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IL-10 Contributes to the Suppressive Function of Tumor Associated Myeloid Cells and Enhances Myeloid Cell Accumulation in Tumors

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Abstract

Studies have revealed that tumor associated myeloid cells (TAMC) are one of the major sources of IL-10 in tumor-bearing mice. However, the significance of TAMC-derived IL-10 in tumor immunity is poorly understood. Here we show that IL-10 blockade or IL-10-deficiency reduces the capacity of TAMC in suppressing the proliferation of P1A-specific CD8 T cells. In the spleen, IL-10-deficient and wild type (WT) mice bearing large tumor burdens have similar TAMC populations. The tumors from IL-10-deficient mice however, have reduced numbers of TAMC compared with tumors from their WT counterparts. IL-10^{-/-}RAG-2^{-/-} mice also had reduced numbers of TAMC compared with tumors from IL-10^{+/+}RAG-2^{-/-} mice; therefore the reduction of TAMC in IL-10-deficient tumors was not due to adaptive immune response in tumors. Adoptively transferred tumor antigen specific CD8 T cells expanded more efficiently within tumors in IL-10^{-/-}RAG-2^{-/-} mice than in tumors from IL-10^{+/+}RAG-2^{-/-} mice. CTL adoptive transfer therapy prevented tumor evasion in IL-10^{-/-}RAG-2^{-/-} mice more efficiently than in IL-10^{+/+}RAG-2^{-/-} mice. Thus, IL-10 enhances accumulation of myeloid cells in tumors and TAMC-derived IL-10 suppresses activation and expansion of tumor antigen specific T cells.

Introduction

Cancer antigen-specific cytotoxic T lymphocytes (CTL) are efficient effectors against cancer cells. However, large established tumors are usually not fully controlled by CTL for at least two reasons. First, the genetic instability of cancer cells often results in selection of antigenic variants by CTLs. Second, large established tumors have an immune suppressive microenvironment that not only inhibits activation and effector function of CTL but also permits tumor progression (1–3). This immune suppressive network is composed of a number of cellular components. In addition to the widely acknowledged Foxp3⁺CD4⁺CD25⁺ T regulatory cells (Treg), tumor associated myeloid cells (TAMCs),

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within the TAMC population, myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) are of particular interest in tumor immune evasion.

MDSCs are phenotypically identified as CD11b⁺Gr-1⁺ and exhibit suppressive activity for T cell activation (4–7). MDSCs are found in significantly high numbers in bone marrow, circulation and peripheral lymphoid organs of mice and humans during tumor progression (8–9). MDSCs can infiltrate tumor-draining lymph nodes to tolerize CD8 T cells (10–12). A number of mechanisms, including IFN- γ -dependent production of nitric oxide (4, 13), arginine depletion through the production of arginase (13–14), production of reactive oxygen species (15–16) and nitration of TCR-CD8 complex (17) have been proposed as responsible for inducing tolerance in CD8 T cells. TAMs represent the major inflammatory component of the stroma of many tumors, and can affect different aspects of the neoplastic tissue (2, 18). TAMs are usually of the M2 phenotype (producing more IL-10) (19–20) and they are known to suppress CTL effector functions and to promote tumor invasion, growth and angiogenesis (2–3, 21). Targeting TAMs alone has been shown to be effective therapy in tumor models (22–23). Studies have revealed that MDSCs can interact with TAMs to convert M1 macrophages into M2 macrophages via production of IL-10 (24). On the other hand, M2 to M1 converted TAMs (devoid of IL-10 production) can become efficient killers of cancer cells (25). These studies have revealed a critical “switching role” for IL-10 in TAMCs. However, the direct role of TAMC-derived IL-10 in tumor immunity has not been clearly demonstrated.

By performing adoptive transfer therapy on mice with large established plasmacytoma J558 tumors using P1CTL (26–27), we have revealed that, P1CTL are very efficient in reducing tumor burden. However, cancer cells can evade P1CTL destruction in vivo via antigenic drift or antigen loss mutations (26–27). In this study we have used the P1A/P1CTL model to evaluate the role of TAMC-derived IL-10 in tumor immunity. We found that TAMC-derived IL-10 inhibited activation of P1CTL cells in vitro and in vivo. IL-10 was not essential for the expansion of TAMC in the peripheral lymphoid organs; however, the IL-10-deficient tumor environment exhibited reduced numbers of TAMC population. The reduced numbers and diminished suppressive function of TAMC in IL-10-deficient tumors allowed enhanced expansion of adoptively transferred CTL, which controlled tumor growth and reduced immune evasion effectively.

