Depleting Intratumoral CD4⁺CD25⁺ Regulatory T Cells via FasL Protein Transfer Enhances the Therapeutic Efficacy of Adoptive T Cell Transfer

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Abstract

One strategy for improving adoptive therapy is preconditioning the host immune environment by depleting CD4⁺CD25⁺ regulatory T cells (Treg) suppressive to antitumor responses. Given that Treg increase, or selectively accumulate, within tumors and are sensitive to FasL-mediated apoptosis, we tested here the hypothesis that inducing apoptosis of intratumoral Treg using FasL may improve adoptive T cell therapy. We show that FasL applied intratumorally via protein transfer decreases intratumoral Treg via inducing apoptosis in these cells. Significantly, we show that the use of FasL, prior to the infusion of tumor-reactive CD8⁺ T cells enhances the therapeutic efficacy of adoptive T cell transfer against established tumors, which is mediated by persistent, systemic antitumor immunity. Intratumoral FasL protein transfer also results in neutrophil infiltration of tumor. However, we show that intratumoral immunodepletion of neutrophils does not abolish the effect of FasL on adoptive transfer. Rather, the effect of FasL is completely abolished by cotransfer of Treg, isolated from the tumor-draining lymph nodes. Hence, our study shows for the first time that using FasL to predeplete intratumoral Treg provides a useful means for optimizing adoptive therapy.

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Introduction

The limited efficacy of adoptive T cell therapy in clinical trials highlights the need to continually improve this modality. One strategy is to enhance the antitumor efficacy of therapeutic T cells by enhancing their persistence, specificity, tumor-homing ability, resistance to tolerance or immunosuppression, and/or memory function (1).

Another strategy is to generate a host immune environment that is conducive to the function of adoptively transferred therapeutic T cells, using strategies such as depleting host lymphocytes (2–4). Although how lymphodepletion enhances adoptive therapy remains partially understood (5, 6), depletion of suppressor T cells seems to be a major contributing factor (7). In the early 1980s, North showed that depletion of a tumor-induced population of suppressor T cells by cyclophosphamide could enhance the antitumor efficacy of adoptive therapy (8). Later studies showed that depletion of CD4⁺CD25⁺ regulatory T cells (Treg), a recently defined T cell subset playing a critical role in governing self-tolerance (9–11) and inhibiting immune responses against "self-like" cancer cells (12–14), could greatly improve adoptive therapy (7, 15).

Thus far, depletion of Treg has been achieved mainly by systemic use of chemotherapeutic agents such as cyclophosphamide (16, 17) or anti-CD25 monoclonal antibodies (mAb). Nonetheless, such strategies have limitations, including toxicity, induction of autoimmunity due to the systemic elimination of Treg (18), and/or loss of CD25-expressing effector T cells. On the other hand, previous studies have shown that Treg increase even selectively accumulate, within tumors (12, 19, 20), likely via the function of the chemokine CCL22 produced by tumor cells and tumor-infiltrating macrophages (12). Collectively, these studies emphasize the need to specifically target intratumoral Treg. One strategy would be to induce the apoptosis of intratumoral Treg locally at the tumor site. Interestingly, it has recently been shown in vitro that compared with CD4⁺CD25⁺ effector T cells (Teff), Treg are highly susceptible to FasL-mediated apoptosis (21).

Based on these findings, in the present study, we aim to test the hypothesis that depleting intratumoral Treg using FasL, prior to adoptive T cell transfer, may improve the antitumor efficacy of adoptive therapy. Our results show for the first time that predepleting intratumoral Treg using FasL provides an alternative means for optimizing adoptive therapy.

Materials and Methods

Mice and tumor cell lines. BALB/c and DBA/2 mice (6–8 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME) and used in accordance with the institutional guidelines for animal care. EG.7 and L5178Y tumor cell lines were purchased from American Type Culture Collection (Manassas, VA) and maintained according to the recommendations of the supplier.

