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Low Dose of IL-12 Stimulates T Cell Response in Cultures of PBMCs Derived from Non Small Cell Lung Cancer Patients*

Lucia D'Amico[1], Enrico Ruffini[2], Riccardo Ferracini[3], Ilaria Roato[1#]

[1]CeRMS (Center for Research and Medical Studies), A.O. Città della Salute e della Scienza di Torino, Torino, Italy; [2]Department of Toracic Surgery, A.O. Città della Salute e della Scienza di Torino, Torino, Italy; [3]Department of Orthopaedics, A.O. Città della Salute e della Scienza di Torino, Torino, Italy.
Email: #roato78@libero.it

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ABSTRACT

Cancer induces tolerance by suppressing immune function, modulating the T helper activity and causing an imbalance of cytokines produced by T cells. IL-12 is an immune regulatory cytokine with potent anti-tumor activity and its signalling network leads to polarization of naïve CD4 T cells into Th1. In pre-clinical studies, administration of recombinant IL-12 by intravenous injection or IL-12 plasmid DNA by intra-tumoral injection showed some anti-tumor effects, measurable immunological responses, but also important dose-dependent side effects. We investigated the ability of low doses of IL-12 to modulate the T cell subpopulations in cultures of PBMCs derived from Non Small Lung Cancer (NSCLC) patients and to induce lysis of lung adenocarcinoma cells by T cells. PBMCs were stimulated with different doses of IL-12 and T cell phenotype was evaluated. IL-12 at 0.01 pg/ml significantly increased the number of CD4 and CD8 T cells, in particular of CD4/IFNγ producing cells. IL-12 did not stimulate T regulatory, but it increased the lysis of lung adenocarcinoma cells induced by T cells. Our results showed that low doses of IL-12 modulated T cell subpopulations *in vitro* and it increased their lytic activity on adenocarcinoma cells. Thus we hypothesize the use of low dose of IL-12 as a therapeutic tool against pathologies characterized by a T cell imbalance, in order to promote an immuno-modulation.

Keywords: Interleukin-12; Immuno-Modulation; T Helper; T Regulatory; Anti-Tumor Immune Response

1. Introduction

Under normal conditions, cells of the immune system inhibit tumor growth and progression through the recognition and rejection of malignant cells, a process called immunosurveillance. However, malignant variants may create an immunosuppressive microenvironment, blocking productive antitumor immunity and resulting in a shift from immunosurveillance to immunotolerance to a tumor [1]. Upon metastasis, the biochemical cues secreted by tumor cells can directly interfere with the cellular communication necessary for eliciting an appropriate immune response. Tumor induces tolerance by producing biochemical cues that suppress immune function, including TGF-β, IL-4, IL-6 and IL-10 [2,3]. TGF-β inhibits the biological activities induced by IL-12 [4] through an undefined mechanism [5], whereas IL-4 induces polarization towards a Th2 phenotype, promoting tumor tolerance [6].

Interleukin-12 (IL-12) is an important immune regulatory cytokine that exerts potent antitumor activity. At the cell level, IL-12 is a paracrine cytokine that provides a critical interface between innate and adaptive immunity [7]; within secondary lymphoid organs, IL-12 plays a critical role in promoting antitumor immunity. IL-12 signaling network leads to polarization of naïve CD4 T cells into a Th1 phenotype [8]. Polarization into a Th1 phenotype promotes anti-tumor immunity via cytokine help for CD8 T cell expansion and switching B cell antibody production to isotypes, such as IgG2a in the mouse, that enhance antibody-dependent NK cell-medi- ated cytotoxicity [9]. This dual role, as promoter of Th1 polarization and activator of NK cells, motivates using IL-12 as an adjuvant for antibody-based tumor immuno- therapy [10]. IL-12 stimulates IFNγ expression, which inhibit tumor angiogenesis through the interaction among lymphocyte-endothelial cells, decreasing endothelial cell survival and adhesion. On the other hand, NK cells,

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#Corresponding author.

which are attracted by IL-12, are synergistically citolytic to endothelial cells.

Lung cancer is a leading cause of cancer death worldwide. The large majority of cases are non-small-cell lung cancers (NSCLC), their prognosis is still grim [11] and novel therapeutic approaches are warranted. Airoldi *et al*. demonstrated an anti-tumor activity of IL-12, because it directly inhibits the growth of human lung adenocarcinoma and targets the adjacent normal bronchial epithelial cells [12]. Thus they suggest that IL-12 might be administered to lung adenocarcinoma patients as tumor-targeted formulations to act directly on the tumor microenvironment [13] or systemically to take advantage of the immunomodulatory activity of the cytokine [14].

