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(54) Title: COMPOSITION FOR TREATING CANCER ADAPTED FOR INTRA-TUMORAL ASMINISTRATION AND USES THEREOF

(57) Abstract: The present invention is related to a composition adapted for intra-tumoral administration of a subject suffering from cancer, whereby administration of said composition to said subject induces IMC differentiation by neutralizing a factor implicated in a DC differentiation defect and use thereof .

COMPOSITION FOR TREATING CANCER ADAPTED FOR INTRA-TUMORAL ADMINISTRATION AND USES THEREOF

The present invention relates to a composition for treating cancer and uses thereof.

Failure of T cells from tumor-bearing hosts to effectively recognize and eliminate tumor cells is one of the major factors of tumor escape from immune system control. An effective antitumor immune response requires participation of the host bone marrow antigen-presenting cell (APC) responsible for the presentation of tumor-specific antigens. Dendritic cells (DC) and macrophages are the two most potent groups of APC. These cells are capable of inducing primary immune responses including the cytotoxic T-lymphocyte response.

Recent studies have clearly demonstrated that the immuno-stimulatory characteristics of DC are dependent on their maturation state. Increasing evidence supports the notion that both immune activation and immune suppression depend on antigen presentation by APC.

DC as well as macrophages and granulocytes arise from a common myeloid progenitor that has the ability to capture antigen but lacks the expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules. Mature DC loaded with antigen are highly effective in eliciting a protective immune response against tumors, whereas immature DC may induce the antigen specific inhibition of CD8+ T cell effector function.

It appears that impaired balance between mature and immature myeloid cells is one of the hallmarks of cancer. There is increasing evidence that progressive tumor growth is associated with an accumulation of immature myeloid cells, monocytes/macrophages, and with a decreased number and function of DC in cancer patients as well as in tumor-

bearing mice. The increased presence of immature myeloid cells (IMCs) capable of inhibiting T cells responses could be the major factor responsible for immune suppression in cancer patient.

The growth of many mouse carcinomas is associated with the early development of splenomegaly and the marked accumulation of IMC in the lymphoid organs (JAFFE *et al.*, *Mol. Med.*, vol.2, p:692-701, 1996; KUSMARTSEV *et al.*, *J. Immunol.*, vol.165, p:779-85, 2000). Decreased presence of DC in the peripheral blood of patients with breast, lung, head and neck cancer was associated with the accumulation in the peripheral blood of cells lacking markers specific for mature myeloid and lymphoid lineages (ALMAND *et al.*, *Clin. Cancer Res.*, vol.6, 1755-1766, 2000). About one third of these cells were immature macrophages and DC and the remaining cells were IMC at earlier stages of differentiation (ALMAND *et al.*, *J. Immunol.*, vol.166, 678-689, 2001). The peripheral blood presence of these cells was dramatically increased in patients with advanced stage cancer, but dropped considerably within three to four weeks after surgical resection of the tumor. This finding is consistent with the hypothesis that the generation of IMC is due to the production of soluble factors by tumors.

Consistent with this hypothesis, it has been shown that several tumor-derived factors affect DC maturation from hematopoietic progenitor cells (HPC).

VEGF is produced by most tumors, and its production is closely associated with poor prognosis (TOI *et al.*, *Eur. J. Cancer*, vol.32A, p: 2513-9, 1996). It has been shown that neutralizing anti-VEGF antibody blocked the negative effects of tumour cell supernatants on DC maturation *in vitro* (GABRILOVICH *et al.*, *Nat. Med.*, vol.2, p:1096-103, 1996). Moreover, a continuous *in vivo* VEGF infusion results in a dramatic inhibition of DC production (GABRILOVICH *et al.*, *Blood*, vol.92, p: 4150-4166, 1998).

VEGF inhibits the activation of transcription factor NF-κB in hematopoietic progenitor cells, which is accompanied by alterations in the development of multiple lineages of hematopoietic cells (DIKOV *et al.*, *Cancer Res.*, vol.61, p: 2015-21, 2001). Chronic administration of recombinant VEGF in naïve mice results in an inhibition of DC development and in an increased production of B cells and immature GR-1$^+$ myeloid cells (OYAMA *et al.*, *J. Immunol.*, vol.160, p:1224-32, 1998).

GM-CSF is another factor that has been shown to be responsible for the stimulation of myelopoiesis in tumor-bearing host. The chronic administration of GM-CSF to mice results in the generation of a cell population that morphologically resembles granulocyte-monocyte progenitors that express the granulocyte-monocyte markers Mac-1 and Gr-1 (BRONTE *et al.*, *Blood*, vol.96, p: 3838-46, 2000).

Other tumor-derived factors such as M-CSF, IL-6 (MENETRIER-CAUX *et al.*, *Blood*, vol.92, p: 4778-4791, 1998), and IL-10 (ALLAVENA *et al.*, *Eur. J. Immunol.*, vol.28, p:359-69, 1998; FAULKNER *et al.*, *Immunology*, vol.99, p: 523-31, 2000) or gangliosides (SHURIN *et al.*, *Cancer Res.*, vol.61, p:363-9, 2001) have also been involved in defective DC differentiation *in vitro*. Neutralizing anti-IL-6 and anti-M-CSF antibodies abrogate the negative effect of supernatants from renal cell carcinomas on DC differentiation (MENETRIER-CAUX *et al.*, abovementioned, 1998). However, it appears that these factors do not stimulate myelopoiesis and mostly affect relatively mature cells. Moreover, IL-10 appears to prevent the differentiation of monocytes to DC, but promotes their maturation to macrophages (ALLAVENA *et al.*, abovementioned, 1998). Furthermore, ALMAND *et al.* (2000, abovementioned) has shown that only patients with peripheral blood elevated levels of VEGF showed statistically significant increased

of IMCs after measuring the levels of M-CSF, GM-CSF, IL-6, IL-10, TGF-β, and VEGF.

The characterization of IMCs has shown that these cells actively suppress Ag-specific T cells responses and contributes to tumor nonresponsiveness (ALMAND *et al.*, abovementioned, 2001). Thus, IMCs actively inhibit $CD8^+$ T cell effector function, MHC class-II associated T-specific proliferation and MHC class I-restricted IFN-γ production in the presence of functionally competent DC. It has been suggested that physiologically, IMCs may serve as a defense mechanism that limits the expansion of activated T cells and prevents the development of autoimmune diseases. However, in the case of cancer, the accumulation of IMCs may lead to the profound suppression of immune responses.

Consequently, IMCs depletion or differentiation constitutes actually an important strategy in order to improve immune response in cancer.

Different ways of therapies have been explored by *in vitro* IMCs differentiation experiments. Thus, it has been shown that a combination of ATRA and GM-CSF is able to induce the differentiation of the majority of IMCs into relatively mature DC (ALMAND *et al.*, abovementioned, 2001). ATRA is a naturally occurring isomer of retinoic acid that is successfully used in differentiation induction therapy in patients with acute promyelotic leukaemia (CASTAIGNE *et al.*, *Blood*, vol.76, p:1704-9, 1990).

However, if the general *in vivo* induction of the differentiation of IMCs by an adapted factor could induce a tumor remission, it could also induce the development of an autoimmune response.

The purpose of the present invention is to fulfil this need by providing a composition for inducing IMCs

differentiation within the tumor sites in order to induce tumor remission without any side effects.

Unexpectedly, the inventors have shown that if IMCs effectively accumulate into lymphoid organs, they further accumulate in tumor sites and this tumor accumulation operates at far greater extend than in lymphoid organs.

This specific tumor accumulation was unexpected since IMCs are very poor migrating cells like mature DCs. As an example and for DCs used in immunotherapy, it has been shown that less than 5% of intra-dermally administrated mature DCs reach the draining lymph nodes (DE VRIES *et al., Cancer Res.*, vol.63, p: 12-17, 2003) and several approaches are develop in immunotherapy in order to stimulate this DC migration. Consequently, nothing suggest to one of skill in the art that IMCs are able to migrate to and to accumulate in tumor sites.

