Title: Aptamer-mediated blockade of IL4Rα triggers apoptosis of MDSCs and limits tumor progression.

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ABSTRACT
In addition to promoting tumor progression and metastasis by enhancing angiogenesis and invasion, myeloid-derived suppressor cells (MDSC) and tumor-associated macrophage (TAM) also inhibit antitumor T cell functions and limit the efficacy of immunotherapeutic interventions. Despite the importance of these leukocyte populations, a simple method for their specific depletion has not been developed. In this study, we generated an RNA aptamer that blocks the murine or human IL4 receptor-α (IL4Rα or CD124) that is critical for MDSC suppression function. In tumor bearing mice, this anti-IL4Rα aptamer preferentially targeted MDSC and TAM and unexpectedly promoted their elimination, an effect that was associated with an increased number of tumor-infiltrating T cells and a reduction in tumor growth. Mechanistic investigations of aptamer-triggered apoptosis in MDSC confirmed the importance of IL4Rα-STAT6 pathway activation in MDSC survival. Our findings define a straightforward strategy to deplete MDSCs and TAMs in vivo and they strengthen the concept IL4Rα signaling is pivotal for MDSC survival. More broadly, these findings suggest therapeutic strategies based on IL4Rα signaling blockades to arrest an important cellular mechanism of tumoral immune escape mediated by MDSC and TAM in cancer.
INTRODUCTION

Myeloid Derived Suppressor Cells (MDSC, CD11b\(^+\)Gr1\(^+\)IL4R\(\alpha\)\(^+\)) and Tumor Associated Macrophages (TAM, CD11b\(^+\)Gr1\(^-\)F4/80\(^+\)) are particularly important in promoting tumor growth metastasis and angiogenesis (4, 5) and in suppressing the anti-tumor immune response (6). Although different signaling pathways may be responsible for the MDSC suppressive activity, the IL4R\(\alpha\)-STAT6 pathway seems to be particularly important: it mediates TGF-\(\beta\) production (7), Arginase activity (8) and, in conjunction with STAT3 and STAT1 activation, it allows for the production of peroxynitrite and ROS (6) and for the expression of MMP9 (9).

Although different compounds were shown to inhibit or deplete MDSC (i.e. STAT3 or PDE5 inhibitors, vitamin D, ATRA, nitroaspirin gemcitabine etc (10)) and demonstrated reasonable rates of success, their systemic use leads to undesirable side effects and off target depletion.

Selective targeting of MDSCs is therefore necessary to maximize the efficacy of immune therapeutic approaches while minimizing systemic side effects. To this aim, we selected a new RNA aptamer that specifically targets IL4R\(\alpha\) because of the importance of this receptor in MDSCs suppressive function. RNA aptamers are nanomolecules that can recognize their targets with extremely high affinity. This class of compounds provides unique advantages as therapeutic agents, such as amenability to chemical modifications, ease of production, and low immunogenicity (11, 12).

Here we describe a new RNA aptamer specific for IL4R\(\alpha\)/CD124. This aptamer selectively targets MDSC and TAM \textit{in vivo}, blocks IL4R\(\alpha\) signaling, induces MDSCs apoptosis and significantly delays tumor progression in 4T1 bearing mice.
MATERIALS AND METHODS

Cell lines. 4T1, 4T1HAThy1.1luciferase TS/A and MSC-2 cell lines were previously described (13, 14). MSC-2 were maintained in DMEM (Invitrogen), supplemented with L-glutamine (2mM), HEPES (10mM), 2-mercaptoethanol (20mM), streptomycin (150U/mL), penicillin (200U/mL), and 10% heat-inactivated FBS (Invitrogen).

Aptamer selection. Aptamers were selected with the optimized magnetic epoxy-beads method described in the supplementary material and used in vitro at 150 nM unless otherwise specified.

Mice. All animal experiments were approved by the Division of Veterinary Resources and the Institutional Animal Care & Use Committee (IACUC) of the University of Miami. BALB/c mice were purchased from Harlan Laboratories while IL4Rα-/- mice were obtained from The Jackson Laboratory. All experiments were conducted on 6-10-weeks old mice in pathogen-free facilities.

Aptamer treatment of tumor bearing mice. Mice were injected s.c. with either 500,000 tumor cell. Starting on day 3 after challenge and every other day thereafter, mice received IV injections of either IL4Rα (16pmoles/grams) or an unrelated aptamer in PBS. Tumor growth was evaluated 3 times a week. Data are expressed as tumor size index that is the product of the main diameter with the perpendicular one.

Flow cytometry. Antibodies used are described in the supplementary material. Dead cells were excluded by the analysis by using the yellow-DEAD/LIVE dye (Invitrogen) or DAPI (Sigma). Manufacturer’s instructions were used for Annexin-V-APC/7AAD and PhosphoSTAT6 staining (BD-Pharmingen). Samples were read on LSR-Fortessa-HTR flow cytometer (Becton-Dickinson) or an LSR2 (equipped with the 532 laser to excite the Cy3 fluorochrome). Data were analyzed using FCS3 express software (Denovo-Software). Proliferation analysis was performed using FCS4 professional software (Denovo-Software).
Tumor immunofluorescent analysis. Specimens were fixed with 10% formalin and embedded in paraffin and stained using standard immune-fluorescence methods described in the supplementary methods.

