$CCR8^+FOXp3^+$ T_{reg} cells as master drivers of immune regulation

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The current study identifies $CCR8^+$ regulatory T cells (T_{reg} cells) as drivers of immunosuppression. We show that in human peripheral blood cells, more than 30% of T_{reg} up-regulate CCR8 following activation in the presence of CCL1. This interaction induces STAT3-dependent up-regulation of FOXp3, CD39, IL-10, and granzyme B, resulting in enhanced suppressive activity of these cells. Of the four human CCR8 ligands, CCL1 is unique in potentiating T_{reg} cells. The relevance of these observations has been extended using an experimental model of multiple sclerosis [experimental autoimmune encephalomyelitis, (EAE)] and a stabilized version of mouse CCL1 (CCL1–Ig). First, we identified a self-feeding mechanism by which CCL1 produced by T_{reg} cells at an autoimmune site up-regulates the expression of its own receptor, CCR8, on these cells. Administration of CCL1–Ig during EAE enhanced the in vivo proliferation of these $CCR8^+$ regulatory cells while inducing the expression of CD39, granzyme B, and IL-10, resulting in the efficacious suppression of ongoing EAE. The critical role of the CCL1–CCR8 axis in T_{reg} cells was further dissected through adoptive transfer studies using $CCR8^{-/-}$ mice. Collectively, we demonstrate the pivotal role of $CCR8^+$ T_{reg} cells in restraining immunity and highlight the potential clinical implications of this discovery.

EAE | chemokine | FOXp3 | CCL1 | CD39

Two major populations of $CD4^+$ regulatory T cells (T_{reg} cells), defined by whether they express the forkhead box protein 3 transcription factor (FOXp3), are thought to play a key role in the maintenance of self-tolerance (1–8). Both $FOXp3^+$ and $FOXp3^-$ subtypes participate in the regulation of inflammatory autoimmunity and in the maintenance of self-tolerance by various mechanisms, including regulating the biological function of effector TH1 and TH17 $CD4^+$ T cells (6, 7, 9–12). $CD4^+$ $FOXp3^-$ regulatory T cells can be categorized as T regulatory-1 cells (Tr1), which primarily produce IL-10 (12–15), and Th3, which express high levels of TGFβ (16).

Chemokines are small (∼8–14 kDa) secreted proteins, structurally similar to cytokines, that regulate cell trafficking through interactions with a subset of seven transmembrane G protein-coupled receptors (GPCRs) (17), and many of them are associated with chemotaxis of leukocytes to inflammatory sites (18–20). Almost 15 y ago we showed that, in addition to their role in chemoattraction, chemokines are also involved in directing effector $CD4^+$ T-cell (T_{eff}) polarization, by showing that the CXCR3 ligand CXCL10 directs the lineage development of TH1 cells (21, 22). More recently we identified two different chemokines that are involved in the lineage development of Tr1 cells (23, 24). The current study focuses on $FOXp3^+$ T_{reg} cells and on the interplay between CCR8 and its ligands.

In mouse, the chemokine receptor CCR8 is expressed principally on T_{reg} cells and also notably on small fractions of TH2 cells, monocytic cells, and NK cells (25–28), but not TH1 cells (29, 30). A similar expression pattern is seen in humans, in which CCR8 is additionally found on ∼2% of $CD8^+$ cells (30). CCR8 is known to be critical for T_{reg} function. For example, Coghill et al. recently showed in a graft versus host disease (GVHD) model, donor T_{reg} cells lacking CCR8 were severely impaired in their ability to prevent lethal GVHD (31). However, the underlying mechanisms of such observations remained unclear.

The current study uncovers the mechanistic basis by which the CCR8–CCL1 axis potentiates T_{reg} cells, its relevance to human biology, and explores the clinical implications of these findings using an experimental autoimmune disease of the central nervous system (CNS) (32).

Results

Of the Four CCR8 Ligands, CCL1 Is Unique in Potentiating the Suppressive Function of Human T_{reg} Cells. Human CCR8 has four known ligands: CCL1, CCL8, CCL16, and CCL18 (33). First we examined whether one or more of the four human CCR8 ligands may enhance the suppressive activity of human T_{reg} cells. Fig. 1 summarizes data obtained from 10 different healthy donors, indicating that of the four human CCR8 ligands, CCL1 was unique in potentiating the suppressive function of human T_{reg} cells, detected as an enhanced suppression of T_{eff} proliferation (Fig. 1 *A*–*C*). Because CCR8 is considered to be the exclusive receptor for CCL1 (26), we sought to confirm that these effects were achieved via CCR8 signaling. As shown in Fig. 1*D*, antihuman CCR8 blocking mAb abolished CCL1-mediated effects in this assay.

Independently, each human CCR8 ligand was tested in a fluorometric imaging plate reader (FLIPR) assay (34) configured to detect the induction of intracellular Ca^{2+} flux in response to CCR8 activation in CHO-K1 cells overexpressing human CCR8. As shown in Fig. S1, CCL1 induced a dose-dependent up-regulation of

Significance

The current study identifies $CCR8^+$ regulatory T cells (T_{reg} cells) as drivers of immunosuppression and provides compelling evidence of a self-feeding mechanism by which, at an autoimmune site, CCL1 produced by $FOXp3^+$ T_{reg} cells upregulates the expression of its own receptor, CCR8, on these cells, and potentiates their in vivo proliferation and suppressive activities as driver T_{reg} cells. The suppression of ongoing autoimmunity by a stabilized version of the chemokine (CCL1–Ig) highlights the translational potential of these findings.

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Conflict of interest statement: N.K., Y.B., and G.W. hold a pending patent on CCL1-based therapy of autoimmunity and graft-versus-host disease that has been outlicensed to GlaxoSmithKline.

