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Interleukin-21 expanded NKDC *in vitro* reduces the B16F10 tumor growth *in vivo*

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ABSTRACT

Innate immunity to tumors is mediated mainly by natural killer cells (NKs) and dendritic cells (DCs). The function of these cells is coordinated by cytokines produced during the inflammatory process. NK cells are highly active against tumors, being an important source of IFN-γ. Natural killer dendritic cells (NKDCs) were recently identified as a group of hybrid cells; some studies claim that they have lytic activity, produce IFN-γ and can also stimulate antigen-specific T cells. Interleukin 21 (IL-21) regulates the proliferation capacity and cytotoxicity of NK and T cells. The main objective of this study was to investigate if IL-21 influences the frequency of NKDCs *in vitro* as well as IFN-γ production and also to verify if these cells could enhance the antitumor activity against B16F10 tumor model *in vivo*. Splenocytes from C57BL/6 mice were isolated and the DC were enriched by immunomagnetic beads and cultured for four days with recombinant IL-21 (10, 20, 40 or 100 ng/ml). NKDC population was characterized as $CD11c^{low/med}B220^{+}NK1.1^{+}$. Expanded cells were used to treat B16F10 tumor bearing mice and tumor growth was compared between the doses of IL-21 10 ng/ml and 20 ng/ml. The results indicate that IL-21 increases the expansion of splenic NKDCs *in vitro* in doses of 10 ng/ml and 20 ng/ml and these cells produce IFN-γ. *In vivo*, cells expanded with IL-21 and injected directly into the growing tumor efficiently reduced the tumor size. Together, these results showed for the first time that IL-21 influences the biology and the effector activity of NKDCs.

1. Introduction

One of the main effective responses against tumor cells is cytotoxicity [1,2]. This response is provided mainly by $CD8^{+}$ T lymphocytes and natural killer cells (NKs) [3–5]. NK cells are components of innate immunity acting as killers of target cells independently of TCR stimulation and producing IFN-γ [6], while $CD8^{+}$ T lymphocytes belong to adaptive immunity and kill tumor cells after antigen recognition on MHC class I molecules [7,8]. The cytotoxicity of both NK cells and $CD8^{+}$ T lymphocytes is mediated by granzymes and perforines, and IFN-γ secretion is also increased during the activity of both cells [9]. Recently, a new group of cells was identified with the ability of killing, producing IFN-γ, and probably antigen presentating capacity. These cells were called natural killer dendritic cells (NKDCs) [10] or IFN-γ producing dendritic cells (IKDCs) [11]. NKDCs can be found in lymphoid and non-lymphoid organs and are highly stimulated in presence of CpG to secrete IFN-γ and lyse specific target cells [12]. They represent 1–2% of spleen derived $CD11c^{+}$ cells in unmanipulated C57BL/6 mice (no tumor or infection) [13]. Very low numbers of NKDCs are isolated from mice without any specific enrichment treatment. Some authors have considered the use of molecules such as CpG, IL-15, IL-18 and Flt3 to expand NKDCs *in vivo* or *in vitro*. The synergistic action of CpG plus IL-18 *in vivo* increases by about 6-fold the number of splenic NKDC, in which 90% of cells produce IFN-γ, and this mechanism is dependent on autocrine action of IL-12 produced by NKDC [14].

Interleukin-15 knockout mice lack NKDCs, however this population can be restored with exogenous administration of recombinant IL-15; after this treatment, cells exhibit expressive anti-tumor activity, even higher than NK cells [15]. Interleukin-21 (IL-21) belongs to the same cytokine family as IL-15 (common γ-chain receptor users) that induces proliferation of lymphocytes [16]. IL-21 is mainly produced by activated $CD4^{+}$ T cells [17] and has a pivotal role in NK cell effector function [18–20]. Murine NK cells upon IL-21 stimulation improve the IFN-γ production and killing activity in tumor mouse models [21]. Treatment of human NK cells with IL-21 increases the expression of anti-apoptotic proteins such as BcL-2 and BcL-x(L) and enhances viability of NK cells, without substantial effects on proliferation [22]. This cytokine has been intensively investigated for development and optimization of vaccines and immunotherapy against tumors, mostly based on NK cell induction and activation.

Considering the dependence of NKDC to other cytokines from family of γ chain receptor users, specially IL-15, and the high influence of IL-21 on NK cells biology, we hypothesized that the recombinant IL-21 would have an effect on NKDC frequency and

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antitumor activity. Our results indicate that IL-21 increases specifically the frequency of NKDC cells *in vitro* and these cells also produce IFN-γ. After expansion with IL-21 cells presented killing activity against B16F10 tumor cells *in vitro* and adoptive transfer of NKDC cells resulted in reduction of tumor size *in vivo*. Collectively, our results suggest that IL-21 can be applied to expand anti-tumor NKDC IFN-γ^+ for use in immunotherapy against cancer.

2. Methods

2.1. Mice

C57BL/6 (B6) mice were purchased from Fundação Estadual de Produção e Pesquisa e Saúde (FEPPS) Porto Alegre, RS, Brazil. Mice were housed under pathogen-free conditions at Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) Faculdade de Biociências (FABIO) animal facility with *ad libitum* access to food and water. Female six to eight week old mice were used for all experiments, in groups of $n = 5$ animals and these were conducted with the approval of PUCRS' Ethics Committee on Animal Research (CEUA).

2.2. Antibodies, proteins and reagents

Antibodies were purchased from BD Biosciences. Characterization of NKDC population was conducted by surface staining with Cy-Chrome conjugated anti-CD11c, fluorescein isothiocyanate (FITC) conjugated anti-B220 (CD45R) and phycoerythrin (PE) conjugated anti-NK 1.1. For interferon analysis, FITC conjugated anti-IFN-γ, Cy5 conjugated anti-B220, Cy7 conjugated anti-CD11c, and PE conjugated anti-NK1.1 were used. The expression of IL-21R was determinate using PE conjugated mouse anti-IL-21R. Flow cytometry analysis was done on FACS Canto (BD Bioscience). Recombinant IL-21 and anti-IL-21 was purchased from eBioscience (San Diego, CA, USA). The data were analyzed using FlowJo software (Tree Star).