Moreover, the inventors have demonstrated that this IMC accumulation is correlated with IL-10 tumor expression. In fact, the inventors have demonstrated that a neutralizing anti-IL-10R antibody permits IMC differentiation into immature dendritic cells.

These results were also unexpected since it was thought that IL-10 does not stimulate myelopoiesis –i.e. IMC differentiation- but mostly affects relatively mature cells.

Another major factor of tumor escape from immune system is associated with immuno-supression mechanisms' activation.

The $CD4^+CD25^+$ T cells constitute nearly 10% of $CD4^+$ T cells in naïve animals and also exist in humans (ITOH *et al., J. Immunol.*, vol.162, p:5317-5326, 1999). These cells are also able to suppress $CD4^+$ T-cell-induced organ-specific autoimmune diseases (SAKAGUCHI *et al., J.*

Immunol., vol.155, p:1151-1164, 1995) and immune responses against foreign antigens and pathogens (XU *et al.*, *J. Immunol.*, vol.170, p:394-399, 2003; OLDENHOVE *et al.*, *J. Exp. Med.*, vol.198, p:259-266, 2003). In the context of tumor immunology, the $CD4^+CD25^+$ T cells have been shown to suppress anti-tumor immunity. Augmentation of $CD4^+CD25^+$ T cell number or proportion in tumor sites has been reported in variety of cancer patients (WOO *et al.*, *Cancer Res.*, vol.61, p: 4766-4772, 2001; SASADA *et al.*, *Cancer*, vol.98, p: 1089-1099, 2003). This augmentation of $CD4^+CD25^+$ T cells in tumor sites may result of their expansion induction by antigen-processing dendritic cells (YAMAZAKI *et al.*, *J. Exp. Med.*, vol.168, p: 235-247, 2003).

The depletion of $CD4^+CD25^+$ T cells *in vivo* by an anti-CD25 antibody before tumor challenge enhances natural tumor immuno-surveillance and induces rejection of multiple immunogenic tumors in multiple strains of mice (ONIZUKA *et al.*, *Cancer Res.*, vol.59, p: 3128-3133, 1999; GOLGHER *et al.*, *Eur. J. Immunol.*, vol.32, p: 3267-3275, 2002). However, if different studies show that removing $CD4^+CD25^+$ T cells enhances anti-tumor immunity, sometimes this treatment also induces autoimmune disease (TAGUCHI *et al.*, *Eur. J. Immunol.*, vol.26, p: 1608-1612, 1996; JONES *et al.*, *Cancer Immun.*, vol.2, p: 1, 2002).

So, there is also a recognized and permanent need in the art for new reliable method for neutralizing or diminishing immuno-suppression in order to enhance anti-tumor immunity.

The purpose of the present invention is also to fulfil this need by providing a composition for inducing IMCs differentiation within the tumor sites in order to locally neutralize or diminish the immuno-suppression.

Unexpectedly, the inventors have also shown that an *in vitro* amplification of $CD4^+CD25^+$ T cells is induced by IMC.

Consequently, the IMC tumor accumulation should explain the known $CD4^+CD25^+$ T tumor accumulation.

The constant turnover of blood cells requires the upregulation of proliferation and differentiation events in the hematopoietic tissues resulting in the production of committed progenitors to each of the eight blood lineages. Interleukin-3 (IL-3) has the broadest target specificity of all the hematopoietic growth factors and plays a central role in the production of macrophages, neutrophils, and eosinophils through stimulation of the pluripotent hematopoietic stem cells and their derivatives (BARREDAA *et al.*, *Developmental and Comparative Immunology*, vol.28, p :509–554, 2004). IL-3 together with other inflammatory cytokines like TNF-α and IFN-γ may stimulate surface molecules expression like E-selectin and IL-8, that may in turn facilitate neutrophil transmigration through the epithelium during inflammatory processes, as well as stimulation of MHC class II expression (BARREDAA *et al.*, 2004, abovementioned).

Presently, the inventors have also shown that the stimulation of immature dendritic cells, resulting from the differentiation of IMCs by anti-IL-10R antibody, with CpG oligonucleotides and IL-3 permits their differentiation into mature dendritic cells.

Finally, the inventors demonstrate that the intratumoral expression of a soluble IL-10 receptor, which neutralizes IL-10, with CpG oligonucleotides and IL-3 induces the differentiation of IMC into mature dendritic cells.

In one aspect the present invention relates to the use, for the manufacture of a medicament for use in the treatment of cancer by intratumoral administration to a subject, of a composition comprising:

(i) a protein able to neutralize the binding of IL-10 to its receptor, or

(ii) a nucleic acid encoding for said protein (i), or

(iii) a cell transformed by said vector (ii) and expressing said protein (iii).

As a result the tumor administration of the composition of the invention will induce an IMC differentiation and consequently an inhibition of $CD4^+CD25^+$ T tumor accumulation.

As used herein, the term "subject" denotes a Mammal, such as a rodent, a feline, a canine and a primate; most preferably said subject is a human.

According to a first preferred embodiment, said protein able to neutralize the binding of IL-10 to its receptor is selected in the group comprising antibodies directed against IL-10 or its receptor, soluble receptors of IL-10 and analogues of IL-10. Preferably, said protein is a soluble receptor of IL-10.

As used herein, antibody includes intact molecules or fragments thereof such as Fab and $F(ab')_2$ which are capable of binding to their antigen.

Example of neutralizing antibodies includes IL-10 neutralizing antibodies as described in CORINTI *et al.* (*J. Immunol.*, vol.166, p: 4312-8, 2001) and IL-10R neutralizing antibodies as described in REINEKE *et al.* (*Protein Science*, vol. 7, 951-960, 1998).

Example of soluble receptors includes IL-10 soluble receptors (the 238 amino acids of the extra-cellular domain of IL-10Rα, R&D SYSTEM). Preferably, said soluble receptor is an IL-10 soluble receptor.

As used herein analogues includes peptidic fragments able to neutralize the binding of said factor to its receptor and recombinant protein including such fragments.

Advantageously, said composition also comprises at least one molecule able to potentiate DC differentiation selected in the group comprising cytokines and Toll-like receptors ligands. Preferably, said composition also comprises at least one cytokine and at least one Toll-like receptors ligand.

Examples of cytokines include IL3 and TNF-α. Preferably, said composition comprises at least IL-3.

Examples of Toll-like receptors ligands include CpG nucleotides, lipopolysaccharide (LPS), monophosphoryl lipid (MPL), poly-I:C, RNA double strand (more than 30bp long). Preferably, said Toll-like receptor ligand is CpG nucleotides.

According to a second preferred embodiment said composition comprises a nucleic acid vector encoding for a protein able to bind to Il-10 or to its receptor as described previously.

Said nucleic acid vector contains the necessary elements for the transcription and the translation of the coding sequence.

The coding sequence is operationally linked to a promoter having a constitutive or inductive expression in transfected or infected cell. Examples of adapted promoter include CMV or ferritin promoters. The promoter sequence can be operationally linked to enhancer sequences in order to potentiate the coding sequence expression. Examples of enhancer sequences include SV40 and CMV enhancer sequences.

The coding sequence is also linked to a polyadenylation signal, preferably to a strong polyadenylation signal like the late SV40 polyA.

The coding sequence includes an adapted signal sequence in order to obtain the secretion of the encoded protein.

The nucleic acid vector can include selectable markers that are active both in bacteria and in mammalian cells.

According to a first specific embodiment, the nucleic acid vector of the present invention corresponds to "naked DNA" like plasmids, cosmids or phagemids, preferably a plasmid and more preferably p310R plasmid (SEQ ID NO:1). Such a naked DNA may be injected into a tumor as described in US 5,580,859. The composition may also comprise non lipid cationic polymers (WU and WU, *J. Biol. Chem.*, vol.263, p: 14621-4, 1988) or liposomes (BRIGHMAN *et al.*, *Am. J. Med. Sci.*, vol.298, p: 278-81, 1989) which form complexes with naked DNA and enhance cellular uptake. Preferably, said "naked DNA" is injected without any non lipid cationic polymers or liposomes.

