Allogeneic effector/memory Th-1 cells impair FoxP3+ regulatory T lymphocytes and synergize with chaperone-rich cell lysate vaccine to treat leukemia

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Therapeutic strategies combining the induction of effective antitumor immunity with the inhibition of the mechanisms of tumor-induced immunosuppression represent a key objective in cancer immunotherapy. Herein we demonstrate that effector/memory CD4+ T helper-1 (Th-1) lymphocytes, in addition to polarizing type-1 antitumor immune responses, impair tumor-induced CD4+CD25+FoxP3+ regulatory T lymphocyte (Treg) immunosuppressive function in vitro and in vivo. Th-1 cells also inhibit the generation of FoxP3+ Tregs from naive CD4+CD25−FoxP3− T cells by an interferon-γ–dependent mechanism. In addition, in an aggressive mouse leukemia model (12B1), Th-1 lymphocytes act synergistically with a chaperone-rich cell lysate (CRCL) vaccine, leading to improved survival and long-lasting protection against leukemia. The combination of CRCL as a source of tumor-specific antigens and Th-1 lymphocytes as an adjuvant has the potential to stimulate efficient specific antitumor immunity while restraining Treg-induced suppression.

Introduction

The primary objective of cancer immunotherapy is to promote tumor elimination through the activation of innate and adaptive immune responses. Successful immunotherapy relies on vaccination strategies endowed with the dual capability of inducing tumor-specific lymphocytes while overcoming the mechanisms of immune tolerance. CD4+CD25+FoxP3+ regulatory T lymphocytes (Tregs) critically contribute to the occurrence and persistence of tumor-induced tolerance. An increase in the frequency of these immunosuppressive cells in cancer patients has been widely reported. Treg expansion observed during tumor progression may result from the proliferation of naturally occurring Tregs (nTregs) or from the conversion of CD4+CD25−FoxP3− T cells into CD4+CD25+FoxP3+ Tregs (iTregs). Tregs dampen immune responses by suppressing the function of the effectors CD4+, CD8+, and natural killer (NK) cells and by inhibiting dendritic cell activation. Because Tregs are one of the main barriers for the eradication of tumors by immune cells, their therapeutic depletion or their functional inactivation using drugs or antibodies improves responses to cancer immunotherapy, such as dendritic cell–based vaccines. However, the selective elimination or inactivation of Tregs constitutes a major challenge because these cells share the same surface markers as activated conventional, nonsuppressive T cells. Indeed, antibody-based approaches indistinguishably target both Tregs and activated effector T lymphocytes. Likewise, chemotherapeutic agents such as cyclophosphamide, which are used to eliminate Tregs, do not target these cells selectively.

Several reports have indicated that the adoptive transfer of allogeneic T cells may increase the efficacy of tumor immunotherapy by providing adjuvant/danger signals to the host immune cells. A method has been optimized allowing for the efficient generation in vitro of a large number of allogeneic CD3/CD28 cross-linked T helper-1 (Th-1) memory T cells. Adoptive transfer of these Th-1 lymphocytes stimulates anticancer immunity and significantly improves the survival of mice lethally injected with BCL1 leukemia cells. This effect partly stems from cytokine production by activated T lymphocytes, which foster the establishment of protective type-1 immune responses. However, the effects of type I cytokines, including interferon-γ (IFN-γ), on Tregs have been discrepant in previous studies. As an essential effector cytokine for cell-mediated immunity, exogenous or autocrine IFN-γ has been reported to negatively regulate Treg generation. Other studies have found that IFN-γ enhances activation-induced cell death and that it thereby may regulate the expansion and persistence of effector T cells by promoting apoptosis.

