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Human Peripheral Blood T Regulatory Cells (Tregs), Functionally Primed CCR4$^+$ Tregs and Unprimed CCR4$^-$ Tregs, Regulate Effector T Cells Using FasL[1]

Dolgor Baatar,* Purevdorj Olkhanud,* Kenya Sumitomo,* Dennis Taub,* Ronald Gress,† and Arya Biragyn[2]*

Regulatory CD25$^+$CD4$^+$ T cells (Tregs) play an important role in the control of peripheral tolerance. In this study we demonstrate that human peripheral blood Tregs can be divided into two distinct populations based on the expression of CCR4. The majority (~75%) of freshly isolated Tregs express CCR4 and presumably represent memory-type Tregs. Interestingly, CCR4$^-$ Tregs require anti-CD3 Ab-mediated activation to acquire a regulatory activity, while CCR4$^+$ Tregs appear to be already primed to suppress the proliferation of CD8$^+$ T cells. CCR4 is also expressed on CD25^{low}CD4$^+$ T cells (CCR4$^+$ non-Tregs) that mostly suppress Th1-type polarization without affecting T cell proliferation, presumably via the production of immunomodulatory cytokines like IL-10. In contrast, CCR4$^+$ Tregs express FasL to primarily regulate T cell proliferation via a contact-mediated process involving FasL/Fas signaling, a major regulatory pathway of T cell homeostasis. Finally, we also demonstrate that the depletion of CCR4$^+$ T cells leads to Th1-type polarization of CD4$^+$ T cells and augmentation of CD8$^+$ T cell responses to tumor Ags. ***The Journal of Immunology,*** **2007, 178: 4891–4900.**

Regulatory CD4$^+$ T cells (Tregs)[3] are emerging as key controllers of peripheral tolerance to self-antigens and alloantigens (1, 2). Their dysfunction is associated with the spontaneous onset of autoimmune disorders (3, 4). Tumor-infiltrating Tregs have been shown to suppress immune responses, resulting in a poor disease outcome in cancer patients (5–9). Tregs are primarily defined as CD4$^+$ T cells that express high levels of CD25 (IL-2Rα), CTL-associated Ag 4 (CTLA-4) and scurfin, a Forkhead box P3 gene product (FoxP3). Tregs are classified into natural and adaptive based on their specificity and effector functions (10); at least two subsets of natural Tregs, memory-type CD25$^+$CD4$^+$ Tregs (11, 12) and natural naive CD4$^+$ Tregs (nnTregs) (13) have been shown to exist in humans. They suppress the proliferation of activated T cells and dendritic cells (DCs) involving a cell contact-mediated process (1, 14–16). In addition, two separate groups of Tregs, murine effector-memory Tregs (17) and human T regulatory type 1 (Tr1) cells (18), which acquire regulatory functions by polarizing human CD25$^-$CD4$^+$ T cells, have been also reported (18). Tregs can be also differentiated by the expression of chemokine receptors presumably reflecting differences in the homing of Tregs. For example, CCR7 is expressed by nnTregs (13) that infiltrate secondary lymphoid organs (19) while CXCR4 is thought to participate in the retention of Tregs in bone marrow (20), CCR6 in the recruitment of murine effector-memory Tregs into the skin (17), and CCR4 in peripheral migration, including the skin (6, 7, 21, 22).

The nature of CCR4$^+$ Tregs in human PBL and their mechanism of regulation remain unresolved, particularly considering the fact that up to 20% of circulating CCR4$^+$CD4$^+$ T cells may represent CCR4$^+$ Tregs (CCR4$^+$CD25$^+$CD4$^+$; see Fig. 1*A*). The majority of CCR4$^+$ lymphocytes appear to be represented by Th2-polarized CD4$^+$ T cells (23) that can also exert suppressive functions via the production of immunosuppressive cytokines (24). As a result, CD25$^-$CD4$^+$ non-Tregs, particularly at higher doses, are shown to be suppressive (25, 26). Although Treg-mediated regulation involves cell contact-dependent perforin/granzyme (GZ)-mediated processes, some Tregs (Tr1) suppress T cells using soluble factors such as IL-10 and TGFβ (27, 28). However, the extent of the participation of immunosuppressive cytokines has not been fully elucidated. To address these issues, we have tested Tregs that were depleted of CCR4$^+$ cells by using a chemokine-fused toxin called a chemotoxin. We demonstrate that Tregs in human PBL consist of at least two distinct subsets, memory-type CCR4$^+$ Tregs and naive-type CCR4$^-$ Tregs. Although freshly isolated CCR4$^+$ Tregs presumably represent natural Tregs and appear to be primed to readily suppress T cell proliferation, CCR4$^-$ Tregs require TCR-mediated activation to render them fully active. Although Treg-mediated regulation required a cell contact-dependent process, it did not use a perforin/GZ-mediated process. Instead, Tregs regulate T cell proliferation through a cell contact-dependent process involving FasL/Fas signaling. Moreover, we also demonstrate that an efficient Th1-type of polarization and augmentation of Ag-specific T cell responses can be achieved via strategies that preferentially deplete CCR4-expressing cells.

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[3] Abbreviations used in this paper: Treg, regulatory CD4$^+$ T cell; CM, conditioned medium; DC, dendritic cell; FoxP3, Forkhead box P3; GZ, granzyme; nnTreg, natural naive Treg; PI, propidium iodide; TARC, thymus- and activation-regulated chemokine; Tr1, T regulatory type 1 (cell).

