Article

THE IMMUNE SYSTEM AS A NEW POSSIBLE CELL TARGET FOR AFP 464 IN A SPONTANEOUS MAMMARY CANCER MOUSE MODEL†

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Abstract

Aminoflavone (AFP 464, NSC 710464), an antitumor agent which recently entered phase II clinical trials, acts against estrogen-positive breast cancer (ER+). AFP 464, which has a unique mechanism of action by activating aryl hydrocarbon receptor (AhR) signaling pathway, decreased tumor size and growth rate in the estrogen dependent, Tamoxifen-sensitive spontaneous M05 mouse model. Considering that AhR has recently emerged as a physiological regulator of the innate and adaptive immune responses, we investigated whether AFP 464 modulates the immune response in our mouse model. Studies on the effect of AFP 464 on the immune system were carried in BALB/c mice bearing M05 semi-differentiated mammary adenocarcinomas that express estrogen and progesterone receptors. Splenic cells and tumor inflammatory infiltrates were studied by cytometric analyses. The modulation of splenocytes cytotoxic activity by AFP 464 was also evaluated. We further investigated the effects of AFP 464 on peritoneal macrophages by evaluating metalloproteinase, arginase and iNOS activities. We found that AFP 464 increased splenic cytotoxic activity, diminished the number of systemic and local Treg lymphocytes and MDSCs, and induced a M1 phenotype in peritoneal macrophages of M05 tumor bearing mice. Therefore, we conclude that AFP 464 modulates immune response which collaborates with its anti-tumor activity. Our results place the immune system as a novel target for this anti-cancer agent to strengthen the rationale for its inclusion in breast cancer treatment regimens.

INTRODUCTION

Breast cancer is a pathological malignancy of major incidence in women. Thousands of women die each year because of the development of chemoresistance, which results in relapse and progression of the disease. The anti-estrogen Tamoxifen is the most frequently used agent for the treatment of estrogen receptor positive tumors (ER+). However, 40% of ER+ tumors acquire resistance to this agent (C.K. Osborne and Schiff 2011). A substantial number of (ER+) tumor shows intrinsic resistance to Tamoxifen. Therefore, to find new therapeutic strategies is of most importance. Aminoflavone (AF, NSC686288 and AFP464, NSC 710464) is an anti-tumor agent with proved efficacy in clinical trials for the treatment of ER+ breast cancer patients. This agent presents a unique mechanism of action activating the aryl hydrocarbon receptor (AhR) signaling pathway which has a crosstalk with the ER signaling pathway (Denison and Nagy 2003; Callero and Loaiza-Pérez 2011). In vitro studies have demonstrated that ER+ breast cancer cell lines and renal cancer cell lines are sensitive to AF (Loaiza-Perez et al. 2004; Loaiza-Pérez et al. 2004; M.A. Callero et al. 2012). AF activity has been linked to the presence of AhR in the cytoplasm, nuclear translocation of AhR-AF complex followed by CYP1A1 induction, sulfotransferase 1A1 induction DNA damage and apoptosis. In breast cancer resistant cell lines like MDA-MB-431 (ER-), it has been found constitutive nuclear localization of AhR without induction of CYP1A1/1A2. It has been demonstrated that MCF-7 variants resistant to Tamoxifen are still sensitive to AF (Shelton et al. 2007; Stark et al. 2010). The patients that would benefit with AF in the clinic are the ones with ER+ tumors resistant to endocrine therapy (Shelton et al. 2007). On the other hand, traditional chemotherapies debulk tumors but fail to produce long-term clinical remissions due to their inability to eradicate cancer stem cells(CSCs). In that sense, our group have recently demonstrated that AFP464 in vivo treatment suppressed growth and disrupted mammospheres of the murine mammary M05 ER+ adenocarcinoma that expresses estrogen and progesterone receptors (Brantley et al. 2016).

It has recently become clear that tumor microenvironment, and in particular the immune system, has a crucial role in modulating tumor progression and response to therapy. Indicators of an ongoing immune response, such as the composition of the intratumoral immune infiltrate, have been correlated with therapeutic outcome. Moreover, several anticancer agents, including classical chemotherapeutics and targeted compounds, stimulate tumor-specific immune responses either by inducing the immunogenic death of tumor cells or by engaging immune effector mechanisms. In patients with breast cancer who are treated with neoadjuvant chemotherapy, a high ratio between CD8+ and FoxP3 lymphocytes (immunosuppressive regulatory T (T_{reg}) cells correlates with improved histological response, as well as relapse-free and overall survival(Ladoire et al. 2008; Ladoire et al. 2011).

AhR has recently emerged as a physiological regulator of the innate and adaptive immune responses including effects on T_{regs} and Th17 differentiation. T_{regs} express AhR; this receptor is involved in the differentiation of these cells through induction of TGF- β expression, and there is a crosstalk between the AhR and TGF-β signaling pathways which modulate T_{regs} differentiation (Quintana et al. 2008; Gandhi et al. 2010; Marshall and Kerkvliet 2010). Another potential mechanism by which AhR affect T_{regs} activity involves dendritic cells. The AhR pathway could influence the T_{regs} via modulation of co-stimulatory molecules expression and cytokines secretion (Vorderstrasse and Kerkvliet 2001; Quintana et al. 2010).

Macrophages are important cells of the innate response which produce pro-inflamatory cytokines and contribute to the adaptative response. It has been shown that peritoneal macrophages derived from AhR-/- mice produce higher levels of IL-6, TNF-α, IL-12 and histamine mediated by lipopolisacharide (LPS) stimulation, compared with wild type mice (WT) (Kimura et al. 2009). B lymphocytes are key components of humoral immunity, also regulated by AhR. Some AhR ligands like TCDD induce B cells differentiation and cytokines expression (Vaidyanathan et al. 2016)

Considering all the previous information we intended to investigate whether AFP464 modulates the immune response in the M05 mammary adenocarcinoma mouse model. We thought to determine if AFP464 could be a new therapeutic option for ER+ metastatic breast tumors with an in vivo activity targeting the immune system besides its effect on CSCs.