In the present study, we report that effector-memory CD4+ Th-1 (emTh-1) cells are capable not only of fostering the establishment of type-1 immune responses, but also of critically impairing tumor-induced immunosuppressive Tregs in vitro and in vivo. These Th-1 cells inhibit the conversion of naive CD4+CD25−FoxP3− T lymphocytes into CD4+CD25+FoxP3+ Tregs and skew their differentiation toward a Tbet+GATA-3+ Th-1 profile. IFN-γ has been identified as being primarily responsible for impairing immunosuppressive Tregs. Unlike conventional approaches aimed...
at inactivating/depleting Tregs, emTh-1 cells do not hinder effector T lymphocytes, but rather promote their antitumor function. Furthermore, allogeneic emTh-1 cells are potent adjuvants capable of enhancing the in vivo therapeutic efficiency of a tumor-derived chaperone-rich cell lysate (CRCL) vaccine developed in our laboratory.

Methods

Mice

Mice were housed under specific pathogen-free conditions and cared for according to the guidelines of the University of Arizona Institutional Animal Care and Use Committee. Female BALB/c (H2b), C57BL6 (H2b), severe combined immunodeficiency (SCID; H2b), and Nude (H2b) mice were obtained from the National Cancer Institute (Bethesda, MD). IFN-γ receptor−/− (H2b) mice were purchased from Jackson ImmunoResearch Laboratories. FoxP3EGFP mice that co-express green fluorescent protein (GFP) and FoxP3 under the control of the endogenous promoter were obtained from Jackson ImmunoResearch Laboratories (C57BL6; Thym2aScid). GFP expression allows the accurate identification and isolation of FoxP3+ Tregs. Congenic Thy1.1 mice (CBA/PL(B6)-Thy1aScid) were obtained from Jackson ImmunoResearch Laboratories. These animals carry the T lymphocyte–specific Thy1.1 allele. Donor T cells from Thy1.2 mice can be distinguished from recipient Thy1.1 mouse T cells using anti-Thy1.2 antibodies. Mice were used at the age of 6-8 weeks.

Preparation of allogeneic emTh-1 cells

emTh-1 lymphocytes were generated and activated in vitro as described by Har-Noy et al.19-20 Spleen cells from C57BL6 mice were harvested and treated with a hypotonic buffer (150mM NH4Cl, 1mM KHCO3, 0.1mM Na2EDTA) for lysis of red blood cells. CD4+ T cells were then isolated using CD4+ microbeads and an autoMACS separator device (Miltenyi Biotec). Positively and negatively selected cells were routinely analyzed by flow cytometry to assess the purity of each fraction. The percentage of CD4+ cells in the positive fraction was >95%. These CD4+ T lymphocytes were expanded in RPMI medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), with anti-CD3- and anti-CD28-coated paramagnetic beads (CD3/CD28 T-cell expander beads [Dynabeads]; Invitrogen) at an initial bead:CD4+ cell ratio of 3:1, and in the presence of 20 IU/mL recombinant interleukin-2 (rmIL-2), 20 ng/mL rmIL-7, 10 ng/mL rmIL-12 (Peprotech), and 10 μg/mL anti–mouse IL-4 monoclonal antibody (mAb; Becton Dickinson). Additional media containing rmIL-2, rmIL-7, anti–IL-4 mAb, and T-cell expander beads was added to the culture daily from day 3 to day 6 to maintain constant cell density (0.5-1×10^6 cells/mL). The amount of beads added was calculated to maintain a 1:1 bead:cell ratio as the cells expanded. After 6 days in culture, the expansion of CD4+ T cells was approximately 60 to 100 times the initial number of plated cells at day 0. Cells were harvested on day 6 and de-beaded by physical disruption and passage over a magnet. These cells were either used fresh or stored in liquid nitrogen for future use. A similar protocol was followed to generate emTh-1 cells from C57BL6 mice.