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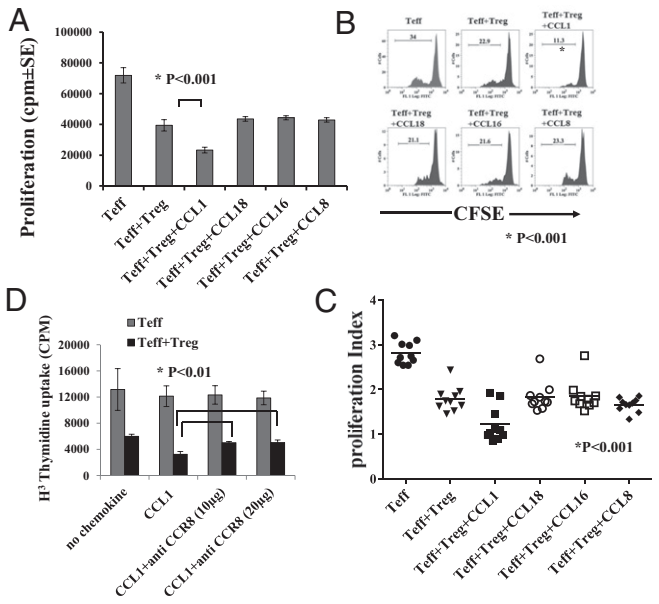

Fig. 1. CCL1 selectively potentiates suppressive function in $CD4^+CD25^+CD127^{low}$ T cells. (*A–C*) CCL1 potentiates the suppressive function of human $CD4^+CD25^+CD127^{low}$ T cells. Freshly isolated human T_{reg} cells were activated in the presence of each of the known CCR8 ligands used in suppression assay (*SI Methods*). Proliferation of T_{eff} cells was detected by incorporation of [^{3}H] thymidine incorporation (results in *A* are shown as mean of triplicates ± SE), or by CFSE staining of effector T cells (*B* and *C*). *B* shows the results of a representative experiment. *C* summarizes CFSE results of all 10 healthy donors as a scattered plot ($P < 0.001$ unpaired Student's *t* test). (*D*) CCL1 directs its function via CCR8. Suppression assays were conducted as described for *A* above, with or without addition of an anti-CCR8 blocking mAb. Results of one of three independent experiments are shown as mean of triplicates ± SE. Significance was determined by two-tailed unpaired Student's *t* test (*$P < 0.01$).

Ca^{2+} flux in response to CCR8 activation, whereas no effect was seen in the presence of CCL8, CCL16, or CCL18. We note that these data differ from a publication showing that CCL18 may also induce Ca^{2+} flux via CCR8 (33).

Collectively, these data show that of the four CCR8 ligands, only CCL1 induces Ca^{2+} flux and potentiates the suppressive activity of these cells.

CCL1 Potentiates Human T_{reg} Cells by Inducing CCR8, FOXp3, CD39, Granzyme B, and IL-10 Expression. At 36 h postactivation of cultured human T_{reg} cells that were, or were not, supplemented with CCL1, they were examined for the transcription of various genes known to be associated with the T_{reg} phenotype (7) by real-time PCR. We observed between 4- and 5-fold increases in the transcription of FOXp3 and CCR8 ($P < 0.0001$), a 3.7-fold increase in the transcription of CD39 and a 2.5-fold increase in granzyme B and IL-10 ($P < 0.01$) (Fig. 2*A*). Results were then confirmed at the protein level by flow cytometry (Fig. 2 *B–D*). We observed that during in vitro activation in the presence of CCL1, the expression of CCR8 on $CD4^+CD25^+C127^{low}$ T_{reg} increases from 2.65% to 36% of the cells (Fig. 2*B*), whereas its expression on $CD4^+CD25^-C127^{high}$ remained at a low level. This finding suggests that T_{reg} cells ($CD4^+CD25^+C127^{low}$) preferentially respond to CCL1 by a profound increase in the expression of CCR8.

An increased expression of FOXp3 (from 89.6% to 95.7%, $P < 0.01$) was also observed in these $CD4^+CD25^+C127^{low}$ T cells (Fig. 2*C*). Further analysis of $FOXp3^+$ T cells (Fig. 2*D*) revealed a significant increase in CD39 (from 4.43% to 16.1%, $P < 0.0001$), granzyme B (from 3.25% to 18.3%, $P < 0.0001$), and IL-10 (from 6.26% to 15.4%, $P < 0.0001$). Further dissection of the differential transcription of these molecules has been conducted in the murine setup and showed preferential early transcription of CCR8 and FOXp3, as discussed later (Fig. S2).

A subsequent set of experiments was conducted to determine whether CCL1 might also increase FOXp3 expression in $CD4^+CD25^-CD127^{high}$ T cells ($FOXp3^-$) to convert them into $FOXp3^+$. We could not find compelling evidence to show that CCL1 may directly convert $FOXp3^-$ T cells into $FOXp3^+$ (Fig. 2*E*).

Taken together, these data imply that CCL1 potentiates the suppressive activity of $CCR8^+$ T_{reg} cells by inducing FOXp3,

