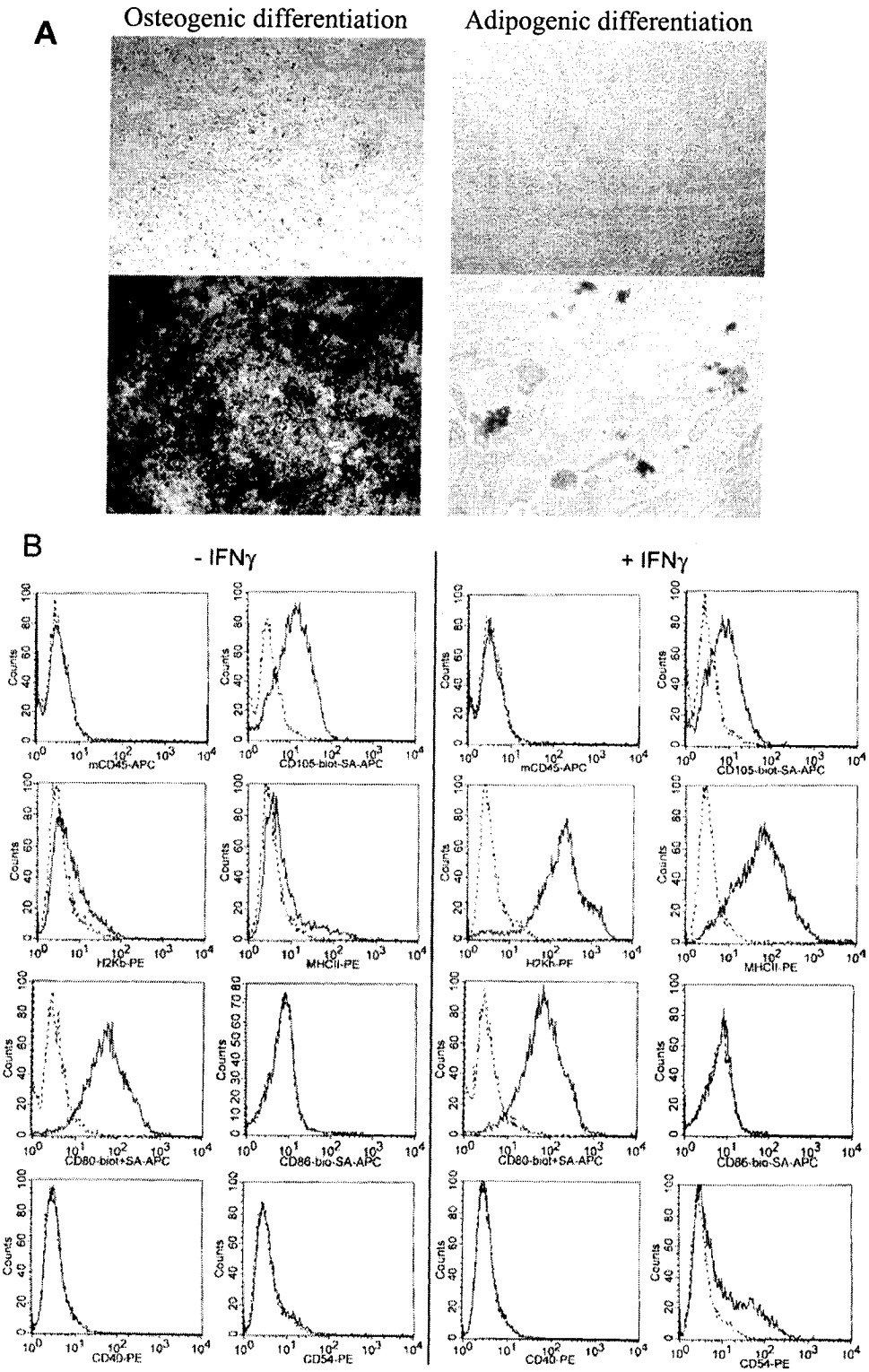
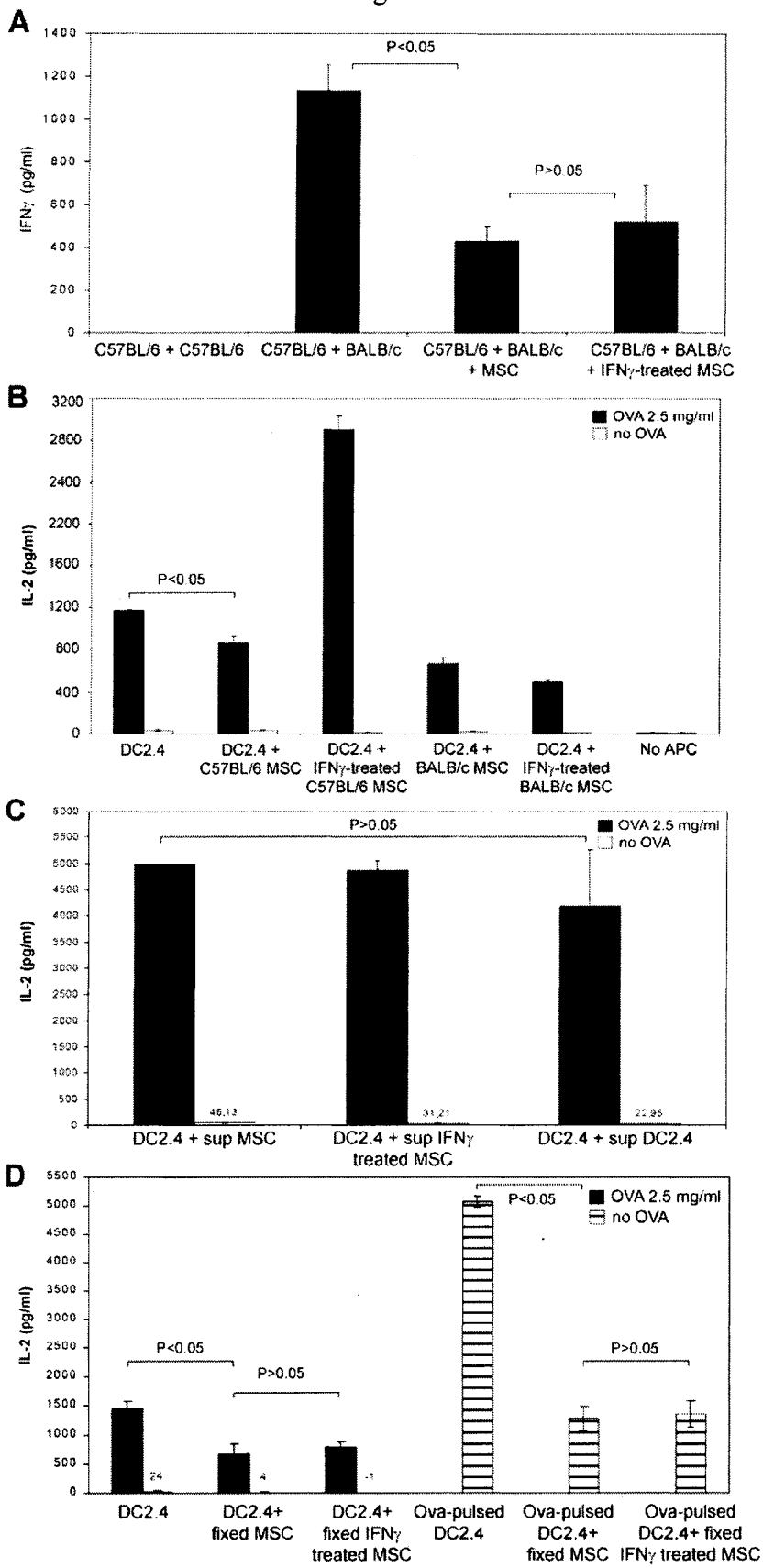

A
Osteogenic differentiation
Adipogenic differentiation
B
- IFNγ
+ IFNγ
Counts
mCD45-APC
CD105-biot-SA-APC
H2Kb-PE
MHCII-PE
CD80-biot+SA-APC
CD86-bio-SA-APC
CD40-PE
CD54-PE

Figure 13: Effect of MSCs on allogeneic and syngeneic immune responses.

(A) Two-way mixed lymphocyte reactions were performed with 10^5 C57BL/6 splenocytes and 10^5 BALB/c splenocytes in the presence or absence of 10^5 C57BL/6 naïve or IFNγ-treated MSCs. After 3 days, supernatant was collected and tested for IFNγ release by ELISA. (B) DC2.4 cells ($5x10^4$ cells) were cocultured for 20hrs with ovalbumin-specific MHC class II-restricted T-T hybridomas (MF2.2D9; 10^5cells) in the presence or not of 2.5mg/ml soluble ovalbumin. Where indicated, $5x10^4$ naïve or IFNγ-pretreated (20hrs) MSCs from C57BL/6 or BALB/c mice were added to the cocultures. After 20hrs, supernatant was collected and tested for IL-2 release by ELISA. (C) Same as (B) and where indicated conditioned supernatant from naïve or IFNγ-treated MSCs were added to the cocultures in replacement of MSCs. (D) Same as (B) and where indicated $5x10^4$ naïve or IFNγ-pretreated paraformaldehyde-fixed MSCs were added to the cocultures in the presence of ovalbumin (filled bars). Alternatively (striped bars), DC2.4 cells were first pulsed with soluble ovalbumin for 20hrs and then cocultured with the indicated cells for another 20hrs (Means of triplicates ± standard deviations of one of two representative experiments are shown).

Figure 13


A
IFNγ (pg/ml)
P<0.05
P>0.05
C57BL/6 + C57BL/6
C57BL/6 + BALB/c
C57BL/6 + BALB/c + MSC
C57BL/6 + BALB/c + IFNγ-treated MSC
B
IL-2 (pg/ml)
OVA 2.5 mg/ml
no OVA
P<0.05
DC2.4
DC2.4 + C57BL/6 MSC
DC2.4 + IFNγ-treated C57BL/6 MSC
DC2.4 + BALB/c MSC
DC2.4 + IFNγ-treated BALB/c MSC
No APC
C
IL-2 (pg/ml)
P>0.05
OVA 2.5 mg/ml
no OVA
DC2.4 + sup MSC
DC2.4 + sup IFNγ treated MSC
DC2.4 + sup DC2.4
D
IL-2 (pg/ml)
P<0.05
P>0.05
P<0.05
P>0.05
OVA 2.5 mg/ml
no OVA
DC2.4
DC2.4+ fixed MSC
DC2.4+ fixed IFNγ treated MSC
Ova-pulsed DC2.4
Ova-pulsed DC2.4+ fixed MSC
Ova-pulsed DC2.4+ fixed IFNγ treated MSC

of IL-2 were produced in an antigen dose-dependent manner as determined by ELISA (Figure 13B). The addition of MSCs to these cocultures significantly inhibited IL-2 release ($P<0.05$ by T-test, performed twice; Figure 13B). However, in marked contrast with the allogeneic mixed lymphocyte reaction (Figure 13A), the addition of IFNγ-treated MSCs to the syngeneic cocultures enhanced IL-2 release ($P<0.05$ by T-test; Figure 13B). Since IFNγ did not, on its own, induce MSCs to release IL-2 (data not shown), this suggested that IFNγ modulated mouse MSCs to become permissive to syngeneic T cell activation.

We thus performed experiments in order to assess the nature of this permissiveness. Specifically, we wanted to determine whether IFNγ-treated MSCs enhanced or failed to suppress DC2.4-mediated antigen presentation. Firstly, we assessed the effect of conditioned supernatant from naïve or IFNγ-treated MSCs on DC2.4-mediated antigen presentation. As shown in Figure 13C, conditioned supernatant from naïve or IFNγ-treated MSCs had no significant effect on DC2.4-mediated antigen presentation ($P>0.05$ by T-test). This suggested that: (i) IFNγ-treated MSCs do not stimulate DC2.4 through a secreted factor, and (ii) the suppressive effect of naïve MSCs on syngeneic antigen presentation is independent of a secreted factor. Secondly, we tested whether naïve or IFNγ-treated MSCs modulated DC2.4 in a contact-dependent manner. As shown in Figure 13D, paraformaldehyde-fixed naïve as well as IFNγ-treated MSCs significantly suppressed DC2.4-mediated antigen presentation ($P<0.05$ by T-test). This suppressive effect was even more pronounced when DC2.4 cells were first pulsed with soluble ovalbumin for 20 hours and then cocultured with fixed MSCs and live hybridoma cells. Taken together, our data suggested that: (i) the suppressive effect of MSCs on syngeneic DC2.4-mediated antigen presentation is contact-dependent, (ii) IFNγ treatment of MSCs does not alter their suppressive effect on DC2.4-mediated antigen presentation, and (iii) despite their suppressive effect, IFNγ-treated MSCs are permissive to syngeneic antigen presentation. We thus hypothesized that IFNγ induced MSCs to acquire antigen-presenting functions.