Antibodies. Anti-mouse CD4-FTTC (GK1.5), anti-mouse CD25-PE (7D4), anti-mouse CD25-PE-Cy5 (PC61.5), anti-mouse Foxp3-PE (JFK-16S), anti-mouse Gr-1-PE (RB6-8C5), anti-human FasL mAb (NOK-1), anti-mouse Gr-1 (RB6-8C5), anti-mouse CD16/32 mAb (clone 93), various isotype controls, and Annexin V-APC were purchased from BD Biosciences (San Diego, CA).

Isolation of T cell subsets. T cell subsets were purified by magnetic cell sorting using various kits (Miltenyi Biotec, Auburn, CA). L5178Y tumor-reactive CD8⁺ T cells were purified from bulk splenocytes using a CD8⁺ T cell kit. CD4⁺CD25⁺ T cells were purified from lymph nodes draining L5178Y tumors using a CD4⁺ T cell kit first, and subsequently, a CD4⁺CD25⁺ cell kit. T cell purity >98% was usually achieved, as determined by flow cytometry.

Determination of FasL-induced apoptosis in vitro. Bulk splenocytes from BALB/c or DBA/2 mice were depleted of RBC and incubated (5 × 10⁶/mL) for 20 h at 80°C with human FasL Fc fusion protein (FasL-Fc; ref. 22), in the absence or the presence of 10 μg/mL of
FasL–neutralizing NOK-1 mAb or control mAb. Cells were stained with anti–CD4-FITC, anti–CD25-PE, and 7-AAD, and analyzed for dead cells (7-AAD+/CD4+/CD25-) by flow cytometry on a FACS Calibur (BD Biosciences). CD4+CD25- and CD4+CD25+ T cell populations were gated, respectively. Data were analyzed using the CellQuestPro software (BD Biosciences).

Depletion of intratumoral Treg cells in vivo via Fasl protein transfer. Procedures for generating palmitated protein A (PPA; ref. 23) and using it for Fc protein transfer (24, 25) have been described previously. Briefly, a conjugate of PPA and Fasl-Fc was first generated by mixing the components at a 1:1 ratio (w/w) in DMEM and incubating the mixture on ice for 30 min. The mixture was injected intratumorally into mice bearing an established intradermal L5178Y tumor. To assess the depletion of intratumoral Treg, the next day, single cell suspensions were prepared from tumors and stained with anti–CD4+FITC and anti–CD25-PE, and analyzed by flow cytometry, gated on the CD4+ population.

Adaptive transfer therapy. To obtain L5178Y tumor-reactive T cells, DBA/2 mice were immunized twice (at 1-week intervals) with 1 × 10^6 mitomycin C–treated L5178Y tumor cells (s.c. injection) and, subsequently, challenged with 1 × 10^6 live L5178Y tumor cells (s.c. injection). Survivors were used as donors for tumor-reactive T cells. One week prior to adoptive therapy, the donors were rechallenged with live L5178Y tumor cells as above. On the day when adoptive therapy was done, the donors were sacrificed, and CD8+ T cells were purified from the spleen.

For adoptive therapy, tumors were established by intradermally injecting 5 × 10^5 L5178Y tumor cells on the right flank of DBA/2 mice (day 1). Palpable tumors (∼5 mm in diameter) usually formed at the injection site after 4 to 5 days. Occasionally, tumors grew into the s.c. area as a result of bad injection; those mice were eliminated before experiments. On days 5 and 6, mice were each injected intratumorally with PPA (2 μg) or PPA:Fasl-Fc conjugate (4 μg total protein). On day 7, PPA-treated mice were each injected with 4 × 10^5 of L5178Y tumor-reactive CD8+ T cells (designated as "CD8+ T cells"). In parallel, PPA:Fasl-Fc conjugate–treated mice either received no further treatment ("Fasl") or were adoptively transferred with the CD8+ T cells, either alone ("Fasl + CD8+ T cells") or together with 2 × 10^5 Preg ("Fasl + CD8+ T cells + Treg"). Treg were isolated from lymph nodes draining progressively growing L5178Y tumors in a separate group of tumor-bearing mice. In some experiments, Fasl protein transfer and depletion of tumor-infiltrating neutrophils were done concurrently. The neutrophil-specific anti–Gr-1 mAb was injected intratumorally (15 μg per tumor) twice a day (at 12-h intervals) during the course of intratumoral Fasl protein transfer.