MATERIALS AND METHODS

Mice and AFP 464 treatment

Inbred 2–4 month old BALB/c female mice were obtained from the Animal Care Division at the Institute of Oncology "Angel H. Roffo". Animal care and manipulation were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals (Pontiggia et al. 2009). The M05 mouse mammary tumor model is described elsewhere (Simian et al. 2009). Tumors growing subcutaneously in mice were measured twice a week with a Vernier caliper (length and width). Mean diameter was calculated using the formula: length × width/2. For animal dosing AFP 464 was prepared as a smooth suspension in saline containing 0.05% Tween 80. AFP 464 was administered intraperitoneally at 12 mg/kg. The treatment schedule followed one daily dose for a total of 5 days (QD × 5), with the first treatment given when the average size of the tumors was about 1 cm^3. Each experiment contained a vehicle non-treated group in parallel with the AFP 464-treated group.

Tumor cell line

The LM05-Mix cell line was derived from the original M05 spontaneous mammary murine adenocarcinome maintained by s.c. transplants. LM05 is maintained as single cell suspension and is composed of a mixture of fibroblastic and epithelial cells. LM05 cells were cultured in DMEM/F12 medium as previously described in detail elsewhere (Pontiggia et al. 2009)

Spleen and tumor single cell suspensions

Spleens were removed and disrupted through a 1-mm metal mesh. M05 tumors were removed, minced and digested in medium containing collagenase I as previously described in detail (Raffo et al. 2013). Red blood cells were lysed using a buffer containing 150 mM NH_4Cl, 10 mM K_2CO_3 and 0.1 mM EDTA. The resulting cell suspensions were filtered through a 40-μm cell strainer and resuspended in PBS.

Isolation of peritoneal macrophages

Macrophages were collected from euthanized animals by peritoneal lavage using 10 mL of ice-cold PBS supplemented with EDTA. Collected cells were washed using PBS and seeded in six well-plates in RPMI 1640 supplemented with 5% FBS, penicillin/streptomycin, and 15 mM Hepes (pH 8.0) and incubated at 37 °C at 5% CO2 for 2 h. Cultures were washed three times with PBS to remove non-adherent cells and remaining adherent cells were left overnight in culture media without FBS.

Adoptive transfer with spleen cells

Mouse splenocytes were isolated, as previously described, fifteen days after treatment (AFP 464 or vehicle). Splenocytes ($3x10^6$) were suspended in saline solution and injected i.p. in a total volume of 1 ml and transferred into tumor bearing mice (TBM). The adoptive transfer schedule followed once weekly dosing for a total of 3 weeks (QW × 3), with the first treatment given when the tumor average size of recipient mice was about 1 cm^2. Tumors were measured twice a week with a Vernier caliper since day one of spleen cells transfer.

Flow cytometry for immunophenotyping

Tumor and spleen cell suspensions were obtained 15 days after treatment and stained with antibodies against various cell surface markers using standard staining methods. The following panel of commercially available and fluorochrome conjugated anti-mouse monoclonal antibodies were used: PE-Cy5-anti-mouse CD4, Alexa 488-anti-mouse CD8, PE-Cy5-anti-mouse CD69, PE-Cy5-anti-mouse -Gr1, Alexa 488-anti-mouse CD11b, PE-anti-mouse CD45.2 (Biolegend). Samples were run on a flow cytometer and data were analyzed using using a Cyflogic software

For intracellular staining, single cell suspensions prepared as described above, were used. After surface staining with PE-anti-mouse CD25 and PE-Cy5-anti-mouse CD4, cells were washed with permeabilization buffer (Biolegend) and incubated with the corresponding mAb (Alexa 488-anti mouse FoxP3) for 1 hr. After washing with PBS, cells were analyzed by flow cytomety.

Cytotoxicity T Lymphocyte (CTL) assay

In order to determine the effect of AFP464 on the specific cytotoxic activity of the spleen cells of TBM, splenocytes from 15-day AFP 464-treated and non-treated mice were activated with LM05-Mix cells pre-incubated with Mitomicin C for 5 days (splenocyte: LM05-Mix cell ratio 10:1). These effector cells obtained from 5-day co-cultures were tested for CTL activity against LM05-Mix cells for 48 h at a splenocyte:tumor cells ratio 10:1 by MTS assay. Each experiment included the culture of isolated splenocytes and LM05-Mix cells separately in order to calculate the cytotoxic activity of splenocytes with the following formula: $\%\ \text{Specific Lysis} = 1 - [(\text{OD splenocytes} + \text{LM05} - \text{Mix cells} - \text{OD Splenocytes}) / \text{OD LM05} - \text{Mix cells}] \times 100$, where OD the absorbance measured at 490/620 nm with an ELISA reader

Antigen-independent cytotoxicity was evaluated with splenocytes activated for 5 days in anti-CD3 pre-coated plates.These effector cells obtained after 5 days were tested for CTL activity against LM05 cells determined by MTS assay as described above.

IFN-γ and IL-10 production in vitro by splenocytes.

IFN-γ and IL-10 levels in splenocytes-LM05 Mix co-culture (mentioned above) supernatants were assessed by ELISA kits for murine IFN-γ and IL-10 (R&D Systems).