Suppressive assays. $10^5$ MDSCs were plated in a 96-well flat bottom plate with $10^5$ CFSE-labelled HA-specific CD8$^+$ T-cells purified by negative selection from the splenocytes of TCRcl4 mice (Jackson laboratories). $10^6$ Thy1.2$^{+/+}$Balb/c splenocytes were added as feeders and T-cells were stimulated with the relevant or an irrelevant peptide. 3 days later, clonotypic T cell proliferation was evaluated by FACS after staining with anti-CD8 and anti-Thy1.1 antibodies.

Statistical analysis. Sigma-Plot was used for statistical analysis. One-way ANOVA or ANOVA on RANKs was performed after normality evaluation by Kolmogorov-Smirnov test. Pairwise post-hoc analysis was performed using the Holm-Sidak’s or the Dunn’s test. Student’s T test was used when two groups were analyzed. Kaplan Meier log-rank followed by the Holm-Sidak post-hoc analysis was used to evaluate the survival differences between groups.
RESULTS

IL4Rα is a functional marker that defines the suppressive MDSCs population in the 4T1 mammary carcinoma.

We have previously shown that IL4Rα (CD124) is up-regulated in many murine tumors and that it plays a key role in MDSCs suppressive function in the mouse CT26 colon carcinoma (2) and A20 lymphoma(15). However it is necessary to determine the importance of a putative functional marker in any model used. To this aim, different population of MDSC were isolated from IL4Rα+/+ or IL4RαKO Balb/c mice bearing the 4T1 mammary carcinoma. Mice were sacrificed at day 16, splenic CD11b+MDSCs were magnetically enriched (Fig.1A,B) and MDSCs populations (CD11b+IL4Rα+, CD11b+IL4Rα−, total CD11b+cells or CD11b+IL4RαKO) were sorted by FACS and the suppressive activity of each population was tested against CFSE labelled HA-specific CD8+ T-cells. CD11b+MDSCs drastically inhibit T-cells proliferation allowing only half of the clonotypic population to divide (Fig.1C). Similar results are obtained with CD11b+IL4Rα+ cells but not with CD11b+IL4Rα− nor with CD11b+IL4RαKO cells (Fig.1C, D). CD11b+ and CD11b+IL4Rα+ cells reduced also the proliferative capacity of the dividing cells as shown by the reduced proliferative index, while no inhibition was seen when CD11b+IL4Rα− or CD11b+IL4RαKO cells were used. These data indicate that CD124 expression (together with CD11b) not only identifies the MDSCs suppressive subpopulation in the 4T1 model, but also indicates that IL4Rα is required for their suppressive function.

Isolation of an IL4Rα specific aptamer through optimization of SELEX.

The results described in Fig.1 not only confirm that IL4Rα plays an important role in MDSCs-mediated immune suppression, but also suggest that it may be used to target MDSCs in mammary carcinoma. To generate aptamers that bind specifically to IL4Rα via SELEX (16), the
traditional selection method needed to be optimized since at physiological pH the commercially available recombinant IL4Rα (IgG1-IL4Rα) has a net negative charge (Suppl.Fig.1) and most of the positively charged pockets are present in the IgG1 portion. Under these conditions the isolation of the aptamers specific for the IL4Rα portion of the recombinant protein is inhibited while, despite a counter-selection step, isolation of anti-IgG1 or anti-linker aptamer is facilitated. Indeed, selections procedures (15 cycles each) based on the original protocol (Suppl.Fig.2A) generate aptamer against IgG1 or the linker. To overcome these limits, a new method for aptamer selection was developed (Suppl.Fig.2B). This method (described in the supplementary methods) is based on the use of epoxy-magnetic beads directly linked to enzymatically cleaved IL4Rα portion. The epoxy-magnetic beads mediated SELEX was used to screen a combinatorial RNA library of approximately 10¹⁴ species for aptamers capable of binding to the extracellular domain of murine IL4Rα. The starting library and the RNA from the subsequent cycles were generated with 2′-fluoro-pyrimidines that were shown to protect RNA from RNase. After 5 cycles of amplification, PCR products from the selected aptamers were cloned and single aptamers sequenced (Fig.2). 28% of the sequenced clones had an identical sequence that was named cl.42 (Fig.2A). Interestingly, however, additional 23% of the sequences showed a strong similarity to the cl.42 aptamer.

