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 macrophages (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 IL-4 receptor-α (IL4Rα or CD124) that is critical for MDSC suppression function. In tumor-bearing mice, this anti–IL4Rα aptamer preferentially targeted MDSCs 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 MDSCs confirmed the importance of IL4Ra–STAT6 pathway activation in MDSC survival. Our findings define a straightforward strategy to deplete MDSCs and TAMs in vivo, and they strengthen the concept that IL4Rα signaling is pivotal for MDSC survival. More broadly, these findings suggest therapeutic strategies based on IL4Ra signaling blockades to arrest an important cellular mechanism of tumoral immune escape mediated by MDSCs and TAM in cancer. Cancer Res; 72(6); 1373–83. ©2012 AACR.

Introduction

Myeloid-derived suppressor cells (MDSC, CD11b⁺Gr1⁺IL4Rα⁺) (1, 2) and tumor-associated macrophages (TAM, CD11b⁺Gr1⁺F4/80⁺; ref. 3) are particularly important in promoting tumor growth metastasis and angiogenesis (4, 5) and in suppressing the antitumor immune response (6). Although different signaling pathways may be responsible for the MDSC suppressive activity, the IL4Rα–STAT6 pathway seems to be particularly important: it mediates TGF-β 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 matrix metalloproteinase 9 (9).

Although different compounds were shown to inhibit or deplete MDSCs (i.e., STAT3 or PDE5 inhibitors, vitamin D, ATRA, nitroaspirin gemcitabine, and others; ref. 10) and showed 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 IL4Rα because of the importance of this receptor in MDSC 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 IL4Rα/CD124. This aptamer selectively targets MDSCs and TAM in vivo, blocks IL4Rα signaling, induces MDSCs apoptosis, and significantly delays tumor progression in 4T1-bearing mice.

Materials and Methods

Cell lines

4T1, 4T1HATHy1.1Luciferase TS/A, and MSC2 cell lines were previously described (13, 14). MSC2 was maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 1-glutamine (2 mmol/L), HEPES (10 mmol/L), 2-mercaptoethanol (20 mmol/L), streptomycin (150 U/mL), penicillin (200 U/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 nmol/L 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 whereas IL4R knockout (KO) mice were obtained from The Jackson Laboratory. All experiments were conducted on 6- to 10-week-old mice in pathogen-free facilities.

**Aptamer treatment of tumor-bearing mice**

Mice were injected subcutaneously with either 500,000 tumor cell. Starting on day 3 after challenge and every other day thereafter, mice received intravenous injections of either IL4Rx (16 pmol/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 4',6-diamidino-2-phenylindole (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 done 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**

A total of $10^5$ MDSCs were plated in a 96-well flat bottom plate with $10^5$ CFSE-labeled HA-specific CD8+ T cells purified by negative selection from the splenocytes of TCRcl4 mice (Jackson laboratories). A total of $10^5$ Thy1.2+/+Balb/c splenocytes were added as feeders and T cells were stimulated with the relevant or an irrelevant peptide. Three days later, clonotypic T-cell proliferation was evaluated by fluorescence-activated cell sorting (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 done after normality evaluation by Kolmogorov–Smirnov test. Pairwise post-hoc analysis was carried out using the Holm-Sidak’s or the Dunn’s test. Student t test was used when 2 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**

IL4Rx is a functional marker that defines the suppressive MDSCs population in the 4T1 mammary carcinoma

We have previously shown that IL4Rx (CD124) is upregulated 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 MDSCs were isolated from IL4Rx+/- or IL4Rx KO Balb/c mice bearing the 4T1 mammary carcinoma. Mice were sacrificed at day 16, splenic CD11b+MDSCs were magnetically enriched (Fig. 1A and B) and MDSCs populations (CD11b+IL4Rx+; CD11b+IL4Rx-, total CD11b+ cells or CD11b+ IL4Rx+KOBalb/c) were sorted by FACS and the suppressive activity of each population was tested against CFSE-labeled HA-specific CD8+ T cells. CD11b+ MDSCs drastically inhibit T cells proliferation allowing only half of the clonotypic population to divide (Fig. 1C).