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Materials and Methods

Plasmid constructs

The cloning strategy for chemokine-fused Ags has been described elsewhere (29). Briefly, the mature sequence for the thymus-and activation-regulated chemokine (TARC) was cloned using RT/PCR from human thymus total RNA. The primers used were: PRhTARCM-1 (5′-ATCACCAT GGCACGAGGGACCAACGTGGGCCGGGAGT-3′) and PRhTARC-GC-R3 (5′-ATACTCGAGGCTACCCCCACCGCCAGAGCCACCACC ACCAGACCTCTCAAGGCTTTGCAGGTA-3′). A truncated form of *Pseudomonas* exotoxin (PE38) that does not have a cell binding and internalization domain (30) was recloned from the pOND21-2 plasmid (gift of Dr. I. Pastan, National Cancer Institute, Bethesda, MD) by PCR using primers PRPE38-3 (5′-ATAACCATGGAAGCTTCTGGAGGTCCCGAG GGCGGCAGCCTGGCCGCGCTGA-3′) and PRPE38-R2 (5′-TATAGA TCTCTTCAGGTCCTCGCGCGGCGGTTTGCCGGGCTGGCT-3′). TARC was genetically fused in frame with PE38 (TARC-PE38) or with irrelevant tumor Ag OFA-iLRP (TARC-OFA) and expressed from bacterial expression vector pET11 (Stratagene). All constructs were verified by DNA sequencing (Keck DNA Sequencing, Yale University, New Haven, CT). Production of chemokine-fused proteins from TARC-PE38 or TARC-OFA-containing BL21(DE3) *Escherichia coli* cells (Stratagene) was described elsewhere (31). The integrity and purity (>90%) of recombinant proteins was tested by SDS-PAGE under reducing conditions and verified by Western blot hybridization with monoclonal 9E10 anti-c-*myc* Ab (Sigma-Aldrich).

Human peripheral blood cell isolation

Human peripheral blood samples were collected from healthy donors in accordance with Human Subject Protocol no. 2003054 by the Health Apheresis Unit and the Clinical Core Laboratory of the National Institute on Aging. $CD4^+$ cells were isolated from PBMCs by negative selection using a human CD4 subset column kit (R&D Systems) after Ficoll-Paque (GE Healthcare) density gradient separation according to the manufacturers' instructions. $CCR4^+CD4^+$or $CCR4^-CD4^+$ cells were selected as described previously (32). Briefly, 1×10^8 $CD4^+$ cells (in 900 μl of PBS containing 0.5% BSA and 2 mM EDTA) were stained with 100 μl of anti-CCR4-FITC Ab (R &D Systems) for 45 min at 4°C, and separated using anti-FITC microbeads (Miltenyi Biotec) and MS columns (Miltenyi Biotech). To achieve the highest purity, two consecutive MS columns were used and $CCR4^-$ cells were further depleted using LD columns (Miltenyi Biotec). $CD25^+$ cells were selected from $CD4^+$ cells or $CCR4^+CD4^+$and $CCR4^-CD4^+$ fractions using anti-CD25 Ab-coated microbeads (Dynabeads CD25; Dynal Biotech/Invitrogen Life Technologies). Beads were removed from cells using DETACHaBEAD CD4/CD8 reagent (Dynal Biotech/Invitrogen Life Technologies). Cell purity was determined by FACS with the following results: 94–98% for $CD25^{high}CCR4^+CD4^+$ ($CCR4^+$ Tregs) and 83–89% for $CD4^+CD25^{high}CCR4^-$cells ($CCR4^-$ Tregs; a lower purity of $CCR4^-$ Tregs was presumably due to slightly lower CD25 expression). $CD8^+$ cells were selected using a human T cell CD8 subset column kit (R&D Systems). Monocyte/macrophage-enriched PBMCs were isolated by plastic adherence and were irradiated with 4500 rad before use as APCs for MLR or feeder cells.

Flow cytometry analysis

Expression of *s*urface markers was detected by staining with the appropriate fluorochrome-conjugated Abs followed by FACS analysis using a FACScan flow cytometer and CellQuest Pro software (BD Biosciences). The following Abs against human Ags were purchased from R&D Systems: anti-CCR4-FITC, anti-CD45RO-PE, anti-CD25-PE, and anti-CCR7-FITC. Anti-CLA-FITC, anti-CD45RA-PE, anti-CD62L-PE, and anti-CD95L (FasL) Abs were from BD Pharmingen. Anti-GZ-A-PE (BD Pharmingen) and anti-GZ-B-PE (Caltag Laboratories) Abs were used for intracellular staining. Cells were fixed and permeabilized with a Fix&Perm cell permeabilization kit (Caltag Laboratories). To study the effect of activation, some cells were stimulated with *MACSi*Bead particles-coupled with anti-CD3/CD28/CD2 Abs (human T cell activation/expansion kit; Miltenyi Biotec) according to manufacturer's instructions. Murine cells were stained with anti-mouse CD25-PE (R&D Systems) and anti-mouse Foxp3-PE Abs (eBioscience).

RNA isolation and RT-PCR

Total RNA from flow-sorted fractions of $CD4^+$ cells was isolated using the TRIzol reagent (Invitrogen Life Technologies) and an RNeasy kit (Qiagen). RT-PCR was performed with primers reported by others: human FoxP3 (33) (5′-GAAACAGCACATTCCCAGAGTTC-3′ and 5′-ATGGCCCAGCGGA TGAG-3′) and human GAPDH (34) (5′-TGTGGAAGGGCTCATGACCACA GTCCAT-3′ and 5′-GCCTGCTTCACCACCTTCTTGATG-3′).

