

Absence of surface expression of CD137 (4-1BB) on Myeloid-derived suppressor cells

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AUSENCIA DE EXPRESIÓN DE CD137 (4-1BB) EN CÉLULAS MIELOIDES SUPRESORAS

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RESUMEN

Las células mieloides supresoras (*Myeloid-derived suppressor cells*, MDSC) pertenecen a un subtipo de leucocitos causantes de inmunosupresión en individuos portadores de tumor. En ratones, estas células han sido definidas como CD11b⁺GR1⁺IL-4Rα⁺ y, debido a la presencia de tumores en modelos experimentales, se acumulan tanto en la lesión tumoral como en los órganos linfoides.

CD137 (4-1BB) es un receptor de coestímulo de la familia de receptores del factor de necrosis tumoral (TNF) principalmente expresado sobre la membrana de linfocitos T y de células NK (*Natural Killer*) activados, aunque también se encuentra en la superficie de otros leucocitos de estirpe mieloide como mastocitos, granulocitos, macrófagos, células dendríticas y endotelio. Anticuerpos agonistas frente a CD137 incrementan la respuesta inmune antitumoral potenciando los CTLs antitumorales. En este trabajo, células de carcinoma de colon C26 transfectadas para expresar GM-CSF se inocularon por vía subcutánea a ratones singénicos debido a sus propiedades inductoras de un gran aumento en el número de las MDSCs. Mediante citometría de flujo multicolor hemos confirmado un notable aumento en el número de estas células CD11b⁺GR1⁺IL-4Rα⁺ tanto en el estroma tumoral como en el bazo de los ratones portadores de tumor. La expresión de CD137 en este subtipo celular sin embargo, mostró resultados negativos. Por tanto, se pueden excluir los efectos directos de los mAbs sobre MDSCs como mecanismo de acción de la inmunoterapia con anticuerpos anti-CD137. Según esto las terapias dirigidas a disminuir el número o función de MDSCs podrían sinergizar con anticuerpos inmunoterapéuticos anti-CD137 ya que actúan sobre dianas diferentes.

ABSTRACT

Myeloid-derived suppressor cells (MDSC) are a subset of leukocytes associated with local and systemic T-cell immunosuppression in tumor-bearing hosts. In mice these cells are best defined as CD11b⁺GR1⁺IL-4Rα⁺ and their numbers increase in response to the presence of an experimental malignancy both at the tumor lesion and in lymphoid organs. CD137 is a co-stimulatory receptor that belongs to the tumor necrosis factor (TNF) receptor family characteristically expressed on activated T cells and NK cells. Its expression has also been reported on myeloid cells such as mastocytes, granulocytes, macrophages, dendritic cells, and on endothelium. Agonist antibodies against CD137 are known to increase the antitumor immune response through augmenting the intensity of antitumor CTLs. In this study C26 colon carcinoma cells transfected to express GM-CSF were subcutaneously implanted in syngeneic mice because of its properties as the most potent inducer of MDSCs. Indeed, multicolor flow cytometry confirmed a dramatic numeric increase in CD11b⁺GR1⁺IL-4Rα⁺ cells both in the tumor stroma and in the spleen of tumor-bearing mice. Analysis of CD137 expression on this cell subset rendered completely negative results. Therefore direct effects of immunotherapeutic anti-CD137 monoclonal antibodies on MDSCs can be excluded as a mechanism of action, thus indicating that therapies aimed at decreasing MDSCs might synergize with immunotherapeutic anti-CD137 antibody as a result of dealing with different targets.

KEY WORDS: MDSCs / CD137 / Immunosuppression.

PALABRAS CLAVE: MDSCs / CD137 / Inmunosupresión.