![Figure 1](image-url)
Similar results are obtained with CD11b+IL4Rα+ cells but not with CD11b+IL4Rα– nor with CD11b+IL4RαKO cells (Fig. 1C and D). CD11b+ and CD11b+IL4Rα+ cells reduced also the proliferative capacity of the dividing cells, as shown by the reduced proliferative index, whereas no inhibition was seen when CD11b+IL4Rα+ or CD11b+IL4RαKO cells were used. These data indicated 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 confirmed that IL4Rα plays an important role in MDSCs mediate immune suppression but also suggested 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 was needed to be optimized because at physiologic pH the commercially available recombinant IL4Rα (IgG1–IL4Rα) has a net negative charge (Supplementary Fig. S1) 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 whereas, despite a counterselection step, isolation of anti-IgG1 or anti-linker aptamer is facilitated. Indeed, selections procedures (15 cycles each) based on the original protocol (Supplementary Fig. S2A) generate aptamer against IgG1 or the linker. To overcome these limits, a new method for aptamer selection was developed (Supplementary Fig. S2B). 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). Twenty-eight percent of the sequenced clones had an identical sequence that was required for their suppressive function.

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 tetranaucleotide using the T4–RNA ligase, a modification unlikely to change the tertiary RNA structure. The biotin-conjugated cl.42 aptamer was then incubated with epoxy beads loaded with IL4Rα, with VCAM (as negative control) or left unconjugated (Fig. 2C). As additional control, an irrelevant, biotin-labeled aptamer was used. Streptavidin–phycoerythrin (SA-PE) conjugate was used to visualize the aptamers bond to the beads’ surface via FACS. Whereas the irrelevant aptamer failed to recognize any beads, the cl.42 aptamer successfully bound to the IL4Rα epoxy beads 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 $K_A$ (14 nmol/L, Fig. 2D) was within the range of anti–IL4Rα antibodies (17) and 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 physiologic condition, the Cy3-conjugated cl.42 or irrelevant aptamer were incubated with the IL4Rα+MSC2 cell line in cell media at 37°C. The anti–IL4Rα aptamer effectively recognizes the MSC2 cell line as determined by immunofluorescence microscopy (Fig. 2E) or by FACS (Supplementary Fig. S3A) with an apparent $K_D$ of 788 nmol/L (Supplementary Fig. S3B). Only background fluorescence was seen when an irrelevant aptamer was used. FACS evaluation of different MDSC preparations ($n = 7$; Fig. 2F) indicated a high degree of correlation ($R^2 = 0.994$) 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 –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 nonhematopoietic cells (19, 20). WT or IL4Rα–/– Balb/c 4T1 bearing mice (with tumor diameter of approximately 0.5 cm) were injected with the Cy3-labeled anti–IL4Rα aptamer or with an irrelevant aptamer. Two hours later spleens, livers, lungs, 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 and B). A higher percentage of Cy3+ cells was detectable in the tumor specimens from 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– F4/80+/-/ monocytic MDSCs

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CD11b\(^+\)F4/80\(^+\)Gr1\(^{\text{low/}}\)/C0 macrophage seemed to be preferentially bound by the anti–IL4R\(\alpha\) aptamer (Supplementary Fig. S5A), whereas in the spleen monocytic MDSCs are the main target of the aptamer (Supplementary Fig. S5B). 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) three populations of cells can be identified based on the Gr1 and F4/80 expression (Fig. 3C): Gr1\(^{\text{high/}}\)MDSCs, Gr1\(^{\text{low/}}\)MDSCs, and Gr1\(^+\)TAM. Analysis of Cy3 fluorescence reveals that the anti–IL4R\(\alpha\) aptamer binds to all 3 myeloid populations, whereas only background signal is obtained when the irrelevant aptamer is used. A similar gating strategy (Fig. 3D and 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 suggested that the anti–IL4R\(\alpha\) aptamer specifically binds its ligand in vivo, and also suggest preferential targeting of MDSCs and TAM in cancer-bearing mice.
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 biologic function (21). Thus, we investigated whether chronic administration of anti–IL4Rα aptamer may influence tumor growth. IL4Rα+/- or IL4Rα-/- 4T1 bearing mice were treated intravenously every other day with the anti–IL4Rα or an irrelevant aptamer (16 pmol/L/g). Tumor progression was significantly inhibited in mice treated with the IL4Rα-specific aptamer (Fig. 4, Supplementary Fig. S6). 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 were obtained when the mammary carcinoma TSA (data not shown) or the 4T1-Thy.1.1-HA-luciferase (Fig. 4B) were used. This 4T1-derived cell line allows the detection of the metastatic disease by different means. In particular, when Thy.1.2 Balb/c mice are challenged, neoplastic cells can be detected by FACS using the Thy.1 marker that, in our hands, seemed 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 IL4Rα, as the effect is lost when IL4Rα-/- 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–IL4Rα aptamer-treated group (Fig. 5A), suggesting that the antitumor effect occurs through modulation of the immune response. This result was confirmed by the staining for CD8 and Gr1 (Fig. 5B) that showed a higher infiltration of CD8+ cells and a significant reduction of Gr1+ cells in the group treated with anti–IL4Rα aptamer. Interestingly, a population of CD8+ Gr1+ cells (a phenotype associated with the peripheral homing of central memory T cells; ref. 22) is also detectable and marginally (Fig. 5B insert) increased by the anti–IL4Rα aptamer treatment. CD11b and Gr1 staining (Supplementary Fig. S7) confirmed a significant decrease of MDSCs and showed 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 conducted on tumor-derived single cells suspensions. The dead cells were excluded using the vital dye LIVE/DEAD yellow.
The previous section indicates that anti–IL4Rα 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. Whereas the MSC2 offers a uniform and stable source of MDSCs, with only minimal spontaneous apoptosis, freshly isolated MDSCs closely mimic the MDSCs derived from tumor-bearing mice. As MDSCs, TAMs, and Tregs were significantly decreased by the treatment with the IL4Rα-specific aptamer, effector T cells appeared increased. Moreover, a higher number of T cells expressing the activation marker CD69 were detectable in the anti–IL4Rα aptamer group compared with the irrelevant (Fig. 5C).