2.3. Cell isolation

Cellular isolation was done as previously described [12]. Briefly, animals were euthanized by CO_2 inhalation and splenocytes were isolated from spleen. After lysis of erythrocytes with cell lysis buffer (8.26 g ammonium chloride; 1 g potassion bicarbonate; 0.037 g EDTA/liter) cells were enriched based on CD11c expression with immunonagnetic beads using the manufacturer's protocol MAC's (Milteniy Biotec). The purity of preparation was analyzed in FACS using Cy-chrome conjugated anti-CD11c antibody.

2.4. Expansion of NKDC in vitro

Cells recovered after CD11c enrichment were cultured in 24 well plate (1×10^6 cells/ml) using Dulbecco's Modified Eagle's Medium (DMEM – Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS – Gibco, Carlsbad, CA, USA) Gentamicin 80 mg/l (Novafarma, Anápolis, Brazil), Fungisone – Anfotericin B, 5 mg/l (Bristol Myers Squibb, New York, NY, USA), 2 mM L-Glutamine (Sigma, St. Louis, MO, USA) and 1× MEM amino acid solution (Invitrogen Corporation, Carlsbad, CA, USA). The medium was supplemented with recombinant IL-21 (IL-21) or only saline. Concentrations of 10 ng/ml, 20 ng/ml, 40 mg/ml and 100 ng/ml of IL-21 were used for *in vitro* treatment, based on our previous studies [23]. Cells were incubated in 37 °C with atmosphere of 5% CO_2. Four days after culture, total cells were removed from the plate, rinsed with PBS and stained with anti-CD11c, anti-NK1.1 and anti-B220. The frequency of NKDCs ($CD11c^{low/med}$, $NK1.1^+$, $B220^+$) was compared among NKDC cultured only with DMEM/saline and IL-21 treatments. To determine, if the effects observed after treatment of NKDC with IL-21 were specific, an antibody neutralization method was used. The media described above were treated for one hour with different concentrations of anti-murine IL-21 antibody (2, 5 or 10 μg/ml) to neutralize the IL-21 protein and block the binding to its receptor on cells surface.

2.5. Cytokine analysis

Interferon gamma production by IL-21 treated NKDC was analyzed through intracellular staining. Briefly, dendritic cells were isolated as described in Section 2.3 and cultured with recombinant IL-21 (10 or 20 ng/ml) in DMEM media supplemented with 10% FBS, DMEM/saline was used as control. Four days after culture in CO_2 incubator the total expanded cells were treated with Golgi plug (Becton and Dickson) for four hours and the cells stained. For this analysis the total expanded cells were stained with anti-CD11c, Cy-5 conjugated anti-B220 and anti-NK 1.1. After staining cells were permealized and again stained with anti-IFN-γ.

2.6. Tumor cell lines

B16F10 melanoma cells, a kind gift from Dr. Peter Henson (National Jewish Center for Immunology, Denver, CO, USA) were cultured DMEM supplemented with 10% FBS in conditions of 37 °C and atmosphere of 5% CO_2.

2.7. In vitro cytotoxic assay with B10F10

The *in vitro* cytotoxic assay was performed as previous described by Jedema and collaborators [40]. Briefly, NKDCs (effectors cells) were enriched from a culture of dendritic cells using IL-21 as stimuli (10 or 20 ng/ml), meanwhile cell suspension of B16F10 cells were stained with CFSE (target cells). After expansion 6×10^4 cells were cultured with CFSE stained B16F10 stimulated at different ratios for four hours in CO_2 incubator. The proportion of effectors and target cells used was E/T 1:6 (1×10^4); 1:12 (0.5×10^4) and 1:24 (0.25×10^4).

2.8. IL-21 treatment and tumor injections

Naïve C57BL/6 mice were inoculated subcutaneously with 10^5 B16F10 melanoma cells in the right thigh diluted in 100 μl of phosphate-buffered saline (PBS). After 4 days of tumor growth 1×10^5 IL-21 treated CD11c enriched cells were injected subcutaneously into the tumor site. For all animal experiments, tumor size was measured every 3 days using a caliper. All subcutaneous injections were done after anesthesia with 83 of mg/kg ketamine and 17 mg/kg of xilazine. Mice were killed in a CO_2 chamber 20 days after tumor injection. Tumor volume (V) (mm^3) was calculate using $V = d^2 \times D \times 0.5$, in which d = minor diameter and D = major diameter.

2.9. Statistical analysis

The Mann Whitney test was used to groups comparison. Statistical analysis and graphs were performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Interleukin-21 increases the frequency of splenic NKDC in vitro

Considering previous observations about the activity of IL-21 on NK cells, we investigated if this cytokine could also influence the

expansion of NKDC *in vitro*. Mice were euthanized and CD11c$^+$ cells enriched by magnetic beads sorting. NKDC cells were characterized as CD11c$^{low/med}$B220$^+$NK1.1$^+$. For this characterization, we gated on CD11c$^{low/med}$ cells and inside this gate only cells positive for B220 and NK1.1 were selected (NKDC). NKDCs represented 0.95% from total isolated CD11c$^{low/med}$ cells in a culture without any stimuli (Fig. 1A–C). To test IL-21 as an inducer of NKDC, CD11c$^+$ isolated cells were counted and 1×10^6 cells were cultured in DMEM 1X supplemented with IL-21 (10 ng/ml, 20 ng/ml, 40 or 100 ng/ml), media with saline was used as control. When splenic purified DCs were cultured in media with IL-21 (20 ng/ml), the frequency of NKDC increased reaching a magnitude of 3.66% from the total CD11c$^+$, comparing with untreated cells a fold increase of 3.85 was observed (Fig. 1D). This result suggested that IL-21 can increase the numbers of splenic NKDC *in vitro* from a bulk culture of splenic DC. The population of CD11c$^{low/med}$B220$^+$ cells that were negative for NK1.1 (plasmacytoid DC), which also are present in the CD11c$^+$ purified cells, did not change frequency upon treatment with any amount of IL-21 (data not shown). To investigate if the expansion of NKDC with IL-21 is a dose dependent phenomenon, higher quantities of the cytokine were used. As shown in Fig. 1E, an expansion was observed with the doses of 10 and 20 ng/ml, but decreased when higher doses were used.