To evaluate whether the cl.42 aptamer was able to specifically recognize the IL4Rα protein, the aptamer was covalently linked at the 3′ terminus with biotin-conjugated tetranucleotide using the T4-RNA-ligase, a modification unlikely to change the tertiary RNA structure. The biotin conjugated cl.42 aptamer was then incubated with epoxybeads loaded with IL4Rα, with VCAM (as negative control) or left unconjugated (Fig.2C). As additional control, an irrelevant, biotin-labeled aptamer was used. Streptavidin-phycoeritrin (SA-PE) conjugate was used to visualize
the aptamers bond to the beads’ surface via FACS. While the irrelevant aptamer failed to recognize any beads, the cl.42 aptamer successfully bound to the IL4Rα-epoxybeads but not to the unconjugated beads or the ones loaded with the unrelated protein VCAM. Similar results were obtained when aptamers were directly labeled with the Cy3 dye. The calculated Kd (14nM, Fig.2D) is within the range of anti-IL4Rα antibodies (17) and is considered to be indicative of a good affinity for aptamers (18).

To evaluate whether the anti-IL4Rα aptamer can recognize not only the recombinant protein but also the native protein under physiological condition, the Cy3-conjugated cl.42 or irrelevant aptamer were incubated with the IL4Rα+MSC-2 cell line in cell media at 37°C. The anti-IL4Rα aptamer effectively recognizes the MSC-2 cell line as determined by immunofluorescence microscopy (Fig.2E) or by FACS (Suppl.Fig.3A) with an apparent Kd of 788nM (Suppl.Fig.3B). Only background fluorescence was seen when an irrelevant aptamer was used.

We next evaluated whether the aptamer raised against murine IL4Rα could cross-react with the human CD124. Briefly, human umbilical cord blood cells enriched of CD33+IL4Rα+MDSCs by a 4 day culture with recombinant GM-CSF and G-CSF were stained with anti CD33 antibody and with either the cl.42 aptamer or the anti-IL4Rα antibody (Fig.2F and Suppl.Fig.4). FACS analysis revealed that anti-IL4Rα aptamer recognized human CD33+MDSCs, while only background signal was present when an irrelevant aptamer was used. FACS evaluation of different MDSC preparations (n=7) (Fig.2F) indicates an high degree of correlation (R²=0.994) between the data obtained using the anti-IL4Rα antibody or the cl.42 aptamer suggesting the capacity of the latter to recognize human MDSCs.
The anti-IL4Rα aptamer cl.42 preferentially recognizes MDSCs and tumor associated macrophages in mice bearing the 4T1 mammary carcinoma.

We next evaluated whether the IL4Rα specific aptamer can bind its ligand in vivo and if preferential targeting of cell subset occurs in tumor bearing mice. In fact, although IL4Rα is highly expressed in MDSCs and M2 macrophages, the receptor is also found in most hematopoietic and non-hematopoietic cells (19, 20). WT or IL4Rα−/−Balb/c 4T1 bearing mice (with tumor diameter of approximately 0.5cm) were injected with the Cy3-labelled anti-IL4Rα aptamer or with an irrelevant aptamer. Two hours later spleens, lungs, livers and tumors were harvested labeled with antibodies and evaluated by FACS. Cy3 signal was detectable in all the organs examined from the WT-Balb/c group treated with anti-IL4Rα aptamer (Fig.3A); in striking contrast, only baseline signal is present in the IL4Rα−/−mice treated with the anti-IL4Rα aptamer or in the IL4Rα+/+mice treated with the irrelevant aptamer (Fig.3A,B). A higher percentage of Cy3⁺cells was detectable in the tumor specimens of WT mice treated with IL4Rα specific aptamer, suggesting a preferential targeting of this tissue (Fig.3A). FACS analysis of the tumor specimens revealed that the Cy3⁺population is positive for the myeloid marker CD11b and is characterized by low expression of Ly6G, high expression of Ly6C, and a bimodal expression of the macrophage marker F4/80. In particular, in the tumor, CD11b⁺Gr1⁺Ly6C⁺F4/80low/monocytic MDSC and CD11b⁺F4/80⁺Gr1low/macrophage seemed to be preferentially bound by the anti-IL4Rα aptamer (Suppl.Fig.5A) whereas in the spleen monocyteic MDSC are the main target of the aptamer (Suppl.Fig.5B).

To better determine the specificity of the aptamer binding in vivo, a different gating strategy was used. After gating on singlet alive CD11b⁺cells (Fig.3B) 3 populations of cells can be identified based on the Gr1 and F4/80 expression (Fig.3C): Gr1highMDSC, Gr1lowMDSC and Gr1⁺TAM.
Analysis of Cy3 fluorescence reveals that the anti-IL4Rα aptamer binds to all 3 myeloid populations while only background signal is obtained when the irrelevant aptamer is used. A similar gating strategy (Fig.3D,E) does not reveal cl.42 aptamer binding on CD4+ and CD8+T-cells, on B-cells (CD19+) on NK (DX5+) and on DC (CD11c+). These data suggest that the anti-IL4Rα aptamer specifically binds its ligand in vivo, and also suggest preferential targeting of MDSC and TAM in cancer-bearing mice.