Suppression assays

To test Treg suppression, the responder $CD8^+$ T cells (5×10^4 each) were labeled with 0.5 μM CFSE (CellTrace cell proliferation kit; Molecular Probes/Invitrogen Life Technologies) in PBS for 10 min at 37°C. The CFSE-labeled cells from a single batch were equally divided and used in assays in triplicate. Cells were then cultured with autologous APCs (5×10^4) and titrated amounts of Tregs for 4–5 days in the presence of soluble anti-CD3 Ab (0.5 μg/ml) in 96-well plates. Cells were stained with anti-CD8-PE Ab or propidium iodide (PI; Roche Diagnostics) and analyzed by FACS. $CD8^+$ cells that underwent divisions and became low in CFSE expression (compared with unstimulated and nonproliferated cells) were considered proliferated cells. In separate experiments, anti-CD3 Ab (0.5 μg/ml) was present or absent in the culture medium throughout the experiment with $CD8^+$ cells and allogeneic APCs in a MLR assay. Some Tregs (or control non-Tregs) were incubated overnight with soluble anti-CD3 Ab (1 μg/ml), washed twice with PBS, and used as suppressors in MLR assays. To neutralize FasL, anti-human FasL/CD95L Ab (10 or 20 μg/ml NOK-1; BD Pharmingen) or isotype-matched control Ab (BD Pharmingen) were used. Tregs or control cells were preincubated with Abs for 1 h at 37°C before being added to responder cells. To test the Fas-mediated killing activity of Tregs, CFSE-labeled Jurkat cells (2.5×10^4) were incubated with titrated amounts of Tregs pretreated for 30 min with anti-CD95L/FasL or control Abs) for 2 days. The concentration of anti-CD95L/FasL Ab in culture medium was 5 μg/ml. Cell death was considered a proportion of $CFSE^+PI^+$ cells.

Depletion of $CCR4^+$ cells using TARC chemotoxin

Human peripheral blood $CD4^+$ and $CD8^+$ cells were treated with 10 μg/ml TARC-PE38, TARC-OFA, or PBS for 2 days in complete RPMI 1640 medium supplemented with 10% FBS and 5% human AB serum. Expressions of CCR4 and cell death were evaluated using anti-CCR4-FITC Ab and PI (Roche Diagnostics), respectively. The viability of purified $CD4^+$ cells was assessed using the cell proliferation reagent WST-1 (Roche Diagnostics).

Effects of $CCR4^+$ cell depletion on $CD4^+$ cell activation and proliferation in response to polyclonal stimulation

After overnight treatment with 10 μg/ml TARC-PE38, TARC-OFA, or PBS, $CD4^+$ cells were washed and activated with 5 μg/ml soluble anti-CD3 (BD Pharmingen) and 1 μg/ml anti-CD28 Abs (BD Pharmingen) for 3 days followed by 4 days of incubation in the presence of 10 U/ml IL-2 (PeproTech). Secreted cytokines were measured using Multiplex (Bio-Rad); cells were stained with anti-CD25-PE and anti-CCR4-FITC Abs and analyzed by FACS. Cell proliferation was tested as an uptake of 100 nM BrdU (colorimetric cell proliferation ELISA; Roche Diagnostics/Roche Applied Science). To study the role of Th2-type non-Tregs, $CD4^+$ cells depleted from $CD25^+$ cells were treated overnight with 10 μg/ml TARC-PE38, TARC-OFA, or PBS, extensively washed in PBS, and activated with plate-bound anti-CD3 Ab (0.5 μg/ml) and 1 μg/ml soluble anti-CD28 Ab. After 3 days, 3×10^6 cells/ml were plated and cultured for another 3 or 5 days in the presence of IL-2 (10 U/ml) to determine the level of secreted cytokines. To evaluate the contribution of $CCR4^+$ Tregs, magnetically selected $CD25^+CD4^+$ Tregs were depleted from $CCR4^+$ cells overnight by treatment with 10 μg/ml TARC-PE38 and mixed with 50,000 CFSE-labeled $CD25^-CD4^+$ responder cells. Tregs treated with TARC-OFA or PBS and $CD25^-CD4^+$ non-Tregs were used as controls. Cells were stimulated with soluble anti-CD3 Ab (0.5 μg/ml) and irradiated autologous APCs (1×10^5). After 4 days, the percentage of divided cells among responder $CD4^+$ cells was measured by FACS.

Effect of $CCR4^+$ cell depletion on Ag-specific T cell proliferation

The following synthetic peptides (purity >99%; Peptide Technologies) were used: human $gp100_{25-33}$ (KVPRNQDWL) and MOPC-315 Ig_{91-101} (ALWFRNHFVFGGGTK) (35). Pmel mice were vaccinated s.c. twice at 3-wk intervals with 10 μg of human $gp100_{25-33}$ peptide emulsified in 100 μl IFA (Sigma-Aldrich). Three weeks after the second vaccination splenocytes were harvested and treated with PBS, TARC-PE38, or TARC-OFA (both 10 μg/ml) for 24 h. After washing with PBS, splenocytes were cultured in complete RPMI 1640 medium containing 10% FBS, 20 IU/ml recombinant human IL-2, and 1 μg/ml corresponding peptide ($gp100_{25-33}$