Materials and Methods

Experimental animals

Transgenic mice expressing a TCR specific for the tumor rejection antigen H-2L^d:P1A35-43 complex (P1CTL) have been described (28). P1CTL TCR transgenic mice were backcrossed with BALB/c mice for at least 15 generations before they were used for this study. BALB/c mice with a targeted mutation of the RAG-2 gene were purchased from Taconic Farms (Germantown, New York, USA). Through breeding P1CTL TCR transgenic mice with RAG-2^{-/-}BALB/c mice we have generated RAG-2-deficient P1CTL TCR transgenic mice (RAG-2^{-/-}P1CTL). Through breeding IL-10^{-/-}BALB/c mice with RAG-2^{-/-}BALB/c mice we have generated RAG-2 and IL-10 double deficient mice (RAG-2^{-/-}IL-10^{-/-}). PCR was used for genotyping of IL-10-deficiency and the primers used were mIL10: 5'-ATA GAC TTG CTC TTG CAC TAC CAA AG-3' (forward) and 5'-CTC ATG GCT TTC CCT AGG ACT CTC TA-3' (reverse). All mice were maintained in OSU laboratory animal facilities that were fully accredited by Institutional Animal Care and Use Committee.

Tumorigenesis and T cell adoptive transfer therapy of mice with established tumors

For tumor establishment *in vivo*, 5×10^6 of J558 cells were injected into each mouse subcutaneously. Tumor volumes were measured for length (a) and width (b) every three days and calculated as $ab^2/2$ (29). For CTL therapy of mice with established tumors, pools of spleen and lymph node cells from P1CTL-transgenic mice were incubated with a cocktail of mAbs (anti-CD4 mAb GK1.5, anti-FcR mAb 2.4G2 and anti-CD11c mAb N418). After removal of unbound mAbs, cells were incubated with anti-IgG coated magnetic beads (DynaL Biotech). The antibody-coated cells were removed by a magnet. The unbound cells consisted of more than 90% CD8⁺ T cells, with no detectable CD4⁺ T cells. The purified CD8⁺ T cells (5×10^6 /mouse) were injected intravenously (i.v.) into mice bearing established tumors.

Antibodies and flow cytometry

FITC-, PE-, APC- or Percp- labeled antibodies to CD11b, Gr-1, CD8 α , V α 8.3 and isotype control antibodies were purchased from BD Biosciences (San Diego, CA). For staining of cell surface markers, cells (cancer cells, splenocytes and single cell suspensions of tumors) were stained with various antibodies in staining buffer (PBS with 1% FCS) and incubated on ice for 30 min. After washing with staining buffer, cells were fixed in 1% Paraformaldehyde in PBS. Cells were analyzed on a FACSCalibur flow cytometer. Data were analyzed using the flowjo software (Tree Star, Inc., OR).

Real time PCR

Quantitative real-time PCR was performed using an ABI 7900-HT sequence system (PE Applied Biosystems) with the QuantiTect SYBR Green PCR kit (Qiagen) in accordance with the manufacturer's instructions. PCR was done using previously determined conditions (26). The following primers were used for amplifying specific genes: mIL10: 5'-ACA GCC GGG AAG ACA ATA AC-3' (forward) and 5'-CAG CTG GTC CTT TGT TTG AA-3' (reverse). The HPRT gene was simultaneously amplified as endogenous control. The primers were 5'-AGCCTAAGATGAGCGCAAGT-3' (forward) and 5'-TTACTAGGCAGATGGCCACA-3' (reverse). Each sample was assayed in triplicate and the experiments were repeated twice. The relative amount of mRNA was calculated by plotting the C_t (cycle number) and the average relative expression for each group was determined using the comparative method ($2^{-\Delta\Delta C_t}$).

Cytokine ELISA

ELISA kit for the detection of IL-10 was obtained from BD Biosciences. Standard procedures were followed to detect release of cytokine in culture supernatants in a variety of settings (detailed in Figure legends to each experiment).