Taken together, these results indicated that the anti–IL4Rα aptamer treatment significantly altered the tumor microenvironment, reducing the number of the negative regulators of the immune response whereas increasing the number and the activation status of effector T cells.

The anti–IL4Rα aptamer promotes MDSC apoptosis

The previous section indicates that anti–IL4Rα 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. Whereas the MSC2 offers 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 to 4 days when cultured in vitro in normal media (Fig. 6A; ref. 23). This pattern is not altered when the control aptamer is added to the culture. However, when cells were treated with the anti–IL4Rα aptamer a significant decrease in MDSCs viability was 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 MSC2 apoptosis and a concomitant reduction of viable cells was observed in the wells treated with the anti–IL4Rα aptamer (Fig. 6B). These data suggested a proapoptotic 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 prosurvival signaling in MDSCs**

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; refs. 24–28) or activation of RNA-dependent protein kinase (PKR; refs. 29–33). Alternatively, aptamer binding may modify some signaling pathways in MDSCs affecting their survival. Because only the IL4Rα-specific aptamer, but not an irrelevant aptamer, triggers MDSCs apoptosis at the concentration used (150 nmol/L, Fig. 6), it is unlikely that the first 2 pathways are majorly involved in this effect. Moreover, although a proapoptotic effect of the irrelevant aptamer on MDSCs 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

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**Figure 4.** Chronic administration of anti–IL4Rα aptamer represses the 4T1 mammary carcinoma progression. A. WT (top) or IL4Rα−/− (bottom) mice were challenged with the 4T1 cells on day 0. Starting on day 3 mice received anti–IL4Rα aptamer (16 pmol/L/g, white dots) or an irrelevant fluorinated RNA (black dots) 3 times a week. Data derived from 2 independent experiments (n = 4) conducted by 2 different investigators representative of 2. Eighteen days after challenge, tumors were surgically removed and weighed. B, Thy1.2−/−Balb/c mice were challenged with the 4T1/HAThy1.1-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+ T cells. As additional control, naive 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 per group) representative of 2.
or mimic the anti–IL4Rα aptamer-induced MDSCs apoptosis (Supplementary Fig. S8). Because 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. Because 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.
whereas the irrelevant aptamer did not significantly alter pSTAT6 signaling, the IL4Rα aptamer drastically reduced pSTAT6. To verify that the anti-IL4Rα aptamer acts as antagonist in the absence of possible artifacts associated with MSC2 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+ cells, the IL4Rα-specific aptamer significantly decreased the IL-13–dependent STAT6 phosphorylation, whereas 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 (Supplementary Fig. S9). Whereas the irrelevant aptamer failed to modulate arginase activity, the anti–IL4Rα prevented Arg1 activation (Supplementary Fig. S9). These data indicated that the anti–IL4Rα aptamer is a blocking aptamer that prevents IL4Rα signaling; however, they do not provide proof whether this is the underlying cause of MDSCs apoptosis. To determine whether IL4Rα signaling promotes MDSCs survival; two complementary strategies were adopted: (i) Because MSC2 cells autocrinally secrete low doses of IL-13 (data not shown), MSC2 survival in the presence of IL-13 neutralizing antibodies was evaluated; (ii) 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, MSC2 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 7AAD and Annexin V staining and data were normalized for the number of cells recovered. IL-13 neutralization resulted in high mortality of MSC2 (Fig. 7C), suggesting that the autocrine secretion of this cytokine acts as a survival signal for MSC2. 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 as 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 suggested that IL4Rα expression and its engagement could promote prosurvival signaling in MDSCs, and that the anti–IL4Rα aptamer can counteract this effect.