Tumor size was measured twice a week. Mice were euthanized when they became moribund or when the tumors exceeded 400 mm^2 in size. Cured mice were each rechallenged (i.p.) with 5 × 10^5 L5178Y tumor cells at least 8 weeks after the initial tumor inoculation.

Cytotoxic T lymphocyte assay. Bulk splenocytes from cured mice, prepared 4 to 6 weeks after tumor rechallenge, were restimulated with mitomycin C–treated tumor cells at a 10:1 ratio for 5 days. Viable cells (used as effectors) were cultured with CFSE-labeled, mitomycin C–treated tumor target cells. After 12 h, the cultures were stained with 7-AAD and analyzed for dead target cells (CFSE+/7-AAD-) by flow cytometry. Specific lysis was calculated as described previously (26).

Detection of cytokines. Bulk splenocytes from cured mice were prepared and restimulated with L5178Y tumor cells as described above, and the conditioned media were analyzed using a flow cytometry-based bead array kit for Th1/Th2 cytokines (BD Biosciences), following the manufacturer's instructions.

Results

Treg are more sensitive than their CD4+CD25- counterpart to Fasl-induced apoptosis in vitro. First, under our experimental settings, we compared the susceptibility to Fasl-mediated apoptosis in vitro of murine Treg versus their CD4+CD25- counterparts (naïve effector T cells, Teff). Purified Treg expressed high levels of Fopx3 and were suppressive to T cell proliferation (data not shown). To ensure that the Treg and Teff subsets were compared under the same conditions, bulk splenocytes from BALB/c mice were directly incubated with recombinant Fasl, the Fasl, Fc fusion protein (FasL-Fc; ref. 22); apoptosis in each subset of T cells was then determined by staining the cells with anti–CD4+, anti–CD25, and 7-AAD and analyzing by flow cytometry, gated on CD4+CD25- and CD4+CD25+ populations, respectively. Fasl effectively elicited apoptosis in Treg, but had a minimal effect on Teff (Fig. 1A). The specificity of Fasl was verified by the blockade of apoptosis by Fasl-neutralizing NOK-1 mAb, but not by control mAb. Fasl titration experiments verified that Treg are more sensitive than Teff to Fasl (Fig. 1B). A similar susceptibility to Fasl was confirmed in splenocytes from another strain of mice, the DBA/2 (Fig. 1C). Together, these results show that Treg are more sensitive than Teff to Fasl-mediated apoptosis in vitro.

Intratumoral Fasl protein transfer leads to depletion of Treg in vivo. As a prelude to applying Fasl to adoptive therapy, we assessed the capacity of Fasl to induce apoptosis in Treg in vivo in the L5178Y lymphoma model. Consistent with prior findings by others (27), we showed that L5178Y tumor cells in our laboratory were resistant to Fasl-induced apoptosis (Fig. 2A). Hence, the use of the L5178Y tumor model excludes the possibility of direct killing of the tumor cells by Fasl.

To clearly define the lineage of the CD4+CD25- cell population within established L5178Y tumors, we assessed the intracellular expression of Fopx3, the Treg lineage marker, on these cells. As shown in Fig. 2B, a majority (>85%) of the CD4+CD25- T cells within tumors coexpress Fopx3, confirming that these T cells are, indeed, Treg.

We next wanted to assess the susceptibility of these intratumoral Treg to Fasl. To minimize the toxicity associated with the use of recombinant Fasl (28, 29), we confined Fasl-Fc within tumors using the protein transfer (or protein "painting") method we previously developed (24). In this method, protein A, after being chemically derivatized with palmitate in a simple reaction, is first incorporated into cell membranes; in turn, this membrane-anchored PPA serves as a "trap" for secondarily added Fc fusion proteins. Moreover, in the L5178Y tumor model, we showed that Fc fusion proteins, after being preconjugated with PPA in vitro, can be directly "painted" and localized for sufficiently long times (up to 18 h) in the tumor bed, thereby efficiently generating cancer vaccines in situ (25).