4.3.3 Activation of MHC class II-restricted hybridomas by IFNγ-treated MSCs

In order to investigate whether IFNγ-treated MSCs could behave as syngeneic antigen presenting cells, soluble ovalbumin was added at increasing doses to cocultures of IFNγ-treated MSCs and MHC class II restricted ovalbumin-specific T-T hybridoma cells. When IFNγ-treated MSCs were exposed to soluble ovalbumin at doses of 2.5, 1.25 and 0.625mg/ml and cocultured for 20 hours with class II restricted hybridomas, significant levels of IL-2 was detected in the supernatants as measured by ELISA (respectively 867, 722 and 551 pg/ml of IL-2; Figure 14A). On the other hand, unstimulated MSCs failed to induce IL-2 release in identical conditions. IL-2 levels were below sensitivity of the assay (<2pg/ml) when IFNγ-treated MSCs were cocultured with hybridomas without ovalbumin, or when the hybridomas were cultured with ovalbumin without MSCs. This experiment was performed 5 times, each in triplicates, with similar results.

In order to rule out the possibility that the observed MSCs-mediated antigen presentation was the result of an idiosyncratic effect, distinct clonal (Figure 14B) and polyclonal (Figure 14C) populations of C57BL/6-derived MSCs were tested with comparable results. Phenotypically, the distinct MSCs populations were very similar (supplementary data), with the exception of MSC clone 10 that constitutively expressed MHC class II and failed to upregulate MHC class II upon IFNγ treatment.

Our data suggested at this point that mouse MSCs can process ovalbumin into MHC class II restricted peptides and activate ovalbumin-specific T-T hybridomas. To exclude the possibility that free peptides in the ovalbumin preparation could have mediated antigen presentation in the absence of antigen processing as others have reported[383], the above-mentioned experiments were repeated using paraformaldehyde-prefixed MSCs subsequently exposed to ovalbumin. As shown in Figure 15A, prefixed IFNγ-treated MSCs did not induce IL-2 release when cocultured with hybridomas and ovalbumin. This suggested that processing of ovalbumin is required for MSCs-mediated antigen presentation. To assess whether processing of ovalbumin was the result of endosomal

Figure 14: MSC-mediated activation of ovalbumin-specific T-T hybridomas

(A) C57BL/6 MSCs, DC2.4 or MEF ($5x10^4$ cells) were cocultured for 20hrs with ovalbumin-specific MHC class II-restricted T-T hybridomas (MF2.2D9; 10^5cells) in the presence of increasing doses of soluble ovalbumin. Where indicated, recombinant mouse IFNγ (50ng/ml final) was added to the cocultures. After 20hrs, supernatant was collected and tested for IL-2 release by ELISA (Means of triplicates ± standard deviations are shown of one of five experiments). (B) Same as (A), except that clonal MSCs obtained by limiting dilution from the initial preparation were used. (C) Same as (A), except that distinct polyclonal C57BL/6-derived MSCs preparations were used.

Figure 14

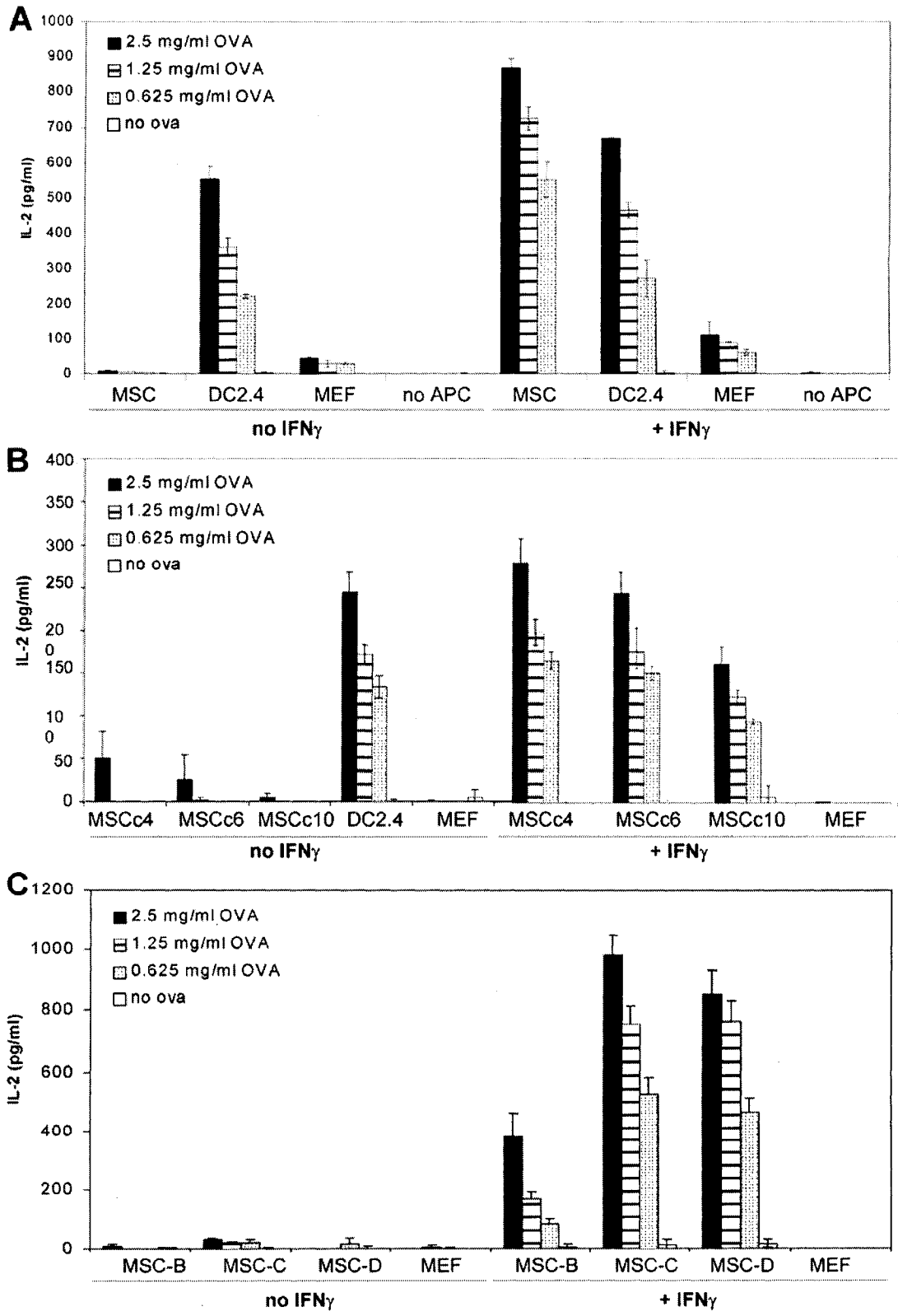
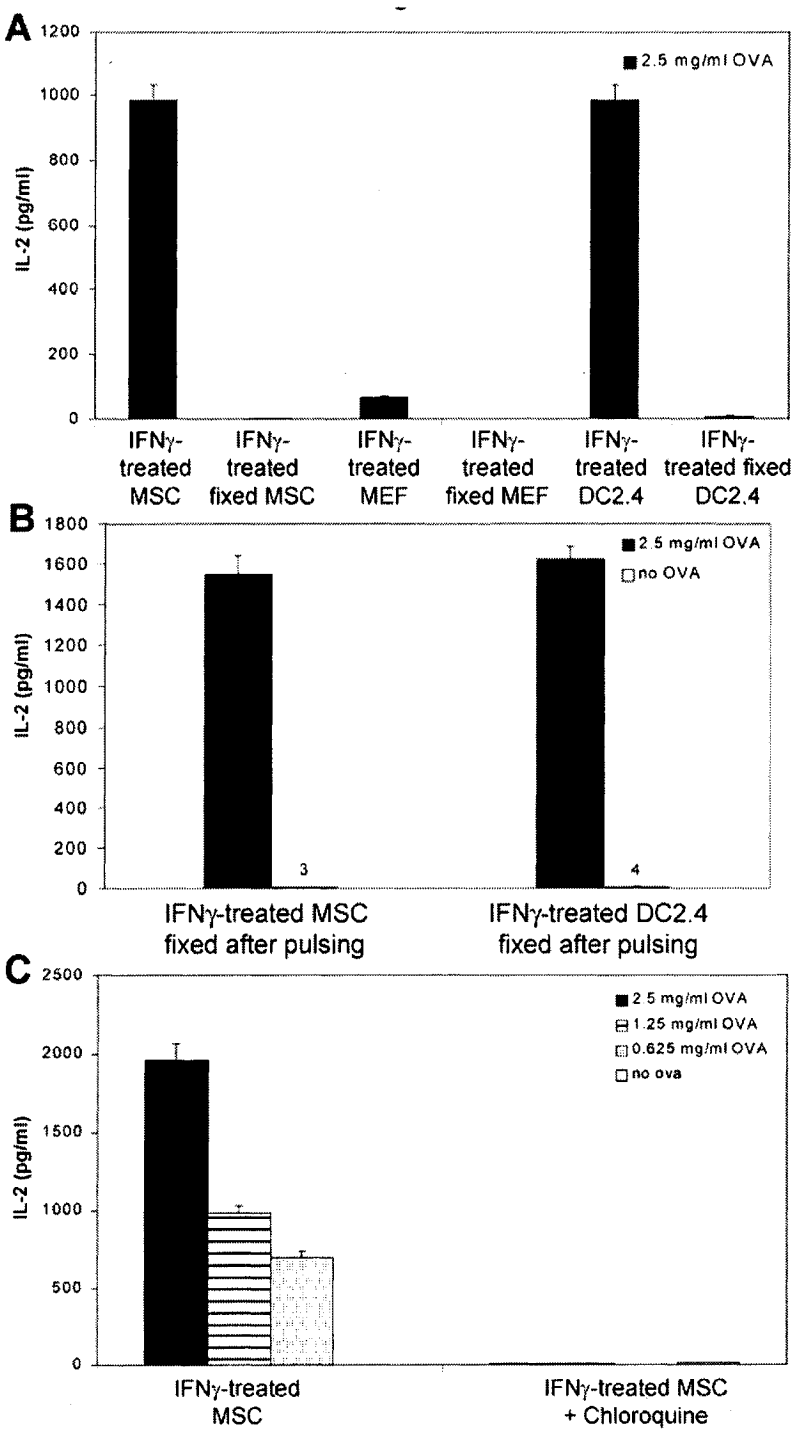

A
2.5 mg/ml OVA
1.25 mg/ml OVA
0.625 mg/ml OVA
no ova
IL-2 (pg/ml)
MSC
DC2.4
MEF
no APC
no IFNγ
+ IFNγ
B
MSCc4
MSCc6
MSCc10
C
MSC-B
MSC-C
MSC-D

Figure 15: Antigen processing for MSC-mediated antigen presentation.

A) C57BL/6 MSCs, DC2.4 or MEF ($5x10^4$ cells) were cocultured for 20hrs with ovalbumin-specific MHC class II-restricted T-T hybridomas (MF2.2D9; 10^5cells) in the presence of 2.5mg/ml of soluble ovalbumin. Where indicated, MSCs were treated with IFNγ (50ng/ml final). Where indicated, MSCs were first fixed with paraformaldehyde prior to coculture. After 20hrs, supernatant was collected and tested for IL-2 release by ELISA. (B) C57BL/6 MSCs or DC2.4 ($5x10^4$ cells) were first incubated with soluble ovalbumin (2.5mg/ml) and IFNγ (50ng/ml final) for 20hrs, then fixed with paraformaldehyde and cocultured for 20hrs with MF2.2D9 hybridomas (10^5 cells). (C) MSCs ($5x10^4$ cells) were cocultured for 20hrs with MF2.2D9 cells (10^5cells) in the presence of increasing doses of soluble ovalbumin. Where indicated, MSCs were treated with chloroquine (100μM) 30min prior to and during antigen exposure (Means of triplicates ± standard deviations of one of two representative experiments are shown).

Figure 15


A
1200
1000
800
600
400
200
0
IL-2 (pg/ml)
2.5 mg/ml OVA
IFNγ-treated MSC
IFNγ-treated fixed MSC
IFNγ-treated MEF
IFNγ-treated fixed MEF
IFNγ-treated DC2.4
IFNγ-treated fixed DC2.4
B
1800
1600
1400
1200
1000
800
600
400
200
0
IL-2 (pg/ml)
2.5 mg/ml OVA
no OVA
3
4
IFNγ-treated MSC fixed after pulsing
IFNγ-treated DC2.4 fixed after pulsing
C
2500
2000
1500
1000
500
0
IL-2 (pg/ml)
2.5 mg/ml OVA
1.25 mg/ml OVA
0.625 mg/ml OVA
no ova
IFNγ-treated MSC
IFNγ-treated MSC + Chloroquine

protein proteolysis[383], we treated MSCs with chloroquine. Chloroquine is known to prevent protein hydrolysis by raising the pH in the endosomal and lysosomal compartments[384]. As shown in Figure 15B, treatment with chloroquine inhibited the presentation of ovalbumin peptides on MHC class II molecules.

