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.

G enetic instability, one of the hallmarks of tumor biology, ensures a changing landscape of mutated or overexpressed tumor-associated Ags (TAAs). TAAs serve as targets for T cell immunity, and the presence of tumor-specific lymphocytes in tumors can be seen as evidence that the host is not ignorant of the developing tumors. Despite this, established tumors rarely generate endogenous immunity leading to successful tumor eradication. In part, this is because TAAs are considered “self” and therefore do not elicit strong immune responses such as those elicited by foreign “danger signals.” However, another major reason for poor TAA immunity is that the tumor microenvironment becomes progressively more immunosuppressive (1–4) as the tumor develops, inhibiting any possible antitumor immune activity.

The immunosuppressive milieu within tumors is brought about by the presence of a variety of suppressors cells such as T regulatory cells (Tregs), myeloid-derived suppressor cells (5), and subsets of plasmacytoid dendritic cells (6) and tumor-associated macrophages (7). Tregs directly inhibit T cells (8), NK cells (9), NK T cells (10), and dendritic cells (DCs) (11). Intratumoral and peripheral Tregs are increased in a growing list of mouse and human tumor types (12), and elevated Treg levels are linked with accelerated tumor progression (13) and reduced overall survival. There are two mechanisms of Treg enrichment within tumors: first, through the conversion of CD4+ T cells by DCs (14) or TGF-β (15, 16), and second, through the proliferation of tumor-specific Tregs after Ag recognition. In fact, cancer vaccines designed to activate tumor specific T effectors, can preferentially expand tumor-specific Tregs (17), leading to exacerbated immune suppression. During tumor progression, the increasing tumor burden leads to a shift from local tolerance within the tumor to a more systemic one, which includes the peripheral lymphoid organs. Under these tolerizing conditions, it becomes evident why current tumor immunotherapy strategies aimed at boosting TAA recognition alone, such as DC vaccination and TAA-vector vaccination have such low success rates.

Several Treg depletion strategies have been explored to enhance immunotherapy. Transient depletion through anti-CD4, anti-CD25, IL-2-immunotoxin and cyclophosphamide results in tumor rejection in mice (18, 19); however, these strategies have serious drawbacks. First, T effector cells are also depleted, thereby reducing antitumor activity in mice (20). Secondly, reducing Tregs systemically increases the risk of autoimmune reactions (21). Thirdly, Treg numbers quickly increase after the transient depletion and this is particularly true for tumor-associated Tregs (22), reestablishing an even more immunosuppressive environment.

Functional blockade of Tregs can be achieved through stimulation of TLR signaling in DCs (23). Vaccines that include costimulation with TLR ligands can reverse Treg-mediated tolerance.

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Address correspondence and reprint requests to Dr. Joseph Lustgarten, Mayo Clinic Arizona, 13400 East Shea Boulevard, Scottsdale, AZ, 85259. E-mail address: lustgarten.joseph@mayo.edu

The online version of this article contains supplemental material.

Abbreviations used in this paper: cpm, counts per minute; DC, dendritic cell; i.t., intratumoral; ITGαE, integrinαE; LN, lymph node; TAA, tumor-associated Ags; TDLN, tumor-draining lymph node; TIL, tumor-infiltrating lymphocyte; Treg, T regulatory cell.

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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). 2 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 blockage 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 (NCS720836) 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 integrinαEβ7 (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, Minneapipois, MN) and BD Bioscience (San Diego, CA), respectively. CD4+FoxP3+ populations (CD4+CD25+ cells from BALB-neuT mice) were incubated with freshly isolated CD8+ cells (7.5 × 106) 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, Sheltion, 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 by 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 Cy3 and Cy5. Dye incorporation and amplified RNA amounts were verified with the Nanodrop. Each sample (850 ng) was hybridized to a × 4 × 44 mouse whole genome chip, 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 reverted 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 performed 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)s of four to eight mice in each treatment condition. Protein was quantified with the BCA Protein Assay kit (Thermo Scientific). Each sample (850 ng) was hybridized to a 15% Tris-HCl gels (BioRad, Hercules, CA), transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA) and probed with anti–CCL1 Ab (R&D Systems) and anti-GAPDH (Sigma-Aldrich, St. Louis, MO) was used as a loading control.

**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...
transcriptional changes in Tregs are reversed or prevented by the addition of IL-6 (compare the second column with the third column, Table I). Values derived from Tregs treated with IL-2 and IL-6 (fourth column) are generally comparable to those of Tregs treated with IL-2 (first column). This verifies that the selected genes are transcriptional targets of IL-6 signaling in newly converted TGF-β Tregs, but that they are not targets of IL-6 signaling in natural Tregs (IL-2 population). Our target gene list of 50 candidates (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 blockade of CCL1 perturbs Treg function, we evaluated whether in vivo blockade 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 arise in humans, specifically those driven by Her2 mutations. The TUBO mouse model has been an important tool in the study of tumor immunology and has been used to evaluate the efficacy of various immunotherapeutic strategies.