**The anti-IL4Rα aptamer restrains 4T1 tumor progression, promote CD8+T cell infiltration and reduces the number of MDSCs infiltrating the tumor.**

Aptamers not only can bind their ligands, but may also trigger a biological function (21). Thus, we investigated whether chronic administration of anti-IL4Rα aptamer may influence tumor growth. IL4Rα+/+ or IL4Rα−/− 4T1 bearing mice, were treated IV every other day with the anti-IL4Rα or an irrelevant aptamer (16pM/g). Tumor progression was significantly inhibited in mice treated with the IL4Rα-specific aptamer (Fig.4, Suppl.Fig.6). In sharp contrast, no effect was observed when IL4Rα−/− mice were used. If mice were not euthanized and treatment was continued, a plateau phase was observed after which the tumor started to grow again and eventually the mice needed to be euthanized.

Similar results are obtained when the mammary carcinoma TSA (data not shown) or the 4T1-Thy1.1-HA-luciferase (Fig.4B) are used. This 4T1 derived cell line allows the detection of the metastatic disease by different means. In particular, when Thy1.2 Balb/c mice are challenged, neoplastic cells can be detected by FACS using the Thy1.1 marker that, in our hands, appears more sensitive than the use of luciferase. As shown in Fig.4B, the anti-IL4Rα aptamer not only inhibited the progression of the primary tumor, but also drastically reduced the number of
neoplastic cells found in the lung; both effects are likely to depend on its specific binding to IL4Ra, since the effect is lost when IL4Ra−/− mice or an irrelevant fluorinated RNA are used. CD3 immune fluorescence analysis of the 4T1 tumor specimens revealed a significantly higher infiltration of T-cells in the anti-IL4Ra aptamer treated group (Fig.5A) suggesting that the anti-tumor effect occurs through modulation of the immune response. This result is confirmed by the staining for CD8 and Gr1 (Fig.5B) that shows a higher infiltration of CD8+ cells and a significant reduction of Gr1+ cells in the group treated with anti-IL4Ra aptamer. Interestingly, a population of CD8+Gr1+ cells (a phenotype associated with the peripheral homing of central memory T-cells (22)) is also detectable, and marginally (Fig.5B insert) increased by the anti-IL4Ra aptamer treatment. CD11b and Gr1 staining (Suppl.Fig.7) confirms a significant decrease of MDSCs and shows a similar reduction of CD11b+Gr1− macrophages. To confirm these data through quantitative analysis and to expand the leukocytes studies to other populations, the experiments were repeated and multicolor FACs analysis was performed on tumor-derived single cells suspensions. The percentages of granulocytic MDSCs (CD11b+Gr1high), monocytic MDSC (CD11b+Gr1low), TAM (F4/80+CD11b+Gr1+), Treg (CD3+CD4+CD25+Foxp3+), CD3−CD4+ and CD3−CD8+ T-cells were evaluated within live (DAPI negative) cells. While MDSCs, TAMs and Tregs were significantly decreased by the treatment with the IL4Ra specific aptamer, effector T-cells appeared increased. Moreover, a higher number of T-cells expressing the activation marker CD69 were detectable in the anti-IL4Ra aptamer group compared to the irrelevant (Fig.5C).

Taken together, these results indicate that the anti-IL4Ra aptamer treatment significantly alter the tumor microenvironment reducing the number of the negative regulators of the immune response while increasing the number and the activation status of effector T-cells.
The anti-IL4Rα aptamer promotes MDSC apoptosis.

The previous section indicates that anti-IL4Ra aptamer reduced the number of MDSCs and TAM in vivo. However, the question remained whether this effect is mediated by alterations in the recruitment/maturation or in the survival of this leukocyte population. To better understand the effect of the anti-IL4Rα aptamer on MDSCs, the immortalized MSC2 cell line and MDSCs freshly isolated from tumor bearing mice were used. While the MSC2 offer a uniform and stable source of MDSCs with only minimal spontaneous apoptosis, freshly isolated MDSCs closely mimic the in vivo setting, but are more heterogeneous and data interpretation may be compromised by a higher level of spontaneous apoptosis, by the isolation procedure, or by spontaneous in vitro differentiation of cell subsets. In the first setting, splenic CD11b+ cells magnetically purified from 4T1 bearing mice were incubated with the anti-IL4Rα aptamer or with the control aptamer. Viability was evaluated after 1, 2, 3 or 4 days. CD11b+ cells tend to die within 3-4 days when cultured in vitro in normal media (Fig.6A) (23). This pattern is not altered when the control aptamer is added to the culture. However, when cells are treated with the anti-IL4Rα aptamer a significant decrease in MDSC viability is observed starting on day 1. In the second setting, MSC2 were incubated for 2 days with either the IL4Rα-specific or an irrelevant aptamer. A significant increase in MSC-2 apoptosis and a concomitant reduction of viable cells was observed in the wells treated with the anti-IL4Rα aptamer (Fig.6B). These data suggest a pro-apoptotic effect of the anti-IL4Rα aptamer on MDSCs that is in keeping with the observation of aptamer-mediated reduction of MDSCs at the tumor site.

IL4Rα mediates a pro-survival signaling in MDSC.