Since we observed that IL-21 expands NKDC *in vitro*, we decided to investigate if this is a specific phenomenon induced by this cytokine. The media with IL-21 was treated for one hour with three different concentrations of murine anti-IL-21 antibody (2 μg/ml, 5 μg/ml or 10 μg/ml) and then added to supplement the culture of purified CD11c$^+$ cells. Four days after culture, NKDCs were analyzed. As seen in Fig. 1F, the percentage of NKDC in the wells treated with anti-IL-21 decreased compared to wells without neutralizing treatment. The NKDC percentage decreased

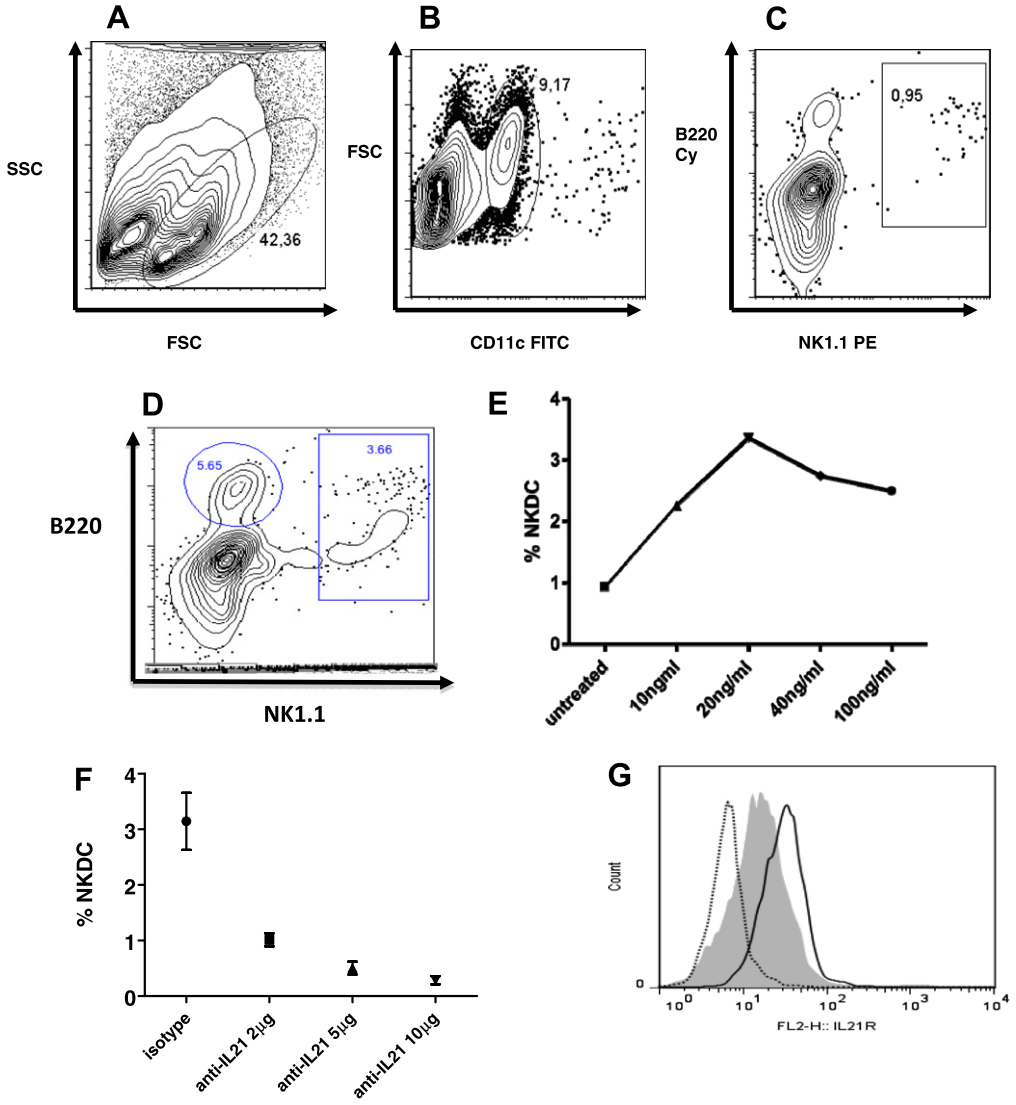

Fig. 1. NKDC characterization and expansion with IL-21. Isolated splenocytes from C57BL/6 were treated with RBC buffer and cells were enriched based on CD11c expression with immunonagnetic beads. Cells were stained with CD11c FITC, B220 Cy-chrom and NK 1.1 PE and analyzed by FACS. The NKDC population were identified with a first gate on SSC × FSC (A), inside this population a second gate with CD11c$^+$ cell was done (B), and finally the B220$^+$ NK1.1$^+$ cells were taken from CD11c$^+$ cells (C). (D) Purified CD11c cells (1×10^6) were cultured with 20 ng/ml of IL-21 for four days and cells stained with the same antibodies used for characterization. The percentage of NKDCs reached 3.66% from the total CD11c$^+$ cells; pDCs (Circled population). (E) Purified CD11c$^+$ cells were treated with different doses of IL-21 (10, 20, 40 or 100 ng/ml) and the percentage of NKDCs compared. (F) Interleukin-21 (20 ng/ml) was incubated with anti-IL-21 (2 μg, 5 μg or 10 μg) by one hour and used to culture CD11c$^+$ cells four days after the percentage of NKDCs were analyzed. (G) Isolated splenic DCs were stained with CD11c Cy7; B220 Cy-chrom; Nk1.1 FITC and IL-21R PE. Cells were analyzed using FACS and the expression of IL-21R was determined in unstimulated lymphocytes (dashed); pDCs (tinted) and NKDCs (solid). The histogram represents the MFI from these three populations, lymphocytes (4.27) pDCs (10.78) and NKDCs (24.95).

proportionally to the amount of anti-IL-21 added. Taken together these results shows that *in vitro* treatment of splenic isolated CD11c$^+$ cells with recombinant IL-21 increases specifically the numbers of NKDCs.