After encouraging results in pre-clinical studies, many Phase I-II trials were initiated with recombinant IL-12 by intravenous injection [10,15,16] or IL-12 plasmid DNA by intratumoral injection [17-19], showing some anti-tumor effects, measurable immunological responses, but also important dose-dependent side effects.

Recently, evidences of efficacy of low doses of IL-12 on modulation of Th1 vs Th2 has been demonstrated in an asthma pre-clinical model [20], suggesting a novel therapeutic approach to diseases which involve a Th1/Th2 imbalance. In this study Gariboldi *et al*. tested different doses of IL-12 for the treatment of asthma in mice and identified 0.01 pg/ml as the lowest and more effective dose [20].

Considering these last results and the previous clinical trials, our study aims to investigate the ability of low doses of IL-12, (in particular 0.01 pg/ml) to modulate the T cell subpopulations in cultures of PBMCs derived from patients affected by NSCLC.

2. Material and Methods

2.1. Patients and Cell Cultures

After informed consent, we obtained 20 peripheral blood samples from patients affected by NSCLC and subjected to surgical treatment in the Department of Toracic Surgery, A.O. della Salute e della Scienza of Torino. We isolated peripheral blood mononuclear cells (PBMCs) from the blood samples after centrifugation over a density gradient, Lymphoprep (Nycomed Pharma, Norway) and cultured in α-MEM, supplemented with 10% FBS, penicillin 100 U/ml and streptomycin 100 μg/m (Cambrex, Bio Science, Walkersville, MD), for 10 days. PBMCs were plated in 24-well plates (2×10^6/well) in the presence of different concentration of IL-12. All dilutions and preparation of cytokines were prepared starting from a concentrated solution (1 μg/ml) of recombinant human IL-12 (Sigma-Aldrich, St. Louis, MO). Different dilution were performed using a 30% hydro-alcoholic solution as diluent to obtain the following dosages: 10 ng/ml, 1 pg/ml, 0.01 pg/ml. These dosages and the placebo were tested on the PBMCs derived from all the 20 patients. After 5 days of culture, medium was refreshed without discarding cells. IL-12 at different doses was added daily for 9 days.

2.2. Flow Cytometry

To monitor the different number of CD4, CD8 and particularly of T regulatory and IFNγ-producing T cells, we analysed these sub-populations after PBMC isolation, at day 5 and 10 of culture.

To perform intracellular staining necessary to detect the expression of CD4/IFNγ, we utilized the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD, Bedford, MA), according to the manufacturer's instructions. For T regulatory (CD4/CD25^{high}/FoxP3) phenotype analysis, we used Human Regulatory T cell staining kit (eBioscience, San Diego, CA). Anti-human PeRCP-CD8 and the relative isotype controls were purchased by BD.

Samples were analyzed in a FACsCalibur instrument and elaborated by CellQuest software (both from, BD Bedford, MA). Data represent a percentage of positive cells, determined by subtracting the percentage value of the appropriate isotype controls from each sample.

2.3. Cytotoxicity Assay Using the CFSE Fluorescent-Based Dye

To evaluate the ability of T cells stimulated with low dose of IL-12 to actually kill cancer cells, we performed a cytotoxicity assay using carboxy-fluorescein diaceate succinimidyl ester (CFSE) labelling of target cells H1373, a human cell line of lung adenocarcinoma (American Type Culture Center, ATCC). Both PBMCs and H1373 were cultured in RPMI 1640 (GIBCO, Invitrogen, Paisley, UK) supplemented with 10% FBS, penicillin 100 U/ml and streptomycin 100 μg/m (Cambrex, Bio Science, Walkersville, MD).

H1373 target cells were labelled with CFSE 5 μM according to manufacturer's instructions (CellTrace CFSE Cell proliferation Kit by Invitrogen, Paisley, UK). After 7 days of culture, we collected PBMCs and plated them on H1373, previously labelled with CFSE, at a ratio 1/10 (*i.e.* 1×10^5 target cells and 1×10^6 effector cells, PBMCs) at 37˚C for 24 hours. H1373 were plated alone with culture media in order to quantify the spontaneous death, whereas the maximum level of death was obtained after Triton lysis. The cytotoxic activity of patients' PBMCs was evaluated by flow cytometry based on CFSE staining of H1373 target cells, and identification of dead cells by propidium iodide staining of CFSE-labelled target cells, as previously reported [21]. Acquisition of

CFSE-labelled H1373 was performed through FACsCalibur instrument (BD Bedford, MA).