The anti-IL4Rα aptamer-mediated apoptosis of MDSCs observed in the previous experiments can be triggered by different processes such as RNA engagement of TLRs (i.e. TLR3) (24-28) or
activation of RNA-dependent protein kinase (PKR) (29-33). Alternatively, aptamer binding may modify some signaling pathways in MDSC affecting their survival. Since only the IL4Rα specific aptamer, but not an irrelevant aptamer, triggers MDSCs apoptosis at the concentration used (150nM, Fig.6) it is unlikely that the first two pathways are majorly involved in this effect. Moreover, although a pro-apoptotic effect of the irrelevant aptamer on MDSC is detectable when the concentration is increased 100 times (data not shown), experiments using PKR-specific inhibitors or the TLR3 ligand poly(I:C) failed to, respectively, inhibit or mimic the anti-IL4Rα aptamer-induced MDSCs apoptosis (Suppl.Fig.8). Since the activation of the IL4Rα-STAT6 pathway has been shown to exert either pro- or anti-apoptotic effects(34-37), depending on the cellular contest, we evaluated the effect of the anti-IL4Rα aptamer on this pathway. Splenic CD11b+ cells from 4T1 bearing mice were stimulated with IL-13 in the presence of the anti-IL4Rα aptamer or an irrelevant aptamer. Since IL4Rα engagement induces STAT6 phosphorylation (38, 39), cells were labeled with antibodies against phosphoSTAT6 (pSTAT6) or with the isotype control and analyzed by FACS. The addition of IL-13 activates the IL4Rα pathways and increases STAT6 phosphorylation (Fig.7A). While the addition of the irrelevant aptamer does not significantly alter pSTAT6 signaling, the IL4Rα specific aptamer drastically reduced pSTAT6. To verify that the anti-IL4Rα aptamer acts as antagonist in the absence of possible artifacts associated with MDSCs purification MSC2 were used and cultured with or without IL-13. Anti-IL4Rα aptamer, irrelevant aptamer or no aptamer were added to the cultures. phosphoSTAT6 was evaluated by FACS 2 hours later (Fig.7B). As observed for the freshly isolated CD11b, the IL4Rα specific aptamer significantly decreased the IL-13-dependent STAT6 phosphorylation while no effect was observed when the irrelevant aptamer was used. These data are further substantiated by the evaluation of arginase activity after induction with IL-13.
While the irrelevant aptamer failed to modulate arginase activity, the anti IL4Rα prevented Arg1 activation (Suppl.Fig.9). These data indicate that the anti-IL4Rα aptamer is a blocking aptamer that prevents IL4Rα signaling; however, they do not proof if this is the underlying cause of MDSCs apoptosis. To determine whether IL4Rα signaling promotes MDSCs survival, 2 complementary strategies were adopted: 1) Since MSC2 cells autocrinally secrete low doses of IL-13 (data not shown), MSC2 survival in the presence of IL-13 neutralizing antibodies was evaluated; 2) CD11b+ cells isolated from tumor bearing mice were treated with IL-13 to evaluate whether this cytokine can promote their survival.

In the first set of experiments, MSC-2 were cultured alone, in the presence of IL-13 neutralizing antibody or isotype control. Media with fresh antibodies was changed on day 2. Viable cells on day 4 were evaluated by 7ADD and annexin V staining and data were normalized for the number of cells recovered. IL-13 neutralization resulted in high mortality of MSC-2 (Fig.7C), suggesting that the autocrine secretion of this cytokine acts as a survival signal for MSC-2. No effect was observed when the isotype control was used.

In the second strategy (Fig.7D), CD11b+ cells isolated from 4T1 tumor bearing mice were cultured in 24 well plates for 2 days in the presence of IL-13, IFN-γ, both cytokines or none. IFN-γ was used since it stimulates the expression of IL4Rα in MDSCs (2). Only half of the cells initially plated were recovered from the wells in which cytokines were not added (Fig.7D). Addition of either IFN-γ or IL-13 did not significantly increase MDSC viability; however, when both cytokines were used, a significant increase of cells recovered was observed. Taken together, these data suggest that IL4Rα expression and its engagement could promote pro-survival signaling in MDSCs, and that the anti-IL4Rα aptamer can counteract this effect.
DISCUSSION