We evaluated whether the antitumor response observed in mice treated with CpG-ODN and α-CCL1 completely rejected the tumor, manifesting no detectable mass 6 mo after the treatment ended. Only one of nine mice in the CpG-ODN and α-CCL1 group showed tumor growth, whereas all mice in the CpG-ODN alone and α-ITGEm group rejected tumors. Tumor growth curves for individual mice in each cohort show that CpG-ODN delayed tumor growth over control tumors (Fig. 3C), and that CpG-ODN and α-CCL1 significantly slowed tumor growth over CpG-ODN treatment alone. Tumors treated with CpG-ODN and α-ITGEm grew at a similar rate to those from CpG-ODN–treated mice, and tumors from mice treated only with the blocking Abs in the absence of CpG-ODN grew at the same rate as control tumors. Furthermore, after subcutaneous rechallenge with surviving CpG-ODN–treated mice to a TUBO rechallenge, 100% of them rejected the tumor. 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 correlated with changes in Treg number or function. Analysis of levels of CD4+FoxP3+ cells in tumor-infiltrating lymphocytes (TILs) showed that i.t. CpG-ODN and β-CCL1 treatment significantly reduced IL-6 sensitivity index (the ratio of fluorescence levels of TGF-β and IL-6 Tregs), which was used to rank genes in order of the highest impact of the addition of IL-6 on the genes’ transcriptional levels.