4.3.4 CD80-dependent activation of OT-II cells by IFNγ-treated MSCs

We next assessed whether IFNγ-treated mouse MSCs could activate primary transgenic T cells. Ovalbumin-specific CD4+ T cells were isolated from the spleens and lymph nodes of transgenic OT-II mice and purified by negative selection (>80% purity, data not shown). When purified CD4+ OT-II cells were cocultured for 48 hours with ovalbumin-pulsed IFNγ-treated MSCs, we observed significant levels of IL-2 production (Figure 16A). We then investigated whether CD80 expression on mouse MSCs was required for OT-II activation. As shown in Figure 16A, the addition of a blocking antibody to CD80 inhibited by 90% the activation of CD4+ OT-II cells ($P<0.05$ by T-test).

4.3.5 B7-H1 expression is induced on mouse MSCs following IFNγ treatment

We investigated by flow cytometry the expression levels of other costimulatory molecules on naïve and IFNγ-stimulated MSCs. Unstimulated as well as IFNγ-treated mouse MSCs were found to be negative for CD86, CD40, CD28, ICOSL, 41BBL and B7-DC surface expression (data not shown). However, after IFNγ stimulation, mouse MSCs robustly upregulated surface expression of B7-H1 molecules (Figure 16B).

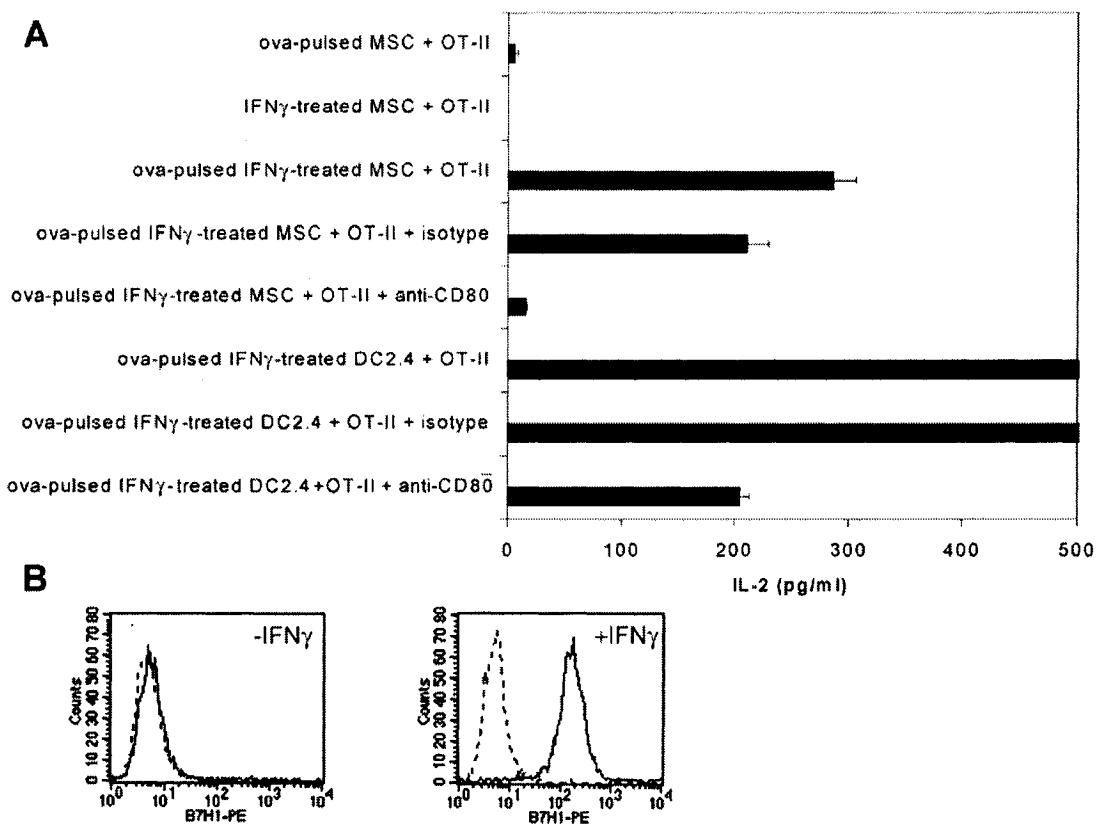
4.3.6 MSCs cannot induce antigen cross-presentation

We further tested whether mouse MSCs could induce activation of MHC class I restricted hybridomas in response to soluble ovalbumin. This experiment essentially measured the

Figure 16: MSC-mediated activation of primary OT-II CD4+ T cells

(A) C57BL/6 MSCs or DC2.4 were pre-treated with recombinant mouse IFNγ (50ng/ml) and soluble ovalbumin (2.5mg/ml) for 20hrs and then cocultured ($5x10^4$ cells) for 48hrs with ovalbumin-specific purified CD4+ T splenocytes (10^5cells; >80% purity) from OT-II transgeneic mice. Where indicated, MSCs and DC2.4 were first incubated with a blocking antibody to mouse CD80 or an isotypic control 30min prior to and during coculture. After coculture, supernatant was collected and tested for IL-2 release by ELISA (Means of triplicates ± standard deviations are shown). (B) C57BL/6 MSCs were analyzed by flow cytometry for B7-H1 surface expression before and after recombinant IFNγ treatment (50ng/ml for 20hrs). Plots show isotype control IgG staining profile (doted line) versus specific Ab staining profile (thick line).

Figure 16


A
ova-pulsed MSC + OT-II
IFNγ-treated MSC + OT-II
ova-pulsed IFNγ-treated MSC + OT-II
ova-pulsed IFNγ-treated MSC + OT-II + isotype
ova-pulsed IFNγ-treated MSC + OT-II + anti-CD80
ova-pulsed IFNγ-treated DC2.4 + OT-II
ova-pulsed IFNγ-treated DC2.4 + OT-II + isotype
ova-pulsed IFNγ-treated DC2.4+OT-II + anti-CD80
0
100
200
300
400
500
IL-2 (pg/ml)
B
-IFNγ
+IFNγ
Counts
B7H1-PE

ability of MSCs to induce cross-presentation of exogenous antigens. While DC2.4 cells induced significant antigen cross-presentation as previously shown[382], unstimulated and IFNγ-stimulated MSCs could not induce IL-2 release (Figure 17). In order to determine whether MSCs could still process exogenous ovalbumin into MHC class I-associated peptides without inducing IL-2 production, we performed flow cytometry analysis of ovalbumin-pulsed MSCs using a monoclonal antibody specific for the SIINFEKL/H-2K^b complex (clone 25D1.16)[385]. While the antibody positively labelled IFNγ-treated MSCs pulsed with 10μM of the synthetic SIINFEKL peptide, unstimulated as well as IFNγ-stimulated MSCs pulsed with soluble ovalbumin were not detected by the antibody (data not shown). Our results therefore suggested that mouse MSCs cannot perform exogenous antigen cross-presentation via the MHC class I pathway.

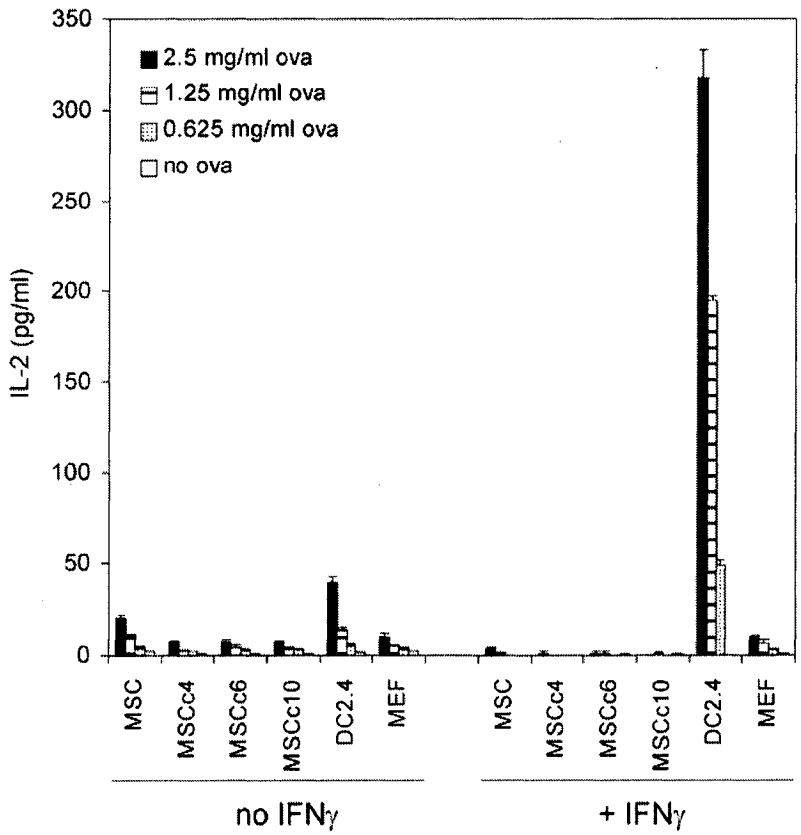
4.3.7 IFNγ-treated MSCs pulsed with soluble ovalbumin induced antigen-specific *in vivo* immune responses

Next, we investigated the ability of IFNγ-treated MSCs to induce antigen-specific *in vivo* immune responses. Polyclonal MSCs or control MEF (both from C57BL/6 origin) were stimulated with recombinant IFNγ and soluble ovalbumin, IFNγ only or ovalbumin only for 20hrs, washed with PBS and injected intraperitonealy into syngeneic C57BL/6 mice. Two weeks later, the mice were injected a second time with the corresponding cells and one week after, ovalbumin-specific immune responses were assessed. Firstly, we assessed whether mice injected with ovalbumin-pulsed IFNγ-treated MSCs could generate anti-ovalbumin antibodies. Although few mice developed anti-ovalbumin antibodies, we observed no significant differences between MSC-injected versus MEF-injected mice (Figure 18A). Secondly, we investigated whether mice injected with ovalbumin-pulsed IFNγ-treated MSCs could generate ovalbumin-specific cytotoxic T lymphocytes (CTL). For this, splenocytes from immunized mice were restimulated *in vitro* with mitomycin C-treated ovalbumin-expressing E.G7 cells and five days later, CD8+ T cells were isolated by negative selection (>90% purity, data not shown) and used as effectors in annexin-V-based CTL assays. Mice immunized with ovalbumin-pulsed

Figure 17: Soluble ovalbumin antigen cross-presentation

C57BL/6 MSCs, DC2.4 or MEF ($5x10^4$ cells) were cocultured for 20hrs with ovalbumin-specific MHC class I-restricted T-T hybridomas (RF33.70; 10^5cells) in the presence of increasing doses of soluble ovalbumin. Where indicated, recombinant IFNγ (50ng/ml final) was added to the cocultures. After 20hrs, supernatant was collected and tested for IL-2 release by ELISA (Means of triplicates ± standard deviations are shown).

Figure 17


350
300
250
200
150
100
50
0
IL-2 (pg/ml)
2.5 mg/ml ova
1.25 mg/ml ova
0.625 mg/ml ova
no ova
MSC
MSCc4
MSCc6
MSCc10
DC2.4
MEF
MSC
MSCc4
MSCc6
MSCc10
DC2.4
MEF
no IFNγ
+ IFNγ

IFNγ-treated MSCs developed a significant CD8+ ovalbumin-specific cytotoxic response (Figure 18B). This experiment was repeated once with similar results. In order to test whether MSC-mediated immunization induced systemic protective immunity, immunized mice were challenged with a subcutaneous injection of a tumorigenic dose ($2x10^6$ cells) of ovalbumin-expressing E.G7 tumor cells. Strikingly, 10 out of 10 mice immunized with ovalbumin-pulsed IFNγ-treated MSCs were fully protected against E.G7 tumors (Figure 18C). In contrast, 1 out of 10 mice immunized with ovalbumin-pulsed IFNγ-treated MEF cells was protected ($P<0.001$ by Log Rank). Taken together, our data indicated that mouse MSCs can induce protective *in vivo* antigen-specific cellular immune responses.

4.3.8 Activation of MHC class II-restricted hybridomas by IFNγ-treated human MSCs

We assessed the ability of human MSCs to acquire antigen-presenting functions following IFNγ stimulation. Human MSCs were isolated from healthy donors, culture expanded and characterized by flow cytometry. Polyclonal MSC populations from donors were HLA-typed, and the cells derived from a HLA class II DR1+ individual were used in the following experiments. When DR1+ IFNγ-treated human MSCs were cocultured for 24hrs with influenza matrix protein 1-specific DR1-restricted T-cell hybridomas in the presence of purified influenza matrix protein 1, significant levels of IL-2 was detected in the supernatant (Figure 19A). This indicated that IFNγ-stimulated human MSCs can efficiently process exogenous antigens and present antigen-derived peptides to MHC class II restricted T cells. Human MSCs also significantly upregulated surface expression of the costimulatory molecule B7-H1 upon IFNγ stimulation (Figure 19B). Taken together, our data suggested that human MSCs may behave as conditional antigen-presenting cells in syngeneic immune responses.