Two are the main approaches that are currently being pursued to target MDSC in tumor bearing host: the first one focuses on compounds that alter MDSCs differentiation either by halting MDSCs recruitment or maturation, or by forcing MDSCs differentiation into a more mature, not immunosuppressive cell type. The second approach instead is aimed at inhibiting the molecular mechanisms that MDSCs use to affect tumor immunity. Specific depletion of MDSCs from the periphery is a third possible approach but it is currently limited by the absence of reagents that selectively target this population. We have previously shown that IL4Rα is a good candidate, since it is expressed on MDSCs in many murine tumor models (2), and its expression is particularly important for the MDSC-mediated immunosuppression in the colon carcinomas CT26s and C26GM, in the fibrosarcoma MCA203 (2) and in the A20 lymphoma (15). Moreover, CD124 is one of the first markers to be upregulated in bone marrow cells under cytokine regimes that generate human and mouse MDSCs (40). Finally, its expression in the mononuclear MDSCs population of patients with Colon cancer and Melanoma correlates with the suppressive function of this population (41). Nevertheless, in the some models (i.e. EL-4 (42)), IL4Rα⁺ and IL4Rα⁻ MDSCs do not differ in their suppressive activity. Here we confirm the functional importance of IL4Rα in the 4T1 mammary carcinoma (Fig.1) and we isolate a new CD124 specific RNA aptamer that selectively depletes MDSCs from tumor bearing mice. The anti-IL4Rα aptamer-dependent MDSC depletion does not rely, as is the case with most antibodies, on complement-mediated cell clearance, because of the absence of the Fc region, and it is also unlikely that cell-mediated cytotoxicity is activated, because of the low immunogenicity that characterizes RNA aptamers. Instead, the IL4Rα specific aptamer promotes MDSCs apoptosis upon binding. By analyzing the mechanisms of depletion, we focused our attention on the IL4Rα pathway since
preliminary experiments seemed to exclude the involvement of both TLR3 and PKR, the two main proteins that may trigger 2′fluoro-dsRNA mediated apoptosis (Suppl. Fig. 8). Indeed, the addition of the IL4Rα-specific aptamer to MDSC cultures was sufficient to prevent IL-13-mediated STAT6 phosphorylation, indicating the antagonist properties of this molecule.

IL4Rα and STAT6 play important roles in MDSC activation and in the maintenance of their suppressive activities by regulating Arg1 expression and TGF-β secretion (8, 43, 44). Furthermore, genetic inactivation of STAT6 restores immune surveillance allows for the rejection of spontaneous metastasis in the 4T1 mammary carcinoma model.

Our data suggest an additional important role of this receptor: IL4Rα engagement could promote MDSCs resistance to apoptosis (Fig. 6 and 7). The anti-apoptotic role of IL4Rα was elucidated in many cell types including B-cells, T-cells, masT-cells, and myeloid cells but, until now, not in MDSC. Interestingly, IL-13 pretreatment has been shown to protect human synoviocytes from nitric oxide induced apoptosis by inducing IRS phosphorylation and PKC activation (45). Although we haven’t yet confirmed this pathway in MDSCs, it is possible that IRS activation by IL4Rα is necessary for protecting MDSCs from the autocrinally produced NO and that the aptamer-mediated blockade of this pathway prevents this protective mechanism. In any case, in this paper, three main evidences suggest an important anti-apoptotic role of IL-13 on MDSCs: 1) aptamer-mediated blockade of IL4Rα signaling correlates with an increase apoptosis in MDSCs (Fig. 6 and 7); 2) the antibody mediated neutralization of IL-13 in cultured MSC2 promotes their apoptosis (Fig. 7C) and, 3) addition of IL-13 to the culture media of freshly isolated MDSCs promotes their survival (Fig. 7D). These in vitro results seem to be holding true also in vivo since the administration of the anti-IL4Rα aptamer correlates with a marked decrease of MDSCs and TAM at the tumor site (Fig. 5).
Interestingly, despite the fact that IL4Rα is not a specific marker of MDSCs, \textit{in vivo} administration of the anti-IL4Rα aptamer showed specificity for MDSCs and tumor associated macrophages (Fig.3). The reasons for this preferential \textit{in vivo} targeting of MDSCs are currently unknown but can be related to cell specific post-translational modification of CD124 that can differentiate the IL4Rα expressed in tumor associated MDSC from the one expressed in other cell types. It is important to note that the recombinant IL4Rα toward which the aptamer has been selected is isolated from the Balb/c myeloma cell line NSO and as such is characterized by several post-translational modifications including differential patterns of glycosaminoglycans typical of tumor and tumor stroma (46, 47). Alternatively, the small differences in the membrane density of CD124 or in the receptor recycling within the different cell populations may be responsible for the preferential staining of MDSCs and tumor associated macrophages. It is important to note, however, that we cannot definitively rule out binding of the aptamer to cells other than MDSCs and TAM below the detection levels of our analysis.

Particularly interesting are the results obtained by the chronic administration of unconjugated aptamers to mice bearing the 4T1 tumor. In these mice, the IL4Rα specific aptamer selectively depletes MDSCs and macrophages in the tumor as well as splenic IL4Rα⁺MDSCs. This depletion leads to a higher infiltration of CD8⁺ and CD4⁺T-cells in the tumor (most of which express CD69 (Fig.5)) and correlates with a significant reduction of tumor growth in the wild type mice but not in IL4RαKO mice (Fig.4). However, it is important to underline that treatment with the IL4Rα aptamer alone does not lead to tumor eradication but promotes only the temporarily arrest of its growth. These results are similar to what we saw with PDE5 inhibitors in which, despite the priming of a spontaneous antitumor immune response, after a steady state, the tumor grew again because other immune escape mechanisms arose (i.e. antigen loss) (14).
Analysis of the metastatic disease using the 4T1HAThy1.1 model seems to indicate that aptamer treatment is sufficient to reduce the number of neoplastic cells in the lung. However, at the moment, we cannot discriminate whether this effect is determined by a direct action of the aptamer on the metastatic disease or just by the fact that the size of the primary tumor is significantly reduced.

