Blockade of CCL1 Inhibits T Regulatory Cell Suppressive Function Enhancing Tumor Immunity without Affecting T Effector Responses

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Intratumoral accumulation of T regulatory cells (Tregs) creates an immunosuppressive environment that reduces the efficacy of antitumor immunotherapy. The immunosuppressive milieu within tumors is largely brought about by the presence of Tregs, which maintain self-tolerance by directly inhibiting T cells, NK cells, and dendritic cells. Depletion of Tregs enhances antitumor immune responses; however, current depletion therapies also affect the function of CD4 and CD8 T effector cells. Previous studies from our laboratory indicate that intratumoral delivery of CpG-ODN strongly reduces the levels of Tregs within the tumor, which is mainly mediated by IL-6. Because IL-6 promotes growth of some human cancers, alternate pathways to inactivate Tregs were sought through microarray analysis, resulting in gene candidates that can be exploited to modulate the function of Tregs. Analysis of these candidates indicates that neutralization of chemokine (C-C motif) ligand 1 (CCL1) prevented de novo conversion and suppressive function of Tregs without affecting the function of T effector cells. The combination of CpG-ODN and anti-CCL1 treatments induced complete rejection of tumors in BALB-neuT tolerant mice, and result in the generation of long-term protective memory responses. Tumor rejection correlated with changes in the lymphocyte composition within the tumor; we observed decreased Treg numbers and a concomitant accumulation of tumoricidal cells such as CD8+NKG2D+ and NK cells. These studies demonstrate that neutralization of CCL1 can be used as an adjuvant to antitumor immunotherapy, as a means of reversing the immunosuppressive function of Tregs without compromising T cell effector function. The Journal of Immunology, 2010, 184: 6833-6842.
The most widely tested TLR ligand is CpG-ODN, which is currently in multiple phase II clinical trials as combination therapy with existing cancer treatments. We previously found that intratumoral (i.t.) injection of CpG-ODN slowed tumor growth and reduced i.t. and systemic Tregs in both a nontolerant (24), as well as a tolerant murine breast cancer model (24, 25). A more therapeutically relevant tumor-targeted delivery of CpG-ODN was achieved by conjugating it to an anti-Her2/neu Ab, resulting in efficacious tumor rejection in a tolerant system (25). The antitumor effect of CpG-ODN is generated through the binding of TLR-9 expressed by APCs, and the subsequent secretion of IL-6 into the tumor microenvironment. The presence of the proinflammatory IL-6, in turn suppresses Treg function and conversion. Because IL-6 promotes growth in a variety of human cancers, we investigated the effects of IL-6 on Tregs using microarray analysis to identify alternate pathways to inactivate Tregs. Chemokine (C-C motif) ligand 1 (CCL1) was highly differentially regulated in Tregs in the presence of IL-6, and its neutralization both inhibited Treg conversion and suppressive function. However, anti-CCL1 did not affect T effector viability or proliferation. The combination of CpG-ODN and CCL1 blockade significantly slowed tumor growth and resulted in tumor rejection in tolerant BALB-neuT mice; moreover, all the animals that rejected the tumor, formed protective memory responses. Tumor rejection was associated with reduced Treg suppressive capacity, lower i.t. Treg numbers and a substantial increase in cytolytic cell populations. In summary, we identify a novel strategy to block Treg conversion and suppressive function, without compromising the T effector population, resulting in an effective antitumor immune response. We propose that neutralization of CCL1 can be used as an adjuvant to antitumor immune therapies, as a means of reversing Treg-dependent immune suppression within the tumor as well as systemically.