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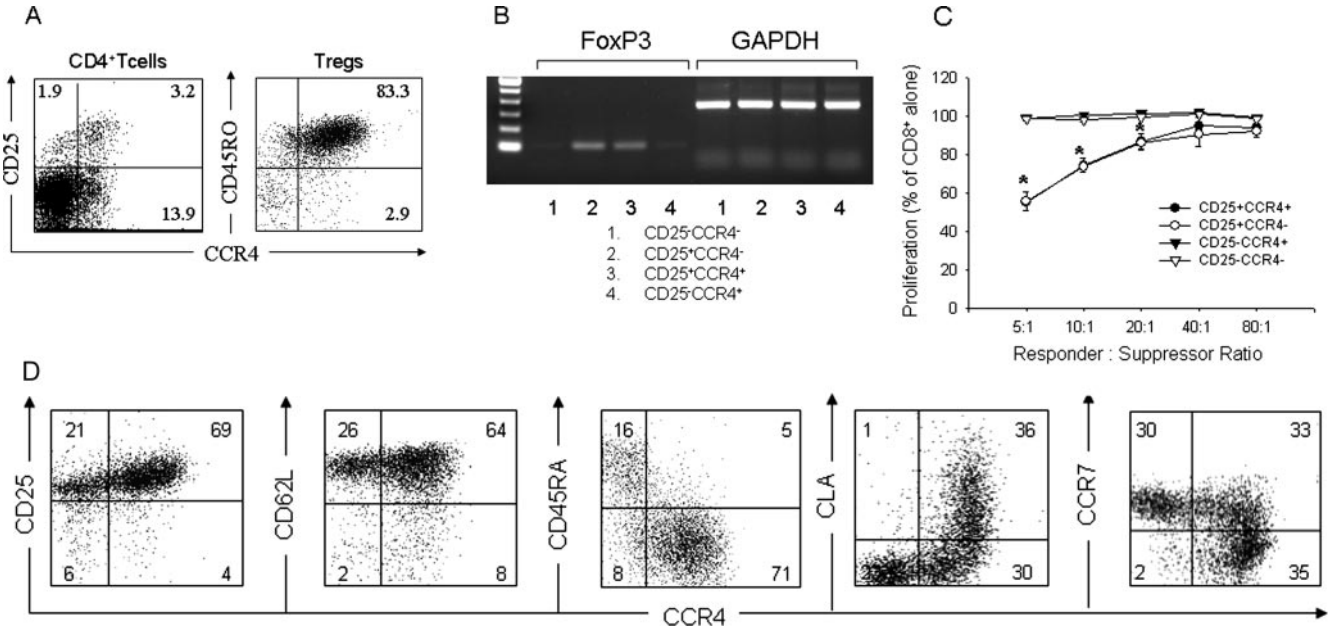

FIGURE 1. Phenotypic characterization of human peripheral blood Tregs. *A*, The majority of Tregs express CCR4 and CD45RO. Freshly isolated $CD4^+$T cells were stained with anti-CD25 and anti-CCR4 Abs (*left panel*) or purified $CD25^+CD4^+$ cells were stained with anti-CD45RO and anti-CCR4 Abs (Tregs; *right panel*). *B*, FoxP3 is only detected in Tregs, but not in non-Tregs. Message levels of FoxP3 and GAPDH were tested by RT/PCR in total RNA extracted from the FACS-sorted human peripheral blood $CD4^+$ cells. *C*, $CCR4^+$ Tregs and $CCR4^-$ Tregs, but not non-Tregs, suppress $CD8^+$ cell proliferation. CFSE-labeled $CD8^+$ cells (responder) were cultured for 4 days with titrated amounts of suppressor cells (Tregs or control cells, indicated) in the presence of anti-CD3 Ab (0.5 μg/ml) and irradiated autologous APCs. Data (mean ± SD of triplicates) represent the proliferation of responder cells compared with the proliferation of $CD8^+$ cells cultured in the absence of suppressor cells ($CD8^+$ alone). *, $p < 0.01$ is for comparisons with the $CD25^-CCR4^+$ group at the corresponding points. *D*, Phenotype of human peripheral blood Tregs. Purified Tregs were stained with Abs to corresponding surface markers (indicated). Numbers represent the percentage of cells in the corresponding quadrant. Shown, representative data from at least three independent experiments.

and MOPC315, respectively) for 3–5 days. The specific activity of T cells was tested on irradiated target cells (DCs from C57BL/6 mice) pulsed with either human $gp100_{25-32}$ or control MOPC315 peptides. Secreted IFN-γ was measured after 24 h of incubation by ELISA. Proliferation of splenocytes was measured by BrdU incorporation following 5 days of culture. DCs were prepared as previously described (29).

For suppression assays, $CD8^+$ and $CD4^+$ cells were isolated from Pmel or C57BL/6 splenocytes, respectively, using murine T cell subset column kits (R&D Systems). $CD4^+$ cells were treated overnight with PBS, TARC-PE38, or TARC-OFA (both 10 μg/ml), washed, and mixed at a 1:1 ratio with Pmel $CD8^+$ cells. They were then stimulated with $gp100_{25-32}$ peptide-pulsed irradiated APCs. Cell proliferation was evaluated after 5 days by BrdU incorporation.

Animals

All mice were bred and housed at the National Institute of Aging animal facility in Baltimore, MD. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 86-23, 1985). TCR transgenic Pmel-1 (Vα1Vβ13 TCR for H-$2D^b$-restricted mouse and human gp100 epitope) mice have been described previously (36). C57BL/6 mice were purchased from The Jackson Laboratory.

Statistical analysis

All data are representative of at least two experiments. For all graphs the data represent the mean ± SD of triplicates. Differences were tested using Student's *t* test or one-way ANOVA followed by a post hoc Scheffe's *F* test. $p < 0.05$ was considered statistically significant.