INTRODUCTION

Myelopoiesis in tumor bearing hosts is altered in such a way that an immunosuppressive cellular subset is increased in the bone marrow, lymphoid tissue and tumor stroma^(1,2). These myeloid-derived suppressor cells (MDSCs) are considered immature myeloid precursors that accumulate in tumor bearing hosts^(1,3). The main activity of MDSCs is contact-dependent inhibition of CD8⁺ T cells specific to peptide antigens presented by MHC-I presented on MDSCs⁽⁴⁾. The result is antigen specific T cell tolerance that seems to imply anergy rather than T-cell apoptosis. MDSCs share surface markers with granulocytes and respond to GM-CSF. The classical definition for MDSCs in mice is CD11b⁺ GR1⁺ myeloid cells that can induce T-cell unresponsiveness. The presence of the IL-4R α -chain further identifies this population and confers responsiveness to IL-13⁽⁵⁾. This point is highlighted by the observation that MDSCs are not suppressive in mice with genetic alterations in IL-13/IL-4R α cytokine pathways^(6,7). The molecular mechanism of action of MDSCs to suppress T-cell function is still a matter of debate and seems to be multifaceted^(1,2). L-arginine metabolism is involved and, importantly, the activity of arginase in these cells is critical for their function in cooperation with the synthesis of oxygen free radicals and nitric oxide^(4,8,9). Recently an elegant paper showed that nitration under oxidative stress of the TCR and the CD8 molecules of a T cell contacting with MDSCs is involved in anergy induction⁽⁴⁾. From an experimental point of view, it has been observed that MDSCs accumulate up to astonishing numbers in mice bearing the C26 colon carcinoma transfected to produce GM-CSF⁽⁵⁾. In sharp contrast to many examples in which GM-CSF transfection leads to antitumor immunity via stimulation of dendritic cells presentation, GM-CSF enhances the growth rate of GM-CSF C26 transfectants as a result of MDSCs function^(5,10).

In humans it is known that tumor associated macrophages favour tumor growth and locally repress cellular immunity (11). However the exact nature of MDSCs in humans is less well defined and many nomenclature issues arise⁽³⁾. In spite of this confusion, interesting observations suggest also a role for L-arginine metabolism in human cancer patients⁽⁸⁾.

4-1BB (CD137) is a member of the TNF receptor family involved in costimulation of T cells⁽¹²⁾. Ligation of 4-1BB with agonistic antibodies costimulates T cells *in vitro* and potently increased antitumor and antiviral CTL responses *in vivo*⁽¹³⁾. To a lesser extent, tumor immunity can be enhanced by transfecting tumor cells to express the natural ligand (4-1BBL or CD137L) or a membrane-bound anti-CD137 single chain antibody⁽¹⁴⁾. The mechanism of action of the antitumor effects are ascribed to CD8⁺ T cell and NK cell stimulation, but other cellular players also expressing 4-1BB can not be

excluded at the present point of time. Impressive therapeutic data in mice⁽¹⁵⁾ have driven a humanized anti-CD137 antibody into clinical trials (ClinicalTrials.gov Identifiers: NCT00461110, NCT00351325, NCT00309023). Intriguingly, anti CD137 treatments in mice have shown potential to ameliorate CD4⁺ T cell-mediated autoimmune conditions through not well understood mechanisms involving the repression of self reactive CD4⁺ T cells *in vivo*^(16,17).

4-1BB (CD137) is also expressed in various non-lymphoid bone marrow derived cells. For instance, it is expressed on activated dendritic cells⁽¹⁸⁾, proangiogenic endothelium⁽¹⁹⁾, mastocytes⁽²⁰⁾ and reportedly on granulocytes⁽²¹⁻²³⁾ (which are phenotypically close relatives of MDSCs). Therefore we reasoned that MDSCs might express 4-1BB and that ligation of CD137 on these cells might interfere with their suppressive function. However, conclusive multicolour flow cytometry negative results exclude mouse MDSCs as a site of action of anti-CD137 mAbs in tumor immunotherapy.

MATERIALS AND METHODS

Cell line

The C26-GM cell line was derived from the C26 colon carcinoma (H-2d) genetically modified to release GM-CSF, and was a kind gift from Dr. Mario Colombo (Milano, Italy)⁽⁵⁾. C26-GM cells used in this study produced GM-CSF at levels of 10–15 ng/ml from 10⁶ cells in 48 hours. These cell lines were grown in RPMI 1640 + Glutamax (GIBCO), 50 μ g/ml 2-mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% heat-inactivated FBS (SIGMA).

Mice and tumor induction

BALB/c mice (6–10 weeks old) were purchased from Harlan Laboratories (Barcelona, Spain). Mice were inoculated s.c. on the right flank with 5 \times 10⁵ C26-GM tumor cells. All animal procedures were conducted under institutional guidelines that comply with national laws and policies. Tumor diameters were monitored with a precision caliper⁽¹³⁾.