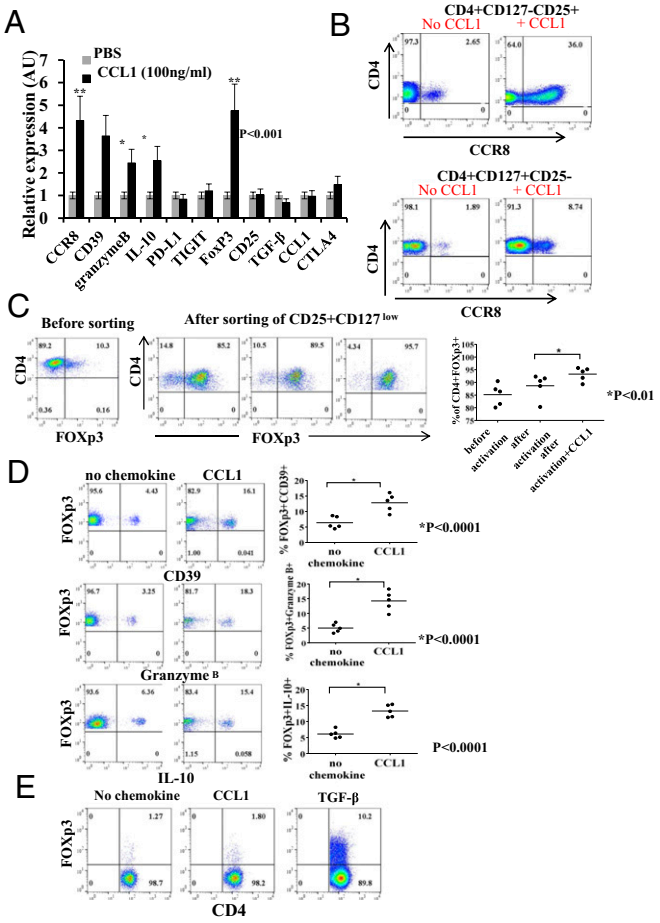

Fig. 2. CCL1 potentiates human T_{reg} cells by inducing CCR8, FOXp3, CD39, granzyme B, and IL-10 expression. (*A*) CCL1 enhances the transcription of FOXp3, CCR8, CD39, granzyme B, and IL-10 in $CD4^+CD25^+CD127^{low}$ T cells. CCL1 was added to cultured $CD4^+CD25^+CD127^{low}$ T cells as described above, and 36 h later, the relative transcription of various genes was detected. Results are shown as mean of triplicates ± SE. Significance was determined by two-tailed unpaired Student's *t* test (*$P < 0.001$). The results represent one of three different experiments with similar observations. (*B*) CC1 preferentially induces the expression of CCR8 on $CD4^+CD25^+CD127^{low}$ T-cells CCL1 was added to cultured $CD4^+CD25^+CD127^{low}$ and $CD4^+CD25^-CD127^{low}$ T cells as described above and expression of CCR8 was detected by flow cytometry. A representative plot is shown together with a scatterplot summarizing five different samples of healthy donors. Significance was determined by unpaired Student's *t* test (*C* and *D*) CCL1 enhances the expression of FOXp3 (*C*), CD39, granzyme B, and IL-10 (*D*) in human $CD4^+CD25^+CD127^{low}$ T cells. CCL1 was added to cultured $CD4^+CD25^+CD127^{low}$ T cells as described above, and 36 h later the expression of various gene products was determined by flow cytometry. A representative plot is shown together with a scatterplot summarizing five different samples of healthy donors. Significance was determined by unpaired Student's *t* test (*B*) $P < 0.0001$, (*C*) $P < 0.01$, and (*D*) $P < 0.0001$. (*E*) CCL1 does not convert $FOXp3^-$ T cells into T_{reg} cells: CCL1 was added to cultured $CD4^+CD25^-$ ($FOXp3^-$) T cells undergoing anti–CD3-induced activation. TGF-β was used as a positive control for induction of iT_{reg} cells.

CD39, granzyme B, and IL-10 in these cells without converting FOXp3$^-$ cells into FOXp3$^+$.

CCL1-Induced Potentiation of T_{reg} Cells Is STAT3 Dependent. Fig. 3*A* shows that CCL1 induces the phosphorylation of STAT3 but none of the other STAT proteins, as determined by phospho-specific detection using flow cytometry (35), and that this phosphorylation is selective to CCR8$^+$ cells. To further validate this observation, we assessed CCL1-mediated effects in the presence or absence of a STAT3 inhibitor. As shown in Fig. 3*B*, flow cytometry analysis confirmed that inhibiting STAT3 prevented the up-regulation of CD39 in FOXp3$^+$ T_{reg} cells (change from 7.25% to 19.8% (CCL1 alone) vs. 13% (CCL1 with inhibitor). Similarly, ELISA detection confirmed abolition of CCL1-induced granzyme B (Fig. 3*C*) and IL-10 (Fig. 3*D*) following STAT3 inhibition.

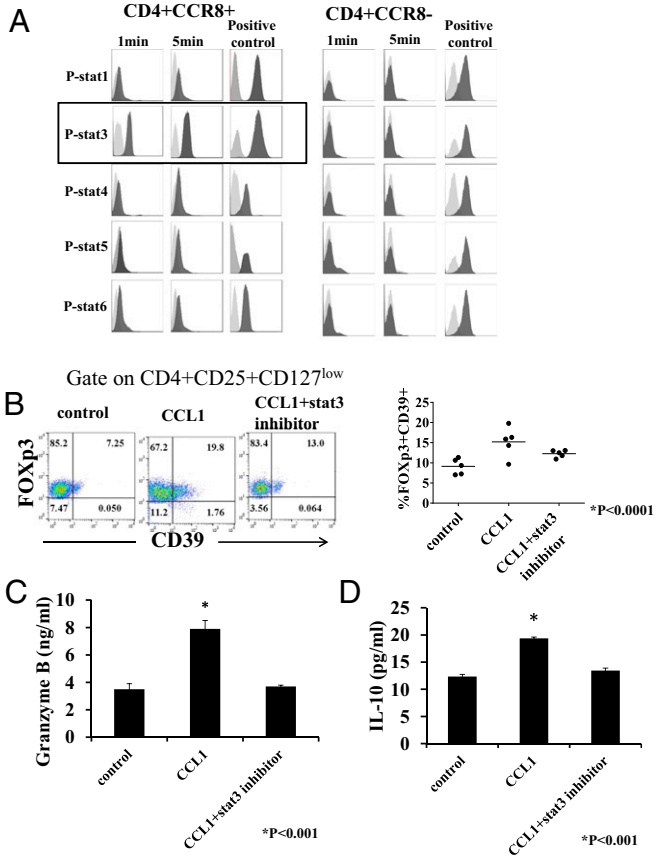

Fig. 3. CCL1 potentiates human T_{reg} cells in a STAT3-dependent manner. CCL1 induces STAT3 phosphorylation. (*A*) STAT phosphorylation was determined in human CD4$^+$CD25$^+$CD127^{low} T cells by flow cytometry analyses. CCL1 was added (100 μg/mL) 24 h after CD3- and anti–CD28-induced activation. The data shown represent one of three independent experiments. (*B*) STAT3 inhibitor reverses the induction of CD39 by CCL1 in FOXp3$^+$ T_{reg} cells. Flow cytometry analyses of the expression of CD39 in cultured CD4$^+$CD25$^+$CD127^{low} T cells undergoing anti–CD3- and anti–CD28-induced activation in the presence of CCL1 with or without addition of a STAT3 inhibitor (CP 690550, Santa Cruz Biotechnology, sc-202818, 20 μM). A representative plot is shown together with a scatterplot summarizing five different samples of healthy donors. Significance was determined by unpaired Student's *t* test ($P < 0.0001$). (*C* and *D*) STAT3 inhibitor reverses the induction of granzyme B and IL-10 by CCL1. Cultured CD4$^+$CD25$^+$CD127^{low} T cells undergoing anti–CD3/anti–CD28-induced activation were supplemented with CCL1 with or without a STAT3 inhibitor. After 72 h, granzyme B and IL-10 levels were recorded by ELISA. Results of one of three independent experiments with similar data are presented as mean triplicates ± SE. Significance was determined by two-tailed unpaired Student's *t* test (*$P < 0.001$).