Human emTh-1 cells were generated from healthy donor peripheral blood lymphocytes isolated by density centrifugation with lymphocyte separation medium (1.077; Eurobio). CD4+ T cells were then isolated using human CD4+ microbeads (Miltenyi Biotec) and cultured with human T-cell expander beads (Dynabeads; Invitrogen) in the presence of 20 IU/mL recombinant human IL-2 (rhIL-2), 20 ng/mL rhIL-7, 10 ng/mL rhIL-12 (Peprotech), and 10 μg/mL anti–human IL-4 mAb (BD Biosciences). Human CD4+ T-cell cultures were maintained as described in the previous paragraph for mouse cells. Human studies were approved by the institutional review board (IRB000005448; WA00004218), with informed consent in compliance with the Declaration of Helsinki.

Magnetic cell sorting

Spleens isolated from BALB/c or C57BL6 mice were harvested and dissociated. CD4+CD62L−, CD4+CD25+, and CD4+CD25− T lymphocytes were purified by magnetic cell sorting using mouse CD4+CD62L− naive T-cell or CD4+CD25+ T regulatory cell isolation kits and an autoMACS separator according to the manufacturer’s instructions (Miltenyi Biotec). We previously reported that CD4+CD25+ T lymphocytes isolated by this technique express high levels of the transcription factor FoxP3 and are endowed with immunosuppressive properties.8,9,14

Conversion of CD4+CD62L+ T cells into CD4+CD25+FoxP3+ Tregs

CD4+CD25−CD62L+ naive T cells were isolated from Balb/c mouse splenocytes as described in “Magnetic cell sorting,” cultured in complete medium in a 96-well plate (1×10^5 cells per well), and activated with T-cell expander beads (T lymphocyte:bead ratio of 1:1) in the presence of transforming growth factor-β1 (TGF-β1; 5 ng/mL) for 72 hours at 37°C. Some wells were treated with emTh-1 supernatant. The percentage of CD4+CD25−FoxP3− and CD4+CD25+FoxP3+ cells was determined by flow cytometric analysis. Blocking antibodies against IFN-γ or tumor necrosis factor-α (TNF-α) were added to the corresponding samples at a concentration of 1 μg/mL.

Flow cytometry analysis and antibodies

Cells (~10^6) were washed in phosphate-buffered saline (PBS) containing 3% heat-inactivated fetal bovine serum and 0.09% sodium azide (Sigma), and were first incubated with an Fe receptor-blocking Ab (BD Biosciences) for 5 minutes and then with saturating amounts of the appropriate combination of fluorescein-conjugated antibody for 40 minutes. Cells were then washed and analyzed using a FACSCalibur (Becton Dickinson). A minimum of 10 000 events was collected for each sample, and data analysis was performed with CellQuest Pro 6.0 (Becton Dickinson). For FoxP3 detection, CD4+CD25+ or CD4+CD25− T cells purified by magnetic cell sorting or converted CD4+CD25− Tregs generated in vitro were fixed, permeabilized, stained using an allophycocyanin anti–mouse FoxP3-staining set following the provider’s instructions (clone FJK-16; eBioscience), and analyzed by flow cytometry. For the monitoring of CD4+CD25− Tregs, cells were first stained with fluorescein isothiocyanate–conjugated anti-CD4 (rat immunoglobulin 2b [IgG2b]; BD Biosciences) and phycoerythrin-conjugated anti-CD25 (rat IgG1; BD Biosciences Pharmingen) antibodies. Cells were then stained using the eBioscience FoxP3 staining set as described. The expression of the transcription factors Tbet and GATA-3 (expressed by Th-1 and Th-2 cells, respectively) was evaluated by intracellular staining using anti–mouse Tbet–phycoerythrin and ant–mouse GATA-3–phycoerythrin monoclonal antibodies (BD Biosciences Pharmingen). Isotype control antibodies were purchased from BD Biosciences (phycoerythrin-conjugated rat IgG1, fluorescein isothiocyanate-conjugated rat IgG2a) or eBioscience (allophycocyanin-conjugated rat IgG1).