Cell isolation

Tumor-bearing or tumor-free mice were sacrificed and their spleens and tumor explants were harvested under sterile conditions. Splenocytes were isolated from both tumor bearing and tumor free mice at the indicated time points. Briefly, spleens were incubated in collagenase and DNase (Roche, Basel, Switzerland) for 15 min at 37°C, and spleens were mechanically disrupted before being passed through a sterile 70 μ m nylon mesh filter (BD Falcon, San Jose, CA). Erythrocytes were lysed with ACK buffer. Tumor nodules

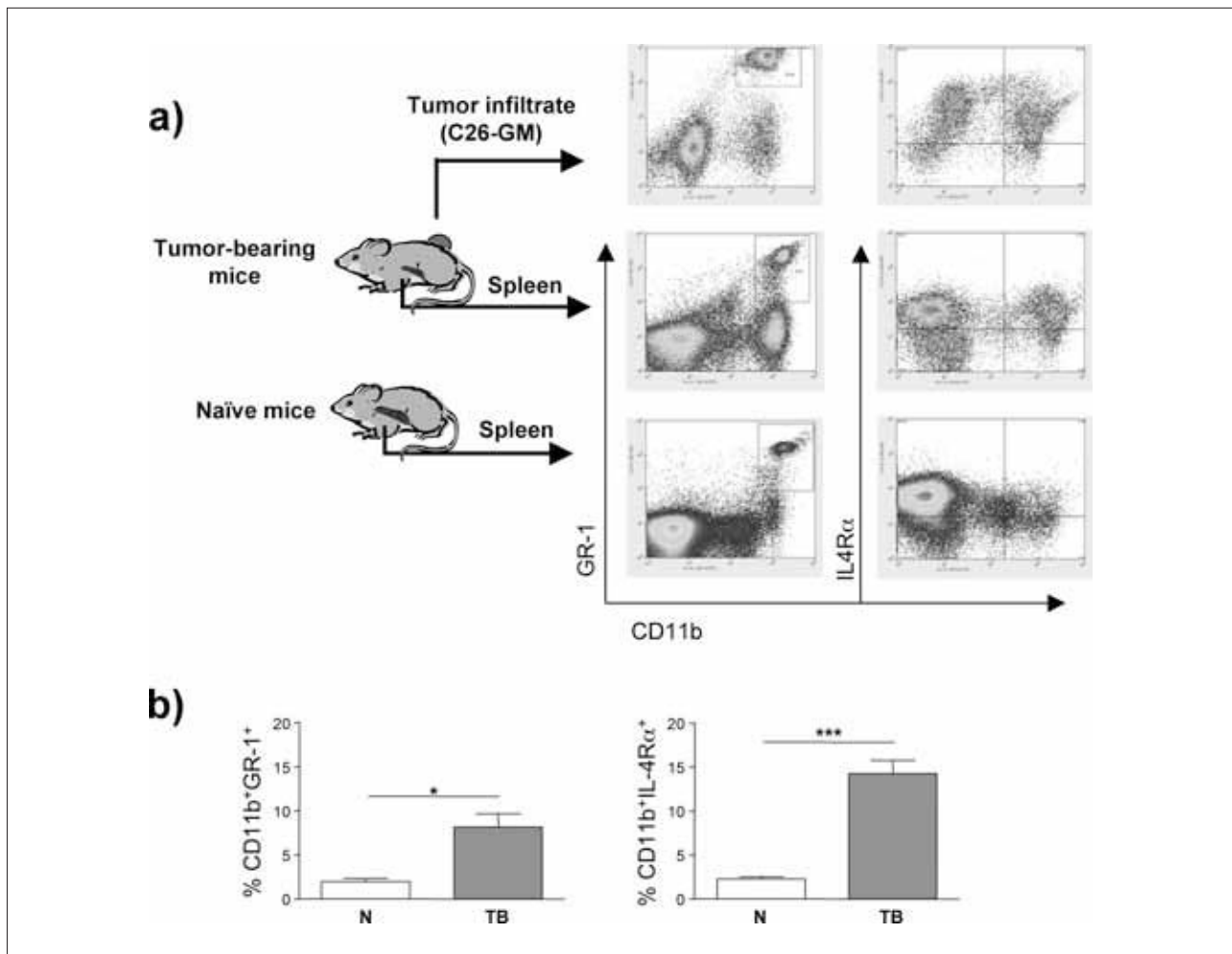


Figure 1. GM-CSF-producing C26 tumors cause the accumulation of MDSCs in syngeneic mice. *a)* Two color FACS analysis of the co-expression of CD11b and GR1 or IL-4R α on cell suspensions obtained from tumor nodules and spleens either from tumor bearing or healthy syngeneic BALB/c mice 8 to 10 days after tumor inoculation. *b)* Percentages of cells that coexpress CD11b and GR1 or IL-4R α were significantly increased in tumor bearing (TB) versus naïve (N) mice (* P < 0.05; *** P < 0.001 using Student's t-test). Data are the mean \pm SEM from 3 experiments.

were similarly processed and both cell suspensions were plated for FACS staining.