Therefore, Fasl-Fc preconjugated with PPA (PPA:Fasl-Fc) was injected (4 μg total protein per tumor) intratumorally into L5178Y tumors. Under these experimental conditions, treated mice seemed healthy, and showed no obvious liver damage as assessed by autopsy (data not shown). The next day, single cell suspensions were prepared from the injected tumors and assessed for the presence of Treg undergoing apoptosis. As shown in Fig. 2C, compared with nontreated control tumors, Fasl-treated tumors contained significantly more abundant Treg undergoing apoptosis, assessed by their Annexin V+ status. The result indicates that Fasl protein transfer could result in the apoptosis of intratumoral Treg in vivo. Of note, we observed that dead Treg or Treg undergoing apoptosis were relatively fragile and readily lysed during the processing of single tumor cell suspensions (data not shown); thus, quantification of these Treg can cause underestimation of Treg depletion. Consequently, we chose to assess the extent of Treg depletion via quantifying live (7-AAD-) Treg and Teff populations.
As shown in Fig. 2D, compared with nontreated or PPA-treated tumors, FasL-treated tumors showed a decrease in the number of live Treg and, thus, a decrease in the ratio of Treg to Teff. Collectively, these results indicate that intratumoral FasL protein transfer could lead to a decrease in intratumoral Treg by inducing apoptosis in these cells. In addition, we also determined whether increasing the quantity of PPA:FasL-Fc conjugate can improve the extent of Treg depletion. As shown in Fig. 2, the dose-dependent response plateaus between 3 and 9 μg per tumor. Hence, the PPA:FasL-Fc conjugate was used at 4 μg per tumor for the entire study.

Next, we determined whether intratumoral FasL treatment can lead to depletion of Treg systemically. At different time points after FasL treatment, cell suspensions prepared from the tumor, tumor-draining lymph nodes, spleen, and peripheral blood were each quantified for live Treg. Again, similar to the results in Fig. 2, the FasL treatment caused a significant decrease in intratumoral Treg, with the maximal decrease (>80%) achieved at 12 h after FasL.

**Figure 1.** Splenic Treg are highly susceptible to FasL-mediated apoptosis in vitro compared with their CD25− counterpart (Teff). A, splenocytes from BALB/c mice were depleted of RBC and incubated for 20 h with 250 ng/mL of FasL-Fc, alone or together with 10 μg/mL of FasL-neutralizing NOK-1 mAb or control mAb. Cells were stained with anti–CD4-FITC, anti–CD25-PE, and 7-AAD, and analyzed by flow cytometry, gated on CD4+CD25− and CD4+CD25+ populations, respectively. B, dose-response curve of FasL-induced apoptosis of CD4+CD25− Treg (■) and CD4+CD25+ Teff (○) populations. C, splenocytes from DBA/2 mice were similarly analyzed for FasL-induced apoptosis as described in (A). Columns, mean of three to four independent experiments; bars, SD.

**Figure 2.** FasL protein transfer leads to a decrease in intratumoral Treg in vivo. A, LS178Y tumor cells were incubated for 20 h with the indicated concentration of FasL-Fc, and the resistance of the tumor cells to apoptosis was determined by 7-AAD staining and flow cytometry. B, single cell suspensions were prepared from intradermal LS178Y tumors established in DBA/2 mice. Cells were first stained with anti–CD4-FITC and anti–CD25-PE-Cy5, and then stained intracellularly with anti–Foxp3-PE. Cells were analyzed by flow cytometry, gated on CD4+CD25− and CD4+CD25+ populations, respectively. B, dose-response curve of FasL-induced apoptosis of CD4+CD25− Treg ( ■) and CD4+CD25+ Teff (○) populations. C, splenocytes from DBA/2 mice were similarly analyzed for FasL-induced apoptosis as described in (A). Columns, mean of three to four independent experiments; bars, SD.
treatment; such a decrease of Treg was sustained even after 24 h (Fig. 3A). In addition, the Fasl transfer also resulted in a decrease (although to a significantly lesser extent) in Treg in the tumor-draining lymph nodes, with the maximal decrease achieved after 12 h (Fig. 3B). In comparison, at the same time points evaluated, the same Fasl treatment did not lead to a decrease in Treg in the spleen and peripheral blood (Fig. 3C and D). Collectively, these data indicate that intratumoral Fasl protein transfer leads to a decrease in Treg locally, but does not decrease Treg systemically.