3.2. Purified NKDC express IL-21R on cell surface

Since we have shown that culture of splenic purified CD11c$^+$ with IL-21 can increase NKDC numbers we should also consider that this observation could be related with other bystander effect produced by any factor released from cells in culture. To eliminate this hypothesis we investigated if fresh NKDC from spleen expresses the IL-21 receptor on surface. Again, splenic CD11$^+$ cells were isolated by immunonagnetic beads stained for CD11c, B220, NK1.1 and IL-21R. The expression level of IL-21R on NKDC was compared with the pDC population that previously did not expanded with any concentration of IL-21, as negative control unactivated lymphocytes were used. Fig. 1G shows the histogram of these three populations analyzed. By one hand NKDC expressed higher levels of IL-21R than pDC that did not grow with IL-21, by the other hand unactivated lymphocytes did not express IL-21R compared with pDC and NKDC. The MFI of IL-21R for NKDC cells was 24.95, for pDC it was 10.78 and for lymphocytes it was 4.27. This data indicates that the fresh population of NKDC, inside total isolated CD11c$^+$ from spleen expresses IL-21R and it is ready to respond to recombinant IL-21.

3.3. IL-21 expanded NKDC produces IFN-γ in vitro

We observed that addition of IL-21 in a culture of splenic purified CD11c$^+$ cells stimulated the expansion of NKDCs. Because one of the main characteristics of these cells is the production of IFN-γ, we investigated if the expanded NKDC with IL-21 would also produce IFN-γ. An intracellular staining experiment was performed using total cells recovered after expansion. When cells were cultured only with media and saline, 18.4% from CD11c$^{low/me}$B220$^+$ NK1.1$^+$ (NKDC) were IFN-γ positive (Fig. 2A). The addition of recombinant IL-21 in the culture lead to a slight increase in this population, since when 10 ng/ml was used the percentage of NKDC IFN-γ$^+$ cells increased from 18.4% to 24.6%. When a concentration 20 ng/ml was used NKDC IFN-γ$^+$ increased to 32.7%. The fold increasing for 10 ng/ml and 20 ng/ml of IL-21 were 1.3 and 1.8, respectively, however, only the concentration of 20 ng/ml increased significantly (Fig. 2B). This data indicate that NKDCs expanded with IL-21 *in vitro* produced IFN-γ.

3.4. NKDC expanded with interleukin-21 kills B16B10 tumor cells in vitro and reduce the growth of the tumor in vivo

Our data indicated that NKDC were expanded from a culture of splenic CD11c$^+$ with IL-21. In order to show that these expanded NKDCs directly kill the tumor cells we used an *in vitro* CFSE-based cytotoxic assay with B16F10 tumor cells line as a target. As shown in Fig. 3A there was a reduction in B16F10 CFSE$^+$ cells when they were incubated with NKDC expanded with IL-21, and even though there was no significant difference between cells treated with 10 ng or 20 ng of IL-21, a slight reduction with the higher dose of IL-21 was observed.

We then investigated if NKDC recovered after IL-21 treatment could also inhibit the growth of B16F10 tumors *in vivo*. In order to do that, B16F10 cell were injected subcutaneously in the right thigh in C57BL/6 mice. Four days after injection, mice were treated with 1×10^5 NKDC recovered from cultures with media or media supplemented with IL-21 (10 ng/ml or 20 ng/ml) intratumorally. The leg volume was analyzed every three days. Up until the 15th day after injection we did not observe any difference between the groups. However, after 20 days, tumor growth was clearly impaired in the group of mice that were treated in the tumor site with NKDC stimulated with 10 ng/ml or 20 ng/ml of IL-21, compared with the group of mice treated with NKDC cultured only with media (Fig. 3C–E). The mean tumor volume was 326.1 mm^3 in the group treated with NKCDs cultured with 20 ng/ml of IL-21, while the mean volume was 1646.1 mm^3 in NKDC untreated group (Fig. 3F), a 5 fold significant difference. This result indicated that a preparation of cells in which NKDC were stimulated and enriched with IL-21 has anti-tumoral effect.

4. Discussion

In this report we showed that IL-21 increases the numbers of NKDCs in a splenic DC culture and the expanded cells also improved the antitumor activity against B16F10 tumor cells. Although this is an already terminally differentiated population, treatment with this cytokine increased frequency of these cells *in vitro* and they are also producing IFN-γ. To our knowledge, this is the first study that evaluated the action of IL-21, the most recently described member from γ-chain user family, over NKDC, both *in vitro* and *in vivo*.

For characterization of NKDC we set up a combination of cells markers. The NKDCs were characterized as CD11c$^{low/med}$B220^{+}-NK1.1$^+$ since CD11c is highly expressed on DC populations. B220 is always expressed by NKDC and NK1.1 is the marker that these cells share with NK cells. To characterize NKDC, others have previously used different combinations of markers. Ullrich and colaborators characterized these cells as CD3$^-$CD11c$^+$B220$^+$NK1.1$^+$; Himoudi used CD11c^{int}B220$^+$CD49$^+$ [24]; Vosshenrich used CD11c^{low}B220$^+$ [25]; Geurtsvankessed considered NKDC as CD11c^{int}B220$^+$Nk1.1$^+$ [26] and Plitasused CD3$^-$CD11c$^+$NK1.1$^+$ [27]. However, NKDCs are better characterized as a population that express low or intermediate levels of CD11c, high expression levels are more appropriated to characterize conventional DCs instead of NKDCs [25,28].