Antibodies and flow cytometry

Cells isolated from tumor or spleens (10^6 per sample) were pre-treated with anti-CD16/32 (clone 2.4G2; BD Biosciences-Pharmingen) to reduce non-specific staining. The following monoclonal antibodies were used for flow cytometry staining: fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD11b (M1/70; eBioscience), biotin-conjugated rat anti-mouse IL-4R α (mIL4R-M1; BD Biosciences-Pharmingen), phycoerythrin (PE)-conjugated hamster anti-mouse CD137 (17B5) and appropriate isotype control (BD Biosciences). Biotinylated antibodies were visualized with allophycocyanin (APC)-

conjugated Streptavidin (BD Biosciences). Labeled cells were analyzed on a FACSCalibur flow cytometer with the use of CellQuest software (BD Biosciences).

RESULTS

CD11b⁺GR1⁺IL-4R α ⁺ cells intensely accumulated in mice harboring C26-GM

As an experimental model to study tumor-related MDSCs, we subcutaneously inoculated mice with 5×10^5 cells from a C26 stably transfected to express GM-CSF at high levels, as described by Mario Colombo and coworkers⁽⁵⁾. Tumor nodules formed at the site of injection at day 4-5 after

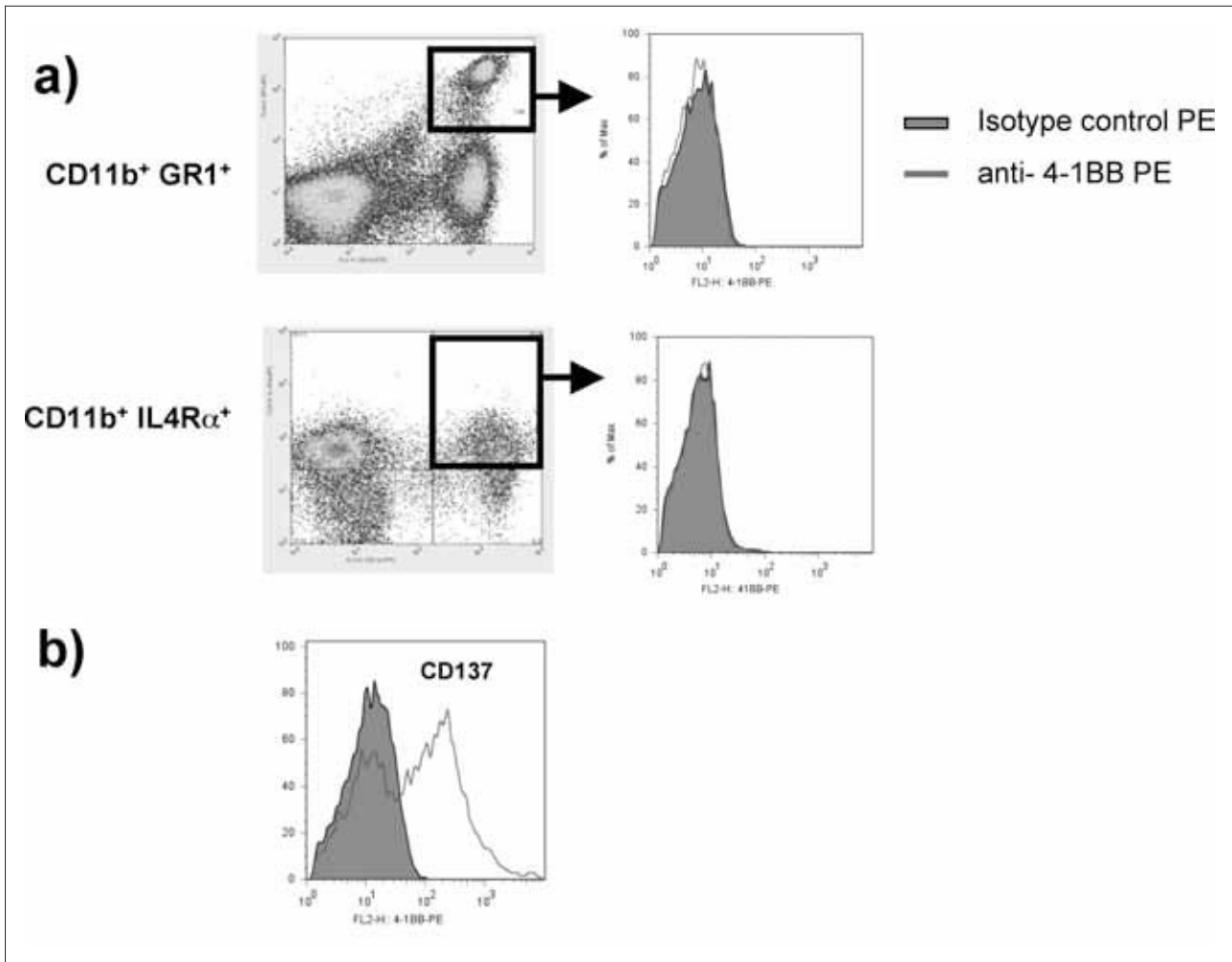


Figure 2. MDSCs do not express surface CD137. a) Analysis of CD137 expression in FACS histograms from cells electronically gated as CD11b⁺GR1⁺ or CD11b⁺IL-4Rα⁺. b) Expression of surface CD137 on CD3⁺CD8⁺ gated cells from cultures of BALB/c splenocytes stimulated with a combination of anti-CD3 and anti-CD28 monoclonal antibodies.

inoculation of cancer cells and steadily progressed to 4 to 8 mm of diameter at day 9. At this stage mice were euthanized and cell suspensions were obtained from the spleen and the tumor. These cells suspensions were stained with antibodies marking CD11b, GR1 and IL-4Rα. When compared to splenocytes of littermate healthy mice we could notice a strong augment of cells depicting a MDSCs phenotype (Figure 1a). Upon comparative quantitative analysis of a series of mice (n=5) statistical differences (Figure 1b) in agreement to published reports were observed.

Myeloid-derived suppressor cells fail to express surface CD137

The abundance of MDSCs in C26-GM tumor bearing mice permitted multicolor flow cytometry on this gated population