**Table I. Target gene list of 50 candidates**

| Symbol | Gene Name | IL-2 | TGF-β | IL-6 | IL-6 Effect
<table>
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<tr>
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<td>Ccl1</td>
<td>Chemokine ligand 1</td>
<td>272 660</td>
<td>1265 3726</td>
<td>175 353</td>
<td>102 295</td>
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<td>Prok1</td>
<td>Prokineticin 1</td>
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<td>31 130</td>
<td>11 13</td>
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<td>Fbn2</td>
<td>Fibulin 2 isoform a</td>
<td>53 69</td>
<td>210 691</td>
<td>50 83</td>
<td>36 67</td>
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<tr>
<td>Igf1</td>
<td>Insulin-like growth factor 1 isoform 1</td>
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<td>13 67</td>
<td>12 12</td>
<td>11 16</td>
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<tr>
<td>Pfn</td>
<td>Profilin 2</td>
<td>101 172</td>
<td>410 2255</td>
<td>218 410</td>
<td>133 162</td>
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<tr>
<td>Navi</td>
<td>Neuron navigator 1</td>
<td>18 44</td>
<td>41 181</td>
<td>29 35</td>
<td>12 26</td>
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<tr>
<td>Adams12</td>
<td>ABAMTS-like 2</td>
<td>28 46</td>
<td>62 294</td>
<td>56 76</td>
<td>33 32</td>
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<tr>
<td>Tlr2</td>
<td>TLR-2</td>
<td>87 86</td>
<td>136 1117</td>
<td>95 318</td>
<td>81 67</td>
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<tr>
<td>Dgkα</td>
<td>Diacylglycerol kinase, η</td>
<td>78 154</td>
<td>245 677</td>
<td>170 192</td>
<td>71 102</td>
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<td>Rcd1</td>
<td>RCSD domain containing 1 isoform a</td>
<td>203 558</td>
<td>179 822</td>
<td>113 242</td>
<td>137 528</td>
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<td>Igfip7</td>
<td>Insulin-like growth factor binding protein 7</td>
<td>222 775</td>
<td>786 2214</td>
<td>137 645</td>
<td>87 408</td>
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<td>Wnt12</td>
<td>Wingless-related MMTV integration site 10b</td>
<td>44 35</td>
<td>27 148</td>
<td>12 44</td>
<td>19 32</td>
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<tr>
<td>Epha5</td>
<td>Ephrin A5 isoform 1</td>
<td>88 389</td>
<td>88 372</td>
<td>60 121</td>
<td>151 298</td>
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<td>Dock4</td>
<td>Dedicator of cytokinesis 4</td>
<td>10 19</td>
<td>43 96</td>
<td>38 31</td>
<td>12 13</td>
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<td>Ikarf2</td>
<td>IKAROS family zinc finger 2</td>
<td>205 3382</td>
<td>219 534</td>
<td>127 235</td>
<td>296 3459</td>
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<td>Ifitm3</td>
<td>IFN-induced transmembrane 3</td>
<td>1524 1376</td>
<td>1825 5160</td>
<td>1781 2226</td>
<td>1628 1504</td>
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<td>IL-3</td>
<td>IL -3</td>
<td>568 263</td>
<td>934 2815</td>
<td>336 1307</td>
<td>239 95</td>
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<td>Npm2</td>
<td>Neuripine 2</td>
<td>23 106</td>
<td>676 1475</td>
<td>499 682</td>
<td>24 97</td>
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<td>Cntn7</td>
<td>CKLF-like MARVEL transmembrane domain containing 7</td>
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<td>3021 6290</td>
<td>2035 3044</td>
<td>1904 3486</td>
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<td>21 55</td>
<td>19 28</td>
<td>14 10</td>
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<tr>
<td>Npm1</td>
<td>Neuripine 1</td>
<td>270 296</td>
<td>234 558</td>
<td>90 290</td>
<td>183 308</td>
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<td>Mia3</td>
<td>Melanoma inhibitory activity 3</td>
<td>34 22</td>
<td>20 56</td>
<td>27 32</td>
<td>35 37</td>
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<tr>
<td>Itgα</td>
<td>Integrin, α E, epithelial-associated isoform 1</td>
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<td>617 7384</td>
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<td>182 2057</td>
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<td>Sla2</td>
<td>SMAD-interacting zinc finger protein 2</td>
<td>188 328</td>
<td>135 312</td>
<td>59 187</td>
<td>165 338</td>
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<tr>
<td>Itgβ8</td>
<td>Integrin β 8</td>
<td>13 198</td>
<td>13 54</td>
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<td>Cxcr7</td>
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<td>24 36</td>
<td>24 158</td>
<td>34 98</td>
<td>15 38</td>
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<tr>
<td>Neil3</td>
<td>Neil-like 3 (E. coli)</td>
<td>829 1140</td>
<td>482 1794</td>
<td>680 1228</td>
<td>732 784</td>
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<td>Fse</td>
<td>Feline sarcoma oncogene</td>
<td>229 356</td>
<td>646 2057</td>
<td>691 1397</td>
<td>324 512</td>
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<td>Cad1</td>
<td>Cadherin 1</td>
<td>12 13</td>
<td>66 510</td>
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<td>Cdc40</td>
<td>TNFR-S5 isoform 3</td>
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<td>Tiam2</td>
<td>T cell lymphoma invasion and metastasis 2</td>
<td>53 114</td>
<td>98 33</td>
<td>52 59</td>
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<td>Il-24</td>
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<td>2329 1431</td>
<td>647 360</td>
<td>758 617</td>
<td>4317 2543</td>
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<td>Mpa2l</td>
<td>Macrophage activation 2</td>
<td>7575 6558</td>
<td>3193 1636</td>
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<td>Igf1r</td>
<td>Insulin-like growth factor 1 receptor</td>
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<td>133 193</td>
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<td>Il-4</td>
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<td>Sens3</td>
<td>Stratin 3</td>
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<td>5903 2898</td>
<td>12371 7211</td>
<td>12320 11991</td>
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<td>Pppf4</td>
<td>Protein tyrosine phosphatase, receptor, F</td>
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<td>Tgf7</td>
<td>Transcription factor 7, T cell specific</td>
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<td>Kras12</td>
<td>Killer cell lectin-like receptor A, 12</td>
<td>434 157</td>
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<td>Gem</td>
<td>GTP binding protein (gene overexpressed in skeletal muscle)</td>
<td>557 521</td>
<td>447 251</td>
<td>1303 729</td>
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<td>Pouqf1l</td>
<td>POU domain, class 2, associating factor 1</td>
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<td>2988 1150</td>
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<td>63 17</td>
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</table>

**Notes:**

- IL-6 sensitivity index (the ratio of fluorescence levels of TGF-β Tregs over TGF-β and IL-6 Tregs), which was used to rank genes in order of the highest impact of the addition of IL-6 on the genes’ transcriptional levels.