TAMCs suppression assay

Purification of TAMCs and their subsets were performed by staining single cell suspensions of tumors and/or spleens using PE-anti-GR-1 (RB6-8C5; BD Biosciences) or PE-anti-CD11b (BD Biosciences), followed by magnetic antibody cell separation using anti-PE microbeads (Milenyi Biotec). Typically the purity of the resulting CD11b⁺ or Gr-1⁺ cells was greater than 90%. To assess the suppressive activity of the purified TAMCs, 1×10^6 /ml of lymphocytes (spleen and lymph node cells) from RAG-2^{-/-}P1CTL mice were cultured in the presence of P1A35-43 (0.1 μ g/ml) with graded numbers of TAMCs for 48–72 h. For detection of proliferation of P1CTL cells, ³H-Tritium was added in the culture for 12 h and incorporation of ³H-Tritium was quantified using a scintillation counter.

Statistics

Student's t test was used to compare differences of various parameters between two groups. A chi square (χ^2) statistic was used to determine differences for numbers of mice with tumor recurrence. $P < 0.05$ was considered significant.

Results

Tumor associated myeloid cells are the major producers of IL-10

Studies using IL-10 reporter mice have revealed that T cells and myeloid lineage of cells are the major producers of IL-10 under various conditions (30). To understand the roles of IL-10 in tumor associated myeloid cells, we analyzed TAMCs in spleens and tumors from BALB/c mice with large established J558 tumors. As shown in Figure 1a, the spleens of J558 tumor bearing mice contained a large population of CD11b⁺ cells, and these cells almost exclusively expressed the Gr-1 marker. J558 tumors contained about 2% of CD11b⁺Gr-1⁺ cells. The CD11b⁺Gr-1⁺ cells have been termed as myeloid-derived suppressor cells (MDSCs) (6–7). Using PE-labeled anti-CD11b antibody and anti-PE MACS beads, we purified tumor associated myeloid cells from spleens and tumors. As shown in Figure 1a, the purity of CD11b⁺Gr-1⁺ cells was about 90% in spleens and 70% in tumors, respectively. Real time PCR analysis of IL-10 gene expression revealed that TAMCs from tumors expressed more IL-10 than TAMCs from spleens (Figure 1b). Upon activation, CD11b-positive cells from J558 tumors produced higher amount of IL-10 than that of spleens. In contrast, activated CD8 T cells specific for a mouse tumor antigen P1A, produced minimal amount of IL-10 compared with J558-tumor associated myeloid cells (Figure 1c). Thus, tumor associated myeloid cells are a major source of IL-10.

TAMC-produced IL-10 inhibits activation of tumor antigen specific CD8 T cells

To test if IL-10 was required for the suppressor function of TAMCs, we purified TAMCs from spleens and tumors of J558-tumor bearing mice. We tested whether blocking IL-10 could affect the suppressive activity of TAMCs on tumor antigen specific T cells. The splenocytes from RAG-2^{-/-} P1CTL mice, whose CD8 T cells recognize tumor rejection antigen P1A, were co-cultured with TAMCs at different E/T ratios in the presence of P1A peptide, with or without anti-IL-10 antibody. As demonstrated in Figure 2, anti-IL-10 antibody significantly inhibited the suppressive activity of TAMCs on P1CTL cells. TAMCs from spleen were less efficient suppressors compared with TAMCs from tumors, since high TAMC/P1CTL ratios were required for them to achieve more significant suppression of P1A specific proliferation of P1CTL cells. However, IL-10 blocking resulted in enhanced proliferation of P1CTL cells in both cases. To further validate this effect, we injected J558 tumor cells into IL-10^{-/-} BALB/c and wild type BALB/c mice. As demonstrated in Figure 3a, both wild type and IL-10^{-/-} BALB/c mice bearing large established J558 tumors contained high numbers of TAMCs. In spleens of tumor bearing mice, TAMC populations were similar between WT and IL-10^{-/-} mice (Figure 3b). However, numbers of TAMCs were consistently lower in tumors from IL-10^{-/-} mice (Figure 3c and Figure 3d). Using CD11b-MACS beads, we purified TAMCs from IL-10^{-/-} and wild type mice with large established J558 tumors. As shown in Figure 4a and Figure 4c, greater than 90% of TAMCs from spleens and 80% of TAMCs from tumors expressed CD11b. We compared the IL-10-deficient TAMCs with TAMCs from wild type mice for their suppressive effects on the proliferation of P1CTL cells. As shown in Figure 4b and d, we found that IL-10-deficient TAMCs had significantly reduced suppressive function compared with TAMCs from wild type mice. Similar effects were observed for both spleen TAMCs (Figure 4b) and tumor TAMCs (Figure 4d).