Predepleting intratumoral Treg using Fasl enhances the efficacy of adoptive T cell transfer. Having documented the capacity of Fasl applied intratumorally to induce apoptosis in local Treg in vivo, we next determined whether predepleting intratumoral Treg using Fasl can improve the efficacy of adoptive T cell therapy. First, in the L5178Y tumor model, we evaluated whether therapy combining Fasl with adoptive T cell transfer could elicit more effective antitumor responses compared with adoptive therapy alone. Then, we determined whether the effect of Fasl on adoptive therapy was attributable to the depletion of intratumoral Treg.

To that end, mice bearing established L5178Y tumors were injected intratumorally with PPA alone or with PPA:Fasl-Fc conjugate. Subsequently, mice were adoptively transferred with L.5178Y tumor-reactive CD8+ T cells, alone or together with Treg. Of note, Treg used in these therapy experiments were isolated from lymph nodes draining progressively growing L5178Y tumors in a separate group of tumor-bearing mice; these Treg expressed Foxp3 and efficiently suppressed T cell proliferation in vitro (data not shown).

As negative controls, nontreated mice all showed progressive tumor growth and died, whereas a portion of mice treated with either Fasl alone or adoptive therapy alone showed delayed tumor growth (Fig. 4A). On average, Fasl treatment alone resulted in complete tumor regression in 12% of treated mice compared with 9% in adoptive therapy alone (Table 1). In comparison, when mice were treated with both Fasl and adoptive T cell therapy, tumor growth was significantly retarded in a substantial portion of treated mice (Fig. 4A), and on average, complete tumor regression was observed in 53% of treated mice (Table 1). These results indicate that therapy combining Fasl with adoptive T cell transfer has stronger antitumor efficacy than that with either Fasl or adoptive transfer alone.

Significantly, post-Fasl treatment, when Treg were cotransferred with tumor-reactive CD8+ T cells, tumors grew aggressively, even to a slightly greater extent than those in nontreated mice (Fig. 4A); on average, tumor regression was observed in 7% of treated mice, which is comparable with that (9%) in mice treated with adoptive therapy alone (Table 1). Hence, the addition of Treg during adoptive T cell transfer completely abolished the effect of Fasl on adoptive therapy, which points to the fact that Fasl enhances the antitumor efficacy of adoptive therapy via, at least partially, predepleting intratumoral Treg.

Establishment of persistent, systemic immunity in mice treated with Fasl and adoptive therapy. Having documented the local tumor regression in a substantial percentage of mice treated with both Fasl and adoptive T cell transfer, we next assessed the establishment of long-term, systemic antitumor immunity in treated mice. To this end, cured mice were rechallenged with L.5178Y tumor cells injected at sites (i.p.) distant from the original tumor at least 2 months after the initial tumor inoculation. All of these cured mice were resistant to the rechallenge, whereas naïve mice inoculated with the same tumor cells all died of tumors (Fig. 4B). It is especially notable that all of the cured mice subjected to i.p. tumor rechallenge >6 months after the initial tumor cell inoculation were still resistant to tumor (data not shown). These rechallenge experiments point to a persistent, systemic antitumor immunity that is established in mice treated with therapy combining Fasl with adoptive T cell transfer.

To further support the establishment of persistent, systemic immunity, we recovered bulk splenocytes from these cured mice, 3 to 6 weeks after tumor rechallenge, and checked for cytokine and cytotoxic T lymphocyte (CTL) responses. Upon in vitro restimulation with the tumor cells, the splenocytes produced high levels of Th1 cytokines, including interleukin 2, IFN-γ, and tumor necrosis factor α (Fig. 4C). The splenocytes showed CTL activity against the L.5178Y tumor cells (Fig. 4D). The CTL activity was L.5178Y tumor cell–specific, as the lysis of irrelevant, control EG.7 tumor cells was at a significantly lower level. Thus, specific CTL can be recovered from a secondary lymphoid organ distal from the tumor cells at the treatment site.