Figure 18: MSC-induced antigen-specific immune responses *in vivo*.

C57BL/6 MSCs or MEF cells were treated *in vitro* with recombinant IFNγ and soluble ovalbumin for 20hrs, washed with PBS and injected ($0.1x10^6$ cells) intraperitonealy into syngeneic C57BL/6 mice. Two weeks later, the mice were injected a second time with the corresponding cells ($0.2x10^6$) and one week after, ovalbumin-specific immune responses were assessed. (A) Serum samples of immunized mice were collected at day 20 after the first immunization, added at different dilutions to ovalbumin-coated 96-well plates and titered for anti-ovalbumin antibodies. (B) Splenocytes were isolated from immunized mice at day 21 after the first immunization and restimulated *in vitro* with mitomycin C-treated ovalbumin-expressing E.G7 cells. Five days later, CD8+ T cells were purified from the reactivated splenocytes (>90% purity) and used as effectors in annexin-V-based CTL assays against EL4 or E.G7 target cells. (C) Immunized mice were challenged at day 21 after the first immunization with a subcutaneous injection of $2x10^6$ ovalbumin-expressing E.G7 tumor cells.

Figure 18

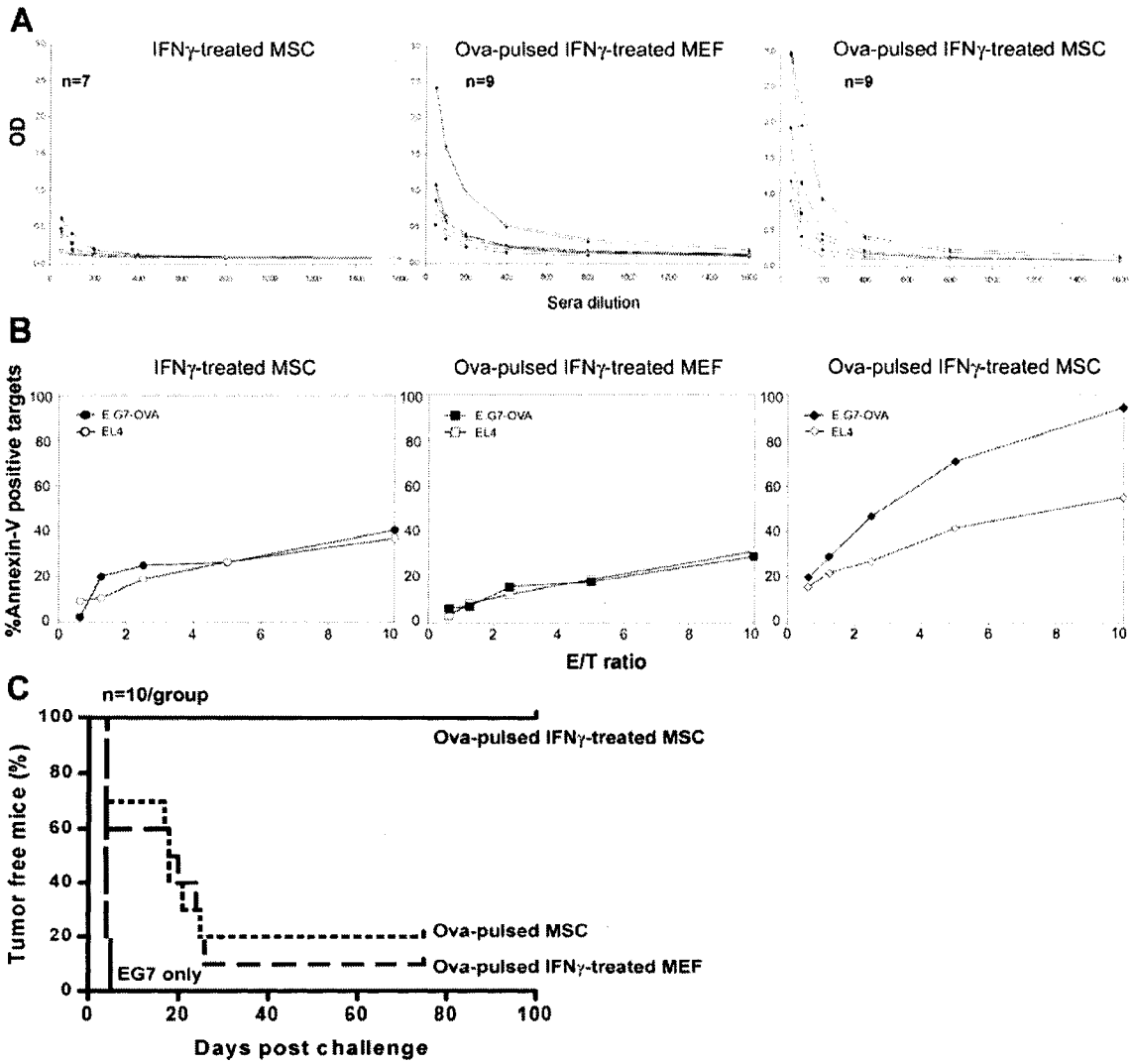
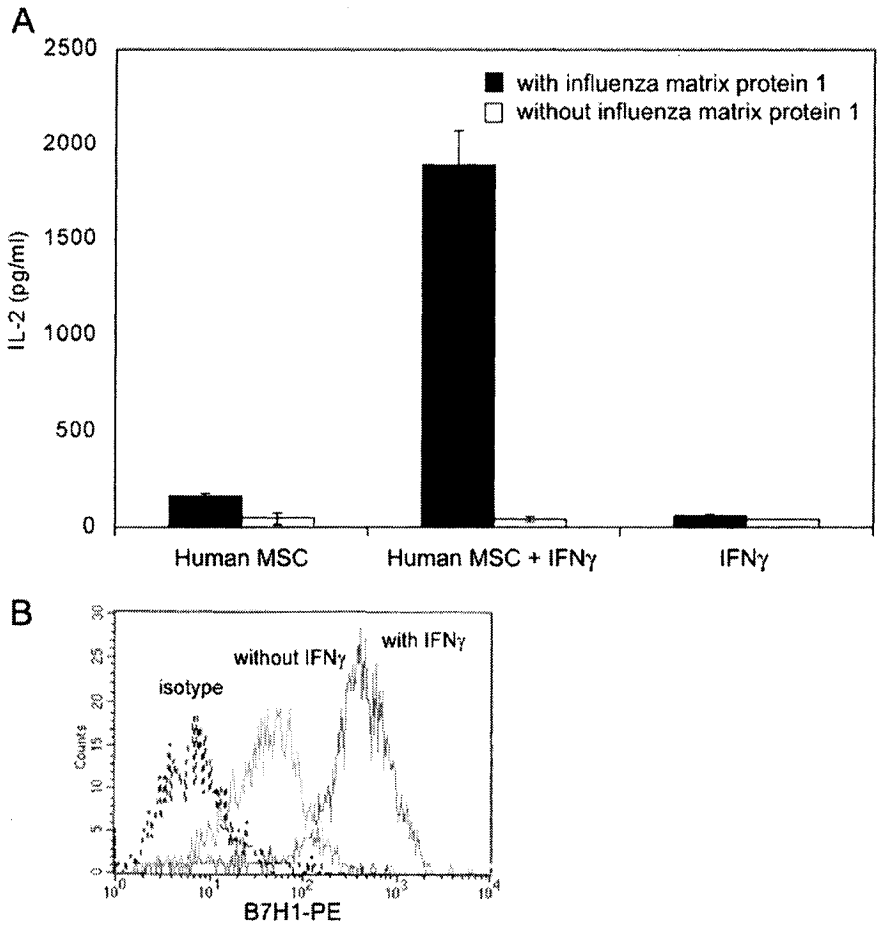

A
IFNγ-treated MSC
Ova-pulsed IFNγ-treated MEF
Ova-pulsed IFNγ-treated MSC
n=7
n=9
n=9
OD
Sera dilution
B
IFNγ-treated MSC
Ova-pulsed IFNγ-treated MEF
Ova-pulsed IFNγ-treated MSC
E.G7-OVA
EL4
%Annexin-V positive targets
E/T ratio
C
n=10/group
Ova-pulsed IFNγ-treated MSC
Ova-pulsed MSC
Ova-pulsed IFNγ-treated MEF
EG7 only
Tumor free mice (%)
Days post challenge

Figure 19: Human MSC-mediated activation of influenza matrix protein 1-specific T-T hybridomas.

(A) Bone marrow-derived DR1 positive human MSCs were treated or not for 24hrs with recombinant human IFNγ (100ng/ml) and subsequently cocultured for 24hrs with influenza matrix protein 1-specific DR1-restricted T cell hybridomas in the presence or not of 100μg/ml purified influenza matrix protein 1. Supernatant was collected and tested for IL-2 release by ELISA (Means of duplicates ± standard deviations). (B) Human MSCs were analyzed by flow cytometry for B7-H1 surface expression before and after recombinant IFNγ treatment (100ng/ml for 24hrs). Plots show isotype control IgG staining profile (doted line) versus specific Ab staining profile (thick lines).

Figure 19


A
2500
2000
1500
1000
500
0
IL-2 (pg/ml)
with influenza matrix protein 1
without influenza matrix protein 1
Human MSC
Human MSC + IFNγ
IFNγ
B
without IFNγ
with IFNγ
isotype
Counts
B7H1-PE

4.4 DISCUSSION

The use of MSCs for regenerative and transgeneic cell therapy is generating promising results for the treatment of diseases such as osteogenesis imperfecta, cardiovascular diseases, stroke, spinal cord injuries, Parkinson's disease and lung diseases[293-310]. The rising interest in MSCs-based therapies comes from their described plasticity and from the fact that they can be easily harvested and expanded to clinically relevant numbers. Depending on the desired therapeutic applications, MSCs have the potential to be used for both autologous and allogeneic transplantations[307]. After allogeneic transplantation, MSCs have been described to induce suppression of allogeneic immune responses[324]. In autologous conditions, however, the immune modulatory effects of MSCs have been largely unexplored. In this article, we investigated the immune modulatory properties of MSCs during syngeneic antigen-specific immune responses.

Specifically, we studied the effect of MSCs on the syngeneic activation, *in vitro* and *in vivo*, of ovalbumin-specific immune responses using previously described ovalbumin-specific mouse T cell activation assays. MSCs were isolated from C57BL/6 mice and characterized *in vitro* prior to assessing their immune modulatory effects. Functional characterization of the cells confirmed a MSCs phenotype as demonstrated by mesenchymal plasticity and immunosuppressive effects when cocultured with allogeneic mixed lymphocytes *in vitro*. Flow cytometry analysis of the isolated MSCs was also in agreement with their reported phenotype[307, 316].

It had been reported that allogeneic MSCs exert more suppressive activity on mixed lymphocyte reactions compared to autologous MSCs with respect to responder cells[322]. Therefore, we hypothesized that the immune modulatory effect of MSCs during syngeneic T cell activation would be distinct from their effect on allogeneic T cell activation. We first observed that in the absence of inflammatory stimuli, mouse MSCs significantly suppressed DC-mediated syngeneic T cell activation. Djouad and colleagues[317] have suggested that MSCs need to be first "activated" – e.g. by allogeneic splenocytes – in order to release the soluble factor(s) responsible for their allogeneic

suppressive effect. However, in our experimental setup, conditioned media from MSCs cultures did not induce inhibition, suggesting a mechanism of suppression independent of secreted soluble factors. On the other hand, paraformaldehyde-fixed MSCs were capable of immunosuppression. Since others have shown that formalin-fixed MSCs fail to inhibit allogeneic T cell activation[323], we propose that unstimulated MSCs use distinct pathways to suppress allogeneic versus syngeneic T cell activation, possibly relying mainly on secreted soluble factors for the former and cell-contact mechanisms for the latter. In sum, we have shown, as have others, that non-activated MSCs – as a default setting – can contact suppress lymphocyte activation in an antigen-independent manner.

We next investigated whether IFNγ could modulate the syngeneic immune properties of MSCs. IFNγ is known to upregulate MHC class I and induce MHC class II expression on MSCs[307]. Strikingly, in contrast to unstimulated MSCs, IFNγ-treated MSCs were permissive to syngeneic DC-mediated T cell activation as determined by IL-2 release. Intriguingly, paraformaldehyde-fixed IFNγ-treated MSCs maintained their *in vitro* suppressive effects. Furthermore, the addition of conditioned media from IFNγ-treated MSCs had no effect of DC-mediated T cell activation, suggesting soluble factors were not in play. We consequently hypothesized that IFNγ directly induced MSCs to acquire antigen-presenting functions. The hypothesis that MSCs could behave as antigen-presenting cells was not totally inconsistent with previous studies. Beyth *et al.*[320], for instance, reported that human MSCs cocultured with purified human CD4+ T cells and SEB superantigen can activate CD4+ T cells. Notwithstanding that antigen processing was not required in these experiments to induce T cell activation, it suggested that human MSCs were able to provide sufficient MHC class II and costimulation signaling to induce CD4+ T cell activation. Based on these reported experiments and our own observations, we further investigated whether MSCs could behave as conditional antigen-presenting cells.