Nitric oxide (NO) production and Arginase activity

Production of NO was estimated after culturing peritoneal macrophages for 18 hours by measuring the nitrite levels in cell supernatant with the Griess reaction (Bryan and Grisham 2007). Briefly, equal volumes of cell supernatants and Griess reagent [1:1 of 0.1% in N-(1-Naphtyl)-ethylendiamindihydrochloride (NEDA) solution (Merck, Germany), in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid] and sample cell supernatant or NaNO2 standards were incubated together at room temperature for 10 min. Absorbance was read at 540 nm.

Arginase activity of purified macrophages was measured by a colorimetric method as described (Corraliza et al. 1994). Briefly, 10 mM MnCl2 and 0.5 M L-arginine were successively added to macrophage lysates for 1 hr at 37°C.

The reaction was stopped by addition of an acid solution (H2SO4:H3PO4:H2O =1:3:7), and the urea generated by arginase was analyzed by addition of α-isonitrosopropiophenone at 100°C for 45 min. The colored product was quantified by absorption at 550 nm in an ELISA reader. Arginase activity was determined as the amount of urea produced from total protein of peritoneal macrophage, quantified by Bradford Method.

Statistical Analysis

Differences between groups were analyzed using one-way ANOVA with Tukey's or Dunnett post-tests for evaluating three or moregroups. To compare two groups, the unpaired Student's t test with Welch's correctionwas used. For in vivo assays, statistical significance was determined using two way ANOVA. Statistical analysis was performed using GraphPad Prism 4.0, GraphPad software, Inc. San Diego, California, USA, www.graphpad.com. Differences wereconsidered significant at $p < 0.05$.

RESULTS

Adoptive transfer of splenocytes from AFP 464-treated mice decreases mammary tumor size in vivo

We have already demonstrated that AFP 464 inhibited the growth of M05 tumors targeting cells with stem cell-like phenotype by abrogating α6-integrin expression (Brantley et al. 2016). Since AhR is also expressed in most cells of the immune system (Stockinger 2009), we therefore sought to determine whether AFP 464 could also modulate the tumor-associated immune response.

For this purpose, we inoculated female syngeneic BALB/c mice with M05 tumor cells as described in materials and methods. Once tumor average size had reached approximately 1 cm^3, mice were treated with AFP 464 [12 mg/kg] or vehicle, i.p., QD × 5 (Fig 1a). As figure 1b shows, AFP 464 decreased M05 size in vivo. Significative difference respect to non-treated animals was detected from the 15th day after treatment. In order to determine if the immune system was involved in the decrease in tumor growth, we adoptively transferred spleen cells from AFP-treated mice to naive M05 tumor bearing mice. Thus, 15 days after treatment, donor mice were sacrificed, splenocytes were isolated and transferred into M05 tumor bearing mice ($3x10^6$ cells, ip, QW × 3) (Fig 1c). As shown in figure 1d, mice adoptively transferred with splenocytes yielded sustained, significant inhibition of M05 tumor growth. The downregulation of tumor growth was statistically significant from day 21 after adoptive transfer.

AFP 464 modulates spleen immune cells

In order to determine AFP 464 modulation on the distribution of spleen immune cells able to decrease M05 tumors when adoptively transferred, spleen cells from donor mice bearing M05 tumor were isolated and analyzed by flow cytometry with fluorochromes-conjugated specific antibodies. We could not find differences in the percentage of Th (CD4+) or Tc (CD8+) lymphocytes between AFP 464treated and non-treated mice. However, we detected that the frequency of activated CD8+ T cells (CD8+/CD69+) was increased in AFP 464-treated mice respect to non-treated ones (7,92 ± 1,6 % vs 4,47 ± 1,0 %, respectively). Additionally, we also found that AFP 464 modulated the frequency of MDSCs and CD4+CD25+FoxP3+ T_{regs} in the spleen, as a decreased number of these immunosuppressor cells was found in AFP464-treated mice (0,78 ± 0.23 vs 3,98 ± 0.93 and 1.69 ± 0.2 vs 2.49 ± 0.3, respectively) (Fig. 2).

AFP 464 increases splenocytes M05-specific cytotoxic activity

According to previous results, we then evaluated if AFP 464 could also enhance the cytotoxic activity of spleen cells against M05 tumor cells. With this purpose a CTL assay was performed with isolated splenocytes after 15 days AFP 464 or vehicle treatment. Spleen cells were stimulated in vitro with Mitomicin C-treated LM05-Mix cells (cell line derived from M05 tumor) and then challenge against LM05-Mix cells. Figure 3a shows that after 48 h, spleen cells from AFP 464-treated mice exerted an increased specific cytotoxic activity (50% increase) compared to control animals (Fig 3b). Concurrently IFN-γ and IL-10 concentrations were measured in the supernatants of spleen and tumor cell co-cultures. Figure 3c shows and increase in IFNγ levels in cultures with spleen cells obtained from AFP 464-treated mice; also the IFNγ-/IL-10 ratio significantly increased respect to cultures with non-treatedsplenocytes. On the other hand, no differences in antigen-independent activity (evaluated by immobilized anti-CD3 splenocytes activation) was observed between AFP 464-treated and non-treated mice.