Together, these results show a persistent, systemic antitumor immunity that is established in mice cured by therapy combining intratumoral Fasl, protein transfer and adoptive T cell transfer.
Depletion of tumor-infiltrating neutrophils does not diminish the effect of FasL on adoptive transfer. Although the experiments above establish the contribution of depleting local Treg to the antitumor efficacy, they do not exclude the possibility that FasL may enhance the antitumor efficacy via other mechanisms. Especially, FasL has been shown to mediate neutrophil recruitment, which, under certain circumstances, can elicit or enhance antitumor responses (27, 30, 31). Hence, we wanted to determine the contribution of FasL-mediated neutrophil recruitment to the increase in the antitumor efficacy. Consistent with the findings from others (27, 30, 31), intratumoral FasL protein transfer resulted in a significant increase of tumor-infiltrating neutrophils (Gr-1+ population); such an increase was effectively reversed by using the neutrophil-specific anti–Gr-1 mAb, coinjected intratumorally with PPA:FasL-Fc (Fig. 5A). FasL, however, did not significantly change the levels of neutrophils in the blood, assessed at various time points postinjection (Fig. 5B). These results indicate that intratumoral FasL protein transfer, whereas increasing tumor-infiltrating neutrophils, does not affect the quantity of neutrophil systemically.

Next, we determined whether FasL-mediated increase in tumor-infiltrating neutrophils could contribute to the effect of FasL on adoptive therapy. To this end, we depleted tumor-infiltrating neutrophils during the course of FasL treatment. As shown in Fig. 5C, a single injection of anti–Gr-1 mAb was sufficient to efficiently block the increase in tumor-infiltrating neutrophils for up to 24 h. Consequently, for adoptive therapy, we injected anti–Gr-1 mAb twice a day (at 12-h intervals) during the entire course of the FasL treatment, to ensure complete depletion of tumor-infiltrating neutrophils. As shown in Fig. 5D, the depletion of tumor-infiltrating neutrophils did not abolish the antitumor efficacy of the therapy combining FasL with adoptive T cell transfer. On average, the therapy done in the presence of tumor-infiltrating neutrophils and that in the absence of neutrophils showed comparable antitumor efficacies, each resulting in complete tumor regression in ~50% treated mice (Table 1, experiments 6 and 7).
Collectively, these data show that the reversal of FasL-mediated neutrophil increase does not affect the antitumor efficacy of therapy combining FasL with adoptive T cell transfer. This finding, together with the finding that the addition of Treg during adoptive therapy completely abolishes the effect of FasL on the antitumor efficacy (Fig. 4), solidifies our conclusion that intratumoral FasL protein transfer enhances the therapeutic efficacy of adoptive therapy via, primarily, depleting Treg locally.

Table 1. Treatment of L5178Y tumors

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Figure 5. Depletion of tumor-infiltrating neutrophils does not abolish the effect of FasL on the therapeutic efficacy of adoptive T cell transfer. A, intradermal L5178Y tumors were established in DBA/2 mice (day 1). On day 5, mice were injected intratumorally with PPA, PPA:FasL-Fc alone, or PPA:FasL-Fc together with anti–Gr-1 mAb. PPA was used at 2 μg/tumor, PPA:FasL-Fc was used at 4 μg per tumor, and anti–Gr-1 mAb was used at 15 μg per tumor. After 12 h, single cell suspensions from the tumors were analyzed for neutrophils by staining with anti–Gr-1-PE and flow cytometry. Columns, average percentages of neutrophils (in total cells within the tumor from two independent experiments); bars, SD. B, intradermal L5178Y tumors were treated by intratumoral FasL protein transfer, as described in (A). At indicated time points posttreatment, single cells from peripheral blood of treated mice were quantified for neutrophils, as described in (A). Points, mean of results from two independent experiments (B-C). C, intradermal L5178Y tumors were treated with either PPA:FasL-Fc alone (●) or PPA:FasL-Fc together with anti–Gr-1 mAb (○), as described in (A). At indicated time points posttreatment, single cell suspensions from the tumors were quantified for neutrophils, as described in (A). Points, mean of results from two independent experiments (B-C). D, intradermal L5178Y tumors were established (day 1), and mice were divided into three groups. On days 5 and 6, mice in group 1 (control) remained nontreated; mice in group 2 were each treated with 4 μg of PPA:FasL-Fc once; mice in group 3 were each treated with 4 μg of PPA:FasL-Fc once and 15 μg of anti–Gr-1 mAb twice (at 12-h intervals). On day 7, mice in groups 2 and 3 were injected intratumorally with 4 × 10^6 L5178Y tumor-reactive CD8+ T cells. Subsequently, tumor size was measured and graphed, as described in Fig. 4A. Data are representative of two independent experiments.
Discussion