According to a second specific embodiment, the nucleic acid vector is a viral vector adapted for *in vivo* gene therapy protocols. Examples of appropriate viral vectors includes retroviral vectors as described in EP 0871459, EP 0386882 and EP 1222300 and adenovirus vectors as described in US 2004/ 265273 and US 6,638,502. In this case, the internalization of virus occurs through the specific interaction of the viral envelope with a cell surface receptor, followed by receptor-mediated endocytosis of the virus/receptor complex.

Advantageously, said nucleic acid vector also encodes at least one molecule able to potentiate DC differentiation selected in the group comprising cytokines and Toll-like receptors ligands. Preferably, said nucleic acid vector

also encodes at least one cytokine and at least one Toll-like receptors ligand.

Preferably, said nucleic acid vector also encodes at least IL-3.

Preferably, said nucleic acid vector also encodes CpG nucleotides.

According to a third preferred embodiment said composition comprises a cell transformed with a nucleic acid vector as described previously and expressing an effective amount of a protein able to bind to Il-10 or to its receptor as described previously.

Advantageously, said cell is obtained from the subject to treat.

The composition may comprise a vehicle. For example, the composition may comprise emulsions, microemulsions, oil-in-water emulsions, anhydrous lipids and oil-in-water emulsions, other types of emulsions. The composition may also comprise one or more additives (e.g., diluents, excipients, stabilizers, preservatives). See, generally, *Ullmann's Encyclopedia of Industrial Chemistry, 6th Ed.* (various editors, 1989-1998, Marcel Dekker); and *Pharmaceutical Dosage Forms and Drug Delivery Systems* (ANSEL *et al.*, 1994, WILLIAMS & WILKINS).

Said composition may comprise a buffer, water, emulsions or microemulsions. Suitable buffers include, but are not limited to, phosphate buffered saline Ca^{++}/Mg^{++} free (PBS), phosphate buffered saline (PBS), normal saline (150 mM NaCl in water), and Tris buffer.

In a second aspect the present invention relates to a method of therapeutic treatment of a subject suffering from cancer comprising the step of administrating to said

subject directly into the tumor an effective amount of the composition described previously.

An effective amount of a protein for inducing the neutralization of IL-10, which is implicated in a DC differentiation defect, and thus inducing IMC differentiation, depends of the used protein. These effective amounts are well known from one of skilled in the art for many proteins or can be determined without undue experimentation. As an example, the effective amount of a specific IL-10 antibody for inducing the neutralization of IL-10 is at least 10μg/ml (WAKKACH *et al*, *Immunity*, vol.18, p: 605-617, 2003).

An effective amount of a molecule able to potentiate DC differentiation depends of the used molecule. These effective amounts are well known from one of skilled in the art for many molecules or can be determined without undue experimentation. As an example, the DC differentiation effective amounts for CpG and IL-3 are at least 2μM and 10ng/ml respectively.

The invention is further illustrated below by the following Examples, which are not intended to limit its scope.

EXAMPLES

1- IMC accumulation and tumor development:

Mice BALB/C were purchased from Charles River Laboratory (IFFACREDO). All mice were then raised in common mouse pathogen-free conditions and were 4-week-old at the beginning of the experiment. In order to obtain mice with induced tumors (called C26 mice), a first group of mice were injected subcutaneously with cells from the murine colon adenocarcinoma line MCA26 as described in GRI *et al.* (*J. Immunology.*, vol.170(1), p: 99-106, 2003).

Mononuclear cells were purified from spleen (S), liver (L) and tumors of 8-week-old mice according to the protocole described in BLIN-WAKKACH *et al.* (*Leukemia*, vol.18(9), p:1505-11, 2004). The purified cells were then incubated with labelled anti-CD11b and anti-Gr-1 Abs in order to identify the cells expressing the specific IMC CD11b and Gr-1 surface markers. Finally, the percentages of IMCs in these purified cells were established by flow cytometry on FACSCAN flow cytometer® (BECTON DICKINSON) according to the manufacturer's instructions. Non-specific binding was measured using FITC-conjugated isotype-matched mouse Ig.

The figures 1A and 1B represent the expression pattern of CD11b and Gr-1 in Mononuclear purified cells from spleen (S) and liver (L) of normal and C26 mice respectively. The percentages of IMCs expressing characteristic levels of CD11b and Gr-1 in spleen (S) and in liver (L) for wild type and C26 mice are indicated.

The figure 2A represents the expression pattern of CD11b and Gr-1 in Mononuclear cells from tumors of C26 mice.

Unexpectedly, the results show that the development of tumors in C26 mice is correlated with a strong accumulation of IMCs in spleen (7.5% versus 1.9%) and in liver (11.5% versus 1.4%). Moreover, this accumulation of IMCs specifically in tumor is even stronger (24%). Thus, these results show for the first time that IMCs are able to migrate and to accumulate at a high level in tumors.

2- IMC and natural regulatory T cell tumor accumulation:

Mononuclear cells were purified from tumors as described previously. The cells were then incubated with labelled anti-CD25 and anti-CD4 Abs in order to identify

the natural regulatory T cells characterized by the expression of CD4 and CD25 markers. Finally, the percentage of IMC in these cells was established by flow cytometry on FACScalibur flow cytometer® (BECTON DICKINSON) according to the manufacturer's instructions. Non-specific binding was measured as previously.

The figure 2B represents the pattern of expression of CD25 and CD4 in T cells from tumours of C26 mice.

Unexpectedly, the results show that the accumulation of IMC in tumour is correlated with regulatory T cells expansion and recruitment.

3- IMC accumulation and ageing:

Mononuclear cells were purified from spleen and tumours of 33-week-old mice as described previously. The cells were then incubated with labelled anti-CD11b and anti-Gr-1 Abs in order to identify the IMC. Finally, the percentage of IMC in these cells was established by flow cytometry on FACSCAN flow cytometer® (BECTON DICKINSON) according to the manufacturer's instructions. Non-specific binding was measured as previously.

The figures 3A and 3B represent the pattern of expression of CD11b and Gr-1 in mononuclear cells from spleen (S) of normal and C26 33-week-old mice respectively.

The figure 3C represent the pattern of expression of CD11b and Gr-1 in mononuclear cells from tumours of C26 33-week-old mice.

The percentages of IMC expressing characteristic levels of CD11b and Gr-1 in spleen (S) and in tumours are indicated.

The results show that the accumulation of IMC in spleen and in tumour is increased with ageing.

4- Induction and expansion of regulatory T-cells by IMC:

a) Naïve $CD4^+$ cells purification :

Naïve $CD4^+$ cells have been prepared from homozygous DO11.10 transgenic mice obtained from N. Glaishenous (INSERM) as described in WAKKACH *et al.* (2003, abovementioned). These transgenic mice express a specific Ovalbumin T receptor.

b) *in vitro* IMC expansion :

Simultaneously, mononuclear cells were purified from spleen of normal and C26 mice as described previously. The purified cells were cultured with RPMI (INVITROGEN), 5% of SVF (HYCLONE; PERBIO) in the presence of interleukin 3 (IL-3)(10ng/ml) for twelve days long in order to amplify IMCs .

The figures 4A and 4B represent the expression pattern of CD11b and Gr-1 in mononulear cells immediately after purification and after twelve days of culture respectively from spleen of normal mice.

The figure 4C represent the morphology of amplified IMC after twelve days of culture by GIEMSA coloration.

The results show that the experimental culture protocol allows obtaining cell cultures with nearly 80% of IMCs.