AFP 464 modulates immune cell compartments of the tumor microenvironment

We then analyzed the different lymphoid subpopulations in the tumor microenvironment. As figure 4a shows, the percentage of tumor infiltrating lymphocytes (TILs) was higher (4-fold increase) in AFP 464-treated mice compared to the non-treated group (1,73 ± 0,12 vs 0,38 ± 0,09 %). Analysis of the quality of the infiltrate revealed no difference in the percentages of CD45.2+/CD8+ Tc cells between treated and non-treated groups. However, among CD8 cells, 74,03 ± 1,1 % were active (CD69+) in AFP 464-treated mice compared to 55,46 ± 3,4% in non-treated animals (approximately 50% increase). T_{regs} can promote tumor growth by thwarting the development of effective antitumor immune response and promoting tumor-immune escape (Whiteside 2010). Similarly, MDSCs constitute a heterogeneous population of immature myeloid cells with broad immunosuppressive activity within tumor

microenvironments (Gabrilovich and Nagaraj 2009). We found a significant decrease in the frequency of both MDSCs (CD11b+Gr1+) and CD4+CD25+FoxP3+T_{regs} within tumor infiltrates in AFP 464-treated mice compared to non-treated animals (0,37 ± 0,08 vs 0,65 ± 0,04 and 0,085 ± 0,02 vs 0,29 ± 0,07 %, respectively).

AFP 464 induces a M1 phenotype in mice peritoneal macrophages

Macrophages possess phenotypic plasticity that can be classified in immune/inflammatory M1 phenotype and immunosuppresive M2 phenotype. In order to investigate if AFP 464 could modulate macrophage polarization in vivo, we evaluated MMPs gelatinase activity, nitric oxide production and arginase activity in peritoneal macrophages from AFP 464-treated and non-treated mice during 15 days. As figure 5a shows, a decrease in both MMP2 and MMP9 gelatinase activity was measured in peritoneal macrophages isolated from AFP464-treated mice respect to non-treated mice (0.79 ± 0.09 vs 0.25 ± 0.05 and 9.84 ± 0.17 vs 0.46 ± 0.1 respectively). Macrophages isolated from AFP 464-treated mice produced higher levels of nitric oxide compared to untreated mice, suggesting NOS activation by AFP464 (1, 69 ± 0, 41 mg/ml vs 0,84 ± ,023 m g/ml) (Fig 5b). Arginase activity was significantly lower, measured as a decrease in urea, in macrophages isolated from AFP 464-treated respect to non-treated animals (12.71+3.7 vs 1.97+.4 mg/ml, respectively). These data suggested that AFP 464 polarized peritoneal macrophages towards M1 phenotype upon administrations of AFP 464 to M05 tumor bearing mice.

DISCUSSION

The antitumor effect of AFP 464 has been extensively studied in breast, ovarian and renal cancer either in vitro or in vivo and it also has been tested in clinical trials. It activates the AhR signaling pathway, which mediates its cytotoxic effect on tumor cells by inducing cell cycle arrest, ROS formation and apoptosis (McLean et al. 2008). More recently, we have demonstrated that this novel anticancer agent diminishes the quantity of breast cancer stem (Brantley et al. 2016). These findings shed additional insight into the molecular mechanism of AFP 464 anticancer action.

We have previously described that AFP 464 significantly reduced M05 tumor size in vivo whereas in vitro studies showed that only very high concentrations of the compound were necessary to exert cytotoxic effects in vitro on the LM05-Mix cell line, derived from M05 tumor (Brantley et al. 2016). This model has been described as a valuable alternative for the study of estrogen responsiveness and tamoxifen resistance in the context of epithelial-stromal interactions (Pontiggia et al. 2009). In order to better characterize the action of an alternative antitumor agent AFP 464, , we decided to investigate whether the in vivo response could also be associated to an effect of AFP 464 on the immune response. The results presented here reveal that AFP 464 influenced host immune response inducing an anti-tumor profile in tumor bearing mice. We identified multi-target effects, in which AFP 464 treatment increased systemic T cell cytotoxic activity, diminished systemic and local regulatory pro-tumoral response and induced an anti-tumoral macrophage phenotype.

Compelling evidence indicates that AhR is critical in the physiological functions of several immune cell compartments, however, there is controversial data about the role played by this receptor in the immune system because it is posible that different ligands bind to the AhR leading to different, almost opposite outcomes, perhaps based on some conformational change or transcription of different genes.

To elucidate AFP 464 effects on our murine M05 adenocarcinoma model and according to our previous findings, we investigated the activity of the spleen cells isolated from M05 tumor bearing mice after being treated with AFP 464.,We observed that when these cells were adoptively transferred to TBM, they were able to decrease tumor growth in recipient mice. These data strongly suggest that AFP 464 induces an anti-tumoral phenotype in splenic cells. Besides, we also found a higher number of immune cells in the tumor microenviroment of donor mice, resembling a pro-inflammatory response induced by this AhR ligand.

Consequently, we evaluated the distribution of different immune cell subsets in spleens of tumor-bearing mice, as indicator of tumor-induced systemic immune responses. Several studies have demonstrated that carcinogenic AhR ligands like 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), suppresses cytotoxic T lymphocyte (CTL) response (Kerkvliet et al. 2009) and prompts regulatory T cells. However, we observed that AFP 464, an anti-tumor AhR ligand, increases CTL activity in the spleen as measured via IFN-γ and IL-10 production. Nevertheless, no differences were found in cytotoxic T cells frequency between AFP 464 treated and untreated mice either in spleen or in the tumor inflammatory infiltrate. In the current study, this increase in T-cell function was associated with a reduction in MDSC and T_{reg} cell numbers, suggesting that the decrease in both MDSC and T_{reg} subpopulations are contributing to AFP 464 anti-tumor action. Further investigation is therefore necessary to determine whether this is due to the influence of MDSC on T_{reg} formation or rather due to a common target of AFP 464, which is shared by MDSC and T_{reg}.

Our results agree with the data reported by Wuan et al (2013) who demonstrated that Resveratrol, a naturally occurring polyphenol with anti-infective, anti-inflammatory, and anti-oxidant properties, which is an AhR ligand, inhibited T_{reg} expansion to some extent, showing the opposite effects that results from xenobiotic and pharmacologic ligands binding to this receptor.