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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 $\alpha$-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 $\alpha$-CCL1 resulted in further reduction of suppression over control tumors ($p = 0.0001$) (Fig. 4B). This points to a sustained effect of $\alpha$-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 expression of CCL1 levels by Tregs from untreated or tumor-bearing mice treated with CpG-ODN and $\alpha$-CCL1. Taken together, these data show that both CpG-ODN and CpG-ODN–$\alpha$-CCL1 result in lower number of Tregs both within the tumor and systemically. However, functional analysis showed that Tregs derived from CpG-ODN–$\alpha$-CCL1–treated mice are less suppressive than Tregs from untreated or CpG-ODN–treated tumors. In addition, systemic $\alpha$-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 $\alpha$-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 $\alpha$-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–$\alpha$-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 $\alpha$-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–

FIGURE 3. CpG-ODN and α-CCL1 treatment results in slowed tumor growth and significantly higher rates of tumor rejection in BALB-neuT mice. A, Treatment scheme of i.t. injections of CpG-ODN in combination with systemic (i.p.) delivery of α-CCL1 or α-ITGαE. B, Kaplan-Meier survival plot showing that CpG-ODN and α-CCL1 treatment has the greatest impact on survival ($\chi^2$ analysis $p = 0.0001$). Cumulative data from three independent experiments ($n = 6–9$ mice total) are shown. C, Growth curves of s.c. TUBO tumors in BALB-neuT mice (five cohorts of 6–9 mice each) treated as shown in Fig. 4A. Day 0 marks the start of the treatment course, and the arrows point to the end of the 3-wk treatment course. D, Tumor-bearing mice that rejected tumors after CpG-ODN and α-CCL1 treatment are capable of memory response. Kaplan-Meier survival plot showing that all ($n = 6$) CpG-ODN and α-CCL1-treated mice that were rechallenged with TUBO cells rejected the tumor and remained tumor-free >8 mo after the rechallenge.
Total splenocytes of tumor-bearing BALB-neuT mice treated with a 1-wk course of CpG-ODN or CpG-ODN and a•CCL1 were used to investigate these targets further. Interestingly, treatment with a•CCL1 but not with aITGαE inhibited the suppressive function of Tregs. Furthermore, a•CCL1 or a•ITGαE 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 ITGαE play a role in Treg function, we asked whether blockade of CCL1 and ITGαE could enhance the antitumor effect of CpG-ODN treatment. Our results show that only the combination of CpG-ODN and a•CCL1 has a strong antitumor effect in which six of nine animals rejected the tumor. More importantly, six of six CpG-ODN and a•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 costimulation. 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
The 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 further 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⁺ T 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 rejection and a protective memory response. This novel approach should be evaluated for use as an adjuvant to various cancer immunotherapeutic approaches.

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References


Supplemental figure 1
Selection algorithm to derive genes that are differentially regulated in Tregs and sensitive to IL6.
Briefly, an ANOVA was performed to select genes with significant variance between CD4+ cells treated with anti-CD3+IL2, anti-CD3+IL2+TGFβ and anti-CD3+IL2+TGFβ+IL6. 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 TGFβ, and in which the transcriptional change brought about by TGFβ is reverted by the addition of IL6. 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 done in order to avoid potentially affecting the T-effector cells with future therapies directed at Tregs.

START 44000 gene chip

Select genes that are modulated differently by TGFβ
2 fold change between Teff and iTreg in the IL2 TGFβ population
4888 genes

Select genes that change with addition of IL6
Keep genes with \( \geq 1.5 \) fold change in expression between iTreg IL2 TGFβ and iTreg IL2 TGFβ IL6:
1218 genes

Select genes responsive to TGFβ in Tregs only
Remove genes with similar expression in Teff IL2 and iTreg IL2 TGFβ
902 genes

Select genes that revert with IL6
Remove genes whose directionality of expression continues trajectory in iTreg IL2 TGFβ compared to iTreg IL2 TGFβ IL6
700 genes

Remove genes that revert at the same rate in iTeff IL2 TGFβ and iTreg IL2 TGFβ with the addition of IL6
Remove genes in which IL6 addition changes the level of expression in both iTeff and iTregs by the same degree
650 genes

Eliminate low expressers
Keep genes with FI \( > 10 \) in 2 of 4 conditions
544 genes

Eliminate housekeeping/ubiquitously expressed genes
Keep genes relevant to biological question
About 230
Supplemental figure 2.
Quantitative RT-PCR of CCL1 and ITGαE mRNA expression in T-effector cells (FOXP3-) and Treg cells (FOXP3+), subjected to the addition of IL2, IL2+TGFβ, IL2+TGFβ+IL6 or IL2+IL6.

Genes expression levels were normalized to those of two housekeeping genes, GAPDH and H3B, and show significant changes in expression levels with the addition of TGFβ which are either not present or of a lower magnitude with the addition of IL6.
Supplemental figure 3

Proliferation of CD4+ cells from Balb/c and FOXP3-gfp mice is not affected by anti-CCL1 or anti-ITGαE antibodies. CD4+ cells were isolated from spleens and plated on α-CD3 and α-CD28 coated wells for 3 days. Proliferation was measured by incorporation of tritiated thymidine.