Using two distinct models, i.e. ovalbumin-specific T-T hybridomas and primary transgenic OT-II activation assays, we demonstrated that IFNγ-treated C57BL/6-derived MSCs can: (i) efficiently process, via endocytosis, exogenous ovalbumin and present

ovalbumin-derived peptides on MHC class II molecules; (ii) efficiently activate, mainly in a CD80-dependent manner, MHC class II restricted CD4+ T cells inducing IL-2 release; (iii) do not induce exogenous antigen cross-presentation via the MHC class I pathway; (iv) efficiently induce *in vivo* antigen-specific CD8+ T cells; and (v) efficiently induce cellular protective immunity against ovalbumin-expressing tumors when injected as a syngeneic cellular vaccine. We thus provide experimental evidence that IFNγ-treated MSCs can process exogeneous antigens and efficiently activate *in vitro* and efficiently induce *in vivo* antigen-specific immune responses. Furthermore, MSCs isolated from different preparations, as well as distinct clonal MSCs populations were equally effective at activating ovalbumin-specific T-T hybridomas. Though MHC class I and II upregulation is observed following IFNγ stimulation in polyclonal MSCs population, clonal MSCs subsets were found to vary markedly in this general rule, yet acquired robust immunostimulatory properties. Of particular interest, we observed that one of the clonal MSC populations (clone 10) expressed constitutively low levels of MHC class II and CD80 molecules, but failed to upregulate these molecules upon IFNγ stimulation. Regardless, MSCs clone 10 acquired antigen-presenting properties upon IFNγ stimulation, suggesting that contact-dependent molecule(s) – distinct from MHC class I, class II and CD80 – were upregulated by IFNγ and implicated in MSC-mediated antigen presentation. This hypothesis was strengthened by the fact that blocking CD80 costimulation partially – rather than totally – inhibited OT-II activation. When we investigated by flow cytometry the surface expression levels of other known costimulatory molecules on naïve and IFNγ-stimulated MSCs, we found MSCs to be consistently negative for CD86, CD40, ICOSL, 41BBL and B7-DC surface expression. However, after IFNγ stimulation, every population of MSCs tested, including human MSCs, robustly upregulated surface expression of B7-H1 molecules. The exact immune functions of B7-H1 are not fully understood and we can only hypothesize, at the moment, on its role during MSCs-mediated antigen presentation. B7-H1 (PD-L1) belongs to the B7 family members and is a ligand for programmed cell death-1 (PD-1) receptor expressed on activated T, B and myeloid cells[386]. B7-H1 is expressed on resting and upregulated on activated T, B, myeloid and DC, and can be expressed on endothelial cells and other non-lymphoid organs[387-390]. While B7-H1$^{-/-}$ mice suggest an essential role for

B7-H1 in negatively regulating T cell activation[391-392], other studies have demonstrated that B7-H1 expression can provide positive costimulation for T cell priming *in vitro* and *in vivo*[393-394].

An important aspect of our studies is the observation that human MSCs can also acquire antigen-presenting functions, strongly suggesting that both mouse and human MSCs behave as conditional antigen-presenting cells. We made use of a previously described transgenic mouse T-cell hybridoma that is restricted to human HLA-DR1 and specific for influenza matrix protein 1-derived peptides in order to study APC-like properties of human MSCs. Our results suggested that human MSCs can: (i) efficiently process soluble influenza matrix protein 1; (ii) efficiently present influenza matrix protein 1-derived peptides on MHC class II molecules; and (iii) efficiently activate antigen-specific T-cell hybridomas as determined by IL-2 release. It remains to be determined, however, whether human MSCs can provide proper T cell costimulation. When the surface expression of costimulatory molecules on human MSCs was analyzed, we found that B7-H1 was clearly upregulated on human MSCs following IFNγ stimulation in a similar fashion to mouse MSCs.

In summary, our data suggest that MSCs possess a previously unrecognized dichotomy in their role as immune modulators, distinctively affecting allogeneic and syngeneic immune responses. We propose that MSCs constitute a novel subset of non-hematological APCs. Few other cell types have been described to possess similar functions in the presence of pro-inflammatory stimuli. The best described are vascular endothelial cells, which have been shown to activate *in vivo* CD8+ T cells in a CD80-dependent fashion upon IFNγ stimulation[395-396]. Interestingly, IFNγ-treated endothelial cells inhibit T cell activation through B7-H1 and/or B7-DC expression[397], suggesting perhaps an inhibitory role for B7-H1 upregulation on MSCs. Keratinocytes are also known to behave as non-professional APCs[398]. In contrast to endothelial cells, however, keratinocytes can either tolerize or activate T cells depending on the nature of the antigen[399]. Finally in the gut, enterocytes have recently been described as non-professional APCs towards CD4+ T cells[400]. An important aspect of the biology of

MSCs that will further need investigation is whether MSCs-mediated antigen presentation actually occurs in the bone marrow and, if so, whether it plays a significant role during endogenous immune responses. As the bone marrow is being revealed as a unique lymphoid organ able to activate naïve T cells and to induce systemic immunity, in some cases more efficiently than peripheral lymph nodes[401-402], MSCs may represent a previously unrecognized player of physiological immune responses.

Lastly, the unique immune modulation afforded by MSCs in the autologous and allogeneic transplant setting could have important repercussions in the development of MSC-based therapies. For instance, genetically engineered MSCs used for autologous transplantation in regenerative medicine may trigger potent immune rejection of these APC-like cells following inflammatory reactions. The APC-like properties of MSCs should also be taken into account in the development of immunosuppressive strategies based on MSC transplantation for treatment of GVHD. On the other hand, autologous transplantation of antigen-pulsed or genetically engineered IFNγ-treated MSCs could be profitably used to stimulate therapeutic antitumor or anti-infectious immune responses. While DCs are routinely studied in clinical trials for this purpose, MSCs may possess distinct APC-like properties inducing qualitatively divergent immune responses. Our *in vivo* observation that MSC-mediated immune responses were greatly biased towards a cellular type 1 response with minimal humoral response supports this hypothesis. The unique dichotomy of function of MSCs – suppressive as a default and stimulatory upon activation – could further be exploited in the setting of allogeneic bone-marrow transplantation with the purpose of simultaneously suppressing allogeneic GVHD and activating antitumor responses. In conclusion, MSCs have spurned much interest due to their mesenchymal plasticity. We here show that their immunological plasticity merits a fresh introspective in to their role in the physiological immune response in health and disease and the harnessing of their unique properties for treatment of maladies amenable to immune modulation.

4.5 METHODS

4.5.1 Animals and cell lines

Mice were 4-8 weeks old female C57BL/6 or BALB/c purchased from Charles River (LaPrairie, Qc, Canada). C57BL/6 mouse embryonic fibroblasts, EL4 and E.G7 cells were purchased from the American Type Culture Collection (Manassas, VA). DC2.4 cells, MF2.2D9 cells and RF33.70 cells have been described previously[382] and were a generous gift from Dr. Ken L. Rock (University of Massachusetts, Worcester). C57BL/6 OT-II mice were kindly provided by Dr. C. Piccirillo (McGill University, Montreal, Canada). The anti-SIINFEKL/H2-K^b mAb-producing hybridoma 25D1.16[385] was a gift from Dr. Ronald N. Germain (NIH, Bethesda, MD) and the mAb purified using Hi-Trap chromatography column (Amersham Biosciences). Synthetic SIINFEKL peptide was purchased from Sheldon Biotechnology Centre (McGill University). Purified influenza matrix protein 1 as well as humanized DR1-restricted influenza-specific T-T hybridomas have been described previously[403] and were a generous gift from Dr. David Canaday (Case Western Reserve University, Ohio).

4.5.2 Harvest of MSCs

Mouse MSCs were isolated from female C57BL/6 mice as previously described[381]. Briefly, whole marrow from the femurs and tibias was flushed in DMEM (Wisent technologies, St-Bruno, QC, Canada) 10% fetal bovine serum (FBS) (Wisent technologies) and 50 U/ml Pen/Strep (Wisent technologies), plated for 5 days, washed and fresh media added to the adherent cells every 3-4 days. When 80% confluent, adherent cells were trypsinized (0.05% Trypsin, Wisent technologies, at 37°C for 5min), harvested and expanded until a homogenous population was obtain, i.e approximatively 20 population doublings, before being used for antigen presentation assays. Human MSCs were isolated as previously described[321]. Briefly, whole marrow was collected form patients undergoing hip surgery (Dr. J. Antoniou, Jewish General Hospital, Montreal, Canada), diluted in DMEM (Wisent technologies), centrifugated to remove the

fatty layer, added to a Ficoll gradient (Amersham Bioscience, Oakville, ON, Canada) and centrifuged at 900g for 30 minutes. Mononuclear cells were plated at $2x10^5$ cells/cm^2 on $10cm^2$ tissue culture dishes in DMEM 10% FBS 50U/ml Pen/Strep (Wisent technologies). The non-adherent cells were removed after 48hours and media replaced every 3-4 days. When 80% confluent, adherent cells were trypsinized (0.05% Trypsin, Wisent technologies, at 37°C for 5min), harvested and expanded for a minimum of 10 population doublings before being used for flow cytometry analysis and antigen presentation assays. Human MSCs did not express CD45 or CD31, and were positive for CD105 and CD73 surface expression as determined by flow cytometry.

4.5.3 Differentiation of mouse MSCs

For osteogenic differentiation, MSCs were cultured in complete media supplemented with β-glycerol phosphate (10mM), dexamethasone (10^{-8} M) and ascorbic acid 2-phosphate (5μg/ml) (all from Sigma-Aldrich, Oakville, ON, Canada) for 4 weeks renewing the media every 2-3 days. Alizarin Red S (2% pH 4.1 in ammonium hydroxide) was then used to stain calcium in the mineralized extracellular matrix. To induce adipogenic differentiation, MSCs were cultured in complete media supplemented with indomethacin (46μM), 3-isobutyl-methylxanthine (0.5mM), dexamethasone (1μM) and insulin (10μg/ml) (all from Sigma-Aldrich) for 7 days renewing the media twice. Oil Red O (Sigma-Aldrich) was used for lipid droplets staining. Photographs of cells were taken under light microscopy using an Axiovert25 Zeiss microscope attached to a Contax167MT camera.

4.5.4 Flow cytometry analysis

Flow cytometry analysis was performed in PBS 2% FBS (Wisent Technologies) with the following mAbs: R-phycoerythrin (PE)-conjugated anti-mouse CD45 (clone 30-F11), H-2Kb (clone AF6-88.5), I-Ab (clone AF6-120.1), CD40 (clone 3/23), CD54 (clone 3E2), CD28 (clone 37.51; eBioscience, San Diego, CA), B7-DC (clone TY25; eBioscience), B7-H1 (clone MIH5; eBioscience) and 4-1BBL (clone TKS-1; eBioscience). Biotin-conjugated anti-mouse CD105 (clone MJ7/18; eBioscience), CD80 (clone 16-10A1),

CD86 (clone PO3), ICOS-L (clone HK5.3). Isotypic control analyses were performed in parallel. Except where indicated, Abs are from BD Pharmingen (San Diego, CA, USA). Biotinylated Abs were revealed by APC-streptavidin (BD Pharmingen). Flow cytometry was performed using a FACS Calibur cytometer (BD) and analyzed using Cellquest software.

4.5.5 Two-way mixed lymphocyte cultures

Splenocytes were isolated from C57BL/6 and BALB/c mice by mechanical dissociation of the spleens followed by red blood cells lysis (ammonium chloride 8.3g/ml; Sigma-Aldrich). In triplicates, 10^5 C57BL/6 splenocytes and 10^5 BALB/c splenocytes per well were cocultured in a round-bottom 96-well plate in 200µl complete medium (RPMI 10% FBS, 50U/ml Pen-Strep; Wisent Technologies) with or without 10^5 C57BL/6 MSCs, pretreated or not with recombinant mouse IFNγ (50ng/ml; BioSource International, Camarillo, CA) for 20hrs followed by extensive washing in PBS. After 3 days, the cocultures were centrifugated and 100µl of supernatant was collected for measurement of mouse IFNγ using a commercial ELISA kit (R&D Systems, Minneapolis, MO).

4.5.6 Ovalbumin-specific T-T hybridoma assays

DC2.4 or control MEF ($5x10^4$ cells) were cocultured for 20hrs with 10^5 MF2.2D9 cells in flat-bottom 96-well plates in the presence or not of soluble ovalbumin (Sigma-Aldrich) at the indicated concentration in 200µl complete media (RPMI 10% FBS 50U/ml Pen/Strep; Wisent Technologies). Where indicated, $5x10^4$ naïve or IFNγ-treated MSCs (50ng/ml for 20hrs) were added to the cocultures or in replacement of DC2.4 cells. Where indicated, recombinant mouse IFNγ was added to the cocultures (final 50ng/ml). Where indicated, conditioned supernatant from naïve or IFNγ-treated MSCs (50ng/ml for 20hrs) were added to DC2.4 and MF2.2D9 cocultures. Where indicated, naïve or IFNγ-pretreated MSCs were fixed in 1% paraformaldehyde, washed once with DMEM (Wisent technologies), once with 0.125M D-L lysine buffer for 30min (Sigma-Aldrich), four times with DMEM (Wisent technologies) and added to DC2.4 and MF2.2D9 cocultures.