Results

Human peripheral blood contains two distinct subtypes of Tregs

$CD25^+CD4^+$ cells represent up to 5% of human peripheral blood $CD4^+$ T cells that can be further divided into two distinct populations of cells: $CCR4^+CD25^+$ (3.2 ± 0.5%) and $CCR4^-CD25^+$(1.9 ± 0.6%) (Fig. 1*A*). These two populations expressed FoxP3 mRNA (Fig. 1*B*) and suppressed the proliferation of autologous $CD8^+$ T cells (Fig. 1*C*) stimulated with anti-CD3 Ab and autologous APCs. In contrast, $CCR4^-CD25^{-/low}$ and $CCR4^+CD25^{-/low}$ $CD4^+$ T cells (non-Tregs) did not express FoxP3 (Fig. 1*B*) or suppress T cell proliferation (Fig. 1*C*). Thus, both $CCR4^+CD25^+$ and $CCR4^-CD25^+$ cells represent T regulatory cells that we designated $CCR4^+$ Tregs and $CCR4^-$ Tregs, respectively. However, the cells appear to differ in their differentiation states because the majority of $CCR4^+$ Tregs (80–90%) expressed so-called memory type surface markers (Fig. 1*A*, *right* ($CD45RO^+$); Fig. 1*D* ($CD45RA^-$)) while most $CCR4^-$ Tregs expressed CD45RA and belonged to naive cells (Fig. 1*D*). The two cells presumably home to different organs, because the skin-homing marker CLA^+ (22, 37) was exclusively expressed by $CCR4^+$ Tregs Fig. 1*D*) as reported by others (6, 21). The majority of $CCR4^-$ Tregs expressed CCR7 and CD62L markers that are associated with homing to secondary lymphoid organs (19). Interestingly, $CCR4^+$ Tregs also expressed CD62L (Fig. 1*D*, ≥ 90%) and CCR7 (Fig. 1*D*, ≥ 50%), suggesting that Tregs may actually consist of at least three distinct subgroups, namely $CCR4^+CCR7^-$, $CCR4^+CCR7^+$, and $CCR4^-CCR7^+$ (Fig. 1*D*).

$CCR4^+$ and $CCR4^-$ Tregs are functionally different regulatory cells

Because $CCR4^-$ Tregs mostly expressed markers for nonactivated naive cells ($CD45RA^+$) that home to lymph nodes (Fig. 1*D*), we have postulated that they may require an initial activation or instruction to become suppressive cells. Traditional assays, typically performed in the extended presence of anti-CD3 Ab, would also activate Tregs and would not functionally discriminate between $CCR4^-$ Tregs and $CCR4^+$ Tregs (see Fig. 1*C*). Thus, we tested the

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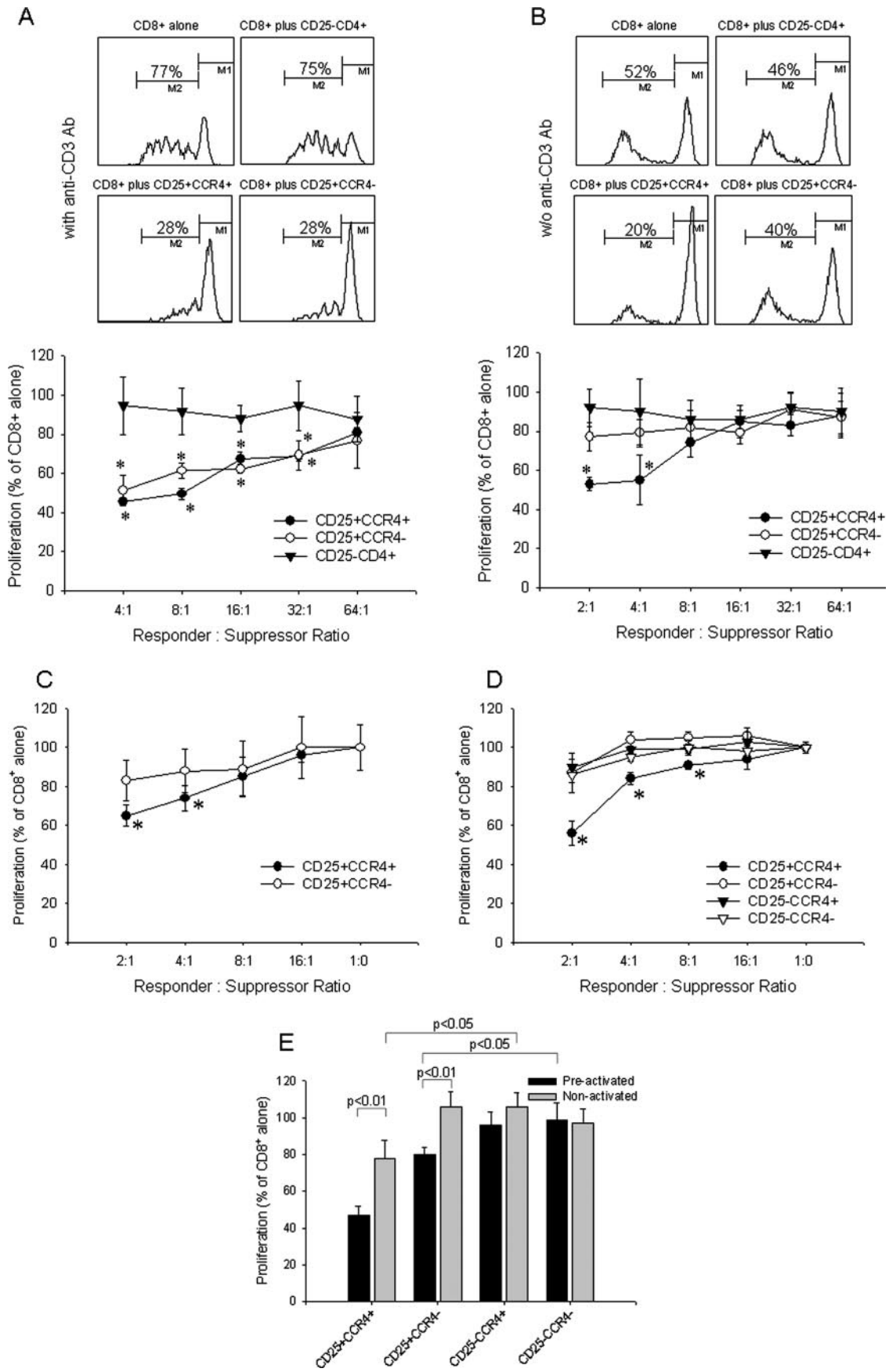