Materials and Methods

Mice, cell line, and reagents

BALB/c mice were purchased from Harlan Breeders (Indianapolis, IN), FoxP3-GFP mice were kindly provided by Dr. Rudensky (Sloan Kettering, New York, NY), and BALB-neuT mice were generated as previously described (26) and housed under specific pathogen-free conditions. TUBO cells are derivatives of a spontaneous mammary carcinoma in BALB/c mice and were obtained from Dr. Forni (University of Torino, Torino, Italy): they were maintained in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 5 × 10⁻⁵ M 2-ME, and 50 μg/ml gentamicin. Recombinant human IL-6 (NCST20836) and IL-2 (Ro-236019, Roche, Madison, WI) were obtained through the National Institutes of Health (Bethesda, MD). CpG-ODN (1826) was obtained from Invivogen (San Diego, CA). Blocking Abs against CCL1 (TCA3, MAB845) and integrin (ITGαE) (CD103, M290) were obtained from R&D Systems (Minneapolis, MN) and BD Bioscience (San Diego, CA), respectively. CD4 and CD8 cells were enriched using the negative selection kits (Invitrogen, Carlsbad, CA).

In vivo tumor studies

Tolerant BALB-neuT mice were implanted with 1 × 10⁶ TUBO cells and treated as previously described (24), with the addition of two weekly α-CCL1 and α-ITGαE (100 μg each) i.p. injections. Mice were treated for 1 wk for FACS analysis, or 3 wk for tumor growth and survival curves. Mice were measured twice weekly and sacrificed when the tumors reached 1 cm³ or showed signs of external necrosis.

Conversion assays

The 1 × 10⁶ CD4⁺ T cells were isolated from spleens of FoxP3⁺ mice through negative isolation kit (Invitrogen), and seeded on plates pretreated with 2 μg/ml anti-CD3. Cells were incubated for 3 d with IL-2 (100 U/ml), TGF-β (R&D Systems) at 4 ng/ml, and IL-6 at 25 ng/ml. The percent of converted Tregs was evaluated by flow cytometry on the third day. For inhibition of conversion, α-CCL1 and α-ITGαE were added at 5 μg/ml.

Flow cytometry and cell sorting

The percentage of Treg cells was determined by staining lymphocytes from spleen, lymph nodes (LNs), and tumor with anti-CD4 (PE) and FoxP3 (FITC) (eBioscience, San Diego, CA). CD8⁺ populations were measured using anti-CD8 (PE) and NKG2D (APC) Abs (eBioscience), NK1.1 (PE) was obtained from BD Bioscience. Pure populations of Tregs for the microarray and suppression assays were derived by cell sorting CD4⁺ FoxP3⁺ splenocytes using FoxP3-GFP–expressing mice with the FACS Aria (BD Bioscience). Tregs from BALB-neuT mice were derived by sorting CD4⁺CD25⁺ cells.

Suppression assays

The 1.85 × 10⁶ cell–sorted Tregs (1.875 × 10⁶, CD4⁺FoxP3⁺ cells from FoxP3 mice, or CD4⁺CD25⁺ cells from BALB-neuT mice) were incubated with freshly isolated CD8⁺ cells (7.5 × 10⁵) in a 1:4 ratio, on plates pretreated with 2 μg/ml α-CD3 (BD Bioscience). After 2 d coculture, 1 μCi [³H]thymidine was added and cells were grown a further 16 h. Incorporated [³H]thymidine was measured using the Top Count instrument (PerkinElmer, Shelton, CT). For inhibition of suppression, IL-6, α-CCL1, or α-ITGαE were added as previously described.