Treg increase, or selectively accumulate, within tumors (12, 19, 20), likely via the function of the chemokine CCL22 produced by tumor-infiltrating macrophages (12). Hence, it is conceivable that it may be advantageous to target intratumoral Treg locally. Supporting this notion, the depletion of intratumoral Treg using anti-CD25 mAb has recently been shown to unmask tumor immunogenicity, leading to the rejection of late-stage tumors (32). In the present study, we show for the first time that depleting Treg locally at the tumor site using FasL likewise enhances the therapeutic efficacy of adoptive T cell therapy.

Other than depleting Treg, FasL may elicit or enhance antitumor responses via other mechanisms. One such mechanism is FasL-mediated recruitment of neutrophils. Thus, we determined whether intratumoral FasL protein transfer could enhance the antitumor efficacy of adoptive therapy via recruiting neutrophils. Our results show that the FasL transfer could cause significant neutrophil infiltration of tumors (Fig. 5A). Nonetheless, depletion of tumor-infiltrating neutrophils does not significantly decrease the antitumor efficacy of therapy combining FasL with adoptive transfer (Fig. 5D). This result is not completely unexpected. Although it has been shown in certain systems that FasL-mediated neutrophil recruitment can result in antitumor responses (27, 30, 31), contradicting observations also exist in the literature. The expression of FasL on tumor cells, although capable of inducing intensive neutrophil infiltration of tumor and inflammation, has been shown to fail to cause tumor rejection (33, 34). This may be attributed to a few factors. For example, transforming growth factor β in the tumor microenvironment has been shown to inhibit neutrophil activation, thereby preventing tumor rejection (30). The antitumor effect of FasL has been shown to be affected by the form (i.e., soluble versus membrane-bound) of FasL expressed on tumors (33). The density of FasL is another contributing factor; tumor cells expressing high levels of FasL, have been shown to impair neutrophil activation (34). Under our experimental conditions, FasL-induced neutrophil infiltration has a minimal effect on the antitumor efficacy of adoptive therapy. This finding, together with the findings that the addition of Treg during adoptive therapy completely abolishes the effect of FasL (Fig. 4A) and that FasL does not induce apoptosis of the tumor cells directly (Fig. 2A), indicates that intratumoral FasL protein transfer improves the antitumor efficacy of adoptive therapy, primarily via depleting Treg locally.

As we have previously reported, intratumorally injected pulsed proteins can be retained in the tumor for up to 18 h (25). The loss of the proteins during or after the 18-h period is likely due to cell metabolism, internalization, and/or degradation. In addition, after protein transfer, we did not observe a significant increase in soluble Fc fusion protein in the blood from treated mice (data not shown). Therefore, protein transfer may provide a safe alternative means for applying immunoregulatory molecules locally to a tumor, particularly for those associated with relatively severe toxicity, such as FasL.

In this study, we observed high susceptibility to FasL-mediated apoptosis by both Treg cultured in vitro and intratumoral Treg in vivo. Our results are consistent with the in vitro study by Fritzsching and colleagues showing that in both humans and mice, CD4+CD25+ Treg cells are highly susceptible to FasL-induced apoptosis (21). Papiernik and colleagues, however, observed resistance of prestimulated CD4+CD25+ Treg to apoptosis induced by anti-Fas mAb (35). One explanation for this discrepancy may be that we and Fritzsching and colleagues used FasL, which might bind and multimerize the Fas receptor differently from the anti-Fas mAb used by Papiernik and colleagues.

In summary, the strategy described here and those developed by others, such as the use of anti-GITR mAbs, now provide a set of tools for targeting host Treg, in order to optimize adoptive transfer and other immunotherapeutic modalities for cancer.

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References

19. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of regulatory T cells is increased in peripheral blood and
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