FIGURE 2. CCR4$^-$ Tregs and CCR4$^+$ Tregs are at different functional stages. *A–D*, Although the proliferation of CD8$^+$ cells can be suppressed equally well with CCR4$^-$ Tregs and CCR4$^+$ Tregs in the presence of anti-CD3 Ab (*A*), it is inhibited only by CCR4$^+$ Tregs when anti-CD3 Ab was omitted (w/o, without) from the MLR assay (*B–D*). Histograms (*top panel*) contain representative experimental data depicting the proportion (percentage) of proliferated CD8$^+$ cells. A suppression assay performed in the absence of anti-CD3 Ab using Tregs from two more human donors is shown in *C* and *D*. Data (mean ± SD

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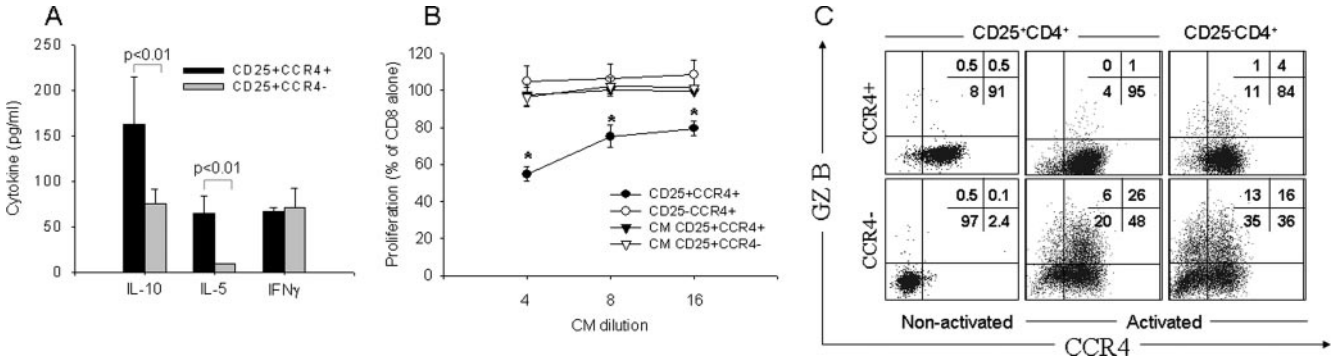

FIGURE 3. Treg-mediated suppression requires cell contact. *A* and *B*, Despite the fact that significant levels of IL-10 and IL-5 were found in the CM of Tregs incubated with $CD8^+$ cells (*A*), they did not affect the proliferation of $CD8^+$ cells (*B*). As shown, unlike Tregs ($CD25^+CCR4^+$), neither non-Tregs ($CD25^-CCR4^+$) or the CM from $CCR4^+$ Tregs and $CCR4^-$ Tregs (CM $CD25^+CCR4^+$ and CM $CD25^+CCR4^-$, respectively) were able to inhibit the proliferation of $CD8^+$ T cells in the presence of anti-CD3 Ab and APCs (*B*). Data (mean ± SD of triplicates) represent the proliferation of responder cells compared with the proliferation of $CD8^+$ cells cultured in the absence of suppressor cells ($CD8^+$ alone). *, $p < 0.01$ is for comparisons with the $CD25^-CCR4^+$ group at the corresponding ratio. *C*, Expression of GZ-B in populations of $CCR4^-$ and $CCR4^+$ $CD4^+$ T cells freshly isolated (nonactivated) or treated with bead-bound anti-CD3/CD28 Abs (activated). The CCR4 Ab-labeled cells were stained intracellularly with anti-GZ-B mAb. Numbers represent percentage of cells in the corresponding quadrant.

suppressive effects of Tregs cultured in the absence or presence of anti-CD3 Ab by incubating them with CFCE-labeled $CD8^+$ T cells and allogeneic APCs for 5 days (MLR). As expected, both $CCR4^-$ and $CCR4^+$ Tregs suppressed the proliferation of $CD8^+$ T cells at almost identical cellular concentrations when MLR was performed in the presence of anti-CD3 Ab (Fig. 2*A*). However, when anti-CD3 Ab was omitted from the assay, only $CCR4^+$ Tregs, but not $CCR4^-$ Tregs, inhibited the proliferation of $CD8^+$ T cells (Fig. 2*B*). Although the degree of inhibition was slightly variable in samples from different human donors, only $CCR4^+$ Tregs consistently inhibited MLR in the absence of anti-CD3 Ab (Fig. 2, *C* and *D*), indicating that this is a general phenomenon. Thus, Tregs appear to consist of two functionally distinct populations primed $CCR4^+$ Tregs that exhibit suppression independent of preactivation and unprimed $CCR4^-$ Tregs that require an initial TCR signaling to become fully active regulatory cells. However, $CCR4^-$ Tregs appear to be presensitized, because pretreatment with a soluble anti-CD3 Ab alone was sufficient to activate their suppressive effects (Fig. 2*E*).