Microarray analysis

CD4⁺ T cells were enriched from LNs of FoxP3-GFP mice and cultured under the following conditions: 1) anti-CD3⁺IL-2; 2) anti-CD3⁺IL-2+TGF-β; 3) anti-CD3⁺IL-2+TGF-β+IL-6; and 4) anti-CD3⁺IL-2+IL-6. On day 4 cells were collected, stained with anti-CD4-PE, and sorted into CD4⁺FoxP3⁺ and CD4⁺FoxP3⁻ populations (>95% purity). RNA was isolated according to manufacturer’s instructions and further purified with RNeasy mini columns (Qiagen, Valencia, CA). RNA quantity and integrity were verified with the Nanodrop (Thermo Scientific, Wilmington, DE) and Bioanalyzer (Agilent, Wilmington, DE) using the nano and pico chips (Agilent). RNA (200 ng per sample) was labeled using the low-input linear amplification kit, two-color (Agilent). Each matched set of FoxP3⁻ and FoxP3⁺ cells for each condition was labeled independently with cyst3 and cyst5. Dye incorporation and amplified RNA amounts were verified with the Nanodrop. A microarray (Genta, Inc.) was hybridized to a 4 × 48 K mouse whole genome slide, washed, and feature extracted according to the manufacturer’s (Agilent) instructions. Data analysis was performed using GeneSpring (GXT) software (Agilent). Briefly, an ANOVA was performed to select genes with significant variance between CD4⁺ cells treated with anti-CD3⁺IL-2, anti-CD3⁺IL-2+TGF-β, and anti-CD3⁺IL-2+TGF-β+IL-6. This gene list was filtered through a series of criteria designed to yield only those genes that are changed in newly converted Tregs through the presence of TGF-β, and in which the transcriptional change brought about by TGF-β is reversed by the addition of IL-6. In addition, genes in which the changes in expression are equal in T effector cells and Tregs derived from the same treatment were also removed. This was preformed to avoid potentially affecting the T effector cells with future therapies directed at Tregs (scheme delineated in Supplemental Fig. 1). The values shown in the heat map (Fig. 1B) are derived from the signal ratio of CD4⁺FoxP3⁺ over CD4⁺FoxP3⁻ cells in each treatment population.

Western blots

CD4⁺CD25⁺ cells were sorted from pooled tumors and tumor-draining lymph nodes (TDLN) of four to eight mice in each treatment condition. Protein was quantified with the BCA Protein Assay kit (Thermo Scientific). Equal amounts of each sample (850 μg) was hybridized to a 4 × 48 K mouse whole genome slide, washed, and feature extracted according to the manufacturer’s (Agilent) instructions. Data analysis was performed using GeneSpring (GXT) software (Agilent). Briefly, an ANOVA was performed to select genes with significant variance between CD4⁺ cells treated with anti-CD3⁺IL-2, anti-CD3⁺IL-2+TGF-β, and anti-CD3⁺IL-2+TGF-β+IL-6. This gene list was filtered through a series of criteria designed to yield only those genes that are changed in newly converted Tregs through the presence of TGF-β, and in which the transcriptional change brought about by TGF-β is reversed by the addition of IL-6. In addition, genes in which the changes in expression are equal in T effector cells and Tregs derived from the same treatment were also removed. This was preformed to avoid potentially affecting the T effector cells with future therapies directed at Tregs (scheme delineated in Supplemental Fig. 1). The values shown in the heat map (Fig. 1B) are derived from the signal ratio of CD4⁺FoxP3⁺ over CD4⁺FoxP3⁻ cells in each treatment population.

Statistical analysis

Statistical significance of data was determined in most cases using the Student t test, and the χ² test was used to evaluate significant differences in survival.

Results

Microarray analysis of the effect of IL-6 on Tregs

It is not known what signals IL-6 provides to T cells to prevent conversion to Tregs, or to inhibit Tregs’ suppressive function. To investigate the events that happen in Tregs after IL-6 treatment, we used expression microarray analysis. For these experiments, CD4⁺ T cells from FoxP3-GFP mice were converted in vitro in the
The presence of IL-2, TGF-β, and/or IL-6. CD4+GFP(FoxP3+) and CD4+GFP−(FoxP3−) cells were sorted from each treatment group (Fig. 1A) and subjected to microarray analysis. All microarray data were deposited in National Center for Biotechnology Information’s Gene Expression Omnibus (27), under the accession number GSE21027 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21027). To isolate genes that encode effectors of IL-6 signaling in induced Tregs, we developed a data analysis algorithm designed to yield those genes that are transcriptionally modulated by both TGF-β and IL-6 (Supplemental Fig. 1), this yielded 310 gene candidates (Supplemental Table I). The final selection of 50 biologically relevant genes (Fig. 1B, Table I) contains genes encoding proteins with a relevant biological function and that could potentially be therapeutically targeted. These include: integrinαE(ITGαE, CD103) and ephrinA5 (EFNA5), which are involved in cell/cell signaling; TLR-2 and chemokine (C-X motif) receptor 3 (CXCR3), which are signal transducers; and chemokine (C-C motif) ligand 1 (CCL1), which could play a role in Treg communication with T effector cells. The values shown in Table I are averages of normalized fluorescence intensities for each treatment population.