Thus, IMC cultures from normal and CD26 mice have been obtained with the protocol described previously.

c) Purification of Splenic DCs:

Splenic DCs have been purified as described in WAKKACH *et al.* (2003, abovementioned).

d) Induction of regulatory T 1 cells (Tr1):

Purified naïve $CD4^+$ cells ($2.5\ 10^5$/ml) obtained in a) have been cultured for seven days with splenic DCs, normal

or CD26 IMC culture (10^5/ml) and with the $OVA_{323-339}$ peptide (SEQ ID NO: 2, ISQAVHAAHAEINEAGR; 0.6 μM). After differentiation, T cells were restimulated with 0.3 μM $OVA_{323-339}$ and irradiated total splenic APCs. The production of IFN-γ, IL-10, IL-4 and was measured by ELISA in supernatants collected at 48 h.

The figures 5A, 5B and 5C represent respectively the expression of IFN-γ, IL-10, IL-4and by primed $CD4^+$ cells cultured under the different conditions described above.

The results show that the stimulation of naïve $CD4^+$ cells by normal or CD26 IMC culture induces an IL-10 strong secretion and an IFN-γ low secretion, which are characteristic of regulatory T cells (Tr1).

e) Expansion of natural regulatory T cells ($CD4^+$ $CD25^+$):

The natural regulatory T cells were purified by FACS sorter FACS VANTAGE (Becton-dickson) from spleen of homozygous DO11.10 transgenic mice based on the expression of CD4 and CD25 ($CD4^+$ $CD25^+$ regulatory T cells: Treg cells).

The figure 6A represent the expression of CD24 and CD25 by the spleen cells of homozygous DO11.10. The 4,5% of regulatory T cells are framed.

Simultaneously, IMC from C26 mice were purified by FACS Vantage as described previously.

Then, 2,5 10^5/ml of Treg cells have been cultured for tree days with IMC (10^5/ml), and with the $OVA_{323-339}$ peptide (0.6 μM).

The Figure 6B represent the expression of CD62L and CD25 –i.e. Tr1 cell markers- after three days of culture.

The results show that IMC were able to expand the Treg cells according to the CD62L cells expression. In fact, the regulatory T cells have strongly proliferated (from 2,5 10^5/ml to 2.5 10^6/ml) in these conditions -i.e 10 fold more-.

The Figure 7 shows that CFSE-labeled splenic OVA-specific $CD4^+CD25^+$ Treg cells from D011-10 mice transferred into normal Balb/c mice or mice bearing C26 tumors were able to proliferate in vivo in the presence of purified IMCs from mice bearing C26 tumors pulsed with OVA peptide.

In conclusion, IMCs induces the specific differentiation of naïve $CD4^+$ cells into Tr1 cells and expand the natural Treg in vitro and in vivo.

The contribution of IL-10 in the expansion of natural Treg elicited by IMCs from mice bearing C26 tumors has been confirmed by neutralizing mouse IL-10 with an anti-IL10 receptor antibody.

5- IL-10 and IMC accumulation:

a) IMC purification and culture:

Purified mononuclear cells from spleen of normal and $IL\text{-}10^{-/-}$ mice were cultured with interleukin 3 (IL-3) for twelve days long as described previously.

Figures 8A and 8B show the morphology of the cells after twelve days of culture by GIEMSA coloration.

As described previously, IMCs were obtained after twelve days of culture of normal mice purified mononuclear cells (cf. figure 8A). For $IL\text{-}10^{-/-}$ mice mononuclear cells, the IMCs were differentiated into dendritic cells after twelve days of culture (figure 8B).

Consequently, these results show that the IL-10 expression is critical for the IMC immature state maintenance.

b) Induction of Tr1 or Th1 cells:

Purified naïve $CD4^+$ cells have been cultured for three days with DCs obtained from IL-$10^{-/-}$ mice or IMC culture and with the $OVA_{323-339}$ peptide as described previously. The supernatant secretion of IL-10 and IFN-γ was then measured by ELISA.

The figures 10A and 10B represent respectively the expression of IFN-γ and IL-10 by naïve $CD4^+$ cells cultured under the different conditions described above.

The results show that the stimulation of naïve $CD4^+$ cells by DCs obtained from IL-$10^{-/-}$ mice induces an IFN-γ strong secretion, which are characteristic of Th1 cells. At the same time, the stimulation of naïve $CD4^+$ cells by IMC culture induces an IL-10 strong secretion and an IFN-γ low secretion as previously.

c) IMC culture with an anti-IL-10R antibody

Purified mononuclear cells from spleen of normal mice were cultured with IL-3 for twelve days long and with or without a neutralizing anti-IL-10R antibody (R&D SYSTEM) and nucleic acids with (2μM) of CpG dinucleotides 1826, (5' TCC ATG ACG TTC CTG ACG TT 3'; SEQ ID NO:3) during the last two days.

The figures 9B and 9A represent the pattern of expression of CD11c and Gr-1 in the amplified IMC cells from spleen (S) of normal and C26 mice at the end of the twelve days with or without anti-IL-10R antibody and CpG dinucleotides respectively.

Consequently, these results confirm that IL-10 expression is critical for the IMC immature state maintenance.

6- *in vivo* IMC maturation induction:

a) p310 R plasmid construction:

T cells derived from BALB/C mice splenocytes were cultured for 12 hours long in RRPMI medium (LIFE TECHNOLOGIES) with 1 μg/ml of concanavalin A.

Total RNA was extracted from 2.10^6 cells using NUCLEOSPIN RNA2® (MACHEREY NAGEL) according to manufacturer's instructions. Single-strand cDNAs was synthesized from 1 μg of total RNA using oligo-dT primers and M-MLV Reverse Transcriptase (PROMEGA) according to the supplier's protocol. The soluble fragment of mouse IL-10 R (NM_034686, N-term 275 amino acids, SEQ ID NO:4) was amplified with probest-DNA polymerase (TAKARA) according to the manufacturer's instructions using sense (IL-10Rs S (SEQ ID NO:5): 5'-**TCT AGA** GAT GTT GTC GCG TTT GCT CC-3') and antisense (IL-10Rs AS (SEQ ID NO:6): 5'-**CCT AGG** CTA AGT GAA ATA CTG CTC CGT CG-3') primers containing XbaI and AvrII restriction sites (bold), respectively.

PCR program was as follows :

Step 1 (1 round):	94°C	2	minutes
Step 2 (35 rounds):	94°C	30	seconds
	58°C	40	seconds
	72°C	1	minutes
Step 3 (1 round)	72°C	10	minutes

The IL-10Rs PCR product and pVIVO2-mcs (INVIVOGEN) were digested with XbaI and AvrII restriction enzymes (BIOLABS). Then, the digested IL-10Rs PCR product was subcloned into XbaI-AvrII-cleaved pVIVO2-mcs. The resulting recombinant expression plasmid (designated pVIVO2-IL-10Rs) was

sequenced to ensure that the insertion was cloned correctly.

The complete mouse IL-3 (NM_010556, complete IL-3 protein SEQ ID NO:7) was amplified as described previously from single-strand cDNA using sense (IL-3 S (SEQ ID NO:8): 5'-**CCA TGG** AGA CAA TGG TTC TTG CCA GC-3') and antisense (IL-3 AS (SEQ ID NO:9): 5'-**GGA TCC** TTA ACA TTC CAC GGT TCC ACG-3') primers containing NcoI and BamHI restriction sites (bold) respectively.

The IL-3 PCR product and the pVIVO2-IL-10-Rs plasmid were digested with Nco I and Bam HI restriction enzymes (BIOLABS) according to the supplier's protocol. Then, the digested IL-3 PCR product was subcloned into NcoI-BamHI-cleaved pVIVO2-IL-10Rs. The resulting recombinant expression plasmid (designated p310R, SEQ ID NO:1) was sequenced to ensure that the insertion was cloned correctly. The figure 11 represents the p310R sequence wherein IL-10 and IL-3 sequences are underlined and in bold respectively.

b) Transient transfection of IMC cell line established in our laboratory

IL-3 and IL-10Rs expression are tested *in vitro* by transiently transfecting GPM-45 cell line cells using Lipofectamine (INVITROGEN) according to the manufacturer's instructions. GPM-45 is an IMC cell line obtained in the laboratory by the isolation of cells expressing CD11b and Gr-1 from a tumor of a $p53^{-/-}$ transgenic mouse. The expression of IL-10Rs and IL-3 were determined by testing the presence of these proteins in the supernatant by using two ELISA kits for detecting IL-10Rs and IL-3 (BECTON-DICKINSON) respectively.

c) *in vitro* IMC cells differentiation

Mononuclear cells were purified from spleen of normal and C26 mice as described previously. The purified cells were cultured with interleukin 3 for 12 days long in order to amplify IMCs.