In conclusion, we report herein on the isolation of a new antagonistic aptamer against mouse and human IL4Ra/CD124 that unveils the importance of the IL4Ra-STAT6 pathway in promoting survival of MDSCs. In light of these new data, the importance of IL4Ra/CD124 in MDSCs biology and as a pharmacological target justifies further research as it may have impact on clinical treatment of cancer.

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FIGURE LEGENDS

Figure 1. IL4Rα identifies the MDSCs suppressive population in the 4T1 mammary carcinoma. A) Experimental scheme. B) Different populations of MDSCs (CD11b⁺, CD11b⁺IL4Rα⁺, CD11b⁺IL4Rα⁻) were isolated by FACS after magnetic pre-enrichment from wt or IL4Rα⁻/⁻Balb/c mice challenged 15 days before with the 4T1 tumor. C) The suppressive activity of each population was evaluated as described in the material and methods. Examples of proliferation analysis are shown while the percentage of divided cells and their proliferation index are reported in D. Sample were run in triplicate and the data refer to one experiment confirmed by another one.

Figure 2. A new aptamer specifically recognizes murine and human IL4Rα. A) different aptamers were cloned after 5 cycle of optimized SELEX and the variable region sequences aligned with Clustalw. B) The secondary structure of the most representative clone (cl.42) was analyzed by RNAstructure 5.2. C), biotin labeled cl.42 was incubated with epoxybeads conjugated with rIL4Rα, with rVCAM or left unconjugated. SA-PE was used to visualize the linked aptamer by FACS. D) Kd was calculated by interpolating the MFI of different concentration Cy3-aptamer with a fixed amount of IL4Rα conjugated beads. E) irrelevant or cl.42 Cy3-aptamer (300 pmoles) were incubated with the IL4Rα⁺MSC2, washed with PBS and cells analyzed by fluorescence microscopy. F) Human umbilical cord bloods (UBC) enriched in CD33⁺IL4Rα⁺MDSC by a 4 days culture with GM-CSF and G-CSF were stained with anti-CD33 antibody and either an irrelevant (left panel) or anti-IL4Rα aptamer cl.42 (middle panel) conjugated with Cy3. Data obtained from the staining with aptamer or antibody of 7 independent UBC-MDSCs preparations are plotted in the right panel and correlation analysis is reported.
Figure 3. Preferential in vivo binding of monocytic MDSC and TAM by the anti-IL4Rα aptamer. A) 4T1 tumor bearing WT or IL4Rα−/−Balb/c were i.v. injected with Cy3 labelled anti-IL4Rα or control aptamer (600 pmoles). 2h later, mice were sacrificed, and single cells suspensions from the indicated organs analyzed by FACS. (A) The percentage (mean+/−SD. n=6) of Cy3+ cells over total cells is reported. B) Gating strategy: FSC-A vs SSC-A gate was designed to exclude debris and aggregates, a hierarchical gate was designed to exclude the duplets using FSC’s and SSC’s –Width and Height. Inside this later gate, dead cells were excluded using the vital dye Live/dead yellow. C) After gating on the alive-singleT-cells, CD11b+ cells were selected and a dot plot using the F4/80 and Gr1 was designed. 3 populations were identified: 1) Gr1F4/80MDSC, 2) Gr1F4/80−MDSC and 3) Gr1−F4/80+macrophages. Cy3 fluorescence on each population was evaluated in the mice treated with the aptamer (open black histogram, middle panel IL4Rα specific aptamer, right panel irrelevant aptamer) or PBS (filled histograms). A similar analysis was performed in (D) after gating on the CD3+ and either CD4+ or CD8+ cells. Finally, Cy3 fluorescence was analyzed on B-cells (CD19+), NK (DX5+) and DC (CD11c+) cells using an additional multicolor antibody panel (E).

Data shown derived from one experiment (5 mice/group) representative of another one.

Figure 4. Chronic administration of anti-IL4Rα aptamer restrains the 4T1 mammary carcinoma progression. A) WT (top panel) or IL4Rα−/− (lower panel) mice were challenged with the 4T1 cells on day 0. Starting on day 3 mice received anti-IL4Rα aptamer (16pM/g, white dots) or an irrelevant fluorinated RNA (black dots) 3 times a week. Data derived from two independent experiments (n=4) performed by two different investigators representative of other 2. 18 days after challenge, tumors were surgically removed and weighed. B) Thy1.2+/+Balb/c
mice were challenged with the 4T1HAThy1.1-luciferase and treated as described in A. On day 17 mice were sacrificed and single cells suspension of the lung analyzed by FACS for the presence of Thy1.1\(^+\) Thy1.2\(^-\) neoplastic cells. As additional control, naïve Thy1.2\(^+/\) Balb/c mice were used. The percentage of Thy1.1\(^+\) neoplastic cells on live cells is reported. Data derived from 1 experiment (5 mice/group) representative of 2.