Blocking CCL1 or ITGαE reduces Treg conversion, but only CCL1 neutralization reduces Treg function

ITGαE and CCL1 were selected for further biological validation, because they are transcriptionally upregulated as CD4+ T cells are converted to CD4+FoxP3+ Tregs through TGF-β and this transcriptional upregulation is reversed by IL-6. After confirming transcriptional changes by quantitative RT-PCR (Supplemental Fig. 2), we assessed whether CCL1 and/or ITGαE play a role in Treg conversion. Addition of blocking Abs against CCL1 or ITGαE inhibited Treg conversion (p = 0.005) to a similar degree as IL-6 (Fig. 2A), whereas the isotype control Ab had no effect. These data suggest that CCL1 and ITGαE both have a role in the process of Treg conversion in vitro.

We also tested the ability of α-CCL1 and α-ITGαE to inhibit the suppressive function of Tregs. The presence of Tregs reduced CD8+ T cell proliferation and the addition of α-CCL1 to Tregs/CD8+ T cell cocultures significantly reversed the suppressive capacity of Tregs (p = 0.0001), in a similar fashion as IL-6 (Fig. 2B). Addition of α-ITGαE had no effect on Treg suppression, and neither blocking Ab reversed Treg anergy. To confirm that targeting CCL1 or ITGαE did not directly affect T effector cells, we measured CD8+ T cell proliferation in the presence of IL-6, α-CCL1, or α-ITGαE, and found no changes from basal proliferation rates (Fig. 2C), nor did they affect CD4+ T cell proliferation (Supplemental Fig. 3). Taken together these data show that blocking CCL1 signaling with α-CCL1 results in both reduced Treg conversion and diminished Treg suppressive function in vitro. In contrast, blocking ITGαE only inhibited Treg conversion.

One of the effects of IL-6 in conjunction with TGF-β is to induce Th17 cells (28). Considering that blocking ITGαE or CCL1 might act in the same or similar fashion as IL-6, we tested whether neutralizing ITGαE or CCL1 in the presence of TGF-β induces Th17 cells. Addition of α-ITGαE or α-CCL1 to TGF-β did not induce Th17 cells, and the blocking Abs did not disrupt the induction of Th17 cells (Fig. 2D). Together, these results indicate that neutralizing ITGαE and CCL1 exclusively targets Tregs and does not have an effect on T effector cells.

Antitumor effect of CpG-ODN plus α-CCL1 or α-ITGαE in BALB-neuT mice

Having demonstrated that blocking CCL1 or ITGαE reduces Treg conversion, and that blockage of CCL1 perturbs Treg function, we evaluated whether in vivo blockage of these molecules enhances the antitumor effect of CpG-ODN treatment of tumor-bearing tolerant BALB-neuT mice. This is a mouse model of breast cancer in which the mice express the rat Her2/neu oncogene under...
transcriptional control of the mouse mammary tumor virus long terminal repeat (29). We used the TUBO murine breast cancer cell line derived from the BALB-neuT mouse to form tumors, which are essentially seen by the mouse immune system as “self.” This mouse model closely reflects the immunobiology of tumors that are essentially recognized by the mouse immune system as “self.” This line derived from the BALB-neuT mouse to form tumors, which grew at the same rate as control tumors. Furthermore, after subcutaneous inoculation of tumor cells, 100% of them rejected the tumor. These mice have remained tumor-free since the time of rechallenge (12 mo to date) (Fig. 3D), indicating that they developed a long-term protective memory response. These results strongly support our hypothesis that targeting CCL1 is a useful strategy to enhance antitumor immune responses.

Analysis of Treg levels and function after in vivo blockade of CCL1 in tumor-bearing mice

We evaluated whether the antitumor response observed in mice treated with Cpg-ODN and α-CCL1 treated mice, and tumors treated only with the blocking Abs in the absence of Cpg-ODN grew at the same rate as control tumors. Furthermore, after subjecting the surviving Cpg-ODN and α-CCL1–treated mice to a TUBO rechallenge, 100% of them rejected the tumor. These mice have remained tumor-free since the time of rechallenge (12 mo to date) (Fig. 3D), indicating that they developed a long-term protective memory response. These results strongly support our hypothesis that targeting CCL1 is a useful strategy to enhance antitumor immune responses.