Then, IMCs cells were transfected using LIPOFECTAMINE® (Invitrogen) according to the manufacturer's instructions with one of the following construction: (1) p310R vector; (2) pVIVO-IL-10Rs vector; (3) pVIVO2 vector; (4) no plasmid DNA.

The transfected cells were cultured for further seven days in the culture medium without IL-3, and in the presence of hygromycin.

5-In vivo expression of p310R plasmid induces IMC maturation into DCs at tumor site:

9 stable clones transfected with p310R were selected after three months of culture, including the clone C-26p310R.

The expression of IL-3 and IL-10Rs mRNAs in said clones has been confirmed by RT-PCR.

At the time, a stable C26-pVIVO2 clone was selected.

5.10^5 C-26p310R and C26 cells were injected subcutaneously to two mice groups (n=10) respectively.

The result shown that the IL-10Rs and IL-3 expression in C-26p310R cells inhibits tumor growth by more than three folds (see Figure 12).

Interestingly, the results have also shown that the expression IL-3 and IL-10Rs in the tumor inhibits the IMC accumulation simultaneously in the tumor and in the spleen and stimulates their differentiation into DCs mature secreting IL-12p70 and interferon gamma: two cytokines

involved in anti-tumoral immune response (see Figure 13 and table I below).

Table I: Cytokines expression in Splenic CD11c cells

ng/ml	C26 + LPS	C26 + CpG	C26-p310 + LPS	C26-p310R + CpG
IL-10	3.7	Nd	1.4	1.9
IL-12p70	Nd	Nd	Nd	5.2
IFN-γ	2.7	Nd	3.7	4.3

e) intra-tumoral administration of IL-3 and IL-10Rs

C26 mice are divided randomly into four groups, and each group consists of 10 mice are injected intratumoraly with one of the following regimens in 100 µl of sterilized normal saline: (1) 100 µg of p310R vector; (2) 100 µg of pVIVO-IL-10Rs vector; (3) 100 µg of pVIVO2 vector; (4) no plasmid DNA. The mice in the last group serve as a challenge infection control. Tumors evolution is controlled in each group following the injection.

Tumor growth is monitored by palpation and measurement using a Calipar three times a week as previously described (GRIC *et al.*, 2003, abovementioned).

CLAIMS

1. Use, for the manufacture of a medicament for use in the treatment of cancer by intratumoral administration to a subject, of a composition comprising:

(i) a protein able to neutralize the binding of IL-10 to its receptor, or

(ii) a nucleic acid encoding for said protein (i), or

(iii) a cell transformed by said vector (ii) and expressing said protein (iii).

2. Use according to claim 1, wherein said protein able to neutralize the binding of IL-10 to its receptor is selected in the group comprising antibodies directed against IL-10 or its receptor, soluble receptors for Il-10 and analogues of IL-10.

3. Use according to claim 2, wherein said antibodies directed against IL-10 or its receptor is selected in the group comprising IL-10 neutralizing antibodies and IL-10 receptor neutralizing antibodies.

4. Use according to claim 2, said protein able to neutralize the binding of IL-10 to its receptor is an IL-10 soluble receptor.

5. Use according to claim 2, wherein said composition comprises a nucleic acid encoding for an IL-10 soluble receptor, preferably said nucleic acid is p310R plasmid (SEQ ID NO:1).

6. Use according to any one of claims 1 to 5, wherein said composition further comprises a molecule

able to potentiate DC differentiation selected in the group comprising cytokines and Toll-like receptors ligands.

7. Use according to claim 6, wherein said cytokines is IL-3.

8. Use according to claim 6, wherein said Toll-like receptor ligand is CpG nucleotides.

9. Use according to any one of claims 6 to 8, wherein said molecule is encoded by a nucleic acid, preferably the p310R plasmid (SEQ ID NO:1).

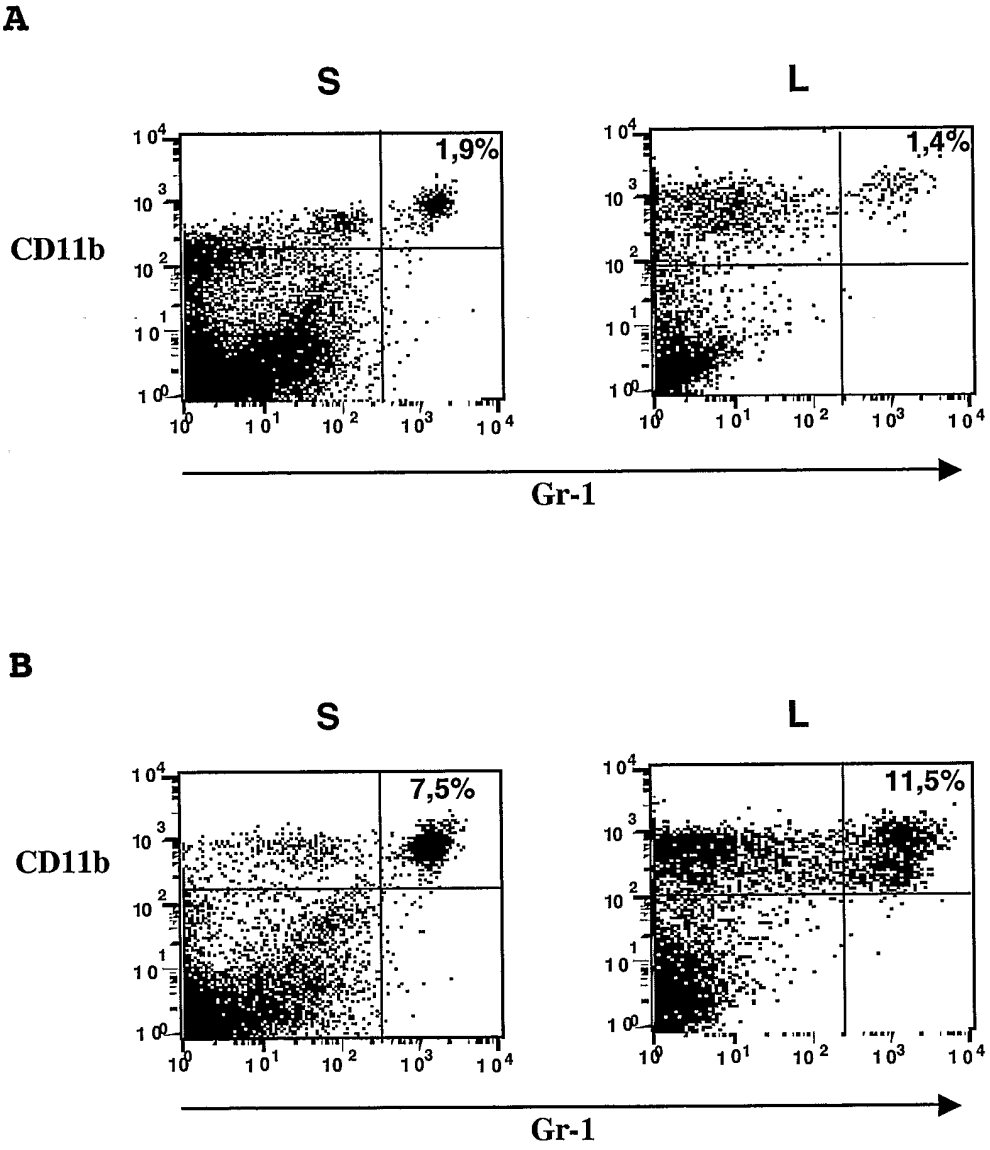

Figure 1

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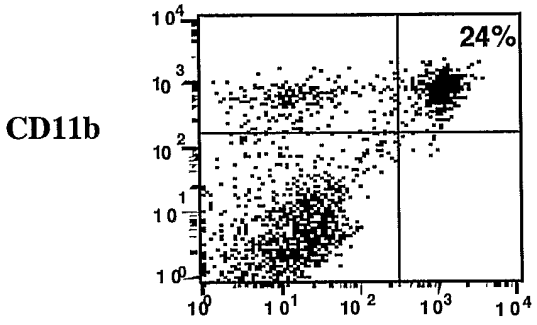