$CCR4^+$ and $CCR4^-$ Tregs use different regulatory pathways

It is widely accepted that Tregs require cell contact to exert their suppressive effects. In concordance, conditioned medium from activated mixtures of Treg/non-Treg cells, which contained significant amounts of IL-10, IL-5 and IFN-γ (Fig. 3*A*), failed to inhibit the proliferation of non-Treg cells (Fig. 3*B*, conditioned medium (CM) $CD25^+CCR4^+$). Recent reports have indicated that Tregs use both GZ-A and GZ-B in the contact-mediated cell suppression (27, 28). However, we did not detect the expression of any GZ-A in Tregs regardless of anti-CD3/CD28 Abs activation (data not shown). Although GZ-B was not detected in non-activated T cells (Fig. 3*C*), it was significantly expressed in $CCR4^-$ non-Tregs and $CCR4^-$ Tregs after activation with anti-CD3/CD28 Abs (Fig. 3*C*). In contrast, $CCR4^+$ Tregs did not express GZ-B even after activation (Fig. 3*C*), indicating that GZs are presumably not used by $CCR4^+$ Tregs.

Fas/FasL (CD95/CD95L) signaling plays a major role in the physiological regulation of T cell homeostasis (38). We hypothesized that it may also be used for the contact-mediated regulation of T cell proliferation. To test this hypothesis, we measured the expression of FasL/CD95L by Tregs. Resting $CD4^+$ T cells have not been reported to express FasL, although it was shown to appear within 8 h of stimulation (38–41). In concordance, we also did not detect FasL on the surface of freshly isolated Tregs (data not shown). Similarly, no FasL was expressed on $CCR4^-$ Tregs (Fig. 4, *A* and *B*) or non-Tregs (both $CCR4^+$ and $CCR4^-$ populations; data not shown). In contrast, significant levels of FasL were detected exclusively on $CCR4^+$ Tregs after a brief in vitro culture without any stimuli (Fig. 4*A*). Upon activation with anti-CD3/CD28 Abs, $CCR4^-$ Tregs also expressed significant levels of FasL but predominantly in the $CCR4^-$ Tregs that were converted to express CCR4 (Fig. 4*B*). Thus, $CCR4^-$ Tregs presumably contain precursor pools that require activation not only to become $CCR4^+$ Tregs but also to use FasL. Furthermore, FasL expressed by Tregs is functionally active, as Tregs were able to mediate the apoptosis of Jurkat cells, which was specifically abrogated by incubation with neutralizing anti-FasL Ab (Fig. 4*C*). In addition, incubation with a neutralizing anti-FasL Ab, but not a control Ab, reversed the suppressive activities of unfractionated Tregs (Fig. 5*A*) as well as $CCR4^+$ Tregs and $CCR4^-$ Tregs (Fig. 5*B*), resulting in a significantly augmented proliferation of $CD8^+$ T cells stimulated with anti-CD3 Ab. However, Tregs appear to inhibit only proliferation T cells, because we did not observe apoptosis of $CD8^+$ T cell even after incubation for 4 days (data not shown). Taken together, these data clearly indicate that Tregs use FasL, at least in part, to regulate T cell proliferation in a contact-dependent manner.

of triplicates) represent the proliferation of responder cells compared with the proliferation of $CD8^+$ cells cultured in the absence of suppressor cells ($CD8^+$ alone). *, $p < 0.01$ is for comparisons with the $CD25^-CCR4^+$ group at the corresponding points (*A*, *B*, and *D*), or with $CD8^+$ T cells alone (*C*). *E*, Anti-CD3 Ab preactivation of Tregs further augments their suppressive potency. Freshly isolated Tregs or non-Tregs (*x*-axis, indicated) were pretreated overnight with anti-CD3 Ab (bars labeled "Pre-activated") or mock treated with PBS (bars labeled "Non-activated") before mixing with $CD8^+$ T cells in the MLR assay as described above. The responder to suppressor ratio was 4:1.

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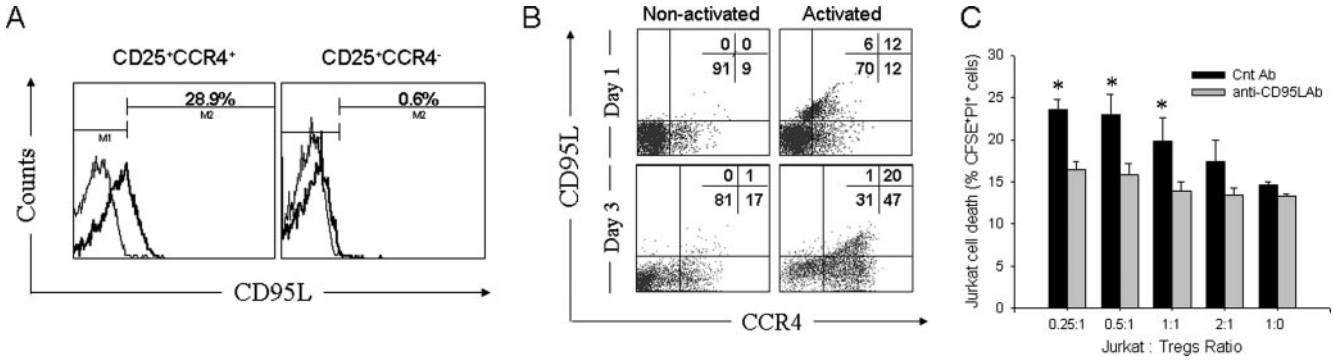

FIGURE 4. Expression of FasL and its role in Treg-mediated suppression. *A*, CD95L/FasL is only expressed by nonactivated CCR4$^+$ Tregs, not by CCR4$^-$ Tregs. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 5% human AB serum for 16 h. Shown is CD95L (thick line) vs isotype-matched control (thin line) in CCR4$^+$ Tregs and CCR4$^-$ Tregs, and numbers represent the percentage of cells in the corresponding gate. *B*, CD95L/FasL is expressed by CCR4$^-$ Tregs upon activation. CCR4$^-$ Tregs were mock treated (nonactivated) or incubated with bead-bound anti-CD3/CD28 Abs (activated) for 16 h (day 1) and 72 h (day 3). Cells were stained with anti-CCR4 and anti-CD95L Abs. Representative data from two independent experiments with similar results are shown. *C*, Tregs express a functionally active CD95L/FasL that is capable of inducing Fas-mediated apoptosis. Twenty-five thousand CFSE-labeled Jurkat cells were cocultured for 2 days with titrated numbers of Tregs in the presence of 20 μg/ml neutralizing anti-CD95L Ab or control (Cnt) Ab. Data (mean ± SD of triplicates) represent dead (PI$^+$CFSE$^+$) cells compared with untreated Jurkat cells alone. *, $p < 0.01$ is for comparisons with Jurkat cells alone.