<table>
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<td>71 102</td>
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Table I. Target gene list of 50 candidates
function of Tregs (TGF-β-cultured for 3 d on plates coated with specified conditions and Treg conversion (number of CD4+FoxP3+ cells) was measured by flow cytometry. Conversion in wells containing or without IL-6, α-CCL1, or α-ITGαE, controls included Tregs alone (or with blocking Abs), and CD8+ T cells alone. The 7.5 × 104 CD8+ T cells were cultured for 3 d on plates coated with αCD3 in the presence or absence of Tregs, blocking Abs, or IL-6. Addition of α-CCL1 strongly reversed the suppressive function of Tregs (p = 0.001); whereas, the presence of α-ITGαE had no effect. C. Blockage of CCL1 or ITGαE does not affect CD8+ T cell proliferation. The effect of the addition of blocking Abs or IL-6 on proliferation was evaluated by measuring levels of [3H]thymidine incorporation in counts per minute (cpm), and values shown are averages of quadruplicate wells ± SD of a representative experiment of three independent replicates (B, C). D. The simultaneous presence of TGF-β and IL-6 leads to the generation of Th17 cells, and is not affected by the presence of α-CCL1 or α-ITGαE. CD4+ T cells were cultured for 3 d in the conditions stated in A. The percentage of CD4+IL-17+ cells in each treatment condition was evaluated by flow cytometry, numbers represent the percentages of CD4+IL-17+ cells of the CD4+ population. All are representative experiments of independent triplicate determinations. cpm, counts per minute.

i.t. Treg numbers (p = 0.01 and p = 0.03, respectively). There was also a trend toward decreased peripheral Treg numbers in treated mice as compared with untreated control tumors (Fig. 4A). Next, we asked whether the presence of α-CCL1 could alter the function of Tregs in vivo. Functional analysis of freshly harvested Tregs derived from tumor-bearing mice showed that Tregs from untreated tumor-bearing mice (control) were highly suppressive; whereas, Tregs from CpG-ODN–treated mice were less suppressive than control Tregs (p = 0.003). The combination treatment of CpG-ODN and α-CCL1 resulted in further reduction of suppression over control tumors (p = 0.0001) (Fig. 4B). This points to a sustained effect of α-CCL1 on Treg function, in which systemic neutralization of CCL1 in vivo results in Treg functional impairment in vitro even in the absence of the Ab. To address the potential of autocrine signaling through CCL1, we evaluated the expression of CCL1 levels by Tregs isolated from tumors and also from TDLNs by Western blot (Fig. 4C). Tregs from untreated tumors and tumors treated with CpG-ODN expressed CCL1; whereas, Tregs from tumor-bearing mice treated with CpG-ODN and α-CCL1 expressed markedly lower levels of CCL1. Expression levels were confirmed by densitometry (data not shown). The TUBO cell line does not express measurable CCL1 nor do the tumors derived from this cell line (D.B. Hoelzinger and J. Lustgarten, unpublished data). Interestingly Tregs from TDLNs from mice subjected to the combination treatment showed the largest drop in CCL1 expression, this is likely due to the systemic (i.p.) delivery of α-CCL1. Taken together, these data show that both CpG-ODN and CpG-ODN–α-CCL1 result in lower number of Tregs both within the tumor and systemically. However, functional analysis showed that Tregs derived from CpG-ODN–α-CCL1–treated mice are less suppressive than Tregs from untreated or CpG-ODN–treated tumors. In addition, systemic α-CCL1 administration reduces the levels of CCL1 expression by Tregs, suggesting that CCL1 may be involved in controlling its own expression.