In some experiments, DC2.4 cells were first pulsed with soluble ovalbumin for 20hrs and then cocultured with the indicated cells for another 20hrs. Where indicated, MSCs were treated with chloroquine (100μM; Sigma-Aldrich) 30min prior to and during antigen exposure. After 20hrs, supernatant was collected from the cocultures and tested for the presence of IL-2 by commercial ELISA (eBioscience).

4.5.7 OT-II antigen presentation assays

C57BL/6 MSCs or DC2.4 were first pre-treated with recombinant mouse IFNγ (50ng/ml) and soluble ovalbumin (2.5mg/ml) for 20hrs. The next day, ovalbumin-specific CD4+ T cells were isolated from the spleens and lymph nodes of transgenic OT-II mice using SpinSep^{TM} kit following manufacturer's instructions (Stem Cell Technologies, Vancouver, Canada). IFNγ-treated ovalbumin-pulsed DC2.4 or MSCs ($5x10^4$ cells) were then cocultured for 48hrs with purified CD4+ OT-II cells in flat-bottom 96-well plates in 200μl complete media (RPMI 10% FBS 50U/ml Pen/Strep; Wisent Technologies). Where indicated, purified anti-mouse CD80 (clone 16-10A1) or isotype control Abs (50μg/ml; BD Pharmingen) were added to the MSCs or DC2.4 30min prior to and during coculture with OT-II cells. We then investigated whether CD80 expression on mouse MSCs was required for OT-II activation. After 48hrs, supernatant was collected from the cocultures and tested for the presence of IL-2 by commercial ELISA (eBioscience)

4.5.8 *In vivo* immunization of mice

C57BL/6 MSCs or MEF cells were treated *in vitro* with recombinant IFNγ (50ng/ml) and soluble ovalbumin (2.5mg/ml) for 20hrs, washed with PBS and injected ($0.1x10^6$ cells) intraperitonealy into syngeneic C57BL/6 mice. Two weeks later, the same mice were injected a second time with the corresponding cells ($0.2x10^6$) and one week after, serum samples and splenocytes of immunized mice were collected. For antibodies titering, serum samples were diluted in PBS, incubated for 2hrs at 37°C onto ovalbumin-coated (10μg/ml) 96-well plates and revealed using anti-mouse Ig-HRP antibody (1:1000 in PBS 10% FBS; BD Pharmingen) and TMB substrate (eBioscience). For cytotoxic T cell assays (CTL), $50x10^6$ pooled splenocytes from immunized mice were restimulated *in*

vitro with 10^6 Mitomycin-C (Sigma-Aldrich) treated E.G7 cells in complete media (RPMI 10% FBS 50U/ml Pen/Strep, 50μM β-mercaptoethanol) for 5 days. Then, CD8+ T cells were purified using SpinSep™ kit (Stem Cell Technologies) and used as effectors in annexin-V-based CTL assays against $5x10^4$ PKH26-labelled (Sigma-Aldrich) EL4 or E.G7 targets and analyzed by flow cytometry as previously described[381, 404].

4.5.9 Human MSC antigen presentation assay

Human MSCs preparations were HLA-typed (Montreal Royal Victoria tissue typing laboratory) and DR1 positive MSCs used in antigen presentation assays. Where indicated, human MSCs were pre-treated for 24hrs with recombinant human IFNγ (100ng/ml; InterMune Pharmaceuticals, Brisbane, CA) and subsequently cocultured for 24hrs with influenza matrix protein 1-specific DR1-restricted T cell hybridomas and/or 100μg/ml purified influenza matrix protein 1 in complete media (RPMI 10% FBS 50U/ml Pen/Strep). After coculture, supernatant was collected and tested for mouse IL-2 release by ELISA (ebioscience).

4.6 ACKNOWLEDGEMENTS

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CHAPTER 5

CONCLUSION

CHAPTER 5: CONCLUSION

Despite recent evidence of the existence of tumor immunosurveillance mechanisms and the identification of anticancer cytokines, only limited immune-based protocols have translated into the clinic. The disappointing outcomes associated with several immune-based clinical trials have highlighted the need to better define the complex cancer-immune system relationship and the necessity to improve upon existing therapeutic strategies. Currently, systemic administration of recombinant IL-2 routinely benefits selected cancer patients[196-197]. On the other hand, GM-CSF-expressing whole-cell cancer vaccines are showing some of the most promising phase III clinical results in inducing systemic and clinically relevant anti-cancer immune responses[188, 231]. However, there is still great room for improvement as response rates remains relatively low. In order to develop new cytokine-based strategies, the following obstacles must be taken into account:

(ii) Cytokine-based strategies exploiting the effect of a single cytokine are confronted by the multi-step requirement for the induction of optimal tumor-specific immune responses. Conversely, when cytokines are administered in combination in an attempt to mimic naturally occurring immune responses, the distinct pharmacokinetic properties of individual cytokines prevent optimal synergistic effects, often inducing unpredictable and paradoxical outcomes.

(iii) Systemic administration of anticancer cytokines often fails to achieve adequate intratumoral levels of cytokines before systemic toxicity arises, thereby limiting the maximum tolerated dose that can be administered. An alternative to this strategy involves cancer-localized cytokine gene expression. However, only limited and often suboptimal methods exist to achieve tumor-localized cytokine expression, including *in vivo* gene delivery methods and *ex vivo* gene-modified whole cell tumor vaccines. *In vivo* tumor-targeted gene delivery is impeded by unpredictable gene transfer or by indiscriminate transduction of normal cells with potential health risks. Although promising,

the injection of *ex vivo* gene-modified cytokine-secreting tumor cells is impeded by the inaccessibility of some tumors, by the necessity to establish cancer cell culture for each patient and by the possible inability to gene modify the patient-derived cancer cells.

(iv) Anticancer immune responses are in essence autoimmune responses and are consequently controlled by naturally occuring processes whose role is to prevent self-destruction. Therefore, a better understanding of the cellular effectors and regulators involved in generating tumor-specific immune responses will enable the development of improved immune-based cancer therapeutics.

In view of these impediments, the main objective of my thesis was to develop novel means in order to improve current cytokine-based immunotherapeutic strategies. The specific research aims of my thesis were:

1. To test the hypothesis that a fusion between the cDNA of two cytokines, specifically GM-CSF and IL-2, can circumvent the limitations associated with the combinatorial use of cytokines and may induce novel anticancer effects;

2. To test the hypothesis that primary marrow stromal cells can be used as a cellular vehicle for the tumor-localized delivery of anticancer cytokines, specifically IL-2, thereby limiting the severe toxicity associated with systemic administration of recombinant cytokines;

3. To test the hypothesis that primary marrow stromal cells are important immune-modulatory cells and can be exploited in order to induce therapeutic antitumor immunity.

Hypothesis 1

The rationale for generating fusion transgenes between anticancer cytokines came from the reported observations that combining the cytokines GM-CSF and IL-2 can induce

paradoxical effects compared to single cytokine therapy. In cancer patients, it was shown that GM-CSF combined to IL-2 can downregulate the functions of monocytes, NK cells and B cells compared to therapy with GM-CSF alone[331]. It another study, it was reported that combined GM-CSF and IL-2 expression abrogated the protective effect against wild type tumor challenge compared to single cytokine expression[332]. The difficulty in predicting the outcome of combined cytokine therapy may come from their distinct pharmacokinetic and biologic properties. Furthermore, cytokines often affect different aspects of the immune system that might "interfere" with each other. For instance, GM-CSF is known to be a potent initiator of the adaptive immune response whereas IL-2 activates the innate effector NK cells. Soliciting these two immune pathways may lead to unheralded antagonism. In support of this is the observation that GM-CSF downregulates the innate immune response mediated by NK cells. Such paradoxical effects may be even more pronounced if production of GM-CSF and IL-2 varies in space and time. We thus hypothesized that a single bi-functional fusion protein translated from a GM-CSF and IL-2 fusion transgene (GIFT) could limit paradoxical effects associated with combined GM-CSF and IL-2 expression. My studies demonstrated that:

i) GIFT gene product is bi-functional;

ii) When expressed by live B16 mouse melanoma cells, GIFT induces complete tumor rejection in immunocompetent syngeneic mice, recapitulating the antitumor effect of IL-2;

iii) When expressed by *prophylactic* irradiated B16 whole cell tumor vaccines, GIFT induces protective immunity of immunocompetent syngeneic mice against wild type B16 tumors, recapitulating the antitumor effect of GM-CSF;

iv) When expressed by *therapeutic* irradiated B16 whole cell tumor vaccines, GIFT induces greater antitumor effects against pre-established wild type B16 tumors than combined GM-CSF and IL-2 at equimolar dose;

v) Antitumor effects mediated by GIFT are dependent upon CD8 and NK cells, but independent of CD4 T cells;

vi) GIFT display novel immunopharmacological properties distinct of GM-CSF and IL-2 used in combination, as revealed by a significantly greater recruitment of macrophages and NK cells.

An important aspect of GIFT-mediated antitumor effects is the induction of distinctive pharmalogical properties compared to the combined use of GM-CSF and IL-2. An important new feature appears to be a greater recruitment of macrophages and NK cells. Traditionally, chemotaxis involves G protein-linked receptor and intracellular Ca^{2+} uptake. However, it is known that GM-CSF and IL-2 receptors are not G protein-linked receptors, rather exploiting the activation of the PI3K pathway in a pertusis-toxin insensitive manner[191]. In an attempt to explain the greater chemotactic effect of GIFT, I hypothesize that GIFT gene product, compared to combined GM-CSF and IL-2, may enhance receptor signaling in cells expressing both receptors due to close proximity of the GM-CSF and IL-2 receptors after binding to GIFT gene product. This hypothesis is supported by the fact that GM-CSF and IL-2 signaling share activation pathways, including the PI3K pathway, and adaptor molecules[114, 162]. Indeed, phosphotyrosine residues on the cytoplasmic tail of both GM-CSF and IL-2 receptors serve as docking sites for the same molecules with PTB or SH2 domains, which then become targets of JAKs, triggering downstream pathways. I thus hypothesize that GIFT-mediated GM-CSF signaling synergizes with GIFT-mediated IL-2 signaling, and vice-versa, through cross-phosphorylation. If this hypothsesis is correct, a greater level of tyrosine phosphorylation of adaptor molecules such as Shc should be observed after binding of GIFT compared to equimolar concentrations of GM-CSF and IL-2. The use of a mutated Tyr-338 on IL-2Rβ (Tyr-338 being essential for IL-2 signaling[114]) could be used to determine if GIFT-mediated GM-CSF signaling can rescue, to some extent, IL-2 signaling. Another hypothesis to explain GIFT-induced chemotaxis is that GIFT gene product induces prolonged signaling of both GM-CSF and IL-2 receptors compared to GM-CSF and IL-2 in combination as a consequence of a greater half-life. However, the observation that GIFT gene product induces similar proliferative responses of IL-2 depedent cells compared to recombinant IL-2 suggests that GIFT's half-life is not significantly different from IL-2's half-life. Finally, GIFT signaling may distinctively trigger the expression of chemokine or chemokine receptors compared to GM-CSF and IL-2 in combination. This hypothesis is supported by the fact that certain cytokines, including IL-2, are known to upregulate several chemokine receptors[405-406].

My studies have thus shown that the nucleotide sequence encoding for the fusion of GM-CSF and IL-2 cDNA can be utilized as a therapeutic transgene for cancer immunotherapy. This was the first report that a fusion between two cytokines can invoke greater antitumor effect than both cytokines in combination when expressed by whole-cell cancer vaccines. We further propose that GIFT fusion cDNA could be used as adjuvant to DNA vaccines when combined to TAAs cDNA. The use of naked DNA to induce prophylactic and therapeutic cancer immune responses as been well established[407]. In addition to their economic advantages, DNA vaccines circumvent the difficulty to generate cancer cell lines, rendering the technology more accessible. For DNA vaccines based on poorly immunogeneic antigens such as TAAs, there is a great need for powerful adjuvants, both strong and safe, that can be used to enhance the immune response. Many adjuvants such as LPS, LT and CT comprise a toxic fragment that is required for adjuvanticity, thus greatly hampering their clinical use[408-409]. The delivery of cytokine genes to enhance TAA-directed immune responses may therefore represent an advantage over conventional adjuvants. The rationale of incorporating GIFT in DNA vaccination strategies comes from the reported observation that co-expression of GM-CSF and IL-2 cDNA induces higher antibody titers and T cell proliferation response than other cytokine genes[410]. In a set of preliminary experiments, I tested the hypothesis that GIFT cDNA will significantly enhance protective immunity against a defined antigen when incorporated into a DNA vaccination strategy.