in order to study the surface expression of additional differentiation antigens. Repeated immunostainings showed completely undetectable expression of 4-1BB (CD137) on the surface of CD11b⁺ GR1⁺ / CD11b⁺ IL-4Rα⁺ cells from tumor bearing mice using various isotype matched negative controls also tagged with PE (Figure 2a). Similar negative data were observed from comparable cells in healthy mice probably reflecting granulocytes (not shown). As a positive control to exclude the possibility that the anti-4-1BB antibody were not functioning staining of activated T cells was carried out in parallel. Spleen cells were activated in culture with a combination of anti-CD3 and CD28 monoclonal antibodies for 24 hours and we readily detected expression of 4-1BB in CD3⁺ CD8⁺ gated T cells (Figure 2b). Accordingly, we can exclude expression of CD137 on MDSCs from tumor bearing mice at a level detectable by FACS.

DISCUSSION

This study was aimed at exploring as to whether CD137 (4-1BB) could be a suitable target for immunostimulatory monoclonal antibodies in MDSCs. If that hypothesis had been correct, we would have added another mechanism of action to explain the anti-CD137 mAbs therapeutic effects, postulating an inhibitory effect on MDSCs activity. This could have been anticipated from the activity of anti-CD137 mAb on mastocytes⁽²⁰⁾ that might be involved in the amelioration of murine asthma models upon anti-CD137 treatment⁽²⁴⁾. Absence of CD137 in MDSCs came as a surprise because its expression had been reported in granulocytes⁽²¹⁻²³⁾. However, we do not detect CD137 expression on CD11b⁺ GR1⁺ IL-4R α ⁺ cells from spleens of healthy mice that should contain granulocytes.

Our results confirm the dramatic accumulation of MDSCs in mice bearing C26-GM tumors both in lymphoid organs and in the tumor stroma⁽⁵⁾. Under these conditions the study of this immunosuppressive population is greatly simplified and would have permitted a detailed functional study of CD137 if it were expressed. However, it is still formally possible that a small number of CD137 molecules under the threshold of detection of sensitive of flow cytometry techniques could play an unlikely functional role during anti-CD137 mAb treatment.

Nonetheless, the news on the lack of CD137 expression on MDSCs encompass the important corollary of possible synergies between anti MDSCs⁽⁹⁾ and anti-CD137 and targeted therapies. This is based on the fact that they would be independent targets sharing a common mechanism of action as they involve antitumor cellular immunity as the final effector therapeutic activity.

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DISCLOSURES

Ignacio Melero consults for Bristol Myers Squibb in the field of development of CD137 therapy.

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