Collectively, these results show that in human cells, CCL1 potentiates T_{reg} cells first by inducing the expression of its target receptor on CD4$^+$CD25$^+$C127^{low} T cells followed by the induction of STAT3-dependent increase of CD39, granzyme B, and IL-10, which are key drivers of the suppressive function of these cells.

We then sought to determine whether similar CCL1-drived pathways exist in mouse, using murine CD4$^+$ T cells. As shown in Fig. S2*A*, of the three known murine ligands for mouse CCR8 (CCL1, CCL8, and CCL16), CCL1 exclusively enhanced the suppressive activities of mouse T_{reg} cells (41% increase, $P < 0.01$) in an ex vivo T_{eff} suppression assay. The dependence on CCR8 of these effects was confirmed using T cells isolated from CCR8-deficient mice. As shown in Fig. S2*B*, murine CCL1 enhanced the suppressive activity of T_{reg} cells from wild-type mice (72% increase, $P < 0.001$) but had no effect on T_{reg} cells obtained from CCR8$^{-/-}$ mice. In experiments corresponding to those in the human system described in Fig. 2, we have similarly shown that mouse CCL1 enhances the transcription of CCR8, FOXp3, granzyme B, CD39, and IL-10 in murine T_{reg} cells (Fig. S2*C*). Here we also compared the relative increase of the transcription of these genes after 16 and 36 h, showing that at the earliest time point, the increased transcription of FOXp3 and CCR8 is mostly dominant over granzyme B, CD39, and IL-10. We also verified these results at the protein level and confirmed that blockade of STAT3 abrogates this increase (Fig. S2 *D* and *E*).

Finally, we examined whether CCL1 can polarize murine FOXp3$^-$ T cells into FOXp3$^+$ [i.e., induction of induced T_{reg} (iT_{reg})], as reported for TGFβ (36). Fig. S2*F* shows that under in vitro conditions, CCL1 does not convert FOXp3$^-$ T CD4$^+$ cells into FOXp3$^+$.

Taken together, these data confirm very close similarity in the mechanistic basis of CCL1-mediated effects on T_{reg} cells between human and mouse.

An Autocrine Role for the CCL1–CCR8 Axis in Potentiating T_{reg} Cells at the Autoimmune Site. To better understand the potential role of CCL1 in autoimmune disease, we sought to investigate the relative transcription of CCL1 in the CNS as a function of disease status in the mouse EAE model. Fig. 4*A* shows a marked elevation of CCL1 levels after the peak of disease (up to 14-fold increase on day 22). At this time CD4$^+$ T cells were isolated from the spinal cord and separated into FOXp3$^+$CD4$^+$ T cells and FOXp3$^-$CD4$^+$ T cells. Each subtype was then analyzed by real-time PCR for the relative transcription of CCL1 (normalized to β2M). CCL1 was up-regulated by 13.8-fold in FOXp3$^+$ T_{reg} cells (Fig. 4*B*), suggesting that these cells were potentially the principal source of CCL1. Comparative analyses showed that within the inflamed CNS, CCL1 is largely transcribed by FOXp3$^+$ T_{reg} cells but not by microglia cells (Fig. 4*C*).

Next, we examined the relative number of CCR8$^+$ cells within the CD4$^+$ FOXp3 subset at the inflamed CNS and compared the expression of granzyme B, CD39, and IL-10 in CCR8$^+$ FOXP3$^+$ and CCR8$^-$ FOXP3$^+$ CD4$^+$ T cells within the CNS (Fig. 4*D*). We found that at the peak of disease, about 9% of CD4$^+$FOXp3$^+$ T cells are CCR8$^+$ and that these cells preferentially express granzyme B (32.5% vs. 8.9% in CCR8$^-$), CD39 (44% vs. 11.5% in CCR8$^-$), and IL-10 (11.3% vs. 5.3% in CCR8$^-$). Collectively, these data suggest an autocrine role for the CCL1–CCR8 axis in potentiating T_{reg} cells at the autoimmune site.

CCL1–Ig Suppress Ongoing EAE by Inducing CCR8 and Potentiating T_{reg} Cells via This Receptor. We sought to investigate whether administration of CCL1 could affect ongoing EAE. The short in vivo half-life of chemokines limits their exposure following systemic administration. Therefore, using similar strategies for extending half-life to those that we have previously used for CXCL12 (24), CXCL10, and CXCL11 (23), we generated a mouse CCL1–Ig fusion protein (Fig. S3 *A* and *B*). This construct retains the biological activities of the native chemokine, including chemoattraction