Discussion

Currently, there are 2 main approaches that are being pursued to target MDSCs 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, as 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 some models (i.e., EL-4; ref. 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 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 IL4Rx–specific aptamer promotes MDSCs apoptosis upon binding. By analyzing the mechanisms of depletion, we focused our attention on the IL4Rx pathway because preliminary experiments seemed to exclude the involvement of both TLR3 and PKR, the two main proteins that may trigger 2′-fluoro-dsRNA–mediated apoptosis (Supplementary Fig. S8). Indeed, the addition of the IL4Rx–specific aptamer to MDSC cultures was sufficient to prevent IL-13–mediated STAT6 phosphorylation, indicating the antagonist properties of this molecule.

IL4Rx 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 and allows for the rejection of spontaneous metastasis in the 4T1 mammary carcinoma model.

Figure 7. The role of IL-13 and STAT6 phosphorylation in aptamer-mediated MDSC death. A total of 2 × 10⁵ CD11b⁺ cells from 4T1-bearing mice (A) or MSC2 (B) plated with or without IL-13 in the presence of anti-IL4Rx aptamer, an irrelevant aptamer or no aptamer. PhosphoSTAT6 was evaluated 2 hours later by FACS. C, MSC2 cells were incubated for 4 days in the presence or in the absence of anti–IL-13 neutralizing antibodies. As comparison anti–IL4Rx 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 (10 ng/mL), IL-13 (10 ng/mL), both cytokines or none. Forty-eight hours later cell viability was evaluated by FACS. Data derived from 1 experiment representative of at least of other 3.
Our data suggest an additional important role of this receptor: IL-4Rα engagement could promote MDSCs resistance to apoptosis (Figs. 6 and 7). The antiapoptotic role of IL-4Rα was elucidated in many cell types including B cells, T cells, mast cells, and myeloid cells but, until now, not in MDSCs. Interestingly, IL-13 pretreatment has been shown to protect human synovocytes from nitric oxide–induced apoptosis by inducing IRS phosphorylation and PKC activation (45). Although we have not yet confirmed this pathway in MDSCs, it is possible that IRS activation by IL-4Rα is necessary for protecting MDSCs from the autocritically produced NO and that the aptamer-mediated blockade of this pathway prevents this protective mechanism. In any case, this article, 3 main evidences suggest an important antiapoptotic role of IL-13 on MDSCs: (i) aptamer-mediated blockade of IL-4Rα signaling correlates with an increase apoptosis in MDSCs (Figs. 6 and 7); (ii) the antibody-mediated neutralization of IL-13 in cultured M2C2 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 as the administration of the anti–IL-4Rα aptamer correlates with a marked decrease of MDSCs and TAM at the tumor site (Fig. 5).

Interestingly, despite the fact that IL-4Rα is not a specific marker of MDSCs, in vivo administration of the anti–IL-4Rα aptamer showed specificity for MDSCs and TAMs (Fig. 3). The reasons for this preferential in vivo targeting of MDSCs are currently unknown but can be related to cell-specific posttranslational modification of CD124 that can differentiate the IL-4Rα expressed in tumor-associated MDSCs from the one expressed in other cell types. It is important to note that the recombinant IL-4Rα toward which the aptamer has been selected is isolated from the Balb/c myeloma cell line NSO and, as such, is characterized by several posttranslational 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 TAMs. 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 IL-4Rα–specific aptamer selectively depletes MDSCs and macrophages in the tumor as well as splenic IL-4Rα+ MDSCs. This depletions 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 IL-4RαKO mice (Fig. 4). However, it is important to underline that treatment with the IL-4Rα 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; ref. 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 IL-4Rα/CD124 that unveils the importance of the IL-4Rα–STAT6 pathway in promoting survival of MDSCs. In light of these new data, the importance of IL-4Rα/CD124 in MDSCs biology and as a pharmacologic target justifies further research, as it may have impact on clinical treatment of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: L. Inverardi, P. Serafini
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