IL-10 enhances accumulation of myeloid cells in tumors

When examining TAMCs in IL-10^{-/-} versus wild type mice, we frequently observed reduced numbers of TAMCs in tumors from IL-10^{-/-} mice (Figure 3c and 3d). Since IL-10-deficient mice have enhanced activation of T lymphocytes and are more susceptible to bowel inflammation, we hypothesized that adaptive immune responses in IL-10^{-/-} mice may affect numbers of TAMCs. Alternatively, IL-10 may be required for the expansion of TAMCs in tumors. To test this issue, we generated IL-10^{-/-}RAG-2^{-/-} mice by breeding IL-10^{-/-} mice with RAG-2^{-/-} mice. We then compared the numbers of TAMCs in J558 tumors from IL-10^{-/-}RAG-2^{-/-} and RAG-2^{-/-} mice. As demonstrated in Figure 5a and Figure 5b, we observed similar numbers of TAMCs in the spleen of mice with similar tumor burden. However in tumors, the TAMC numbers were significantly reduced in IL-10^{-/-}RAG-2^{-/-} mice (Figure 5c and Figure 5d). Thus, adaptive immune responses in tumors of wild type and IL-10^{-/-} mice are not responsible for the differential numbers of TAMCs, and it is likely that IL-10 itself enhances tumor accumulation of TAMC.

Enhanced proliferation and effector function of tumor antigen specific CD8 T cells in IL-10^{-/-}RAG-2^{-/-} mice

To test the consequences of IL-10 deficiency-associated reduction in TAMC on CTL activation and effector function in vivo, we injected J558 tumor cells into IL-10^{-/-}RAG-2^{-/-} and RAG-2^{-/-} mice. Since RAG-2^{-/-} mice are devoid of T and B lymphocytes, in a tumor bearing mouse, the major source of IL-10 should be from the TAMCs. To test if the myeloid cell systems in IL-10^{-/-}RAG-2^{-/-} and IL-10^{+/+}RAG-2^{-/-} mice differ in suppression of tumor antigen specific T cells, we labeled purified P1CTL cells with CFSE, and injected the CFSE-labeled P1CTL cells into mice with established J558 tumors. Given T cells undergo homeostatic proliferation in RAG-deficient mice, which may cause difficulties in distinguishing antigen-induced proliferation versus homeostatic proliferation, we first injected CFSE-labeled P1CTL cells into tumor free IL-10^{-/-}RAG-2^{-/-} and IL-10^{+/+}RAG-2^{-/-} mice. Five days after T cell injection, we found that P1CTL cells only experienced minimal proliferation, as reflected by limited CFSE dilution (Figure 6a). In contrast, three days after T cell injection into tumor bearing mice, P1CTL cells proliferated vigorously. P1CTL cells proliferated substantially faster in IL-10^{-/-}RAG-2^{-/-} recipient mice compared to P1CTL cells in IL-10^{+/+}RAG-2^{-/-} recipient mice, which was reflected by CFSE dilution in more cells (Figure 6b). As a result, more P1CTL cells accumulated in the spleens (Figure 6b) and tumors (Figure 6c) of IL-10^{-/-}RAG-2^{-/-} mice than that of IL-10^{+/+}RAG-2^{-/-} mice. Thus, IL-10-deficiency in myeloid cells enhances proliferation and accumulation of tumor antigen specific T cells in vivo.