Analysis of levels of tumoricidal cells within tumors after CpG-ODN and α-CCL1 treatment

We examined levels of CD8+NKG2D+ cells and NK cells in the same treatment cohorts to determine whether there is a link between tumor rejection and an increase of tumoricidal cells in tumors from mice treated with CpG-ODN and α-CCL1. We observed a modest increase of tumoricidal CD8+ T cells (expressing the cytotoxic NKG2D cellular marker, CD8+NKG2D+) in TILs derived from CpG-ODN–treated mice as compared with TILs from control tumors (from 5% to 8%). TILs derived from CpG-ODN and α-CCL1–treated mice contained 3-fold more (14%) CD8+NKG2D+ T cells than TILs from untreated tumors (p = 0.01) (Fig. 5A, 5B). Levels of NK1.1+ cells were significantly (p = 0.03) increased within the tumors of CpG-ODN and α-CCL1–treated mice (Fig. 5A, 5B). In summary, these data
show that CpG-ODN and α-CCL1 treatment resulted not only in higher numbers of CD8+NKG2D+ T cells, but also in an increase of i.t. NK1.1-expressing cells. This shift of the composition of immune cells within the tumor from a prosuppressive to a tumor-lytic one, correlates with the activation of an antitumor response.

Discussion

It has long been recognized that the genetic instability of tumors not only generates neoantigens, but also leads to over expression of self-Ags that can drive tumor-genesis or tumor-progression. These TAAs can be recognized by the adaptive immune system, yet immune cells are rarely capable of leading to tumor rejection. One salient reason for this is the establishment of an immunosuppressive environment within and around the tumor. Various suppressor cells contribute to this phenomenon: Tregs, myeloid-derived suppressor cells, DCs, and tyrosine-based activation motifs. Tregs are essential components of the immune system that prevent autoimmune disease and control inflammation by maintaining immune tolerance/homeostasis. Their presence in the tumor microenvironment, however, has the unwanted effect of creating an immunosuppressive milieu that reduces the effectiveness of cancer immunotherapy. In this study, we report that targeting CCL1 is a novel mechanism to inhibit Treg suppressive function, which reverses immunosuppression, thereby enhancing the efficacy of immunotherapy.

Activating the innate immune system through i.t. CpG-ODN injections, slows tumor growth in tolerant BALB-neuT mice, and coincides with a decrease in i.t. Tregs. This effect is also true for CpG-ODN conjugated to an anti-Her2/neu Ab, which specifically targets CpG-ODN to murine breast cancer tumors (25). There are currently multiple clinical trials examining CpG-ODN as an immunotherapy adjuvant. Our laboratory and others have observed that CpG-ODN induces APCs to secrete IL-6, and that IL-6 inhibits conversion, and abrogates the suppressive capacity of Tregs (30). However, IL-6 can have both growth promoting and inhibitory effects (31) on human tumors. For example, a majority of breast cancers express IL-6 and its receptors IL-6R and gp130 (32), and high-serum levels of IL-6 correlate with poor outcome. In addition, IL-6 signaling is linked to angiogenesis and promotion of tumor growth (31), consequently IL-6 therapy is poorly translatable to humans.

We sought to identify the mechanism by which IL-6 inhibits the suppressive function and conversion of Tregs by expression microarray. The goal of this analysis was to identify mediators of IL-6 signaling in Tregs that could be exploited as specific anti-Treg therapy without affecting T effector function. The results of the analysis points to potential gene candidates, which encode proteins that can be manipulated to elicit changes in Treg behavior or number, such as ITGαE and CCL1. ITGαE was selected because it is substantially transcriptionally upregulated in TGF-β-induced Tregs. ITGαE is expressed in a highly suppressive (33–
35) subset of natural Tregs present in the gut (34) and skin (36). In addition, ITG\(\alpha\)E+ Tregs migrate to inflamed tissue (37), secrete higher levels of IL-10 and TGF-\(\beta\) than their ITG\(\alpha\)E counterparts, and are able to reverse inflammatory bowel disease in mice (34). The gene expression of the second candidate, CCL1, is very sensitive to both TGF-\(\beta\) and IL-6. CD4+ T cells upregulate CCL1 mRNA by 14-fold in the presence of TGF-\(\beta\), and the addition of IL-6 reverses the expression by 11-fold, making it the gene with the highest IL-6 sensitivity index. As a cytokine, it has the capacity of affecting Tregs themselves in an autocrine manner, or other cells in the vicinity of Tregs, in a paracrine manner. Autocrine effects are likely because Tregs express the CCL1 receptor, chemokine, CC motif, receptor 8 (CCR8), and migrate in response to ligand binding (38). Interestingly, Tregs that migrate in response to CCL1 are also more suppressive (38). This raises the question whether newly converted Tregs secreting CCL1 play a role in Treg homing and accumulation in the tumor. Paracrine signaling is probable because other immune cells also express CCR8 and migrate to CCL1, cutaneous memory sub-sets of CD4+ and CD8+ cells (39), NK cells (40, 41), Langerhans-type DCs (42), peritoneal macrophages (43), and Th2 cells (44). This evidence of the role of ITG\(\alpha\)E and CCL1 in Treg biology led us to investigate these targets further.