**Figure 5. Chronic anti-IL4R\(\alpha\) aptamer treatment modifies the ratio between the leukocytes population infiltrating the tumor.** Paraffin embedded 4T1 tumors isolated from the mice treated with the anti-IL4R\(\alpha\) aptamer or with an irrelevant aptamer were labeled with A) anti CD3 antibody or (B) anti-CD8 and anti-Gr1 antibodies. C) Single cells suspension from the tumor of mice challenged 18 days before and treated either with the IL4R\(\alpha\) specific or the irrelevant aptamer were analyzed via FACS to determine the number of: 1) CD11b\(^+\) F480\(^+\) Gr1\(^{high}\) MDSCs, 2) CD11b\(^+\) F4/80\(^+\) Gr1\(^{low}\) MDSC, 3) CD11b\(^+\) F4/80\(^+\) Gr1\(^{-}\) TAM, 4) CD3\(^+\) CD4\(^+\) Foxp3\(^+\) Treg, 5) CD3\(^+\) CD4\(^+\) T-cells, 6) CD3\(^+\) CD8\(^+\) T-cells. The T-cells expressing the CD69 markers were also evaluated. Data are reported as percentage of live cells using a gating strategy similar to the one described in Fig.3. Data derived from 1 experiment (n=5) representative of another one. In the different plots, each mouse is represented by the same symbol.

**Figure 6. The anti-IL4R\(\alpha\) aptamer induce MDSCs apoptosis.** A) CD11b\(^+\) cells from 4T1 tumor bearing mice were cultured in media alone, in the presence of the IL4R\(\alpha\) specific aptamer or the irrelevant aptamer (30 pmoles). Cells were stained with 7AAD and anti-annexin V and analyzed by FACS. B) MSC-2 cells were cultured for 48h alone, with an irrelevant aptamer or with the anti-IL4R\(\alpha\) aptamer. Cells were stained with 7AAD and anti-annexin V and analyzed by FACS. Data derived from 1 experiment representative of other 4.
Figure 7. The role of IL13 and STAT6 phosphorylation in Aptamer-mediated MDSC death

2x10^5 CD11^+ cells from 4T1 bearing mice (A) or MSC-2 (B) plated with or without IL-13 in the presence of anti-IL4Rα aptamer, an irrelevant aptamer or no aptamer. phosphoSTAT6 was evaluated 2h later by FACs C) MSC-2 cells were incubated for 4 days in the presence or in the absence of anti-IL13 neutralizing antibodies. As comparison anti-IL4Rα or irrelevant aptamer were used. Alive cells were evaluated by FACS. D) Splenic CD11b^+ cells from tumor bearing mice were cultured in the presence of IFN-γ (10ng/ml), IL-13 (10ng/mol), both cytokines or none. 48h later cell viability was evaluated by FACS. Data derived from one experiment representative of at least of other 3.
Figure 1

A

WT Balb/c
IL4RaKO Balb/c

WT MDSC

IL4RaKO MDSC

CD11b APC-A

CD124 PEA

CD11b APC-A

CD11b

HA specific CD8+ T cells

day 15
CD11b
definition

C

HA specific CD8+ T cells

T cell alone

T cell + IL4Ra

T cell + IL4Ra

T cell + IL4Ra

D

% of alive cells

p<0.001

no MDSC

WT CD11b

IL4RaKO CD11b

p=0.001

IL4Ra

CD11b

p<0.001

IL4RaKO CD11b

p<0.001

CD11b

p<0.001

II Cycle

CFSE

proliferation index

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Figure 2

A

B

C

D

E

F
Figure 4

A

IL4RαWT

Tumor size index (mm²; Mean± SD)

Tumor weight (g; Mean± SD)

p=0.004

IL4RαKO

Tumor size index (mm²; Mean± SD)

Tumor weight (g; Mean± SD)

p=0.01

B

Primary tumor
4T1Thy1.1HALuc

Lung metastasis

CTRL anti-IL4Ra aptamer aptamer

% necrotic cells (Thy1.1+ Thy1.2+)

No tumor

p<0.01
Figure 5

A

irrelevant aptamer anti-IL4Rα aptamer

CD3+ DAPI

B

irrelevant aptamer anti-IL4Rα aptamer

CD8+ DAPI Gr1+ DAPI Merged

C

Gr1<sup>high</sup> MDSC Gr1<sup>low</sup> MDSC TAM Treg

% of alive cells

aptamer treatment

CD8<sup>+</sup> CD8<sup>+</sup>CD69<sup>+</sup> CD4<sup>+</sup> CD4<sup>+</sup>CD69<sup>+</sup>

% of alive cells

ctrl anti-IL4R<sub>α</sub> ctrl anti-IL4R<sub>α</sub> ctrl anti-IL4R<sub>α</sub> ctrl anti-IL4R<sub>α</sub>
Figure 6

A

B

no aptamer
irrelevant aptamer
anti-IL4Rα aptamer

p<0.001
p=0.001
p=0.003

# of CD11b+Alveolar cells X 10^6

days in culture

# of MSC-2 Alveolar cells X 10^6

No Aptamer
Irrelevant Aptamer
Anti-IL4Rα Aptamer

p=0.001
Aptamer-mediated blockade of IL4Rα triggers apoptosis of MDSCs and limits tumor progression

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