2.4. Statistical Analysis

The normal distributions of each parameter were determined by Kurtosis's test. The number of CD4 and CD8 T cells were not normally distributed, thus they were compared by means of the Wilcoxon Signed Ranks Test, a non parametric test for paired samples. Pearson's correlation coefficients were used to check univariate associations. The SPSS 17.0 software package was used to process the data with $p < 0.05$ as the significance cut-off.

3. Results

3.1. Low Dose of IL-12 Stimulates CD4 and CD8 T Cells in PBMC Cultures

IL-12 is known for its stimulatory effect on CD4 T cells, thus we analysed the ability of low doses of IL-12 to modulate CD4 subpopulation in patients' PBMC cultures. After PBMC isolation (basal level) from patients' peripheral blood, the mean value of CD4 T cells in patients' PBMC samples was 46.7%. At day 5 of culture, the percentage of CD4 T cells increased compared to the basal, but it was comparable among the different conditions of stimulation (**Figure 1**). At day 10 of culture, we observed that CD4 T cells remained high with 0.01 pg/ml, ($p < 0.05$), whereas they diminished with 1 pg/ml, 10 ng/ml and placebo. We observed a significant increase of CD8 T cells after stimulation with 0.01 pg/ml compared to the basal condition, $p < 0.05$ (data not shown).

3.2. Low Dose of IL-12 Increases CD4/IFNγT Cells

The stimulation of Th1 represents a fundamental step for an efficient anti-cancer response of the immune system, thus we studied the Th1 modulation in PBMC culture, after IL-12 at low doses. At basal level, the mean percentage of CD4/IFNγ T cells was 30.7%. After 5 days of culture, CD4/IFNγ T cells decreased with 1 pg/ml and 10 ng/ml, whereas it was significantly increased with 0.01 pg/ml (48.4%) and placebo (48.3%). At day 10, we observed a further significant CD4/IFNγ T cell stimulation with 0.01 pg/ml compared to the basal level, ($p < 0.01$), whereas there was not a significant difference between basal condition and placebo (**Figure 2**).

3.3. Low Dose of IL-12 Increases Lysis of H1373 Cells

It is known that an increase of IFN-γ released by Th1 can stimulate the activation of cytotoxic CD8 T cells, thus we evaluated the ability of low doses of IL-12 to induce lysis of adenocarcinoma cells. Since we observed an increased number of CD8 T cells after stimulation with 0.01 pg/ml of IL-12 and a contemporary increase of CD4/IFN-γ T cells, we performed a co-culture of patients PBMCs and H1373 cells, detecting a significant increase in H1373 lysis with 0.01 pg/ml of IL-12 compared to the placebo condition, $p < 0.05$ (**Figure 3**).

3.4. Low Dose of IL-12 Suppresses T Regulatory Cells

Since in cancer patients an increase of T regulatory cells has been documented, we analysed the ability of IL-12 at different doses to modulate T regulatory in PBMC cultures. The mean value of T regulatory (CD4/CD25^{high}/FoxP3 positive cells) was 1.7% at the basal level, **Figure 4**. At day 5 of culture, the percentage of T regulatory did not significantly increase after IL-12 stimulation, whereas it increased with placebo. After 10 days of culture, T regulatory showed a trend to decrease, suggesting an inhi-

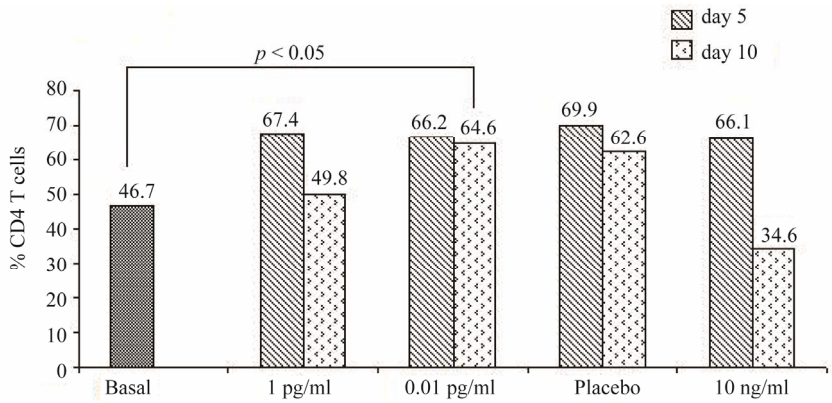

Figure 1. The histogram shows the percentages of CD4 T cells at basal level, after 5 and 10 days of culture. The numbers on the column bars represent the mean value plus standard deviation. At day 5, no significant differences are present among the different culture conditions. At day 10, the number of CD4 T cells was significantly higher with IL-12 at 0.01 pg/ml than the basal level, $p < 0.05$.