Since tumor antigen specific T cells proliferated substantially faster in IL-10^{-/-}RAG-2^{-/-} mice that bear established tumors, we next examined if P1CTL adoptive transfer therapy can eliminate established tumors in IL-10^{-/-}RAG-2^{-/-} mice. Our previous studies (26–27, 31) have revealed that P1CTL therapy failed to control J558 tumors in IL-10^{+/+}RAG-2^{-/-} mice because of tumor antigen mutation-caused immune evasion. 5×10^6 J558 cells or 1×10^6 P815 cells were injected into each IL-10^{-/-}RAG-2^{-/-} and IL-10^{+/+}RAG-2^{-/-} mouse subcutaneously. When tumors were established (9 days after tumor cell injection), 5×10^6 purified P1CTL cells were injected into each mouse intravenously. As demonstrated in Figure 6d, P1CTL therapy resulted in much higher percentages of tumor free survival in IL-10^{-/-}RAG-2^{-/-} mice than in IL-10^{+/+}RAG-2^{-/-} mice. Thus, P1CTL cells show enhanced capacity to control tumor growth in IL-10^{-/-}RAG-2^{-/-} mice compared to their IL-10^{+/+}RAG-2^{-/-} counterparts.

Discussion

Studies using IL-10 reporter mice have revealed that T lymphocytes and innate myeloid cells are the major cell types that produce IL-10 (30). In this study we have created IL-10^{-/-}RAG-2^{-/-} mice where T lymphocytes are absent and thus innate myeloid cells are the major producers of IL-10. This model allowed us to evaluate the in vivo impact of tumor associated myeloid cell-derived IL-10 in tumor immunity. We have made the following two observations in this study. First, myeloid cell-derived IL-10 directly inhibits activation of tumor antigen specific CD8 T cells in vitro and in vivo. Second, although IL-10 does not enhance expansion of myeloid cells in the peripheral lymphoid organs, tumors from IL-10^{-/-} and IL-10^{-/-}RAG-2^{-/-} mice have significantly reduced numbers of TAMCs compared to their wild type counterparts. The reduced quality and quantity of TAMC in IL-10-deficient mice renders IL-10-deficient tumors more susceptible to CTL adoptive transfer therapy. As a result, the tumor immune evasion frequently observed in IL-10^{+/+}RAG-2^{-/-} mice (32), is substantially reduced in IL-10^{-/-}RAG-2^{-/-} mice.

TAMCs are composed of at least two populations of cells: MDSCs and TAMs. Both cell types can suppress CTL effector functions and promote tumor invasion, growth and angiogenesis (2–3, 21). A number of mechanisms, including IFN- γ -dependent production of nitric oxide (4, 13), arginine depletion through the production of arginase (13–14), production of reactive oxygen species (15–16) and nitration of TCR-CD8 complex (17) have been proposed to be responsible for the tolerance of CD8 T cells. The direct effect of IL-10 on the suppressive function of tumor associated myeloid cells has not been reported previously. When using purified myeloid cells to perform suppression assay, we observed significantly reduced suppressive activity if IL-10 secretion was blocked or IL-10-deficient myeloid cells were used. This observation was also confirmed in vivo, where CFSE-labeled P1CTL cells divided significantly faster in IL-10^{-/-}RAG-2^{-/-} mice. Thus, TAMC-derived IL-10 can significantly inhibit activation of tumor antigen specific CD8 T cells both in vitro and in vivo.

In this study we have shown that although the numbers of TAMCs in the peripheral lymphoid organs of WT and IL-10-deficient mice were similar, numbers of TAMCs in tumors from IL-10^{-/-} mice were significantly lower compared with that of WT mice. This observation was also verified in IL-10^{+/+}RAG-2^{-/-} and IL-10^{-/-}RAG-2^{-/-} mouse models. Thus, differential adaptive immune responses in tumors of WT versus IL-10^{-/-} mice is not responsible for the difference. Since NK cells were barely detectable (not shown) in the tumor microenvironment of IL-10-deficient and sufficient J558 tumors, it is unlikely that differential functions of NK cells mediated this effect. Thus, it is likely that IL-10 itself enhanced tumor accumulation of TAMC. Interestingly, IL-10 has been shown to enhance CCL16 (33) and CCL2 expression (34) in monocytes, and these two chemokines are known to be chemotactic to monocytes (34–36). It remains to be tested if this self-regulatory loop exists during TAMC accumulation in tumors and is responsible for the reduced TAMC accumulation in tumors from IL-10-deficient mice.