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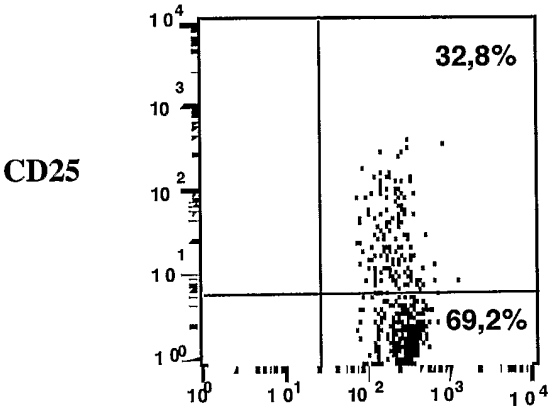

Figure 2

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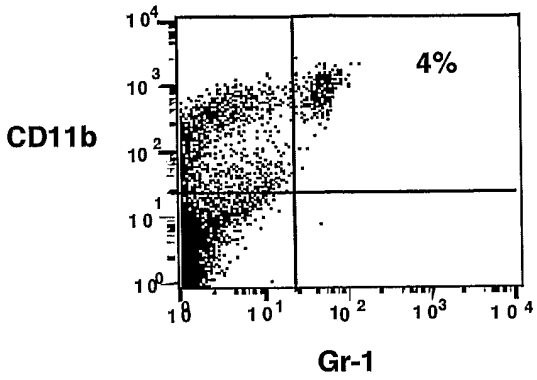

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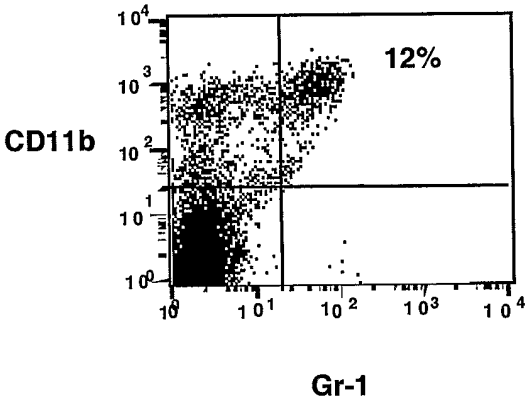

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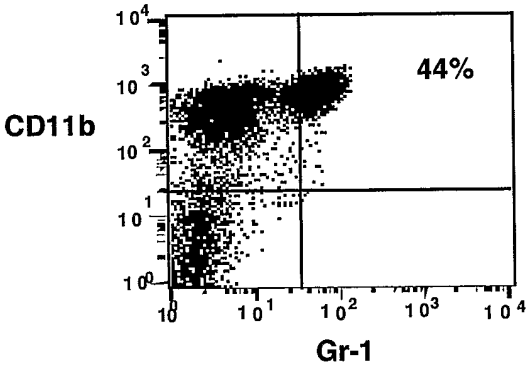

Figure 3

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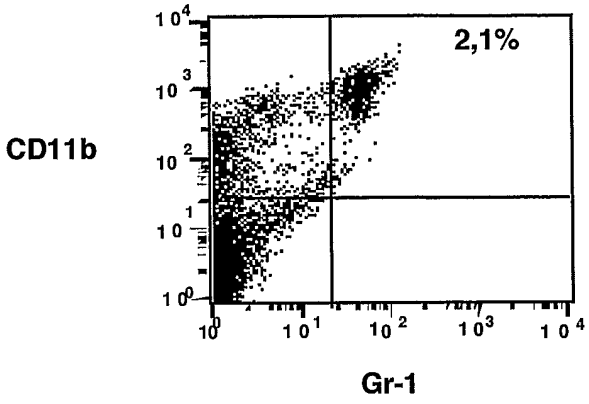

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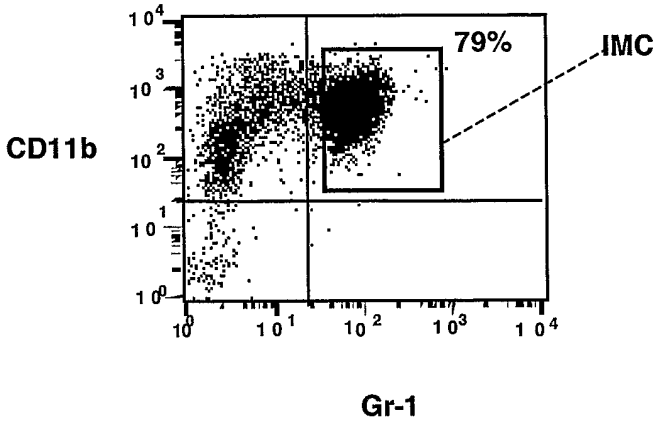
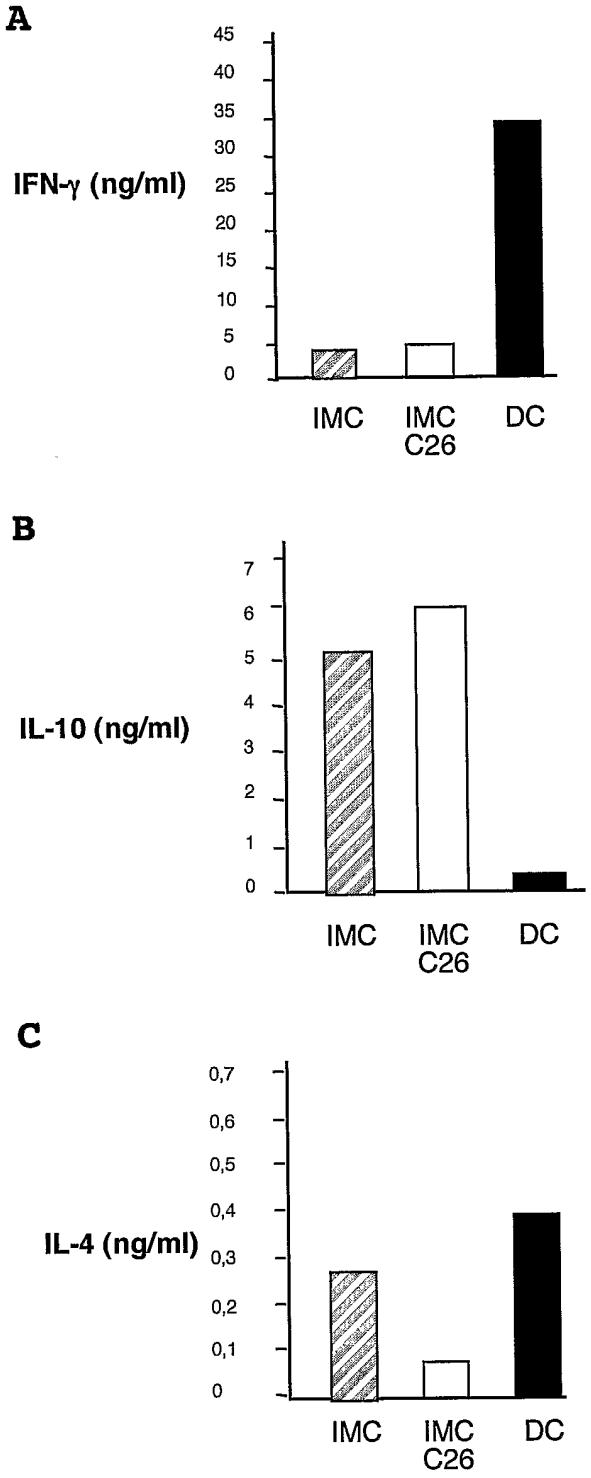