Our findings indicate that the small splenic population of NKDCs was increased by about four times after *in vitro* culture of freshly isolated CD11c$^+$ with IL-21 (0.985% vs 3.66%). This increase is related to the dependence of NKDC from cytokines that use the γ-chain subunit for proliferation and growth, especially IL-15. Previous studies have reported that *in vivo* treatment with this cytokine increased the number of NKDCs in about 50%. Chaudhry and collaborators also showed that in mice knockout to IL-15 gene the percentage of splenic NKDC are even less than 0.5% from total CD11c, implying that these cells are dependent on IL-15 to exist. Injection of IL-15 increased the number of splenic NKDCs in about 3-fold over the baseline [15,30]. Interleukin-2, another γ-chain cytokine, also induces proliferation and anti-tumor activity of NKDCs *in vivo*, when combined with imatinib mesylate, a drug for stromal gastrointestinal cancer [28]. However, culture of NKDC with IL-2 resulted in <20% cells viability by day 3 and near complete cell loss on day 7 [15]. Taken together, these studies indicate that any procedure to increase the NKDC population or function should consider the use of a γ-chain cytokine, as we did using IL-21 to induce these cells. We believe that the previous aspects observed with IL-15 corroborate our observation that NKDC can proliferate in response to IL-21 *in vitro*.

To confirm if the effect observed on NKDCs was a result from direct binding of IL-21 on the cell surface and not from any bystander action of another cell or factor released during the stimulation, we investigated if IL-21R was expressed on expanded NKDC. The pDCs were defined as CD11c^{low}B220$^+$NK1.1$^-$ and were used as negative control for expansion with IL-21 and analysis of IL-21R expression. We favored the use of pDC instead of NK cells as negative control

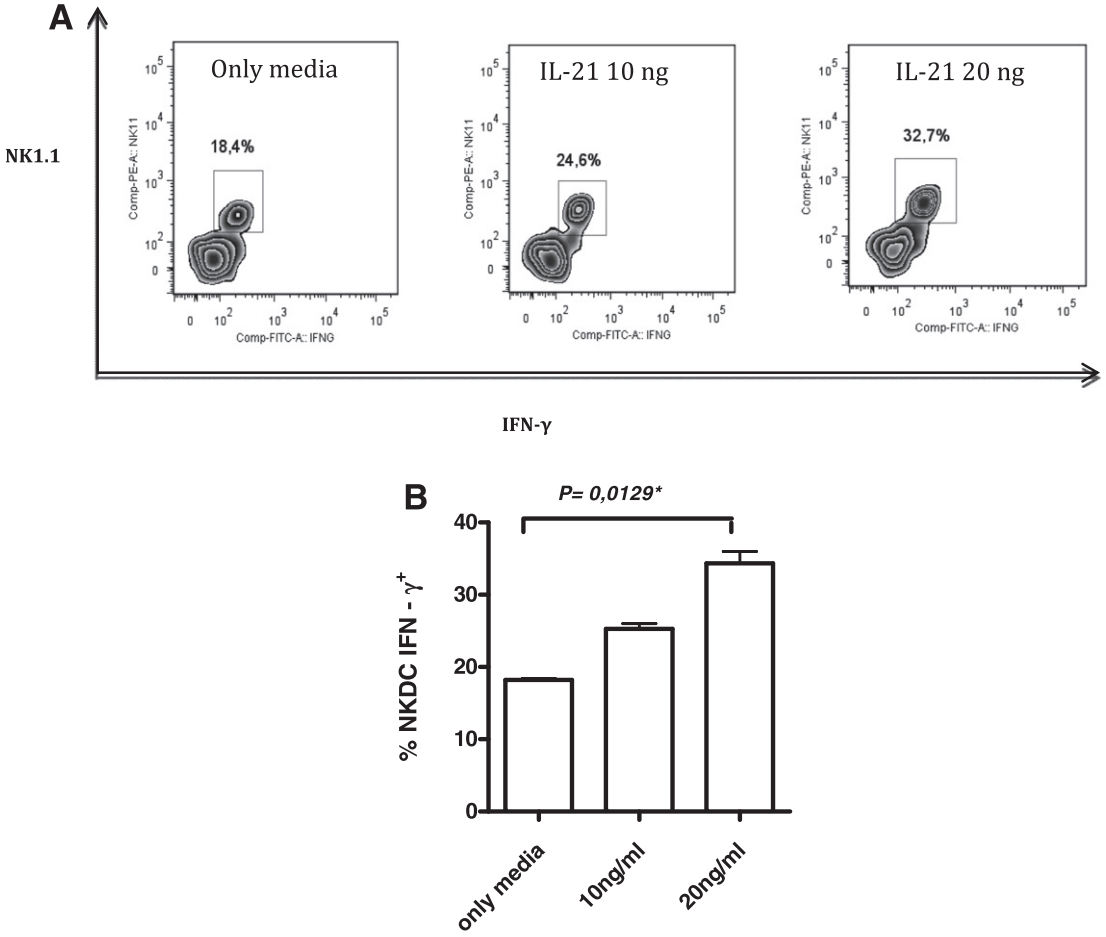

Fig. 2. IFN-γ production by NKDC: splenocytes were isolated from C57BL/6 mice and CD11c$^+$ cells isolated with immunomagnetic beads. Cells were cultured in DMEM 1 × 10% SFB with or without IL-21 (10 or 20 ng/ml) for four days. The IFN-γ was determined by intracellular staining. The cells were stained for surface marker CD11c, B220, NK1.1, and intracellular stained for IFN-γ. (A) Contour plot of NKDC IFN-γ$^+$ cells treated only with media/saline (18.4%), 10 ng/ml (24.6%) and 20 ng/ml (32.7%) of IL-21. (B) Percentage of NKDC IFN-γ$^+$ in all untreated and treated groups; These data represent the mean ± standard error of two independent experiments.$^*p < 0.05$.

because NKDC and pDC share more surface markers (B220, CD11c, CD11b) and arise from close related progenitors [32]. The results showed that freshly purified NKDC expressed more IL-21R on cell surface than pDC. This observation explains why they respond more efficiently to IL-21 treatment. The magnitude of the pDC population did not change with any dose of IL-21 used, possibly because they do not express significant quantities of IL-21R. We did not perform any experiment to investigate if IL-21 induces activation of NKDC *in vitro*, but the presence of IL-21R on freshly isolated cells indicated that they are ready to respond to this cytokine. These observations suggest a direct effect from the cytokine on the cell surface instead of a bystander effect from other factor present in culture.