T-cell proliferation and suppression assays

CD4+CD25− and CD4+CD25+ T cells were purified from splenocytes and lymph node cells using Miltenyi Biotec isolation kits. The cells were cultured for 48 hours in 96-well plates at 37°C either in complete medium or with emTh-1 supernatant, and activated with plate-bound anti-CD3 (5 ng/mL), soluble anti-CD28 (5 ng/mL), and IL-2 (20 IU/mL).

In other experiments, untreated CD4+CD25− T cells, emTh-1 supernatant–pretreated CD4+CD25+ T cells, or freshly isolated CD4+CD25− T cells (1×10^5) were cocultured for 48 hours in rounded-bottom 96-well plates with CD4+CD25− T cells (1×10^5) and exposed or not to emTh-1 supernatant. Anti-CD3/CD28 T-cell expander beads (Dynabeads) were added in all cocultures (cell:bead ratio = 1:1). Bromodeoxyuridine (BrdU; Millipore) was then added for an additional 12 hours. The cells were then fixed and the incorporation of BrdU detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s procedures (Millipore). Cultures were set up in triplicates.
analyze the diagrams.

The murine leukemia cell line 12B1 was obtained by retroviral transformation (eBiosciences). The level of endotoxin in CRCL was lower than that in the media control (< 0.01 EU/µg). 12B1 CRCL was used for in vivo vaccination of mice.

Tumor growth in vivo and combination immunotherapy

BALB/c mice were injected with 5 × 10^3 viable 12B1 cells in the right groin on day 0. Allogeneic (C57BL/6) emTh-1 lymphocytes (10^5 cells/ mouse), 12B1-derived CRCL vaccine (25 µg/mouse), or cells plus CRCL were administered in the footpad in a total volume of 100 µL on days +3, +7, and +14. Tumor growth was monitored every other day, and mice were euthanized when tumor volume reached 4000 mm³. Tumor-free survival was compared among the different treatment groups.

Depletion of immune cells in vivo

Mice were depleted of NK cells by intraperitoneal injection with anti-asialo GM1 antibodies (25 µL/mouse, 1/8 diluted with PBS; Wako Chemicals) on days −1, +3, and +5.

Statistics

Kaplan-Meier curves were generated and analyzed by log-rank statistics to determine survival percentages and differences between the treatment groups. In other experiments, Student t tests were used to determine significant differences (P < .05) between groups.

Results

EmTh-1 supernatant impairs the generation of iTregs induced by tumor cells or TGF-β in vitro

Treg expansion may result from the conversion of CD4^+CD25^-FoxP3^- T cells into CD4^+CD25^+FoxP3^+ cells or from the proliferation of naturally occurring Tregs. The transcription factor FoxP3 is required for the induction of Treg suppressive function, and its expression in nonregulatory cells converts them into immunosuppressive cells. TGF-β1 has been shown to promote the polarization of naive CD4^+ T lymphocytes into Tregs.35 We first examined whether soluble factors produced by emTh-1 cells may negatively regulate the generation of Tregs from naive T cells induced by different TGF-β–secreting tumors in vitro. 12B1 leukemia, B16 melanoma, and 4T1 breast cancer cells secrete TGF-β1 in culture (not shown). The culture of naive CD4^+CD62L^+ T cells with either of these 3 tumor cell lines triggered their differentiation into FoxP3^+ T cells (Figure 1A) endowed with immunosuppressive activity (not shown). The presence of the

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**Figure 1. EmTh-1 cells impair tumor-induced iTreg generation.** CD4^+CD25^-CD62L^- naive T cells were isolated from mouse spleens using magnetic activated cell sorting. The cells were activated using T-cell expander beads (cell:bead ratio 1:1), resuspended in the culture medium from different tumor cell lines (12B1, B16, 4T1), and treated with emTh-1 supernatant. (A) FoxP3 expression was determined by flow cytometry. (B) Pooled data from 3 independent experiments are depicted. Student t tests were used to analyze the diagrams. *P < .001, a significant difference compared with the corresponding group without emTh-1 supernatant.
supernatant of emTh-1 during the differentiation process significantly dampened tumor-induced FoxP3 (Figure 1A-B).