It has been suggested that AhR may affect the balance between the inflammatory M1 phenotype and the alternative anti-inflammatory M2 phenotype, since after inducing macrophages to differentiate (polarize?) towardsthe M1 phenotype, AhR-null cells present a mixed response and demonstrate decreased NO production and phagocytic capacity. Functionally, it may impair cell microbicidal potential (Climaco-Arvizu, 2016). Coincidently with these data,

in this study AFP 464 induced a M1 peritoneal macrophage phenotype in which NO production was increased and arginase activity, as well as MMP2 and MMP9 gelatinases activities were diminished.
In conclusion, we show that the anti-tumor effect of AFP 464 against M05 mammary tumors implies not only a direct cytotoxic effect on tumor cells but also mediates an anti- tumor immune response, both at local and systemic level. In fact, AFP464 treated mice showed an increase in the pro-inflammatory infiltrate with a decrease in the immunosuppressor MDSC and T_{reg} cells in the tumor microenvironment. At systemic level, AFP 464 decreased the number of MDSC and T_{reg} cells in the spleen as well as it induced the production of IFN-γ by spleen cells, in parallel with the induction of a pro-inflammatory macrophage profile. Our data provide a rationale for the continued development of AFP464 as an agent to enhance the therapeutic management of breast cancer. We found that AFP464 not only reduces bulk tumor similar to other P450 pro-drugs (Swanson HI et al. 2010), but also appears to target cells with stem cell-like properties (Brantley et al. 2016) and induces antitumor immune response. In contrast, Tamoxifen appears to increase stemness properties in breast cancer cells (Raffo et al. 2013) and induces a protumoral immune response (Behjati S and Frank MH 2009). This raises questions concerning its overall clinical efficacy. AFP464 could reduce Tamoxifen undesired effects, ongoing studies of our laboratory will elucidate if combination therapy approaches involving AFP464 and endocrine therapy could improve clinical outcomes for breast cancer patients.

In summary, our data suggest that the anti-tumor effect of AFP464 includes not only a direct cytotoxic effects and reduction of CSC, but also modulates the immune response in order to eliminate the tumor. This could position AFP 464 as a novel complementary agent to current breast cancer therapy.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

We declare that we have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Besides, all procedures performed in studies involving animals were in accordance with the ethical standards of the Ángel H. Roffo Oncology Institute at which the studies were conducted

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Figures:

Figure 1.

Modulation of LM05 tumor growth in vivo by AFP 464. Adoptive transfer of Splenocytes. A- Schematic representation of AFP 464 mice treatment. Female virgin syngeneic mice were inoculated with M05 tumor. Once the average size of tumors reached 1 cm2, mice were treated with AFP 464 [12 mg/kg] or vehicle (non-treated animals, NT), i.p., QD × 5. Tumors growing subcutaneously in mice were measured twice a week with a Vernier caliper; B- Effect of AFP on LM05 tumor growth. Data are presented with respect to tumor size median ± SEM of at least three independent experiments (n=6), *p<0.05;(c) Schematic representation of the adoptive transfer assay. Mice were treated as described in C, fifteen days after treatment mice were sacrificed and splenocytes were isolated and inoculated i.p.into mice bearing M05 tumors; D- Results are the median ± SEM of three experiments (n=6 mice per group); *p<0,05 compared to tumors in animals inoculated with splenocytes isolated from untreated animals (NT).

Figure 2.

Immune cell subsets in spleens of AFP 464 tumor-bearing mice. The frequency of different lymphocyte subpopulations was fifteen days after AFP 464 treatment and in untreated mice (NT). The mean percent±SEM of CD8 + T cytotoxic cells (A), CD8 +/ CD69 + activated cytotoxic T cells (B), CD11 +/Gr1 + MDSC (C) and CD4 +/CD25 +/FoxP3 + Treg cells (D) are shown (n=3 independent experiments, and 4 mice per group); * p <0.05 versus NT animals

Figure 3.

Cytotoxic activity of splenocytes isolated from AFP 464-treated animals. A- Mice were treated as described in figure 1a. Fifteen days after AFP treatment, tumor-specific cytotoxic activity of splenocytes was evaluated after their activation with Mitomicin C- treated LM05-Mix cells; B- Antigen-independent cytotoxic activity was evaluated with immobilized anti-CD3 activation. Isolated splenocytes and LM05-Mix cells were cultured separately in order to calculate the cytotoxic activity of splenocytes measured by the MTS assay. Cytotoxic activity was calcualted as% Specific Lysis = 1- [(OD splenocytes+LM05-Mix cells - OD Splenocytes)/ OD LM05-Mix cells] x 100; where OD is the optical density measured with an ELISA Reader at 490/620 nm. C- The amount IL-10 and IFN-γ were evaluated from conditioned media obtained from the co-culture of activated splenocytes with LM05-Mix cells by ELISA assay. The values represent the mean±SEM of three independent experiments (four mice per group); * p <0.05 versus non-treated animals (NT)

Figure 4.

Modulation of tumor-infiltrating immune cells (TIICs) in AFP 464-treated animals. The presence of TIICs was analyzed by flow cytometry in cell suspensions obtained from 15-days AFP 464 treated and untreated control animals. A- Representative forward versus side scatter dot plot, indicating the percentage of TIICs in solid tumors together with the mean value ± SEM of the percentage of TIICs in each group (n=3 independent experiments); * p<0.05 versus control; B- TIICs were identified as CD45.2+/CD8 + cytotoxic T cells, CD8+/CD69+ activated cytotoxic T cells, CD11b+/Gr1+ MDSC and CD4+/CD25+/FoxP3+ Treg cells were quantified. The values represent the percentage mean ± SEM of three independent experiments (four mice per group); * p <0.05 versus non-treated animals (NT)

Figure 5.