We also observed that our expanded NKDCs are producing IFN-γ *in vitro*. The amount of IFN-γ detected after expansion with IL-21 is too low to represent a dose dependent increase, however this analysis corroborates the characterization of IFN-γ producing NKDC. IFN-γ is one of the most reliable markers to indicate effector function of CD8$^+$ and NK cells, and mice deficient in IFN-γ or its receptor are susceptible to infection with intracellular bacteria and virus [33,34]. NKDC produces high amounts of IFN-γ compared with NK cells in murine models of tumor and bacterial infection. The combination of IL-18 with IL-12 was the most potent inducer of IFN-γ by NKDC *in vitro*. IFN-γ is only seen after the incubation of IL-15 pre-treated cells with IL-12 and IL-18 [27,35]. Interleukin-15 improves the response of NKDC to other cytokines, and does not directly increase the production of IFN-γ *in vitro* [15]. In our experiments with IL-21 we also did not see a significant improvement of IFN-γ production with IL-21 alone, in agreement with those findings.

Direct intratumoral injection of purified IL-21 expanded CD11c$^+$ cells abrogated B16F10 tumor growth in C57BL/6 mice. Also, twenty days after injection of IL-21 expanded NKDCs cells into the growing tumor, we observed a significant decrease in leg volume between the group of animals injected only with NKDC treated with the high dose of IL-21 (20 ng/ml) compared with those injected with untreated NKDC. Administration of IL-21 in tumor bearing mice induces the infiltration of NK cells into the tumor, followed by antigen specific CD8+ T cells, however the role of this cytokine in lymphocyte function during tumor immune responses is more restricted to cells housed in the lymphoid organs and antigen-specific T cells [38,39]. Several studies have explored the antitumoral potential of NKDCs and most of them used cytokines from innate immunity to optimize the function of these cells [12,30,31]. However if NKDCs can influence adaptive immunity, as several previous studies have claimed this hypothesis, independently if they present antigen or not it could be predicted that they should also respond to cytokines produced by activated T cells. Indeed, normally T cells located inside the tumor are inefficient to modulating an immunological response [36,37], so the surrounding T cells outside the tumor should be activated and they need tumor fragments to start the adaptive immunity. NKDC can first attach to the tumor, generate and present fragments to T cells that become activated and produce IL-21. The importance of the anti-