In agreement with these results, the data depicted in Figure 2 indicate that emTh-1 supernatant significantly inhibited the TGF-β-induced conversion of naive T cells into FoxP3+ T lymphocytes (Figure 2A-B). Furthermore, the number of activated effector CD25+CD62L+ naive T cells was significantly augmented by the allogeneic emTh-1 supernatant (Figure 2C). This effect was observed in a dose-dependent manner (supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). To further evaluate whether the suppressive function of residual FoxP3-expressing cells was affected by the emTh-1 supernatant, TGF-β-induced conversion of naive CD4+CD25+FoxP3+ cells into iTregs (i.e., GFP-positive) from FoxP3EGFP transgenic mice was performed in the presence or absence of emTh-1 supernatant. GFP-positive (i.e., FoxP3-expressing) cells were then sorted and their suppressive activity evaluated. Our results demonstrate that the function of these residual FoxP3-expressing iTregs was impaired (supplemental Figure 2). In addition, emTh-1 supernatant added to FoxP3+ iTregs that had been previously converted significantly impaired their immunosuppressive function (supplemental Figure 3).

We then evaluated whether the emTh-1 supernatant may skew TGF-β–induced Foxp3+ T-cell differentiation toward another T-lymphocyte lineage. The transcription factors Tbet and GATA-3 are predominantly expressed by Th-1 or Th-2 cells, respectively.36

Figure 2. EmTh-1 supernatant impairs TGF-β–induced iTreg generation and promotes Tbet+ T-lymphocyte differentiation. CD4+CD25−CD62L− naive T cells were cultured for 72 hours with T-cell expander beads (cell:bead ratio 1:1) with or without TGF-β1 (5 ng/mL) in the presence or absence of the emTh-1 supernatant (emTh-1 sup). Cells were then analyzed by flow cytometry. (A-B) Representative dot plots or histogram plots from 10 independent experiments. (C) Percentage of CD4+CD25−FoxP3− activated T cells in total CD4+ T lymphocytes. *P < .01, a significant difference compared with cells cultured without emTh-1 supernatant. (D) The expression of the transcription factors FoxP3, Tbet, and GATA-3 was determined in CD4+CD25−CD62L− T cells cultured for 72 hours with T-cell expander beads with or without TGF-β1 treated or not with emTh-1 supernatant. Results are representative of 3 independent experiments.
These transcription factors not only play a critical role in promoting the permissive lineage fate, but also actively repress the opposite lineage commitment. Flow cytometric analysis indicated that the majority of CD4+ T cells obtained after 72 hours of culture in the presence of TGF-β plus emTh-1 supernatant expressed a low level of GATA-3 and FoxP3, while displaying a Tbet-positive phenotype consistent with Th-1 polarization (Figure 2D). These results indicate that emTh-1 cells produce soluble factors capable of switching TGF-β–dependent polarization of naive T cells from FoxP3+ Tregs to the proinflammatory Th-1 lineage.

**Inhibition of iTreg generation by emTh-1 cells depends on IFN-γ**

To address the mechanism by which emTh-1 cells suppress Treg generation from naive CD4+ T cells, we examined the role of key cytokines produced by Th-1 cells. Consistent with previous studies, high levels of IFN-γ and TNF-α were detected in the supernatants from emTh-1 culture (not shown). Neutralization of IFN-γ but not of TNF-α using blocking antibodies abrogated the effects of the emTh-1 supernatant in restoring TGF-β–induced conversion of naive cells into FoxP3+ T cells (Figure 3A-B). Consistent with these data, recombinant IFN-γ but not recombinant TNF-α impaired the negative modulation of TGF-β–induced generation of FoxP3+ T cells (Figure 3A-B and data not shown). These results were confirmed using IFN-γR−/− mice. The data depicted in Figure 3C and D indicate that the conversion of CD4+ naive T cells isolated from IFN-γR−/− mice into FoxP3+ T cells was not modified by emTh-1 supernatant (Figure 3C-D). Therefore, IFN-γ produced by Th-1 lymphocytes is primarily responsible for the inhibition of FoxP3+ T-cell generation.