To validate this hypothesis, I investigated whether GIFT would enhance the immune response of a well-defined xenoantigen: chicken ovalbumin. The ovalbumin immune response in C57BL/6 mice is one widely used in the field of vaccine immunology. I compared the immune response after intramuscular administration of naked plasmid DNA encoding for full-length ovalbumin +/- GIFT (Figure 20). I consistently found that GIFT plasmid significantly enhanced the cellular immune response to ovalbumin and was superior to the co-administration of GM-CSF and IL-2 cDNA plasmids. Indeed, I found that GIFT increased in vitro IFNγ release by splenocytes isolated from immunized mice (Figure 21A), induced memory CD8+ T cells (Figure 21B) and induced ovalbumin-

Figure 20. DNA vaccination protocol.

Vaccines were administered as described intramuscularly (i.m) using DNA plasmids encoding for: i) control vector; ii) ovalbumin; iii) ovalbumin+gmcsf+il2; or iv) ovalbumin+gift.

Figure 20

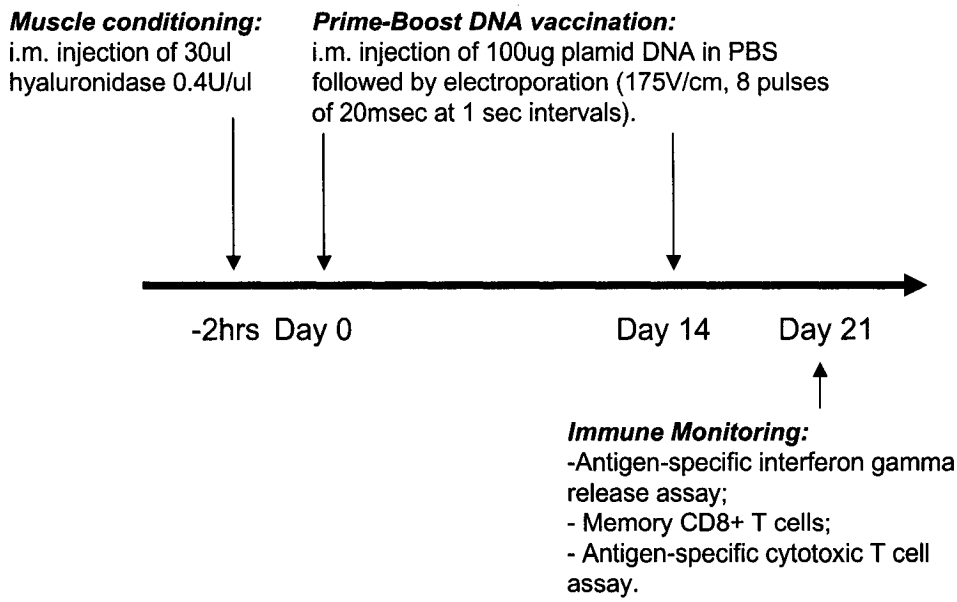
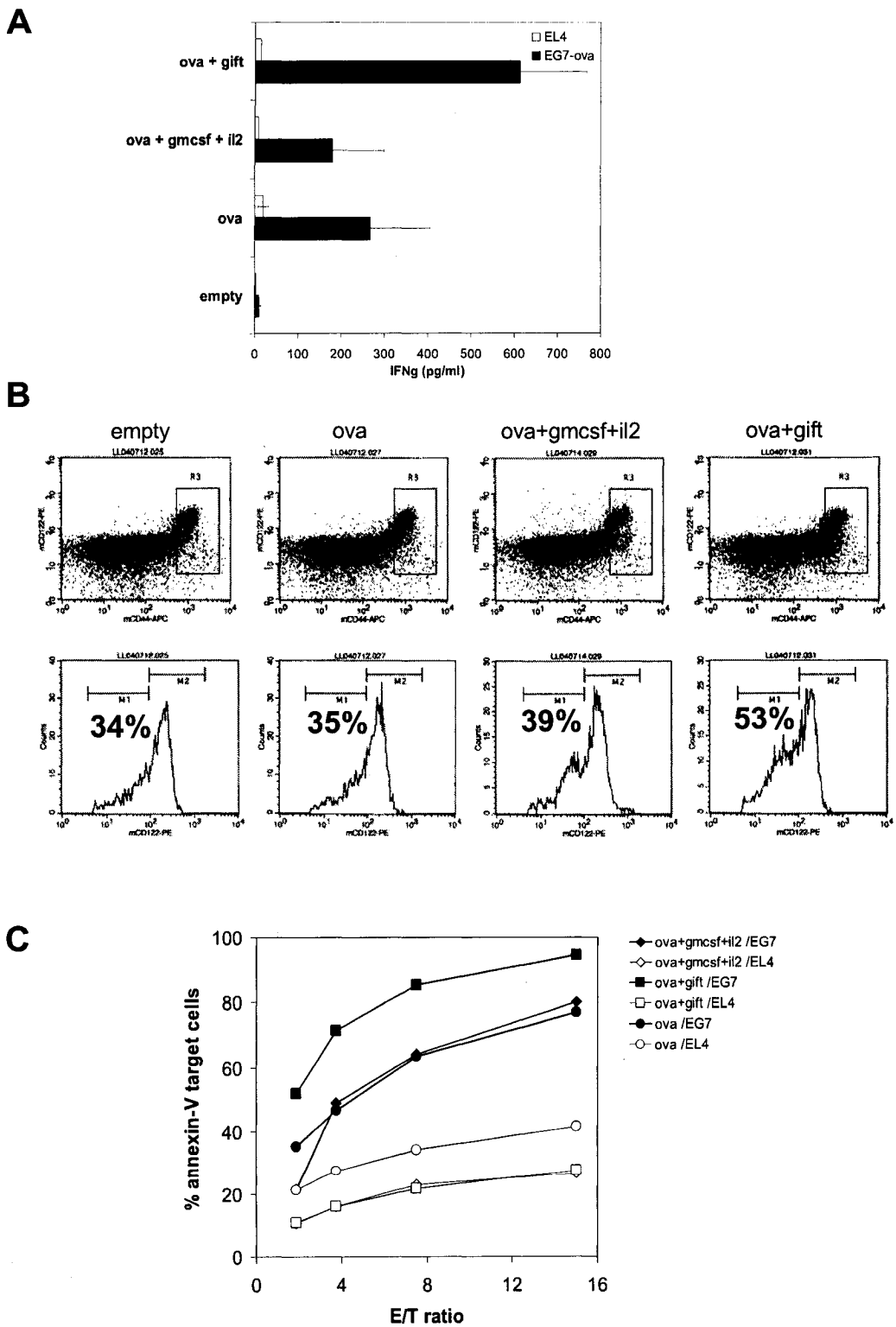

Muscle conditioning:
i.m. injection of 30ul
hyaluronidase 0.4U/ul
Prime-Boost DNA vaccination:
i.m. injection of 100ug plamid DNA in PBS
followed by electroporation (175V/cm, 8 pulses
of 20msec at 1 sec intervals).
-2hrs
Day 0
Day 14
Day 21
Immune Monitoring:
-Antigen-specific interferon gamma
release assay;
- Memory CD8+ T cells;
- Antigen-specific cytotoxic T cell
assay.

Figure 21. GIFT as adjuvant to naked plasmid DNA vaccination.

(A) C57BL/6 mice were injected twice intramuscularly at two-week interval with 100μg total plasmid DNA. Twenty-one days after vaccination, $5x10^6$ splenocytes were isolated from immunized mice (n=4/group) and cocultured with $1.25x10^5$ EL4 cells or ovalbumin-expressing EG7 cells for 24 hours. Following coculture, supernatants were tested by ELISA for the presence of interferon gamma (IFNg). **(B)** C57BL/6 mice were injected twice intramuscularly at two-week interval with 100μg total plasmid DNA. Twenty-one days after vaccination, splenocytes were isolated from immunized mice (n=4/group) and analyzed by flow cytometry for the presence of CD8+ effector memory T cells (CD8+CD44+CD122low). Representative flow cytometry analyses are shown. **(C)** C57BL/6 mice were injected twice intramuscularly at two-week interval with 100μg total plasmid DNA. Twenty-one days after vaccination, $5x10^6$ splenocytes were isolated from immunized mice (n=4/group) and restimulated in vitro with 10^5 irradiated ovalbumin-expressing EG7 cells for 5 days in the presence of recombinant mouse IL-2 (30U/ml). After restimulation, CD8+ T cells were purified, pooled and used as effectors in cytotoxic T cell assays against EL-4 or ovalbumin-expressing EG7 target cells.

Figure 21


A
EL4
EG7-ova
ova + gift
ova + gmcsf + il2
ova
empty
0
100
200
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500
600
700
800
IFNg (pg/ml)
B
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ova
ova+gmcsf+il2
ova+gift
R3
M1
M2
34%
35%
39%
53%
C
% annexin-V target cells
E/T ratio
ova+gmcsf+il2 /EG7
ova+gmcsf+il2 /EL4
ova+gift /EG7
ova+gift /EL4
ova /EG7
ova /EL4

specific cytotoxic CD8+ T cells (Figure 21C). Taken together, my preliminary studies showed that intramuscular injection of the GIFT cDNA when co-expressed with ovalbumin cDNA as part of a DNA vaccine strategy leads to improved immune responses when compared with control.

Hypothesis 2:

As above-mentioned, current cytokine-based therapies are limited by the severe toxicity associated with the systemic administration of these immune modulatory proteins. In order to localize cytokines to a tumor's microenvironment – thus lowering the toxicity induced by high systemic levels – previous studies have investigated the use of delivering cytokine gene-modified cells. Two approaches have been tested: (i) injection of gene-modified tumor cells, and (ii) injection of gene-modified normal somatic cells. Bubenik *et al.*[411] were the first to investigate the use of normal somatic cells to deliver *in situ* anticancer cytokines. Their studies demonstrated that allogeneic or autologous fibroblasts can be gene-modified to produce high levels of IL-2 and when injected at the site of a tumor, can profoundly inhibit tumor growth. This strategy has been since extended to the delivery of other cytokines such as IL-12, which induced long-term protective antitumor immunity[239]. However, several drawbacks associated with the use of terminally differentiated somatic cells such as fibroblasts limit this therapeutic strategy. For instance, fibroblasts have been shown to inactivate introduced vector sequences[358-359]. In addition, pre-programmed replicative-senescence would make it difficult, especially in the aged cancer patients, to culture expand *ex vivo* large amounts of gene-modified somatic cells, especially if clonal populations are to be isolated[360]. We hypothesized that the use of postnatal adult stem cells may address this issue. The ideal cellular vehicle for therapeutic delivery of gene products should be: (i) abundant in humans of all ages; (ii) easy to harvest; (iii) easy to gene modify; and (iv) expandable to clinically relevant numbers. We hypothesized that primary marrow stromal cells (MSCs) fulfill these criteria and can be used for the tumor-localized delivery of anticancer cytokines such as IL-2, thereby limiting the severe toxicity associated with systemic administration of recombinant cytokines.

Several studies have demonstrated, essentially in immunocompromised animals, successful *in vivo* delivery of various proteins by gene-modified MSCs. Our group has shown, in addition, that gene-modified autologous MSCs can be used to generate a subcutaneous removable implant in order to deliver erythropoietin in unconditioned normal hosts[351]. However, the utility of MSCs for the delivery of immunostimulatory proteins had been unexplored and was thought to be uncertain. Indeed, recent observations demonstrated that MSCs possess intrinsic immunosuppressive properties able to suppress allogeneic mixed lymphocyte reactions and to favour tumor growth in allogeneic recipients[311-326]. We demonstrated that primary MSCs did not affect syngeneic tumor growth and, most importantly, that IL-2 producing MSCs could generate CD8 and NK mediated systemic immune responses against B16 cells. Specifically, my studies demonstrated that:

i) Primary mouse MSCs can be gene-modified to secrete IL-2 without affecting their phenotype;

ii) Co-injection of control GFP gene-modified mouse MSCs and syngeneic B16 cells does not alter B16 tumor growth in immunocompetent mice;

iii) Co-injection of IL-2 gene-modified mouse MSCs and syngeneic B16 cells delays B16 tumor growth in immunocompetent mice;

iv) Peritumoral injection of IL-2 gene-modified mouse MSCs embedded in a collagen-based matrix prevents pre-established B16 tumor growth in a localized manner;

v) Antitumor effect of matrix-embedded IL-2 secreting mouse MSCs requires CD8+ T cells and NK cells but not CD4+ T cells, and induces systemic CD8+ immune response.

vi) Matrix-embedded IL-2 secreting mouse MSCs form blood vessel-like structures and a fraction of them differentiate into CD31+ endothelial cells;

vii) Matrix-embedded IL-2 secreting MSCs induce tumor infiltration of NK cells and NKT cells, and downregulate the infiltration of CD4+CD25- T cells compared to control.