Our previous studies (26–27, 37) have revealed that P1CTL therapy rarely leads to complete rejection of unmodified J558 tumors in IL-10^{+/+}RAG-2^{-/-} mice. Tumors almost always grew back after initial tumor rejection due to antigenic mutations. In this study we have found that tumor recurrence was reduced in IL-10^{-/-}RAG-2^{-/-} mice. Complete rejection of large established tumors requires elimination of both antigen positive cancer cells and their antigenic variants (38–39). How did monoclonal P1CTL therapy eliminate both antigen positive cells and antigenic variants in IL-10^{-/-}RAG-2^{-/-} mice? We hypothesize that the following three mechanisms are responsible. First, reduced suppressor activity and reduced numbers of TAMCs enhances CTL activation and accumulation. Thus, in the destruction

phase, tumor antigen positive tumor cells and their stromal cells can be eliminated more thoroughly, which reduces the risk of the tumor recurrence (38, 40–41). Second, more CTL cells will produce more IFN- γ in the tumor bed, which can inhibit tumor angiogenesis (42–43) leading to reduced tumor recurrence. Finally, we have found that IL-10-deficient TAMCs can produce more TNF- α (44) and can potentially kill both antigen-positive cancer cells and their antigenic variants, as TNFR signaling is required for the complete eradication of tumors (41, 45). Consistent with these findings, a recent study (25) has clearly shown that “re-educated” TAMCs (devoid of IL-10 production) can efficiently kill cancer cells rather than promote tumor progression.

Taken together, we have found that IL-10 enhances accumulation and the suppressive function of TAMC. These findings can explain why CTL adoptive transfer therapy has better effect in controlling large established tumors. In addition, our results suggest that targeting TAMC and TAMC-derived IL-10 may be beneficial in T cell therapy of cancer.

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Figure 1.

IL-10 production by tumor associated myeloid cells. **a.** J558 tumor cells were injected into BALB/c mice, large established tumors were then digested using collagenase followed by mechanical dissociation. Single cell suspensions prepared from tumors were stained for CD11b and Gr-1 followed by flow cytometry analysis (upper panel). CD11b⁺ cells were purified from single cell suspensions from dissociated J558 tumors and spleens using CD11b MACS beads (lower panel). **b.** Real time PCR analysis of IL-10 gene expression in TAMC. Total RNA was isolated from purified TAMCs and real time PCR was used for the detection of IL-10 gene expression. **c.** Purified TAMCs were stimulated with PMA (100 ng/ml) and Ionomycin (100 ng/ml) for 48 h, IL-10 release in the culture supernatants were then detected by ELISA. For detection of IL-10 production by P1CTL cells, splenocytes from P1CTL transgenic mice were stimulated with P1A35-43 for 4 days, IL-10 release in the culture supernatants were detected by ELISA. Data shown represents 3–5 experiments with similar results.

Figure 2.

Blockade of TAMC-derived IL-10 enhances proliferation of tumor antigen specific T cells.

a. Splenocytes of RAG-2^{-/-}P1CTL mice were co-cultured with different numbers of TAMCs (from spleen) for 48 h in the presence of P1A35-43. Tridium-incorporation assay was used to measure proliferation of P1CTL cells. **: P<0.01 by student's t test. **b.** The same result presented in **a** was calculated as % inhibition. **c.** Splenocytes of RAG-2^{-/-}P1CTL mice were co-cultured with different numbers of TAMCs (from tumor) for 48 h in the presence of P1A35-43. Tridium-incorporation assay was used to measure proliferation of P1CTL cells. TAMC/P1CTL ratio = 0.5. *: P<0.05 by student's t test. **d.** The same result presented in **c** was calculated as % inhibition.

Figure 3. Number of TAMC in tumors from IL-10^{-/-} and wild type mice. **a.** Flow cytometry analysis of TAMCs in spleens from IL-10^{-/-}BALB/c or IL-10^{+/+}BALB/c mice. Single cell suspensions of spleens were stained for CD11b and Gr-1 followed by flow cytometry analysis. **b.** Quantification of TAMCs in spleens from WT and IL-10^{-/-} mice. Data shown are paired samples of % MDSC (CD11b⁺Gr-1⁺) and % TAM (CD11b⁺Gr-1⁻) in spleens. Paired student's t test was used for the statistical analysis. **c.** Flow cytometry analysis of TAMCs in tumors from IL-10^{-/-}BALB/c and IL-10^{+/+}BALB/c mice. Single cell suspensions of tumors were stained for CD11b and Gr-1 followed by flow cytometry analysis. **d.** Data shown are paired samples of % MDSC (CD11b⁺Gr-1⁺) and % TAM (CD11b⁺Gr-1⁻) in tumors. Paired student's t test was used for the statistical analysis.