**EmTh-1 cells minimally affect nTreg phenotype, but significantly diminish their immunosuppressive function and promote effector T-cell resistance to Treg-mediated suppression**

Because the emTh-1 supernatant impaired the generation of FoxP3+ T lymphocytes from non-Treg precursors, we next sought...
compared with responder CD25+/H11002 cocultured with freshly isolated CD4 were incubated for 48 hours with emTh-1 supernatant and then of CD4 lymphocytes (supplemental Figure 4A).

Figure 4. EmTh-1 supernatant inhibits nTreg immunosuppressive function. (A) CD4+CD25+ nTregs were isolated from BALB/c mouse lymphoid tissues and cultured for the indicated periods of time with plate-bound anti-CD3 (5 ng/mL), soluble anti-CD28 (5 ng/mL), and IL-2 (20 IU/mL) with or without emTh-1 supernatant. FoxP3 expression was then determined using flow cytometry. (B) CD4+CD25+ nTregs were cultured for 48 hours with plate-bound anti-CD3, soluble anti-CD28, and IL-2 with or without emTh-1 supernatant. Cells were then washed extensively with complete medium. Responder CD4+CD25− T-lymphocytes were stimulated with anti-CD3/antiCD28 T-cell expander beads in the absence (CD25−) or presence of untreated nTregs (CD25− + untreated nTreg) or in the presence of emTh-1 supernatant–treated nTregs (CD25− + [nTreg]EmTh-1 sup). Responder CD4+CD25− T-lymphocyte proliferation was determined after 48 hours using BrdU incorporation assays. NS, nonsignificant; *P < .001, a significant difference compared with responder CD25− T cells cultured with untreated Tregs. (C) CD4+CD25− T lymphocytes were first treated ([CD25−]EmTh-1 sup) or not (untreated CD25−) for 48 hours with emTh-1 supernatant, washed extensively with complete medium, and cocultured for 48 hours with freshly isolated CD4+CD25+ nTregs ( − nTreg). Proliferation of responder CD25− T cells was then determined using BrdU incorporation assays. *P < .001. (D) IFN-γ concentration was assessed in the cocultures as described for panel C. *P < .001; **P < .0001.

to evaluate their influence on preexisting nTregs. CD4+CD25−FoxP3+ cells were isolated from spleens and lymph nodes and incubated for 24, 48, or 72 hours with supernatant from emTh-1 cells. Our results demonstrated that FoxP3 expression in nTregs was not impaired by the emTh-1 supernatant (Figure 4A).

We next investigated the influence of the emTh-1 supernatant on Treg immunosuppressive function in vitro. nTregs isolated from mouse lymphoid tissues were activated with anti-CD3 and anti-CD28 antibodies and IL-2. The cells were cultured in either complete medium or emTh-1 supernatant for 48 hours and then washed before being cocultured with freshly isolated CD4+CD25− cells for an additional 48 hours. The ability of emTh-1 supernatant–treated or untreated nTregs to inhibit the proliferation of CD4+CD25− cells was then analyzed using BrdU incorporation assays. Our data indicate that emTh-1 supernatant (Figure 4B) or IFN-γ (not shown) significantly inhibited the capacity of Tregs to suppress CD4+CD25− conventional T-cell proliferation. Similar results were obtained using human CD4+CD25− Tregs and CD4+CD25− responder T cells isolated from peripheral blood lymphocytes (supplemental Figure 4A).