This proof-of-principle study supporting the use of MSCs for sustained local delivery of IL-2 could be extended to the delivery of other interleukins, cytokines and chemokines. Our data is the first to demonstrate that primary MSCs can be used to enhance immune responses in syngeneic immunocompetent hosts. An important aspect of our study in that the analysis of the cellular immune infiltrate demonstrated that control GFP gene-modified MSCs failed to modulate, either negatively or positively, the immune response when transplanted with B16 cells in syngeneic recipients. This is in contrast with the reported immunosuppressive effect of MSCs on B16 growth in allogeneic recipients[317]. On the other hand, our results with IL-2 producing MSCs are consistent with the literature on IL-2 anticancer effects and demonstrate that MSCs can be effectively exploited as a cellular vehicle to stimulate an immune response against poorly immunogeneic tumors. Interestingly, the fact that mice treated with IL-2 producing MSCs could mount tumor-specific systemic immunity is in contrast with what had been reported using IL-2 producing fibroblasts. Indeed, paracrine delivery of high doses of IL-2 by engineered fibroblasts was reported to be ineffective in generating systemic immunity[372]. Given our observations, we formulated a new hypothesis that MSCs in themselves can provide immune modulation that may have acted synergistically with IL-2 in generating a memory immune response.

Hypothesis 3:

In order to assess whether primary MSCs possess such intrinsic properties that may enhance antitumor immunity, we decided to investigate the immunomodulatory effects of MSCs during a well-defined syngeneic antigen-specific immune response. As discussed above, the immunosuppressive effects of MSCs on allogeneic or third party immune responses has been well described. It has been shown that MSCs are able: (i) to suppress the proliferation of allogeneic T cells in response to mitogen or allogeneic cells; (ii) to inhibit the production of IFNγ and tumor-necrosis factor (TNF)-α and increase the production of IL-10; (iii) to induce T cell division arrest anergy; (iv) to inhibit the maturation and function of antigen presenting cells such as monocytes and dendritic cells; (v) to decrease alloantigen-specific cytotoxicity of CD8 T cells and natural killer (NK) cells; and (vi) to favor the differentiation of CD4 T cells with presumed regulatory

activity[311-326]. However, the effect of MSCs on syngeneic immune responses had been unexplored. We tested the hypothesis that MSCs can modulate syngeneic immune responses distinctively from allogeneic immune responses. My studies demonstrated that:

i) Primary mouse and human MSCs, upon IFNγ stimulation, can process soluble exogenous proteins, present antigenic peptides onto MHC class II molecules and activate antigen-specific hybridoma T cells *in vitro*;

ii) Primary mouse MSCs, upon IFNγ stimulation, can activate primary transgenic antigen-specific CD4+ T cells in a CD80-dependent manner *in vitro*;

iii) Primary mouse MSCs, unlike control DCs, cannot perform MHC class I-mediated cross-presentation of exogenous antigens as demonstrated by *in vitro* hybridoma T cell assays;

iv) Injection of primary mouse MSCs, upon *in vitro* IFNγ stimulation and protein pulsing, induce complete protective immunity in immunocompetent syngeneic hosts and CD8+ antigen-specific cytotoxic T cells.

Taken together, our results strongly suggest that in syngeneic conditions, IFNγ-stimulated MSCs behave as conditional antigen presenting cells able to activate antigen-specific immune responses. This is in marked contrast with the allogeneic setting, where MSCs have been shown to be potent immunosuppressors. We propose that MSCs constitute a novel subset of non-hematological APCs with distinct dichotomy of function. Our observation that MSCs can induce a strong CTL response *in vivo* despite being unable to perform cross-presentation *in vitro* suggests a role for host APCs in the generation of CTL response. Indeed, host APCs are known to internalize and present exogenous antigens acquired from other cell types, a phenomenon known as cross-priming. The implication of host APCs in our experiments is supported by the observation that ovalbumin-pulsed non-APC cells such as MEF can induce a specific, albeit limited, immune response protecting 10% of mice against a tumor challenge. However, effective cross-priming of CTL and subsequent secondary expansion of CTL upon antigen re-encounter are dependent upon proper activation of CD4 helper T cells. I thus propose

that CD4 T cell activation by MSCs enhances host-derived CTL cross-priming resulting in the generation of strong antigen-specific protective immunity.

An important aspect of our study is the observation of MSCs with antigen-presenting properties requiring contact molecule(s) distinct from MHC class I, class II and CD80. In addition, the only costimulatory molecule that we found consistently expressed on mouse and human MSCs upon IFNγ stimulation was B7H1. Since the exact immune functions of B7-H1 are currently not fully understood, we can only hypothesize, at the moment, on its role during MSCs-mediated antigen presentation. While B7-H1$^{-/-}$ mice suggest an essential role for B7-H1 in negatively regulating T cell activation, other studies have demonstrated that B7-H1 expression can provide positive costimulation for T cell priming *in vitro* and *in vivo*. More experiments are required at this time in order to determine the function, if any, of B7-H1 expression on MSCs for antigen presentation, using B7-H1$^{-/-}$ MSCs for instance. Conversely, novel yet unidentified costimulatory molecules could be implicated in MSC-mediated antigen presentation. Recently, Laouar *et al.*[412] identified a unique population of APCs of non-hematopoietic origin that are found in the lamina propria of the gut and depend upon CD70 for antigen presentation. CD70 was thus identified as a new costimulatory molecule essential for antigen-presentation for specific APCs and would be worthwile investigating on MSCs. Of particular interest, is the fact that these non-hematopoietic APCs described by Laouar *et al.* display a similar surface phenotype to MSCs.

Another important aspect of the biology of MSCs that will need further investigation is whether MSCs-mediated antigen presentation actually occurs *in vivo* in the bone marrow and, if so, whether it plays a significant role during endogenous immune responses. Experiments based on transplantation of wild type MSCs in MHC class II-deficient mice followed by *in vivo* administration of ovalbumin and adoptive transfer of labelled ovalbumin-specific transgeneic T cells should help clarify the issue.

From a therapeutic perspective, it would be of interest to investigate whether gene-modified MSCs can process intracellularly expressed antigens and behave as antigen-

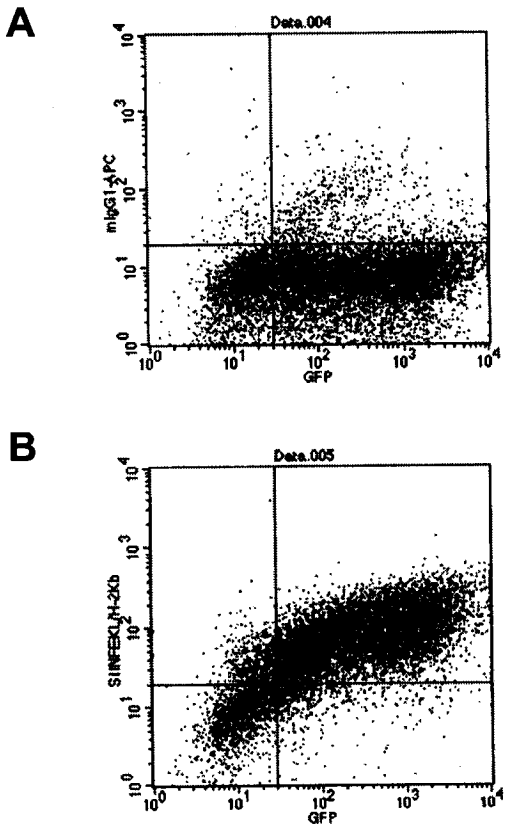
presenting cells upon IFNγ stimulation. Since the intracellular processing of antigens induces presentation via MHC class I molecules to CD8+ T cells, the induction of CTLs effectors by gene-modified MSCs could be enhanced compared to what is achieved with exogenous antigen pulsing. I have obtained preliminary results demonstrating that gene-modified MSCs can indeed activate MHC class I immune responses. MSCs gene-modified with an adenoviral vector encoding for ovalbumin-GFP fusion protein efficiently presented on MHC class I molecules the dominant SIINFEKL epitope (Figure 22A). These results suggest that the use of gene-modified MSCs may represent an alternative method to induce antigen-specific immune responses. An extension of this approach would be to combine antigen gene transfer to cytokine gene transfer into MSCs. Presumably, by doing so, antigen presenting functions of MSCs or downstream effector functions of activated lymphocytes could be enhanced by the release of pro-inflammatory cytokines such as IL-2.

In conclusion, my research has demonstrated that the fusion between two cytokines can markedly enhance the therapeutic value of each, either used alone or in combination, in the development of cancer vaccines. My research also demonstrated that primary MSCs represent a novel cellular subset for the efficient delivery of immunostimulatory gene products such as IL-2. Finally, I provided strong evidence that primary MSCs possess previously unrecognized intrinsic immune modulatory properties and can be used as APCs in order to induce systemic protective immunity against a surrogate tumor antigen. These studies represent novel avenues for the development of new therapeutic strategies in the fight against cancer based on the harnessing of the immune system, and may reveal MSCs as previously unrecognized cellular regulators of physiological immune responses. In view of my research, I propose to pursue investigating the generation of other cytokine chimeric gene products based, for instance, on cytokines such as IL-12 or IL-15. IL-12 and IL-15 have been shown to be potent inducers of NK cell activation and can stimulate the generation of tumor-specific adaptive immune responses, in some cases more efficiently than IL-2 and GM-CSF [241,243]. In addition, I propose to investigate the possibility to couple APC features of MSCs with the immuno-stimulatory abilities of antitumoral cytokines and other chimeric fusions. Presumably, by doing so, APC

functions of MSCs or downstream effector functions of activated lymphocytes could be enhanced by the release of pro-inflammatory cytokines. Finally, I propose to investigate the possibility to combine technological platforms with complementary immune stimulatory features, such as the use of immuno-stimulatory strategies combined to the blockade of anergic mechanisms and reversal of tumor-antigen tolerance. For instance, the use of blocking antibodies to cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)[413] or the depletion of regulatory T cells with low dose cyclophosphomide, fludarabine[414] or monoclonal antibodies[415] would be other venues of investigation in order to enhance immune stimulatory strategies.

Figure 22. MHC class I-mediated antigen presentation by gene-modified MSCs. C57BL/6-derived mouse MSCs were incubated with an adenoviral vector encoding for ovalbumin-GFP fusion protein (a generous gift from Dr. Jonathan Bramson, McMaster University) at a multiplicity of infection of 100 in complete media overnight. The next day, the cells were washed with PBS and incubated for another 24 hours in complete media. MSCs were then trypsinized and incubated with an isotypic control (A) or a purified monoclonal antibody specific to the ovalbumin epitope SIINFEKL presented on H-2Kb molecules (B) as previously described[385] (a generous gift from Dr. Ronald Germain, NIH). The antibody was then revealed with a biotinylated anti-mouse IgG1 antibody and streptavidin-APC.

Figure 22



Contribution to original knowledge

i) I generated and characterized the antitumor effects of a chimeric bi-functional mouse GM-CSF/IL-2 fusion transgene (GIFT).

ii) I demonstrated that tumor expression of GIFT induces complete rejection of mouse melanoma in immunocompetent syngeneic mice, that GIFT induces complete protective immunity against wild type B16 tumors when expressed by a prophylactic irradiated whole cell tumor vaccine, and that GIFT induces greater antitumoral effects against pre-established wild type tumors than GM-CSF and IL-2, alone or in combinasion, when expressed by a therapeutic irradiated whole cell tumor vaccine.

iii) I demonstrated that the GIFT-mediated antitumor effects are CD8 cell-dependent and NK cell-dependent, but independent of CD4 cells, and that GIFT displays novel immunopharmacological properties distinct of those of GM-CSF and IL-2 used alone or in combination.

iv) I demonstrated that primary mouse marrow stromal cells (MSCs) constitute a previously unrecognized source of autologous somatic cells available for ex vivo gene transfer and *in vivo* delivery of IL-2.

v) I demonstrated that peritumoral injection of IL-2 gene-modified mouse MSCs embedded in a collagen-based matrix prevents pre-established B16 tumor growth in a localized manner, requires CD8 and NK cells but not CD4 cells, and induces systemic CD8-mediated immune response.

vi) I demonstrated that primary mouse and human MSCs, upon IFNγ stimulation, can process soluble exogenous proteins, present antigenic peptides onto MHC class II molecules and activate antigen-specific hybridoma T cells *in vitro*.

vii) I demonstrated that primary mouse MSCs, upon IFNγ stimulation, can activate primary transgenic antigen-specific CD4+ T cells in a CD80-dependent manner *in vitro*.

viii) I demonstrated that the injection of primary mouse MSCs, upon *in vitro* IFNγ stimulation and exposure to soluble protein, induces CD8 antigen-specific cytotoxic T cells and systemic protective immunity in immunocompetent syngeneic hosts. My research suggests that in syngeneic conditions, IFNγ-stimulated MSCs behave as conditional antigen presenting cells able to activate antigen-specific immune responses, and may constitute a previously unrecognized player of endogenous immune responses.

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