Figure 4.

IL-10-deficient TAMCs have reduced capacity to suppress proliferation of tumor antigen specific T cells. **a.** Flow cytometry analysis of TAMCs in spleens and tumors from IL-10^{-/-}BALB/c or IL10^{+/+}BALB/c mice bearing large established J558 tumors. Splenocytes and single cell suspensions of tumors were stained for CD11b and Gr-1 followed by flow cytometry analysis. **b.** TAMCs were isolated from spleens and tumors using MACS anti-CD11b beads. **c.** Splenocytes of RAG-2^{-/-}P1CTL mice were co-cultured with different numbers of TAMCs (from spleen) for 48 h in the presence of P1A35-43 (0.1 µg/ml). Tritium-incorporation assay was used to measure proliferation of P1CTL cells. *: P<0.05 by student's t test. **d.** Splenocytes of RAG-2^{-/-}P1CTL mice were co-cultured with different numbers of TAMCs (from tumor) for 48 h in the presence of P1A35-43. Tritium-incorporation assay was used to measure proliferation of P1CTL cells. *: P<0.05 by student's t test. Data shown in figure 4 represents 3–5 experiments with similar results.

Figure 5. Number of TAMC in IL-10^{-/-}RAG-2^{-/-} (DKO) and IL-10^{+/+}RAG-2^{-/-} (RAGKO) mice. **a.** Flow cytometry analysis of TAMCs in spleens from DKO and RAGKO mice. Single cell suspensions of tumors were stained for CD11b and Gr-1 followed by flow cytometry analysis. **b.** Quantification of TAMCs in spleens from DKO and RAGKO mice. Data shown are paired samples of % MDSC (CD11b⁺Gr-1⁺) and % TAM (CD11b⁺Gr-1⁻) in spleens. Paired student's t test was used for the statistical analysis. **c.** Flow cytometry analysis of TAMCs in tumors from DKO and RAGKO mice. Single cell suspensions of tumors were stained for CD11b and Gr-1 followed by flow cytometry analysis. **d.** Quantification of TAMCs in spleens from DKO and RAGKO mice. Data shown are paired samples of % MDSC (CD11b⁺Gr-1⁺) and % TAM (CD11b⁺Gr-1⁻) in tumors. Paired student's t test was used for the statistical analysis.

Figure 6.

TAMC-derived IL-10 inhibits proliferation and accumulation of tumor antigen specific CD8 T cells in vivo. **a.** Purified CD8⁺ T cells from PICTL transgenic mice were labeled with CFSE and were injected into IL-10^{-/-}RAG-2^{-/-} (DKO) and IL-10^{+/+}RAG-2^{-/-} (RAGKO) mice. Five days after T cell injection, spleens were removed and single cell suspensions were stained for Vα8.3 and CD8 followed by flow cytometry analysis. Data shown were gated on CD8⁺Vα8.3⁺ cells. **b.** J558 tumor cells were injected into IL-10^{-/-}RAG-2^{-/-} (DKO) and IL-10^{+/+}RAG-2^{-/-} (RAGKO) mice subcutaneously at a dose of 5×10^6 /mouse. CFSE-labeled PICTL cells were then injected into DKO or RAGKO mice with established J558 tumors. Three days after T cell injection, spleens were removed and single cell suspensions were stained for Vα8.3 and CD8 followed by flow cytometry analysis. **c.** T cell infiltration into tumors. As described in **b**, three days after CFSE-PICTL injection, dissociated tumor cells were stained for Vα8.3 and CD8 followed by flow cytometry

analysis. Data shown in b and c represent at least 3 experiments with similar results. Data shown are mean $\pm$ SE of % P1CTL cells from three spleens (**b**) and tumors (**c**). *: $P < 0.05$ by student's t test. **d**. Enhanced in vivo tumor rejection by P1CTL in IL-10^{-/-}RAG-2^{-/-} (DKO) mice. Purified CD8⁺ cells from P1CTL mice were injected into DKO or RAGKO mice with established J558 or P815 tumors intravenously at a dose of 5×10^6 /mouse. Tumor free survival of mice at day 50 after P1CTL treatment is shown. *: $P < 0.05$ by chi square (X^2) test.