C

Figure 4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40
35
30
25
20
15
10
5
0
IMC
IMC C26
DC
B
IL-10 (ng/ml)
7
6
5
4
3
2
1
0
IMC
IMC C26
DC
C
IL-4 (ng/ml)
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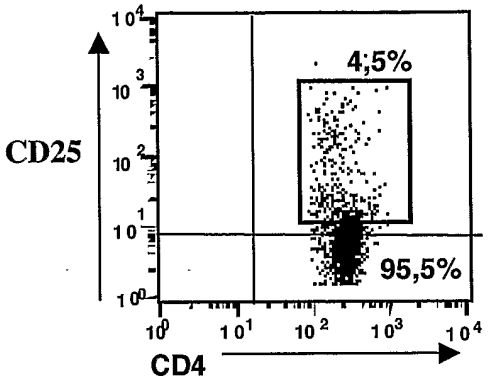
0,4
0,3
0,2
0,1
0
IMC
IMC C26
DC

Figure 5

A



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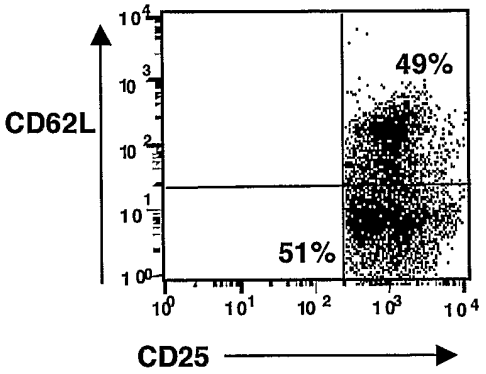


Figure 6

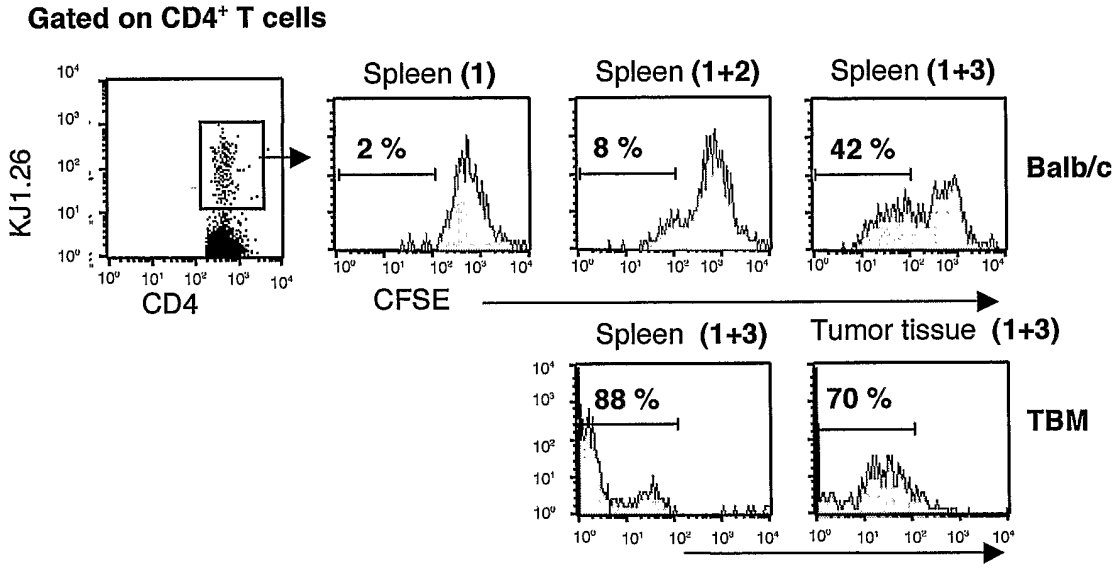
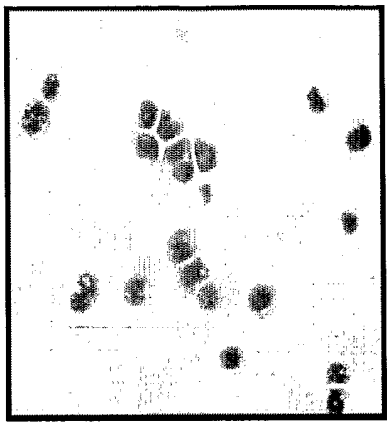

Gated on CD4+ T cells
KJ1.26
CD4
Spleen (1)
2 %
Spleen (1+2)
8 %
Spleen (1+3)
42 %
Balb/c
CFSE
Spleen (1+3)
88 %
Tumor tissue (1+3)
70 %
TBM

Figure 7

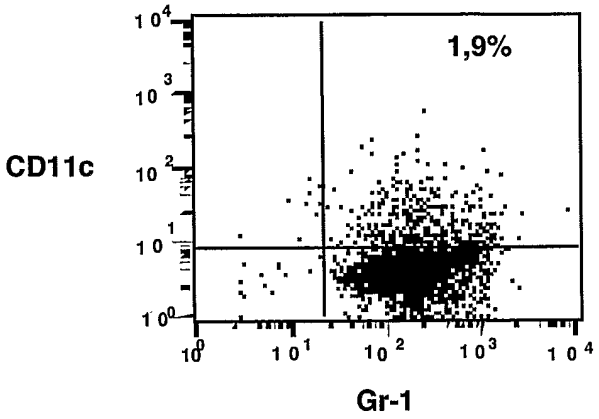
A



B

Figure 8

A



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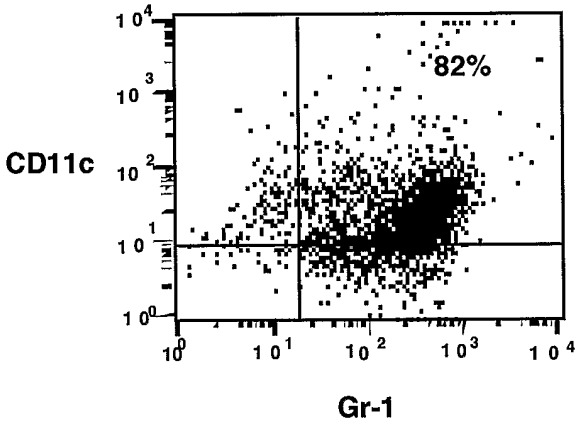
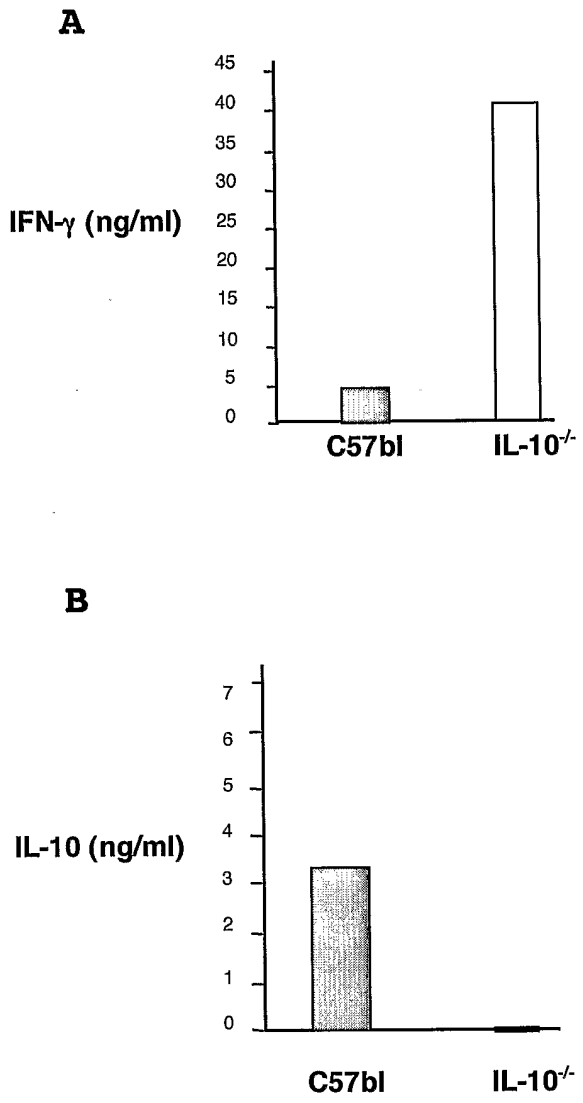