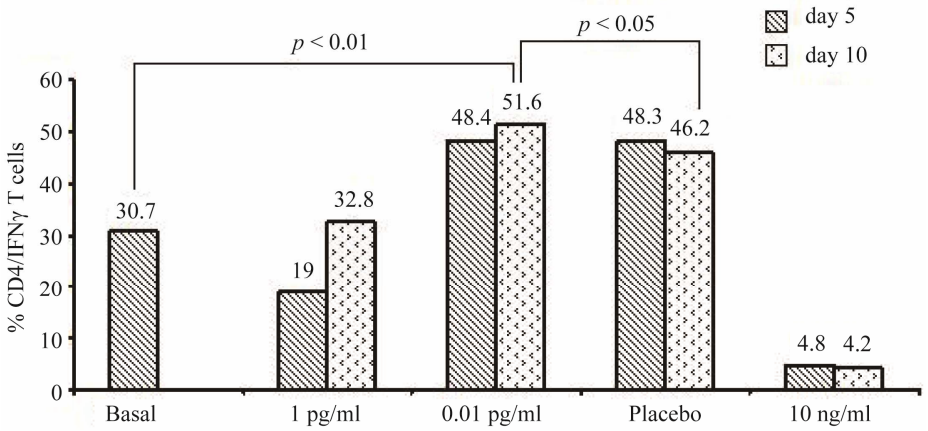

Figure 2. CD4/IFNγ producing T cells increased after stimulation with IL-12 at 0.01 pg/ml compared to the basal level, $p < 0.01$. Moreover, at day 10, CD4/IFNγ T cells were significantly higher with 0.01 pg/ml than with placebo, $p < 0.05$. The numbers on the column bars represent the mean value plus standard deviation.

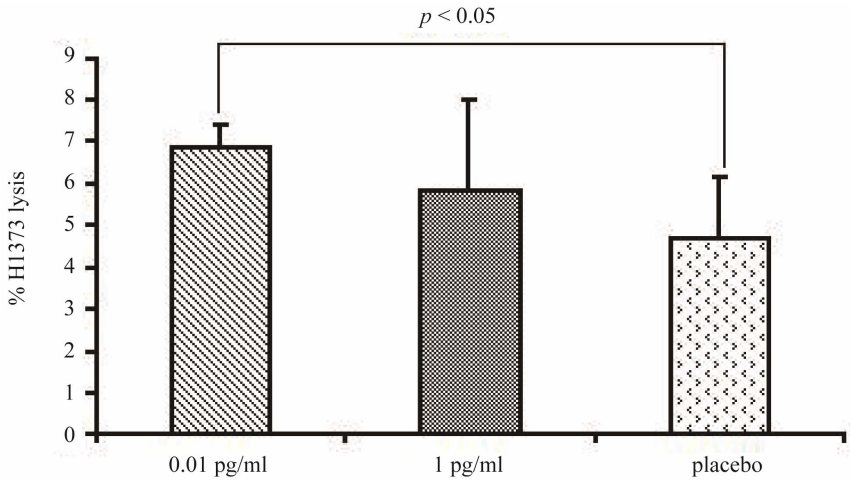

Figure 3. The histogram shows the mean value of H1373 lysis by PBMCs, isolated from 5 NSCLC patients. IL-12 at 0.01 pg/ml enhanced the H1373 lysis induced by PBMCs, $p < 0.05$.

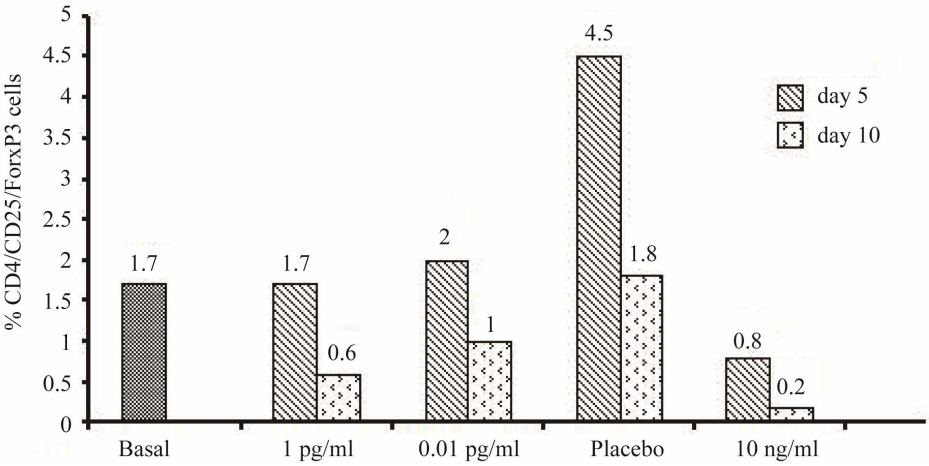

Figure 4. The histogram shows the modulation of T regulatory cells during culture with and without IL-12. The numbers on the column bars represent the mean value plus standard deviation. CD4/CD25/FoxP3 was not increased after IL-12 stimulation, at any dosages and they were reduced at day 10 of culture.

bitory effect of IL-12 on them. In PBMCs treated with placebo, T regulatory decreased, but they were higher than at the basal condition, at day 10 (**Figure 4**).