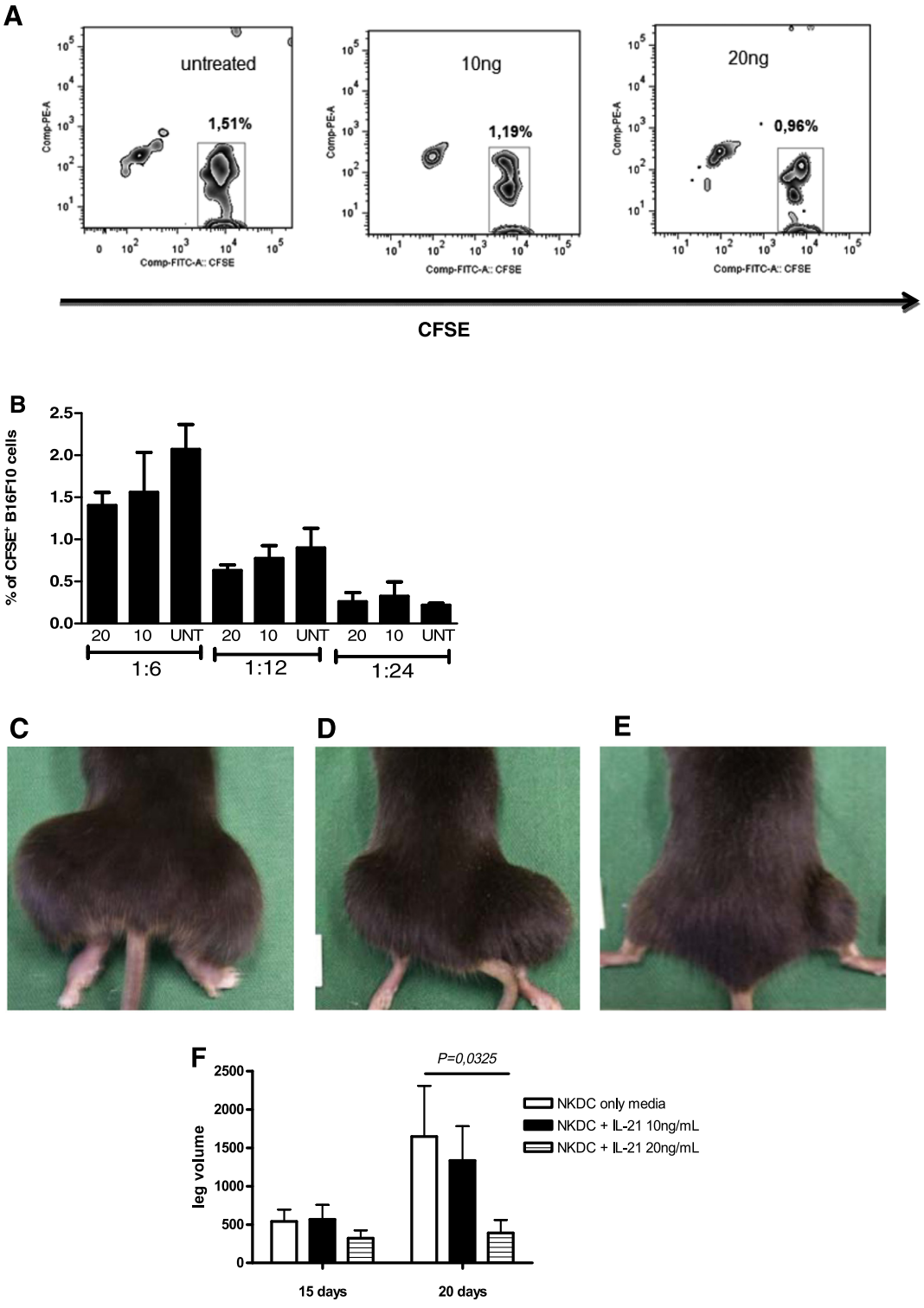

Fig. 3. *In vitro* cytotoxicity against B16F10 tumor cells and Inhibition of tumor growth with expanded NKDC. B16F10 cells were stained with CFSE (target cells) and cultured with NKDC (effectors cells) (6×10^4) stimulated with IL-21 (10 or 20 ng/ml) at different ratios of effectors and target cells E/T 1:6 (1×10^4); 1:12 (0.5×10^4) and 1:24 (0.25×10^4). (A) Contour plot of $CFSE^+$B16F10 cells cultures with NKDC untreated or treated with 10 ng/ml or 20 ng/ml of IL-21; ratio 1:6. (B) Percentage of CFSE + B16F10 cells after culture of NKDC stimulated with 10 or 20 ng/ml and different ratios of effector:target cells. B16F10 cells were cultured till confluence and 1×10^6 cells were injected in subcutaneously in the right thigh using PBS as vehicle, 100 μl by leg. Four days, in the beginning of tumor growing mice were treated with NKDC expanded with 10 ng/ml or 20 ng/ml of IL-21. Fifteen and twenty days after treatment the leg volume was analyzed considering tumor volume (V) (mm^3); $V = d^2 \times D \times 0.5$, where d = minor diameter and D = major diameter. (C) Leg from mouse treated with NKDC expanded in media only. (D) Leg from mouse treated with NKDC expanded in media supplemented with 10 ng/ml of IL-21. (E) Leg from mouse treated with NKDC expanded in media supplemented with 20 ng/ml of IL-21. (F) Blank: cells cultured only with media; black: IL-21 10 ng/ml; lined: IL-21 ng/ml. These data represent the mean ± standard error of two independent experiments.$^*p < 0.05$.

tumor effect of NKDC was previously observed when these cells were removed from bone marrow generated DC and the tumoricidal effect was totally lost; when the cells were added back, the effect was restored [24].

In conclusion, our results support the idea that murine IL-21 can be applied as a proliferative factor for specifically increasing NKDCs inside a population of splenic $CD11c^+$ cells. The expanded cells also maintained phenotypic and effector characteristics. As the anti-tumor action is an intrinsic effector activity of NKDCs, the use of IL-21 to optimize the generation of NKDCs is relevant for development new anti-tumor therapies.