Modulation of peritoneal macrophage phenotype in AFP 464-treated mice. Animals were treated as described in figure 1 a. Fifteen days after, peritoneal macrophage phenotype was characterized by (A) MMPs (2 and 9) gelatinase activity in a zymography assay, (B) nitric oxide production by Griess assay and (C) arginase activity by urea production. The values represent the mean ± SEM of three independent experiments (four mice per group); * p <0.05 versus non-treated animals (NT)

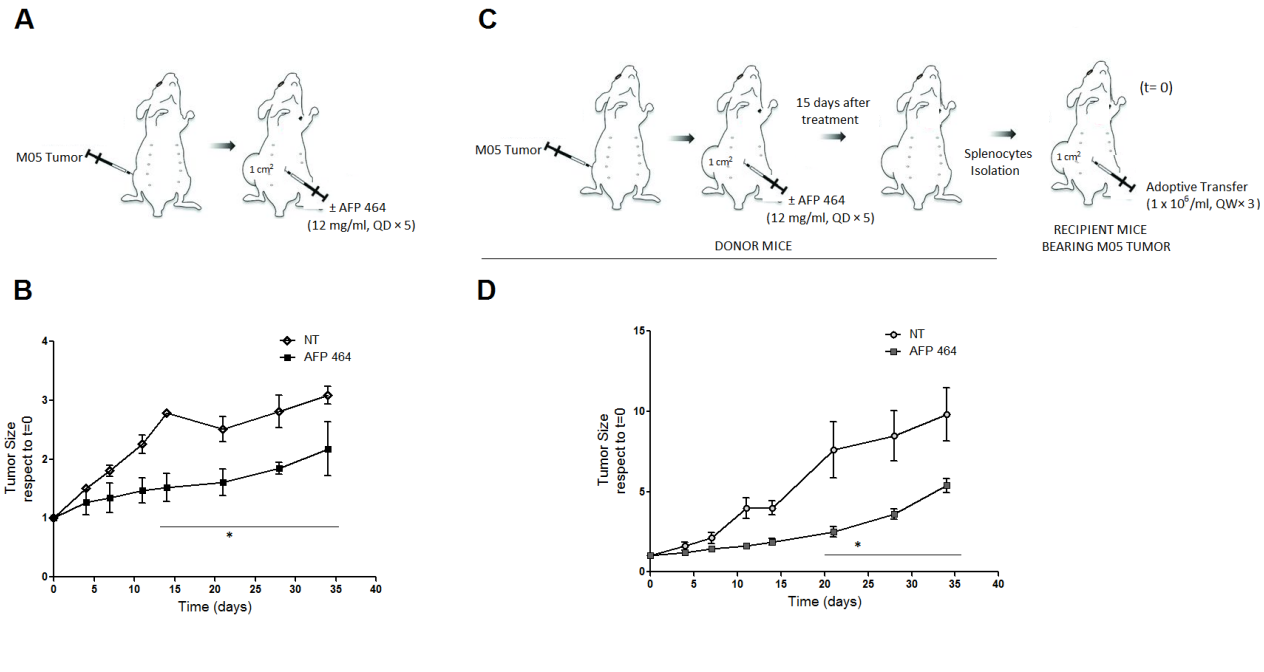

Figure 1

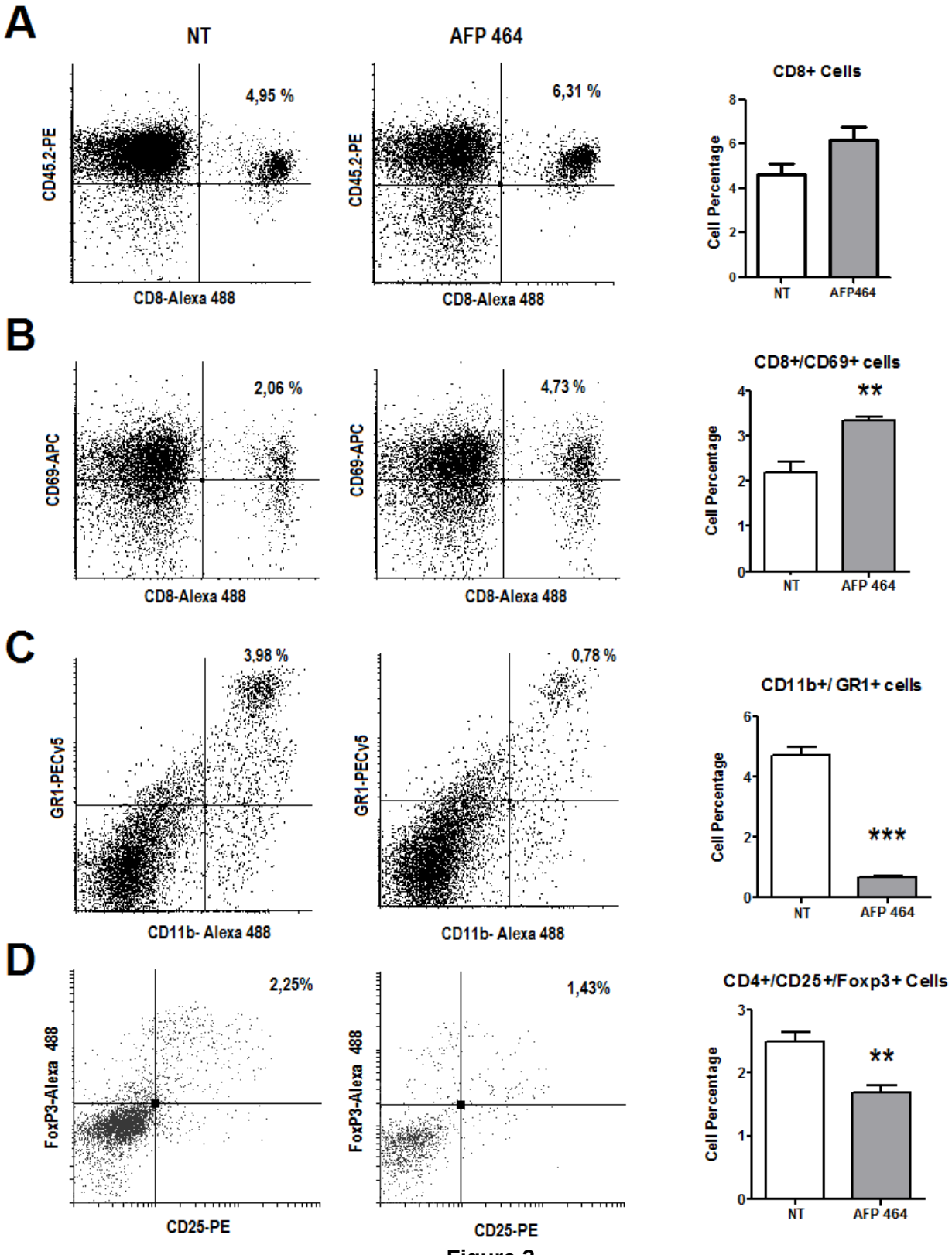

Figure 2

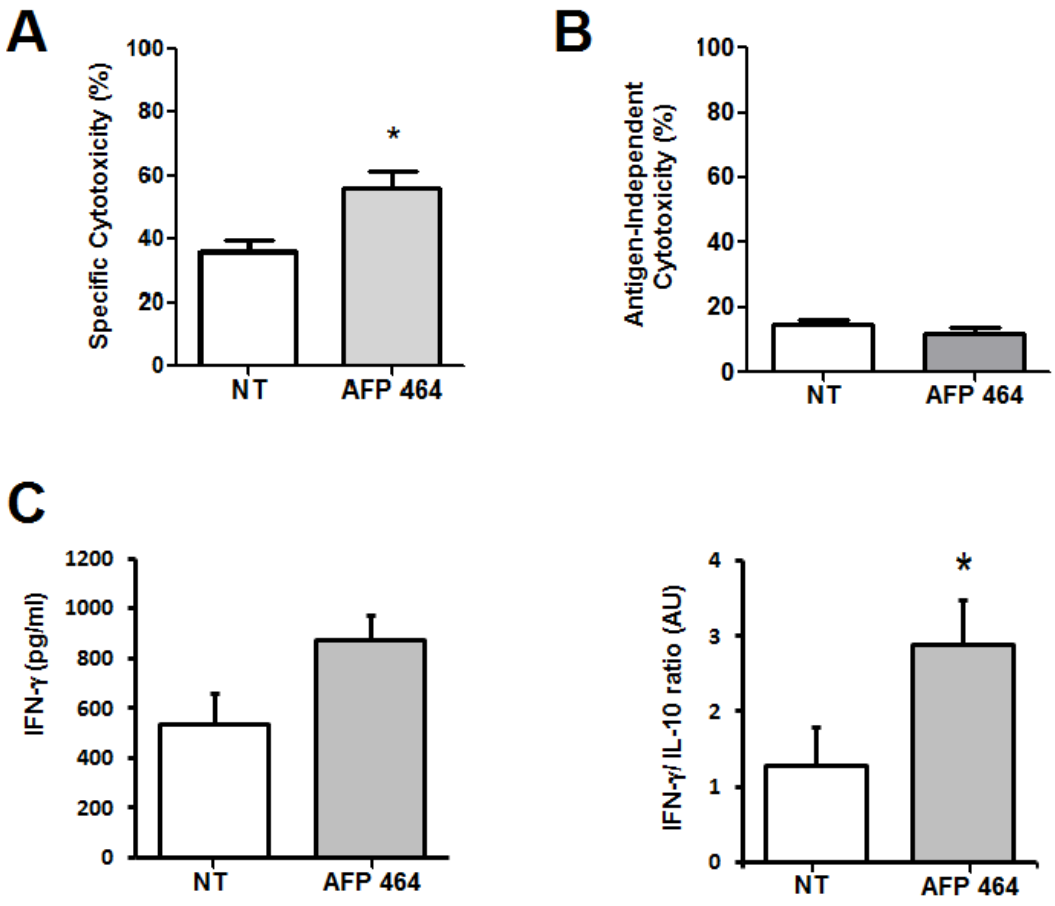

Figure 3

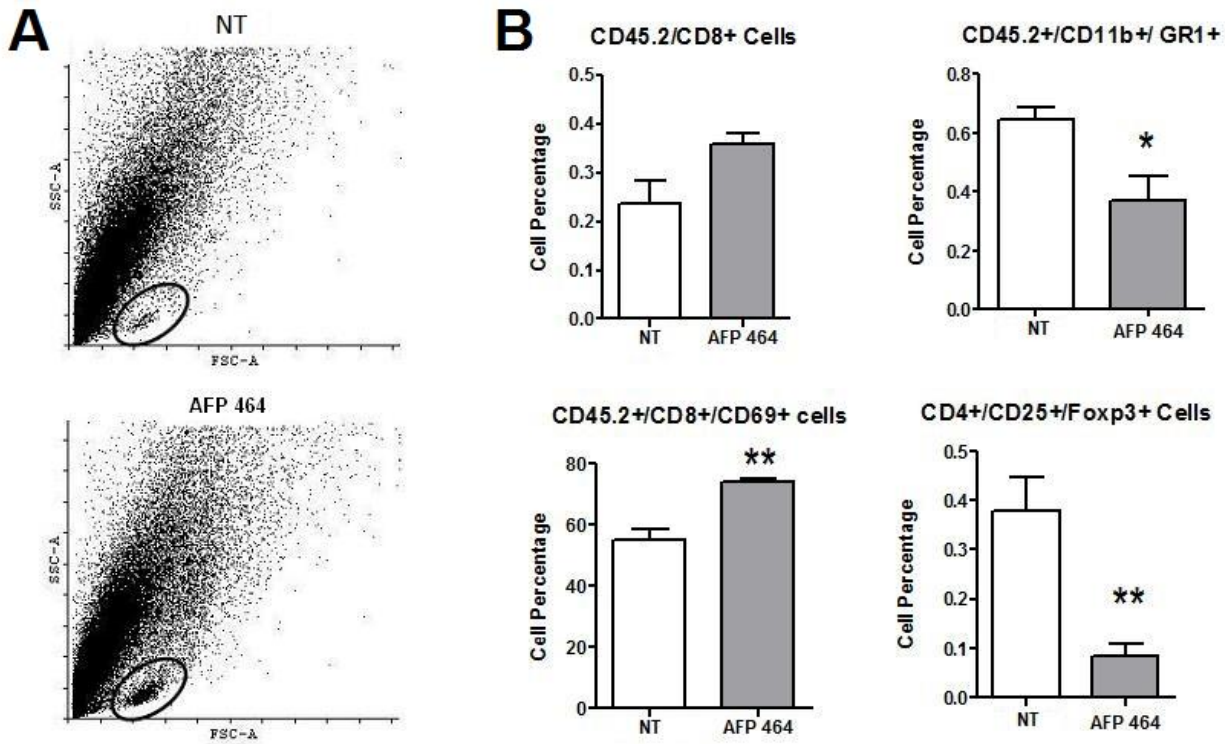

Figure 4

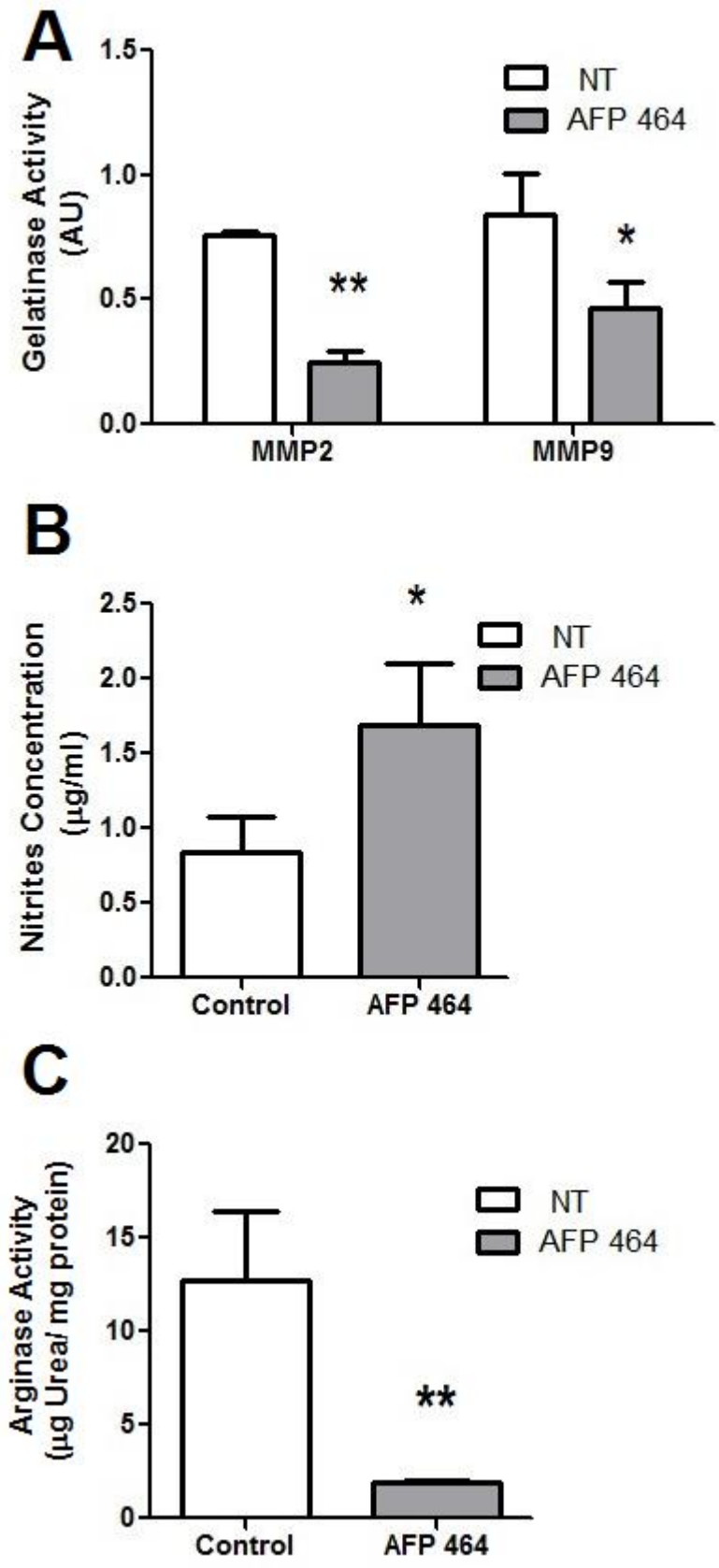

Figure 5