To further define whether emTh-1 may modulate the sensitivity of CD4+CD25− T lymphocytes to Tregs, CD4+CD25− T cells were incubated for 48 hours with emTh-1 supernatant and then cocultured with freshly isolated CD4+CD25+FoxP3+ nTregs. Neither the proliferation (Figure 4C) nor the production of IFN-γ (Figure 4D) of CD4+CD25− T cells pretreated with emTh-1 supernatant was suppressed by Tregs. Similar results were obtained when human CD4+CD25− responder T cells pre-incubated with emTh-1 supernatant were exposed to human CD4+CD25+ Tregs (supplemental Figure 4B).

These data indicate that emTh-1 cells not only impair the inhibitory function of Tregs, but also induce resistance of effector T cells to Treg-mediated inhibition.

EmTh-1 cells can be efficiently combined with a tumor-derived CRCL vaccine to treat mice with 12B1 leukemia

We previously reported on the immune stimulatory and protecting effects of the CRCL vaccine against multiple types of cancers, including 12B1 leukemia,27–30,37 and have documented that the tumor-derived CRCL vaccination can be efficiently combined with Treg elimination to treat established tumors.8 In the present study, we evaluated whether emTh-1 cell–based immunotherapy can improve CRCL vaccination.

Using a therapeutic approach of established 12B1 tumors in naive Balb/c mice, we confirmed that allogeneic emTh-1 cell–based immunotherapy is safe and can be efficiently combined with CRCL immunization, resulting in a significant tumor-free survival.
of treated animals (Figure 5A). Similar results were obtained with a B16 melanoma model that consisted of B16 tumor-bearing C57BL/6 mice treated with B16-derived CRCL plus emTh-1 cells generated from Balb/c mice (supplemental Figure 5).

The protective effects of emTh-1 cells combined with CRCL vaccine are mediated by host T lymphocytes

We next sought to define the role of T lymphocytes in the antitumor responses induced by CRCL plus allogeneic emTh-1 cells. 12B1 tumor–bearing SCID mice were treated with CRCL plus allogeneic emTh-1 cells on days 3, 7, and 14 after tumor cell inoculation, as described in “Tumor growth in vivo and combination immunotherapy.” CRCL plus allogeneic emTh-1 cells did not improve the survival of 12B1 tumor–bearing SCID mice (Figure 5B). Similar results were obtained using Nude mice (data not shown). Therefore, these data indicate that the protective effects of adoptively transferred allogeneic emTh-1 cells with CRCL immunization are host T-lymphocyte dependent. Consistent with these results, anti-asialo-GM1 did not significantly impair the therapeutic efficacy of the allogeneic emTh-1/CRCL vaccine, indicating that NK cells do not play a major role in the antitumor immune responses induced by this combination immunotherapy (Figure 5C).

Surviving mice treated with CRCL plus allogeneic emTh-1 cells were rechallenged with the parental 12B1 tumor cells in the right groin and with an unrelated B-cell leukemia (A20, H-2d) in the opposite groin. A20 tumors developed in all 8 mice in both the treated and control groups (Figure 6A-B), whereas 5 of 8 mice were protected against 12B1 tumor rechallenge in the CRCL plus allogeneic emTh-1 cell group. Two additional mice demonstrated significant tumor growth delay, while all control mice developed 12B1 tumors (Figure 6C-D). Therefore, combination immunotherapy consisting of CRCL plus allogeneic emTh-1 cells can induce long-lasting tumor-specific immunity.

We further analyzed the antitumoral function of T cells isolated from CRCL plus emTh-1 cell–treated mice. Lymphocytes purified from the spleens of animals receiving the combination therapy were capable of specifically killing parental tumor cells but not irrelevant target cancer cells (Figure 7A).

emTh-1 cells were able to skew the differentiation of naive Thy1.2^CD4^CD25^-FoxP3- T lymphocytes transferred to
12B1 tumor–bearing congenic Thy1.1\(^+\) mice toward Thy1.2\(^-\)CD4\(^+\)CD25\(^-\)FoxP3\(^-\) effector T cells rather than Thy1.2\(^-\)CD4\(^+\)CD25\(^-\)FoxP3\(^+\) Tregs (Figure 7B). In addition, the suppressive function of Tregs isolated from tumor-bearing animals treated with emTh-1 cells was significantly reduced (Figure 7C). This confirms that the effects of emTh-1 cells on Tregs observed in vitro also occur in vivo, and also demonstrates that the mechanism by which emTh-1 cells augment the efficacy of CRCL vaccination involves the inhibition of tumor-induced Tregs.