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References

[1] Mukherji B, Chakraborty NG, Sivanandham M. T-cell clones that react against autologous human tumors. Immunol Rev 1990;116:33–62.
[2] Heiskala MK, Stenman UH, Koivunen E, Carpen O, Saksela E, Timonen T. Characteristics of soluble tumour-derived proteins that inhibit natural killer activity. Scand J Immunol 1988;28:19–27.
[3] Balsamo M, Zambello R, Teramo A, Pedrazzi M, Sparatore B, Scordamaglia F, et al. Analysis of NK cell/DC interaction in NK-type lymphoproliferative disease of granular lymphocytes (LDGL): role of DNAM-1 and NKp30. Exp Hematol 2009;37:1167–75.
[4] Cho D, Shook DR, Shimasaki N, Chang YH, Fujisaki H, Campana D. Cytotoxicity of activated natural killer cells against pediatric solid tumors. Clin Cancer Res 2010;16:3901–9.
[5] Tomala J, Chmelova H, Mrkvan T, Rihova B, Kovar M. *In vivo* expansion of activated naive CD8+ T cells and NK cells driven by complexes of IL-2 and anti-IL-2 monoclonal antibody as novel approach of cancer immunotherapy. J Immunol 2009;183:4904–12.
[6] Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol 1996;157:1840–5.
[7] Decman V, Laidlaw BJ, Dimenna LJ, Abdulla S, Mozdzanowska K, Erikson J, et al. Cell-intrinsic defects in the proliferative response of antiviral memory CD8 T cells in aged mice upon secondary infection. J Immunol 2010;184:5151–9.
[8] Vezys V, Masopust D, Kemball CC, Barber DL, O'Mara LA, Larsen CP, et al. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. J Exp Med 2006;203:2263–9.
[9] Colonna M. Cytolytic responses: cadherins put out the fire. J Exp Med 2006;203:261–4.
[10] Homann D, Jahreis A, Wolfe T, Hughes A, Coon B, van Stipdonk MJ, et al. CD40L blockade prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells. Immunity 2002;16:403–15.
[11] Chan CW, Crafton E, Fan HN, Flook J, Yoshimura K, Skarica M, et al. Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity. Nat Med 2006;12:207–13.
[12] Pillarisetty VG, Katz SC, Bleier JI, Shah AB, Dematteo RP. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-gamma via autocrine IL-12. J Immunol 2005;174:2612–8.
[13] Taieb J, Chaput N, Menard C, Apetoh L, Ullrich E, Bonmort M, et al. A novel dendritic cell subset involved in tumor immunosurveillance. Nat Med 2006;12:214–9.
[14] Chaudhry UI, Kingham TP, Plitas G, Katz SC, Raab JR, DeMatteo RP. Combined stimulation with interleukin-18 and CpG induces murine natural killer dendritic cells to produce IFN-gamma and inhibit tumor growth. Cancer Res 2006;66:10497–504.
[15] Chaudhry UI, Plitas G, Burt BM, Kingham TP, Raab JR, DeMatteo RP. NK dendritic cells expanded in IL-15 exhibit antitumor responses *in vivo*. J Immunol 2007;179:4654–60.
[16] Alves NL, Arosa FA, van Lier RA. Common gamma chain cytokines: dissidence in the details. Immunol Lett 2007;108:113–20.
[17] Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 2000;408:57–63.
[18] Strbo N, de Armas L, Liu H, Kolber MA, Lichtenheld M, Pahwa S. IL-21 augments natural killer effector functions in chronically HIV-infected individuals. Aids 2008;22:1551–60.
[19] Sivori S, Cantoni C, Parolini S, Marcenaro E, Conte R, Moretta L, et al. IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors. Eur J Immunol 2003;33:3439–47.
[20] Gays F, Martin K, Kenefeck R, Aust JG, Brooks CG. Multiple cytokines regulate the NK gene complex-encoded receptor repertoire of mature NK cells and T cells. J Immunol 2005;175:2938–47.
[21] Brady J, Hayakawa Y, Smyth MJ, Nutt SL. IL-21 induces the functional maturation of murine NK cells. J Immunol 2004;172:2048–58.
[22] Iannello A, Boulassel MR, Samarani S, Tremblay C, Toma E, Routy JP, et al. IL-21 enhances NK cell functions and survival in healthy and HIV-infected patients with minimal stimulation of viral replication. J Leukocyte Biol 2010;87:857–67.
[23] Rodrigues L, Nandakumar S, Bonorino C, Rouse BT, Kumaraguru U. IL-21 and IL-15 cytokine DNA augments HSV specific effector and memory CD8+ T cell response. Mol Immunol 2009;46:1494–504.
[24] Himoudi N, Nabarro S, Buddle J, Eddaoudi A, Thrasher AJ, Anderson J. Bone marrow-derived IFN-producing killer dendritic cells account for the tumoricidal activity of unpulsed dendritic cells. J Immunol 2008;181:6654–63.
[25] Vosshenrich CA, Lesjean-Pottier S, Hasan M, Richard-Le Goff O, Corcuff E, Mandelboim O, et al. CD11cloB220+ interferon-producing killer dendritic cells are activated natural killer cells. J Exp Med 2007;204:2569–78.
[26] GeurtsvanKessel CH, Bergen IM, Muskens F, Boon L, Hoogsteden HC, Osterhaus AD, et al. Both conventional and interferon killer dendritic cells have antigen-presenting capacity during influenza virus infection. PLoS One 2009;4:e7187.
[27] Plitas G, Chaudhry UI, Kingham TP, Raab JR, DeMatteo RP. NK dendritic cells are innate immune responders to Listeria monocytogenes infection. J Immunol 2007;178:4411–6.
[28] Mignot G, Ullrich E, Bonmort M, Menard C, Apetoh L, Taieb J, et al. The critical role of IL-15 in the antitumor effects mediated by the combination therapy imatinib and IL-2. J Immunol 2008;180:6477–83.
[30] Chaudhry UI, Katz SC, Kingham TP, Pillarisetty VG, Raab JR, Shah AB, et al. *In vivo* overexpression of Flt3 ligand expands and activates murine spleen natural killer dendritic cells. Faseb J 2006;20:982–4.
[31] Ullrich E, Bonmort M, Mignot G, Jacobs B, Bosisio D, Sozzani S, et al. Trans-presentation of IL-15 dictates IFN-producing killer dendritic cells effector functions. J Immunol 2008;180:7887–97.
[32] Welner RS, Pelayo R, Garrett KP, Chen X, Perry SS, Sun XH, et al. Interferon-producing killer dendritic cells (IKDCs) arise via a unique differentiation pathway from primitive c-kitHiCD62L+ lymphoid progenitors. Blood 2007;109:4825–931.
[33] Jouanguy E, Altare F, Lamhamedi S, Revy P, Emile JF, Newport M, et al. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection. N Engl J Med 1996;335:1956–61.
[34] Newport MJ, Huxley CM, Huston S, Hawrylowicz CM, Oostra BA, Williamson R, et al. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N Engl J Med 1996;335:1941–9.
[35] Bonmort M, Ullrich E, Mignot G, Jacobs B, Chaput N, Zitvogel L. Interferon-gamma is produced by another player of innate immune responses: the interferon-producing killer dendritic cell (IKDC). Biochimie 2007;89:872–7.
[36] Shafer-Weaver KA, Anderson MJ, Stagliano K, Malyguine A, Greenberg NM, Hurwitz AA. Cutting edge: tumor-specific CD8+ T cells infiltrating prostatic tumors are induced to become suppressor cells. J Immunol 2009;183:4848–52.
[37] Wang SF, Fouquet S, Chapon M, Salmon H, Regnier F, Labroquere K, et al. Early T cell signalling is reversibly altered in PD-1+ T lymphocytes infiltrating human tumors. PLoS One 2011;6:e17621.
[38] Hsieh C, Hsu S, Shen S, Chen M, Liu S, Chong P, et al. Increased expression of IL-21 reduces tumor growth by modulating the status of tumor-infiltrating lymphocytes. Immunobiology 2011;216:491–6.
[39] Petersen CC, Diernaes JEF, Skovbo A, Hvid M, Deleuran B, Hokland M. Interleukin-21 restrains tumor growth and induces a substancial increase in the number of circulating tumor-specific T cells in a murine model malignant melanoma. Cytokine 2010;49:80–8.
[40] Jedema I, Van der Werff NM, Barge RM, Willemze R, Falkenburg JH. New CFSE-based assay to determine susceptibility to lysis by cytotoxic T cells of leukemic precursor cells within a heterogeneous target cell population. Blood 2004;103:2677–82.

Further reading

[29] Caminschi I, Ahmet F, Heger K, Brady J, Nutt SL, Vremec D, et al. Putative IKDCs are functionally and developmentally similar to natural killer cells, but not to dendritic cells. J Exp Med 2007;204:2579–90.