Figure 9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IFN-γ (ng/ml)
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40
35
30
25
20
15
10
5
0
C57bl
IL-10-/-
B
IL-10 (ng/ml)
7
6
5
4
3
2
1
0
C57bl
IL-10-/-

Figure 10

CCTGCAGGCG TTACATAACT TACGGTAAAT GGCCCGCCTG GCTGACCGCC CAACGACCCC
CGCCCATTGA CGTCAATAAT GACGTATGTT CCCATAGTAA CGCCAATAGG GACTTTCCAT
TGACGTCAAT GGGTGGAGTA TTTACGGTAA ACTGCCCACT TGGCAGTACA TCAAGTGTAT
CATATGCCAA GTACGCCCCC TATTGACGTC AATGACGGTA AATGGCCCGC CTGGCATTAT
GCCCAGTACA TGACCTTATG GGACTTTCCT ACTTGGCAGT ACATCTACGT ATTAGTCATC
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GATCTGGAGA CAGTGCTAAC CTCTAGACCA CCTCAGCCCG CATCTGGCTC CGTCTCTCCT

Figure 11 (1/4)

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Figure 11 (2/4)

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AGTGAAACAG GAAACCTTCA CCCTCACGGT CCCCATAGGG GTGAGAAAGT TTTGTGTCAA
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Figure 11 (3/4)

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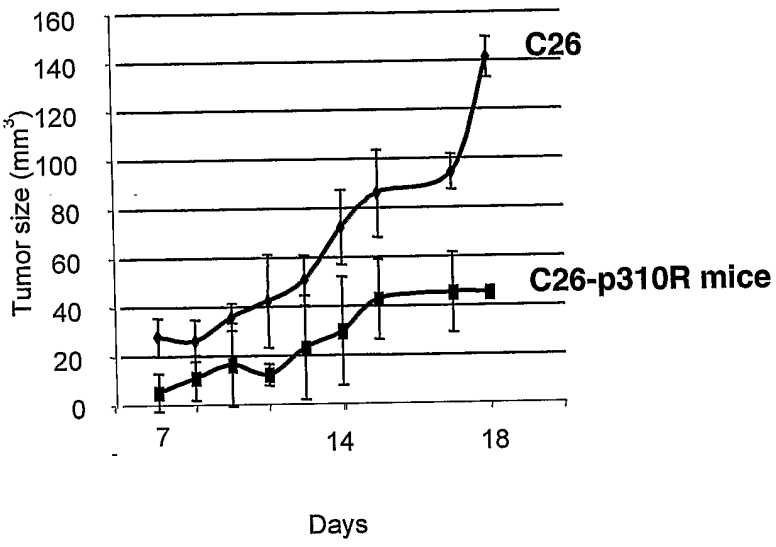
Figure 11 (4/4)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140
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Tumor size (mm3)
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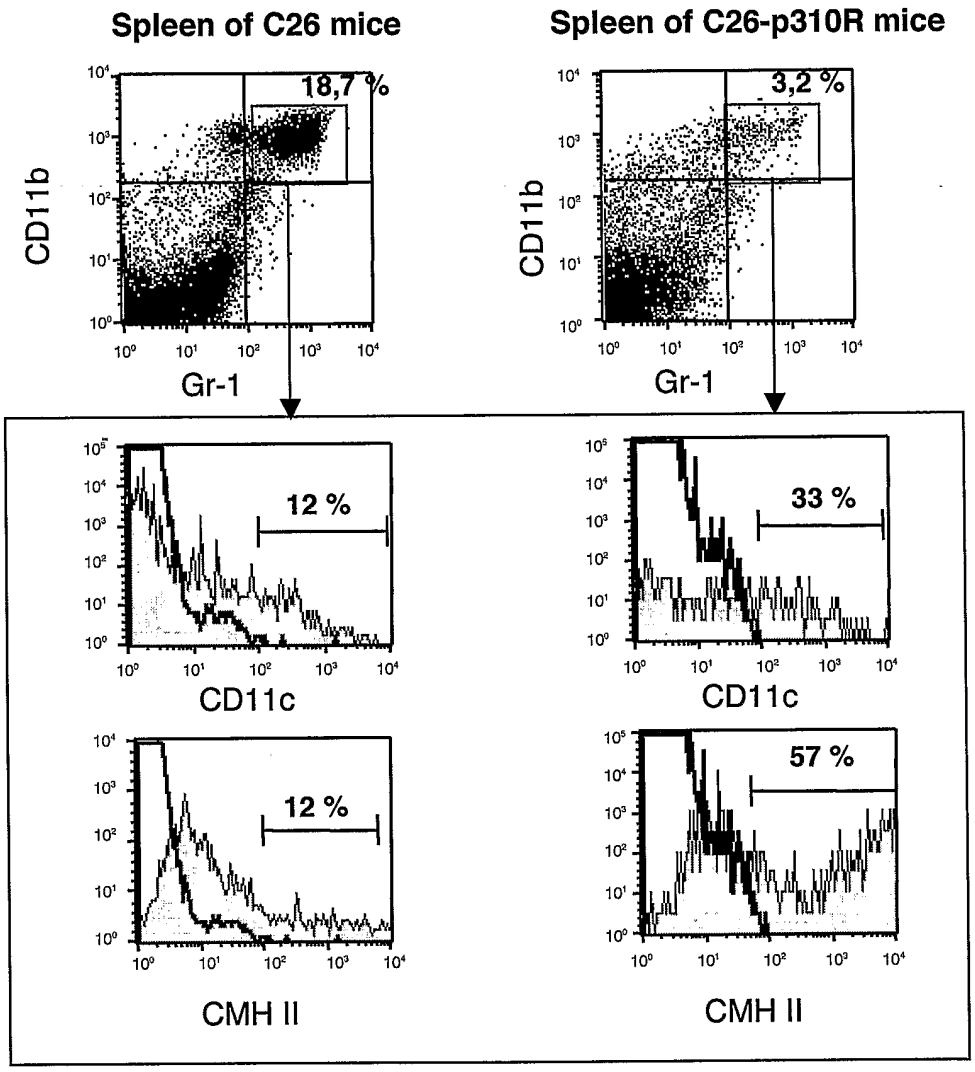
14
18
Days
C26
C26-p310R mice

Figure 12


Spleen of C26 mice
Spleen of C26-p310R mice
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CD11b
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12 %
33 %
CD11c
12 %
57 %
CMH II

Figure 13

SEQUENCE LISTING

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WAKKACH, Abdelilah
BLIN-WAKKACH, Claudine
MOMIER, David
CARLE, Georges

<120> COMPOSITION FOR TREATING CANCER ADAPTED FOR INTRA-TUMORAL ADMINISTRATION
AND USES THEREOF

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<170> PatentIn version 3.1

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Ser Cys Asp Leu Thr Thr Phe Thr Leu Asp Leu Tyr His Arg Ser Tyr
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Gly Tyr Arg Ala Arg Val Arg Ala Val Asp Asn Ser Gln Tyr Ser Asn
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Trp Thr Thr Thr Glu Thr Arg Phe Thr Val Asp Glu Val Ile Leu Thr
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Val Asp Ser Val Thr Leu Lys Ala Met Asp Gly Ile Ile Tyr Gly Thr
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