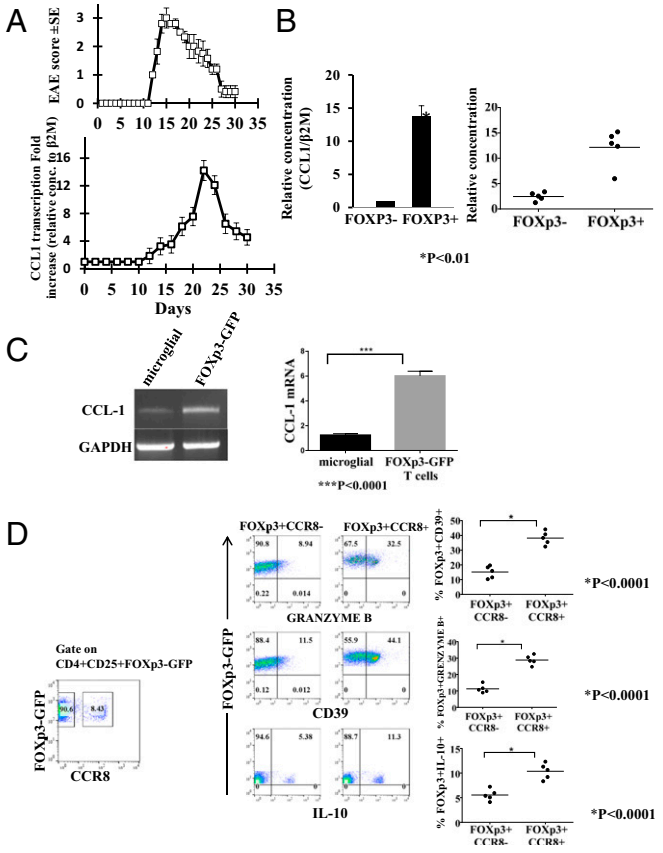

Fig. 4. An autocrine role for the CCL1–CCR8 axis in potentiating T_{reg} cells at the autoimmune site. (*A*) The kinetics of CCL1 expression at the CNS with EAE disease course: C57BL/6 mice (FOXp3^{GFP} transgenic) were subjected to active induction of disease. Representative mice ($n = 3$) were killed at different time points and the relative transcription of CCL1 at the lumbar spinal cord samples was quantitated and normalized to β2M by real-time PCR. (*B*) At the peak of disease in FOXp3–GFP reporter mice FOXp3$^+$CD4$^+$ and FOXp3$^-$CD4$^+$ populations were separated from the lumbar spinal cord. Each subtype was then analyzed by real-time PCR for the relative transcription of CCL1 (normalized to β2M), showing a 13.8-fold increase ($P < 0.001$) in its transcription by FOXp3$^+$ T_{reg} cells. The results of one of five experiments is shown (black bars) and a summary of all five experiments is shown at *Right* ($P < 0.001$ unpaired Student's *t* test). (*C*) At the peak of disease in FOXp3–GFP reporter mice, FOXp3$^+$CD4$^+$ T cells and microglia cells were separated from the lumbar spinal cord and subjected to PCR analyses of CCL1 normalized by GAPDH. (*D*) Analyses of the differential expression of CD39, granzyme B, and IL-10 on CCR8$^+$ and CCR8$^-$ T_{reg} cells in CD4$^+$ T cells isolated from the lumbar spinal cord of EAE mice at the peak of disease. Representative flow cytometry plot is accompanied by scatterplot of five different experiments (unpaired Student's *t* test, $P < 0.0001$).

(Fig. S3*C*) and ERK1/2 phosphorylation of the BW5147 CCR8$^+$ thyoma cell line (37) (Fig. S3*D*). Preliminary pharmacokinetic (PK) analysis in mice confirmed that CCL1 formatted as a Fc-fusion protein had an acceptable exposure and half-life to enable its use in disease models (Fig. S3*E*). Pharmacodynamic (PD) analysis confirmed that it retained biological activity in vivo, achieving a durable induction of FOXP3$^+$CD39$^+$ T_{reg} cells both in the spleen and in the spinal cord (Fig. S4). We were therefore able to investigate the potential therapeutic effect of CCL1–Ig on ongoing EAE. Fig. 5*A* summarizes data from one of three independent experiments with similar observations, showing that administration of CCL1–Ig during ongoing EAE rapidly suppressed the development and progression of disease (day 21 mean maximal score of 1 ± 0.13 compared with 2.5 ± 0.23, $P < 0.01$). Clinical observations were confirmed histologically (Fig. 5*B*, mean histological score 0.5 ± 0.1 compared with 2.6 ± 0.3 in and 2.5 ± 0.3 in control groups, $P < 0.01$).

Protective administration of CCL1–Ig led to a significant increase in the relative number of FOXp3$^+$ T_{reg} cells both in the periphery (spleen) and the CNS, and of the relative expression of CCR8 on these cells (Fig. S5*A*). As the relative increase in FOXp3$^+$ T cells is systemic, it is not likely that it is due to differential migration of cells, but rather increased expression of FOXp3 and/or increased in vivo proliferation of FOXp3$^+$ T cells. We also tested 5-bromo-2′-deoxyuridine (BrdU) uptake in this in vivo model (Fig. S5*B*) showing a significant increase in the proliferative response of FOXp3$^+$ T cells at the CNS and spleen of CCL1–Ig-treated mice ($P < 0.01$) combined with reduced proliferation of FOXp3$^-$ CD4$^+$ T cells ($P < 0.01$), which could be due to the increased suppressive effect of FOXp3$^+$ T_{reg} cells. Furthermore, the relative expression of CD39, granzyme B, and IL-10 significantly increased in these CCR8$^+$ T cells following CCL1–Ig administration (Fig. S5*C*, $P < 0.001$). Collectively, these data suggest that CCL1–Ig suppresses ongoing EAE, in part, by inducing the proliferative response of T_{reg} cells and potentiating their activity via CCR8, which is induced by itself via CCL1.

The in Vivo Suppressive Activity of CCL1 on T_{reg} Cells Is CCR8 Dependent. We conducted a set of experiments to further examine the relevance of the interplay between CCL1 and its receptor CCR8 in restraining EAE. The experimental system included CCR8$^{-/-}$ mice reconstituted with CD4$^+$ T_{reg} cells from either CCR8$^{-/-}$ or WT mice followed by administration of CCL1–Ig. To optimize the methodology of the adoptive transfer protocol, we performed pilot studies in which mice were administered 5×10^5, 5×10^6 , or 5×10^7 T_{reg} cells (WT) and monitored for the development and progression of EAE. Administration of cells in the range of 5×10^6–5×10^7 significantly suppressed the disease without the need to inject CCL1–Ig. Therefore, administration of 5×10^5 cells was selected for further studies.

First, we confirmed that CCR8$^+$ T_{reg} cells from FOXp3–GFP reporter mice (FOXp3^{GFP}) injected i.p. entered the CNS within 48 h (Fig. S6), and that administration of CCL1–Ig to mice lacking CCR8 had no effect on the severity of disease (Fig. S7*A*), in contrast