**Discussion**

The advantages of active immunotherapy include its relative lack of side effects, its specificity against target tumor cells, and the generation of memory responses against tumor-specific antigens. However, even if proven clinically safe, immunotherapy has only sparked moderate enthusiasm because of the relatively limited objective clinical responses that have been observed in cancer patients. This modest therapeutic success stems in part from the immunosuppressive environment created by tumors, with patients. This modest therapeutic success stems in part from the objective clinical responses that have been observed in cancer patients. 

We previously documented that the efficacy of tumor-derived CRCL vaccination can be enhanced by Treg elimination. The advantages of active immunotherapy include its relative lack of side effects, its specificity against target tumor cells, and the generation of memory responses against tumor-specific antigens. However, even if proven clinically safe, immunotherapy has only sparked moderate enthusiasm because of the relatively limited objective clinical responses that have been observed in cancer patients. This modest therapeutic success stems in part from the immunosuppressive environment created by tumors, with patients. This modest therapeutic success stems in part from the objective clinical responses that have been observed in cancer patients. 

Multiple strategies harnessing the adjuvant effect of allogeneic T-cell transfer have been optimized with the goal of skewing the host’s immune responses toward the induction of a “graft-versus-tumor” effect. Recent studies have demonstrated that in vitro–generated allogeneic emTh-1 cells can serve as a potent adjuvant for stimulating type-1 antitumor immunity when used together with a source of tumor antigen. However, significant tumor-free survival of tumor-bearing mice was difficult to achieve with allogeneic emTh-1 cells in association with tumor cell lysate or irradiated cancer cell vaccines. In the current study, we demonstrate that the combination of allogeneic emTh-1 cells with our CRCL vaccine significantly increased the survival of mice bearing established leukemia or melanoma. The demonstrated advantages in efficacy of CRCL over more common vaccine strategies, such as individual tumor-derived chaperone proteins HSP70, gp96, and tumor lysates, have been extensively published by our group. The combination of tumor-derived CRCL plus allogeneic emTh-1 cells demonstrated a superior therapeutic outcome compared with monotherapy. In addition, combination immunotherapy with CRCL plus allogeneic emTh-1 cells promoted durable, tumor-specific, T cell–dependent adaptive immunity.

We previously documented that the efficacy of tumor-derived CRCL vaccination can be enhanced by Treg elimination. The advantages of active immunotherapy include its relative lack of side effects, its specificity against target tumor cells, and the generation of memory responses against tumor-specific antigens. However, even if proven clinically safe, immunotherapy has only sparked moderate enthusiasm because of the relatively limited objective clinical responses that have been observed in cancer patients. This modest therapeutic success stems in part from the immunosuppressive environment created by tumors, with patients. This modest therapeutic success stems in part from the objective clinical responses that have been observed in cancer patients.
selective negative modulation of Tregs by emTh-1 cells represents an effective mechanism by which these transferred cells improve the therapeutic efficacy of the CRCL vaccine. Allogeneic emTh-1 cells thus represent a powerful adjuvant capable of enhancing the therapeutic potential of the CRCL vaccine, and therefore represent a promising translational approach in cancer immunotherapy clinical trials.

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Authorship

Contribution: N.L. and E.K. designed and directed research; N.J., N.L., and E.K. analyzed data; and N.J., M.H.-N., N.L., and E.K. wrote the paper.

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