Functional validation established that blockade of both CCL1 and ITG\(\alpha\)E prevented the conversion of Tregs in a similar fashion as IL-6. Interestingly, treatment with \(\alpha\)-CCL1 but not with \(\alpha\)-ITG\(\alpha\)E inhibited the suppressive function of Tregs. Furthermore, \(\alpha\)-CCL1 or \(\alpha\)-ITG\(\alpha\)E did not affect effector T cell proliferation, reverse Treg anergy, nor did these Abs have an effect on the induction of Th17 cells. Taken together, these results indicate that neutralizing CCL1 exclusively targets Tregs, disrupting the function of these cells, without affecting the function of T effectors or conversion of Th17 cells.

Having found that CCL1 and ITG\(\alpha\)E play a role in Treg function, we asked whether blockade of CCL1 and ITG\(\alpha\)E could enhance the antitumor effect of CpG-ODN treatment. Our results show that only the combination of CpG-ODN and \(\alpha\)-CCL1 has a strong antitumor effect in which six of nine animals rejected the tumor. More importantly, six of six CpG-ODN and \(\alpha\)-CCL1–treated mice developed a protective memory response. During >12 mo observation of these animals, they showed no evidence of developing spontaneous tumors or other diseases. Previous studies from our laboratory and others (45) show that even though the T cell repertoire in BALB-neuT mice is of low avidity (46), an antitumor response can be achieved with adequate cosimulation. Such responses are limited to slowing tumor growth and do not result in tumor rejection, much less memory responses. Our results highlight the importance of perturbing the function of Tregs to achieve tumor rejection and generate long-term protective memory responses in tolerant hosts. Although in these studies, we used i.t. CpG-ODN delivery as a specific strategy to induce antitumor responses, many other vaccination strategies such as DCs, viral vectors, and DNA vaccines have also been used to activate an antitumor immune response. A major reason as to why these vaccination strategies are not as effective as expected is because currently there are no good strategies to deplete or inhibit Tregs without affecting T effector cells. This indicates that inhibiting the suppressive function of Tregs by neutralizing CCL1 could be
potentially combined with multiple vaccination strategies to induce stronger antitumor responses.

Cpg-ODN and Cpg-ODN and α-CCL1 treatments result in a reduction of Treg numbers within the tumor and peripheral lymphoid organs. Circulating Treg migration to tumors or other sites of inflammation is determined by CCR4 (47), CCR2, and CCR5 (48). No role was found for CCR8, the only known receptor for CCL1, in attracting Tregs to tumors. The modest reduction in Treg levels within the peripheral lymphoid organs with Cpg-ODN and α-CCL1 treatment is likely due to the fact that CCL1 is not known to play a role in lymphocyte (and Treg) homing. Current understanding is that CCL1 is expressed in the LN subcapsular sinus and high endothelial venules (49), and plays a role in DC migration to the LN (49). Conversely, Treg migration to the LN is determined by the expression of CCR7 (50) on Tregs and driven by the presence of CCL19 and CCL21 in the LN (51, 52). Although it is possible that CCL1 may therefore also play a role in Treg homing to the LN, it is not known to be a classical LN homing signal. Therefore the lack of significant variance in Treg numbers in LN with anti-CCL1 treatment may indicate that CCL1 is not a factor in Treg homing or conversion in the LN.