4. Discussion

Early *in vitro* studies demonstrated that IL-12 could enhance the cytolytic potential of lymphocytes from patients with a variety of both solid and haematological malignancies: an overnight incubation with low doses of IL-12 induced T-cell-mediated lysis of both leukemic and solid tumor cell lines [22,23]. Based upon encouraging responses in murine models, many clinical trials on different tumors had been performed, showing somewhat modest clinical results and, in some instances, untoward toxicity [15]. Since IL-12 is an important modulator of immune response and mediates various anti-tumor effects, we analyzed the ability of low doses of IL-12 to modulate CD4 subpopulation in cultures of PBMC derived from lung cancer patients. In literature evidences of efficacy of low doses of IL-12 on modulation of Th1 vs Th2 has been demonstrated in an asthma pre-clinical model [20], suggesting the possibility to utilize low doses of IL-12 to act on other immune pathologies characterized by Th1/Th2 imbalance.

Our results show that CD4 T cells proliferate and were viable in culture, at day 5 with or without IL-12 stimulation, whereas IL-12 at 0.01 pg/ml significantly stimulated CD4 at day 10, suggesting that this low dose of IL-12 was able to support CD4 proliferation. Doses of IL-12 higher than 0.01 pg/ml and placebo did not show a statistically significant stimulation in CD4 T cells, thus IL-12 at low doses seems to modulate CD4 population. Also CD8 T cells were increased compared to the basal number, after stimulation with 0.01 pg/ml of IL-12.

To better define the action of the low dose of IL-12 on CD4 T cells, we studied the expression of IFNγ on CD4 T cells after isolation and IL-12 stimulation. IL-12 at 1 pg/ml and at 10 ng/ml failed to stimulate CD4/IFNγ producing cells, in particular IL-12 at 10 ng/ml dramatically down-regulated these cells. IL-12 at 0.01 pg/ml significantly stimulated CD4/IFNγ producing cells, which were significantly higher with 0.01 pg/ml than with placebo. This result demonstrates that IL-12 at 0.01 pg/ml stimulates proliferation of Th1. The increase in CD4/IFNγ T cells is fundamental to activate an anti-cancer response. Here, we showed an increased lysis of adenocarcinoma cells after co-culture with patients' PBMCs stimulated with 0.01 pg/ml of IL-12 compared to placebo. These data suggest that this low dose of IL-12 may be effective for the inhibition of cancer cell proliferation. Since the inhibitory role of T regulatory cells on immune response against cancer has been demonstrated [24], we studied the ability of low doses of IL-12 to down-regulate T regulatory in PBMC cultures. We showed that the number of T regulatory was higher with placebo than with IL-12, which shown an inhibitory effect on T regulatory cells. These data confirm our hypothesis that IL-12 at low doses immuno-modulates T cell sub-populations *in vitro*, stimulating Th1 and inhibiting T regulatory cells. Moreover, our results are in accordance with recent published data concerning the use of IL-12 at low dose to treat asthma [16], but need to be also confirmed also on patients affected by other type of tumors or immunological disorders characterized by unbalance of cytokines and T cell subpopulation.

5. Conclusion

In the present work, we demonstrated that low doses of IL-12 modulate the T cell subpopulations in cultures of PBMCs derived from patients affected by NSCLC. We observed an increased number of CD4 and CD8 after stimulation with low dose of IL-12, in particular of CD4/IFNγ producing cells, whereas there was an inhibition of T regulatory cells. This low dose of IL-12 also promoted the inhibition of the proliferation of lung adenocarcinoma cells *in vitro*. The possibility to utilize low dosages of IL-12 as immuno-modulator opens new perspectives to study also other cytokines at low doses in order to restore the balance in the immune response for diseases characterized by immune system alterations. Further studies, mainly *in vivo*, are mandatory to better characterize the immune effector mechanisms involved in this model of immunological therapy and to define the possible use of IL-12 at low dosage to support the immune response in patients affected by NSCLC.

6. Acknowledgements

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