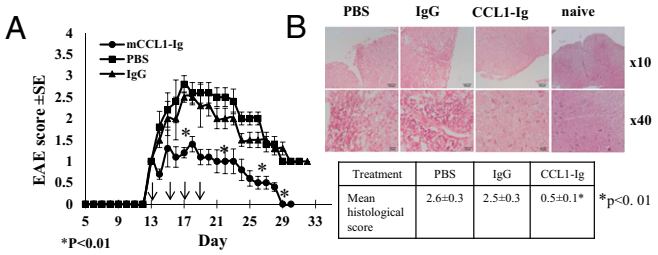

Fig. 5. CCL1–Ig suppress ongoing EAE. (*A*) C57BL/6 female mice were injected with MOG$_{p35-55}$ to induce active EAE and at the onset of disease (day 12) were separated into groups with comparable disease scores ($n = 9$ mice per group, from which three were killed at the peak of disease). On days 13, 15, 17, and 19 after the induction of disease mice were injected (i.p.) with either PBS, 300 μg per mouse of mCCL1–Ig or IgG isotype control. An observer blind to the experimental protocol monitored the development and progression of disease. The results ($n = 9$ mice per each group until day 17 and $n = 6$ from day 17 onward) are shown as the mean maximal score ± SE. The results show one of three independent experiments with similar data. One-way ANOVA for paired data was used to determine the significance of the time–response curves (*$P < 0.01$). The arrows indicate the days of mCCL1–Ig or IgG administration. (*B*) Histopathological evaluation: At the peak of disease (day 17), three representative mice per group were killed and lumbar spinal cord was subjected to histological analysis (18 sections per spinal cord) using a score of 0–3 as described in ref. 63 (see also *SI Methods*). The mean histological score ± SE was calculated for each treatment group. Representative histological sections are shown, and a statistical analysis of all sections is also given. Significance was determined by two-tailed unpaired Student's's *t* test (*$P < 0.001$).

to its effect on WT mice (Fig. S7*B*). Importantly, we observed that in CCR8$^{-/-}$ mice reconstituted with CCR8^{+} T_{reg} cells (Fig. S7*C*) administration of CCL1–Ig rapidly suppressed disease (day 21, 1.5 ± 0.166 compared with 3 ± 0.23, $P < 0.01$), whereas reconstitution with T_{reg} cells from CCR8$^{-/-}$ mice was without effect (Fig. S7*D*). This finding further indicates the pivotal role of the CCL1–CCR8 axis on T_{reg} cells in the regulation of EAE.

Because the mechanism of T_{reg} potentiation includes up-regulation of IL-10, we sought to address whether increased production of IL-10 in T_{reg} cells is the principal mechanism of CCL1-induced potentiation of these cells. An in vitro suppression assay comparing T_{reg} and effector CD4^{+} T cells from WT or IL-10 KO mice revealed that CCL1-mediated potentiation of T_{reg} cells was achieved even in the absence of IL-10 (Fig. S8*A*). Subsequently, we used the adoptive transfer model to show that CCL1–Ig effectively suppresses EAE when acting on T_{reg} cells from IL-10 KO mice (Fig. S8*B*). Collectively, these data imply CCL1 may potentiate T_{reg} cells even in the absence of IL-10.

Our adoptive transfer experiments (Fig. S7) show that the interaction between CCL1 and CCR8 on T_{reg} cells is essential for suppressing EAE. However, this observation still does not exclude the possibility that CCL1 may also affect the development and progression of disease via its interaction on other CCR8^{+} cells, such as CCR8^{+} macrophages or natural killer (NK) cells, or other CD4^{+} T cell subsets. Other than T_{reg} cells, Th2 cells are the only CD4^{+} population that express high levels of CCR8 (38). Th2 cells produce IL-4 to mediate their biological function, so we examined whether CCL1 potentiates IL-4 production in these cells but found no evidence of such an effect (Fig. S9), consistent with the regulatory effect of CCL1 on CD4^{+} T cells being predominantly T_{reg} dependent.

Discussion

The current study focuses on the interplay between the chemokine receptor CCR8 and its ligands, particularly CCL1, and its role in the generation and maintenance of active tolerance. We show that of the known CCR8 ligands, CCL1 is unique in its ability to induce Ca^{2+} flux via this receptor and to potentiate the suppressive activities of T_{reg} cells. We also show that this chemokine up-regulates the expression of its target receptor on these cells (in vitro and in vivo) and by so doing, further induces their suppressive activities in an autocrine loop. Evidence from the EAE model suggests that at the autoimmune site, CCL1 is largely produced by T_{reg} cells to potentiate their suppressive function in an autocrine loop, further emphasizing the key role of this interaction in the regulation of autoimmunity.

We suggest two complementary pathways by which T_{reg} cells, via the CCR8–CCL1 interaction, function as driver suppressor cells in vivo: (*i*) the induction of their proliferation to increase their relative number and (*ii*) an increased expression of key mediators of suppressive immune regulation. These include: the suppressor cytokine IL-10, which suppresses both innate and specific immune activities (39); granzyme B, which is capable of specifically killing antigen-presenting B cells, thus limiting autoimmunity (40); and CD39, which decreases the extracellular concentration of ATP, and has recently been tightly associated with control of autoimmunity within the CNS (41). Interestingly, we showed that CCL1 could effectively potentiate CCR8^{+} T_{reg} cells lacking IL-10 (Fig. S7). This result implies that IL-10 could be important, but not critical, for CCL1-induced potentiation of T_{reg} cells. Future complementary studies using IL-10R KO mice are required to investigate whether there is an alternative cytokine that induces IL-10R–dependent activation of STAT3 in these mice.

Even though CCR8 is expressed by "driver" T_{reg} cells, its expression is not exclusive to these cells (28, 30, 42–45). Our results (Fig. S7) showing that CCL1–Ig rapidly suppresses disease in mice reconstituted with T_{reg} cells from wild-type but not CCR8-deficient mice suggest that T_{reg} cells are the dominant cell type by which CCL1 achieves its beneficial effects. Nevertheless, we do not exclude the possibility that the interaction of CCL1 with other CCR8^{+} cells may also affect the dynamics of disease.

A further question relates to the role of STAT3 in T_{reg} potentiation. The involvement of STAT3 in the polarization of both Th17 and FOXp3^{+} T_{reg} cells (46–49) might explain, in part, the plasticity of the Th17/T_{reg} pathways (10, 50) and its implications in the regulation of immunity, particularly within the gut (51–53). Moreover, in Th17 cells STAT3 phosphorylation is induced by the proinflammatory cytokines IL-6 and IL-23 (48). Taken together, these data imply that STAT3 phosphorylation is induced by different cytokines/chemokines in different cell types and that the biological consequences of this phosphorylation may be cell type specific and/or that alternative signaling pathways, yet to be identified, are required for the CCL1–CCR8-induced potentiation of T_{reg} cells.