The most important aspect of tolerance is not the absolute number of Tregs in the tumor but whether they are functional immunosuppressors. We observed that Tregs derived from the periphery (spleen) of animals treated with Cpg-ODN and α-CCL1 were significantly less suppressive (in vitro) than Tregs derived from animals with untreated tumors. This suggests that a systemic administration of a CCL1-blocking Ab not only has the short-term action of neutralizing CCL1 in circulation during the course of treatment, but also has long-lasting effects on Treg function. Indeed, we observed that Tregs isolated from tumors and TDLN of mice treated with Cpg-ODN and α-CCL1 expressed substantially less CCL1 than Tregs from animals with untreated or Cpg-ODN–treated tumors. These results suggest that CCL1 is part of a positive feedback system in Tregs that in part controls Treg function. Further studies will examine the duration of the effect of anti-CCL1 neutralization on Treg numbers and function.

Tumors treated with Cpg-ODN and α-CCL1 contained substantial populations of effector cells such as CD8+NKG2D+ T cells and NK cells. The number of cytolytic cells in tumors from Cpg-ODN and α-CCL1–treated mice was larger than that of the Cpg-ODN–treated group, and three times more than untreated mice. This increase in cytolytic cell number may be due to Treg functional inhibition with the net result of enhanced CD8+ T cell activation, leading to CD8+NKG2D+ T cell and NK cell proliferation and tumor rejection.

There are two important conditions for tumor rejection: the reversal of the immunosuppressive environment brought about by Tregs and the infiltration or proliferation of immune cells with antitumor activity. Depletion of Tregs alone does not lead to tumor rejection if cytotoxic cells are not stimulated, suggesting that the balance between regulators and T effector cells could be the defining factor in mounting a successful antitumor response. In fact, the i.t. balance between Tregs and cytotoxic cells was found to be predictive of survival in ovarian and hepatocellular cancers (53, 54); thus, again, refocusing the efforts to depleting or inactivating Tregs in such a way as to leave the T effector cell populations intact. We propose that targeting CCL1 may be a more specific strategy to inhibit Treg function in the context of tumor immunotherapy, because it does not interfere with, but furthers the expansion of antitumor effector cells.

CCL1 may have multiple effects on Treg and T effector biology. Therefore, blocking its activity may also have multiple end points: 1) Tregs can educate CD4+CD25+ effector cells to develop suppressive ability, this requires TGF-β and cell–cell contact (55). CCL1 may play a role in coordinating Treg/CD4+CD25+ interactions, therefore blocking CCL1 may influence Treg conversion and reduce the number of i.t. Tregs; 2) because CCL1 drives Treg motility (38), neutralizing its activity may block Treg migration to the tumor itself; 3) intravital microscopy studies of LNs, showed that the presence of Tregs caused T cell swarming (56), reducing the duration of DC/T cell interaction, and inhibiting the establishment of stable contact during the priming phase of naive T cells (57). It was suggested that swarming was not the result of cell–cell contact between Tregs and T effector cells, but was related to the number of Tregs present. Based on these studies, we propose that CCL1 secreted by Tregs could be one of the factors that interrupts DC/T effector interaction. 4) CCL1, in its role as a motility factor, could be necessary to coordinate Treg/T effector or Treg/NK contact to bring about suppression. Studies to elucidate the specific role of CCL1 in Treg suppression are under way in our laboratory. Preliminary results suggest that blocking the CCL1 receptor, CCR8, is as effective in blocking Treg conversion and inhibiting Treg suppressive function.

In this work, we used i.t. injections of Cpg-ODN as a proof of concept to show that blocking CCL1 enhances antitumor responses. Our laboratory has previously demonstrated that Cpg-ODN can be targeted anywhere in the body using an anti–neu-Cpg-ODN hybrid molecule (25). Current studies are evaluating the effect of combining anti–neu-Cpg-ODN and anti-CCL1 to eradicate localized and metastatic tumors. In conclusion, these studies show that blocking CCL1 resulted in a highly specific inhibition of Treg...
function, without affecting T effector cells, leading to efficient tumor regression and a protective memory response. This novel approach should be evaluated for use as an adjuvant to various cancer immunotherapy approaches.

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Disclosures

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References