We note, and draw some parallels with, previous reports that low dose IL-2 rapidly induces the in vivo expansion of T_{reg} cells to suppress type I diabetes in nonobese diabetic mice, even though the in vitro effect of low-dose IL-2 on T_{reg} expansion was very limited (54, 55). These low-dose IL-2 studies were later extended to clinical trials, e.g., for hepatitis C virus-induced vasculitis (56), and GVHD (57). Indeed, the induction of in vivo expansion of T_{reg} cells as a therapeutic strategy for autoimmune disease remains an area of intense interest. However, our data suggest that intervention with a CCL1-based therapy may be preferred to IL-2, because the latter has the potential, even at low dose, to activate effector T cells and NK cells and potentially aggravate disease (58).

From the translational perspective, CCL1–Ig could be a preferred candidate for therapy of autoimmune diseases, because CCR8^{+} T_{reg} insufficiency (functionally and/or numerically) is likely to be an important contributor to a range of diseases (31). Conversely, blockade of the CCR8–CCL1 axis may be used as a strategy to enhance anticancer immunity (59).

Methods

Animals. The 6-wk-old female C57BL/6 mice were purchased from Harlan and maintained under specific pathogen-free conditions in our animal facility. FOXp3^{GFP} mice (internal ribosome entry site–GFP knocked in to the *FOXp3* locus, on the C57BL/6 background) were kindly provided by Vijay Kuchroo, Harvard Medical School, Boston, MA. The generation of CCR8$^{-/-}$ mice has been previously described (by S.A.L.) (29).

The use of animals and experimental protocols were approved by the Animal Care and Use Committee of the Technion.

Antibodies, Cytokines, and Chemokines. Anti-mouse CCL1 neutralizing antibody (AF845), hTGF-β and hIL-2, and all recombinant chemokines (human and mouse) were purchased from R&D Systems.

Human Samples. All human samples were purchased from the Israel Blood Banks. All human biological samples were sourced ethically and their research use was in accordance with the terms of the informed consents.

In Vitro Proliferation Assays. T-cell proliferation was determined either by thymidine incorporation or by carboxyfluorescein succinimidyl ester (CFSE) staining. CFSE labeling studies used a CFSE Cell Division Tracker Kit (423801 BioLegend) according to the manufacturer's protocol and only CD4^{+} T_{eff} cells were labeled.

Cell Separation and Suppression Assays. The basic protocol for the mixed lymphocyte suppression assay was conducted according to Collison and Vignali (60). The detailed protocols for murine and human are specified in *SI Methods*.

Measurement of Intracellular Calcium Mobilization. A Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices) was used to detect calcium flux. Data were analyzed using GraphPad Prism (v5) as specified in detail in *SI Methods*.

Phospho-Specific Flow Cytometry and STAT Inhibitors. Phospho-specific flow cytometry was conducted according to ref. 35. The biological relevance was verified by using STAT-specific inhibitors. All protocols are specified in detail in *SI Methods*.

Cytokine Measurement by ELISA. Methods of cytokine measurement by ELISA are specified in *SI Methods*.

Real-Time PCR Primers. Construction of mIgG plasmid and CCL1–Ig cloning are specified in detail in *SI Methods*.

Expression and Purification of Fusion Proteins. Fusion proteins were expressed and purified using CHO dhfr$^{-/-}$ (DG44) cells (provided by L. Chasin, Columbia University, New York, NY) according to the method described in detail in ref. 61. The protocols are specified in *SI Methods*.

Induction of Acute and Semichronic EAE. Studies were conducted according to ref. 62 and are further detailed in *SI Methods*.

BrdU Uptake. BrdU (Sigma) was added to the drinking water (1 mg/mL) for 14 d according to the manufacturer's protocol. BrdU uptake was conducted by flow cytometry using anti-Brdu mAb (BioLegend, clone Bu20a).

Histopathology. Histopathology was conducted as we previously described (63) and is explained in details in *SI Methods*.

Statistical Analysis. Statistical analysis was done according to the recommendations provided by *Nature* for reporting life sciences research and are specified in *SI Methods*.

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1. Silver PB, et al. (2015) Retina-specific T regulatory cells bring about resolution and maintain remission of autoimmune uveitis. *J Immunol* 194:3011–3019.
2. Joller N, et al. (2014) Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* 40:569–581.
3. van Loosdregt J, et al. (2013) Stabilization of the transcription factor Foxp3 by the deubiquitinase USP7 increases Treg-cell-suppressive capacity. *Immunity* 39:259–271.
4. Laurence A, Belkaid Y, O'Shea JJ (2013) A degrading view of regulatory T cells. *Immunity* 39:201–203.
5. Lowther DE, Hafler DA (2012) Regulatory T cells in the central nervous system. *Immunol Rev* 248:156–169.
6. Chaudhry A, et al. (2011) Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* 34:566–578.
7. Shevach EM (2009) Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636–645.
8. Wing K, Sakaguchi S (2010) Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol* 11:7–13.
9. Arpaia N, et al. (2015) A distinct function of regulatory T cells in tissue protection. *Cell* 162:1078–1089.
10. Durant L, et al. (2010) Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* 32:605–615.
11. Sakaguchi S, et al. (2006) Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212:8–27.
12. Gagliani N, et al. (2013) Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* 19:739–746.
13. Groux H, et al. (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737–742.
14. Roncarolo MG, et al. (2006) Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 212:28–50.
15. Wildbaum G, Netzer N, Karin N (2002) Tr1 cell-dependent active tolerance blunts the pathogenic effects of determinant spreading. *J Clin Invest* 110:701–710.
16. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL (1994) Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis. *Science* 265:1237–1240.
17. Luster AD (1998) Chemokines–chemotactic cytokines that mediate inflammation. *N Engl J Med* 338:436–445.
18. Charo IF, Ransohoff RM (2006) The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 354:610–621.
19. Proudfoot AE (2002) Chemokine receptors: Multifaceted therapeutic targets. *Nat Rev Immunol* 2:106–115.
20. Schall TJ, Proudfoot AE (2011) Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nat Rev Immunol* 11:355–363.
21. Salomon I, et al. (2002) Targeting the function of IFN-gamma-inducible protein 10 suppresses ongoing adjuvant arthritis. *J Immunol* 169:2685–2693.
22. Wildbaum G, Netzer N, Karin N (2002) Plasmid DNA encoding IFN-gamma-inducible protein 10 redirects antigen-specific T cell polarization and suppresses experimental autoimmune encephalomyelitis. *J Immunol* 168:5885–5892.
23. Zohar Y, et al. (2014) CXCL11-dependent induction of FOXP3-negative regulatory T cells suppresses autoimmune encephalomyelitis. *J Clin Invest* 124:2009–2022.
24. Meiron M, Zohar Y, Anunu R, Wildbaum G, Karin N (2008) CXCL12 (SDF-1alpha) suppresses ongoing experimental autoimmune encephalomyelitis by selecting antigen-specific regulatory T cells. *J Exp Med* 205:2643–2655.
25. Freeman CM, et al. (2005) CCR8 is expressed by antigen-elicited, IL-10-producing CD4+CD25+ T cells, which regulate Th2-mediated granuloma formation in mice. *J Immunol* 174:1962–1970.
26. Roos RS, et al. (1997) Identification of CCR8, the receptor for the human CC chemokine I-309. *J Biol Chem* 272:17251–17254.
27. Inngjerdingen M, Damaj B, Maghazachi AA (2000) Human NK cells express CC chemokine receptors 4 and 8 and respond to thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309. *J Immunol* 164:4048–4054.
28. Inngjerdingen M, Damaj B, Maghazachi AA (2001) Expression and regulation of chemokine receptors in human natural killer cells. *Blood* 97:367–375.
29. Chensue SW, et al. (2001) Aberrant in vivo T helper type 2 cell response and impaired eosinophil recruitment in CC chemokine receptor 8 knockout mice. *J Exp Med* 193:573–584.
30. Soler D, et al. (2006) CCR8 expression identifies CD4 memory T cells enriched for FOXP3+ regulatory and Th2 effector lymphocytes. *J Immunol* 177:6940–6951.
31. Coghill JM, et al. (2013) CC chemokine receptor 8 potentiates donor Treg survival and is critical for the prevention of murine graft-versus-host disease. *Blood* 122:825–836.
32. Zamvil S, et al. (1985) T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. *Nature* 317:355–358.
33. Islam SA, Ling MF, Leung J, Shreffler WG, Luster AD (2013) Identification of human CCR8 as a CCL18 receptor. *J Exp Med* 210:1889–1898.
34. Princen K, Hatse S, Vermeire K, De Clercq E, Schols D (2003) Evaluation of SDF-1/CXCR4-induced Ca2+ signaling by fluorometric imaging plate reader (FLIPR) and flow cytometry. *Cytometry A* 51:35–45.
35. Krutzik PO, Trejo A, Schulz KR, Nolan GP (2011) Phospho flow cytometry methods for the analysis of kinase signaling in cell lines and primary human blood samples. *Methods Mol Biol* 699:179–202.
36. Chen W, et al. (2003) Conversion of peripheral CD4+CD25$^-$ naive T cells to CD4+ CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875–1886.
37. Spinetti G, et al. (2003) The chemokine receptor CCR8 mediates rescue from dexamethasone-induced apoptosis via an ERK-dependent pathway. *J Leukoc Biol* 73:201–207.
38. D'Ambrosio D, et al. (1998) Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J Immunol* 161:5111–5115.
39. Saraiva M, O'Garra A (2010) The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 10:170–181.
40. Shevach EM, et al. (2006) The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol Rev* 212:60–73.
41. Mascanfroni ID, et al. (2013) IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39. *Nat Immunol* 14:1054–1063.
42. Eruslanov E, et al. (2013) Expansion of CCR8(+) inflammatory myeloid cells in cancer patients with urothelial and renal carcinomas. *Clin Cancer Res* 19:1670–1680.
43. Qu C, et al. (2004) Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J Exp Med* 200:1231–1241.
44. Zingoni A, et al. (1998) The chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells. *J Immunol* 161:547–551.
45. Sebastiani S, et al. (2001) Chemokine receptor expression and function in CD4+ T lymphocytes with regulatory activity. *J Immunol* 166:996–1002.
46. Elias KM, et al. (2008) Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* 111:1013–1020.
47. Nurieva R, et al. (2007) Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448:480–483.
48. Taflin C, et al. (2011) Human endothelial cells generate Th17 and regulatory T cells under inflammatory conditions. *Proc Natl Acad Sci USA* 108:2891–2896.
49. Chalmin F, et al. (2012) Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression. *Immunity* 36:362–373.
50. Chaudhry A, et al. (2009) CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* 326:986–991.
51. Ciofani M, et al. (2012) A validated regulatory network for Th17 cell specification. *Cell* 151:289–303.
52. Littman DR, Rudensky AY (2010) Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* 140:845–858.
53. Hovhannisyan Z, Treatman J, Littman DR, Mayer L (2011) Characterization of interleukin-17-producing regulatory T cells in inflamed intestinal mucosa from patients with inflammatory bowel diseases. *Gastroenterology* 140:957–965.
54. Grinberg-Bleyer Y, et al. (2010) IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med* 207:1871–1878.
55. Tang Q, et al. (2008) Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* 28:687–697.
56. Saadoun D, et al. (2011) Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* 365:2067–2077.
57. Koreth J, et al. (2011) Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* 365:2055–2066.
58. Bluestone JA (2011) The yin and yang of interleukin-2-mediated immunotherapy. *N Engl J Med* 365:2129–2131.
59. Hoelzinger DB, et al. (2010) Blockade of CCL1 inhibits T regulatory cell suppressive function enhancing tumor immunity without affecting T effector responses. *J Immunol* 184:6833–6842.
60. Collison LW, Vignali DA (2011) In vitro Treg suppression assays. *Methods Mol Biol* 707:21–37.
61. Cacciatore JJ, Leonard EF, Chasin LA (2012) The isolation of CHO cells with a site conferring a high and reproducible transgene amplification rate. *J Biotechnol* 164:346–353.
62. Tompkins SM, et al. (2002) De novo central nervous system processing of myelin antigen is required for the initiation of experimental autoimmune encephalomyelitis. *J Immunol* 168:4173–4183.
63. Wildbaum G, Zohar Y, Karin N (2010) Antigen-specific CD25- Foxp3- IFN-gamma(high) CD4+ T cells restrain the development of experimental allergic encephalomyelitis by suppressing Th17. *Am J Pathol* 176:2764–2775.