CCR4$^+$CD4$^+$ non-Treg cells control Th1 polarization while CCR4$^+$ Tregs regulate the proliferation of T cells

In human peripheral blood we detected expression of CCR4 on two types of cells, Tregs and Th2-type CD4$^+$ non-Treg cells, with the latter exerting suppression by production of the immunomodulatory cytokine IL-10 (24). Therefore, to assess their regulatory functions we have developed a specific method that depletes CCR4-expressing cells using chemotoxin, a recombinant chemokine TARC/CCL17 fused with a truncated exotoxin PE38 (TARC-PE38). Preliminary experiments demonstrated that CCR4$^+$ T cells can be specifically killed by treatment with low amounts (ng/ml) of TARC-PE38 (Fig. 6*A*), but not control TARC fused with an irrelevant tumor Ag (TARC-OFA). To test the effects on CCR4$^+$ T cells, purified CD4$^+$ T cells were pretreated overnight with PBS, TARC-PE38, or TARC-OFA. Then the cells were extensively washed with PBS and stimulated with soluble anti-CD3/CD28 Abs. As shown in Fig. 6*B*, CD4$^+$ T cells pretreated with TARC-PE38, but not cells treated with TARC-OFA or PBS, proliferated significantly better (Fig. 6*B*, $p < 0.01$). The TARC-PE38 pretreatment, in contrast to treatment with TARC-OFA or PBS, also induced significantly higher IFN-γ (Fig. 6*C*; $p < 0.01$) and lower IL-10 and IL-5 secretions (data not shown; also see Fig. 6, *D* and *E*). Given that TARC-PE38 specifically and selectively killed CCR4$^+$ T cells (see Fig. 6A), these data suggest that CCR4$^+$ cells keep both the proliferation and Th1 polarization of CCR4$^-$ cells transiently suppressed. In support of this conclusion, purified/sorted CCR4$^-$CD4$^+$ T cells alone (Fig. 6, *B* and *C*, CD4$^+$CCR4$^-$; contained <5% contaminating CCR4$^+$ cells) proliferated and produced IFN-γ at similar levels as T cells pretreated with TARC-PE38. Conversely, highly purified CCR4$^+$CD4$^+$ T cells proliferated poorly and failed to secrete IFN-γ (Fig. 6, *B* and *C*, CD4$^+$CCR4$^+$). Taken together, these data indicate that CCR4$^+$ T cells are primarily responsible for control of the Th1 polarization of CCR4$^-$CD4$^+$ T cells.

To further dissect the roles of CCR4$^+$ Tregs and CCR4$^+$ non-Tregs, CD4$^+$ T cells were depleted of CD25-expressing cells (Tregs) using anti-CD25 Ab-coupled beads. As expected, the remaining non-Treg CD4$^+$ cells (≥95% pure) proliferated well and did not inhibit the proliferation of other T cells (see Fig. 1*C*, CD25$^-$CCR4$^+$), indicating that Tregs are a primary regulator of

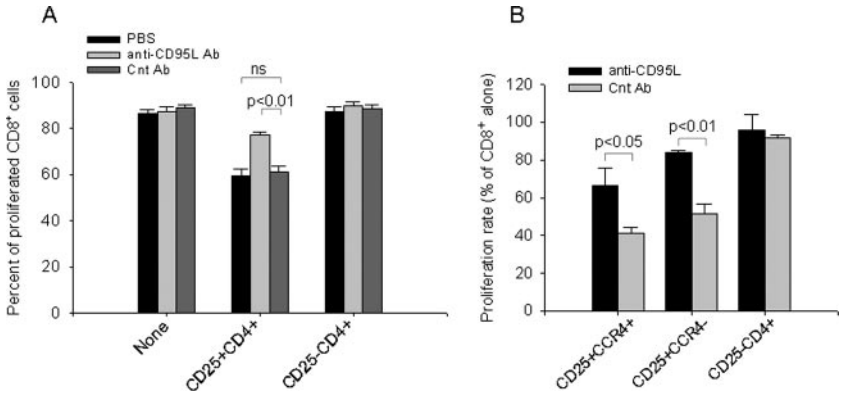

FIGURE 5. CD95L/FasL is used in Tregs-mediated T cell regulation. *A*, Suppressive effects of Tregs are reversed in the presence of the neutralizing anti-CD95L Ab but not in that of the control (Cnt) isotype-matched Ab. Mock treatment with PBS and Abs does not affect the proliferation of CFSE-labeled CD8$^+$ T cells alone (none) or mixed with non-Tregs (CD25$^-$CD4$^+$). However, anti-CD95L Ab, but not control Ab, reversed a suppressive state of CD8$^+$ T cells induced by Tregs (CD25$^+$CD4$^+$). The suppression assay is as described for Fig. 1. *B*, CD95L/FasL is used by both CCR4$^+$ Tregs and CCR4$^-$ Tregs to suppress CD8$^+$T cell proliferation. Anti-CD95L Ab or control Ab (20 μg/ml each) were incubated in the suppression assay as described for Fig. 1. Shown are representative data from at least three independent experiments.

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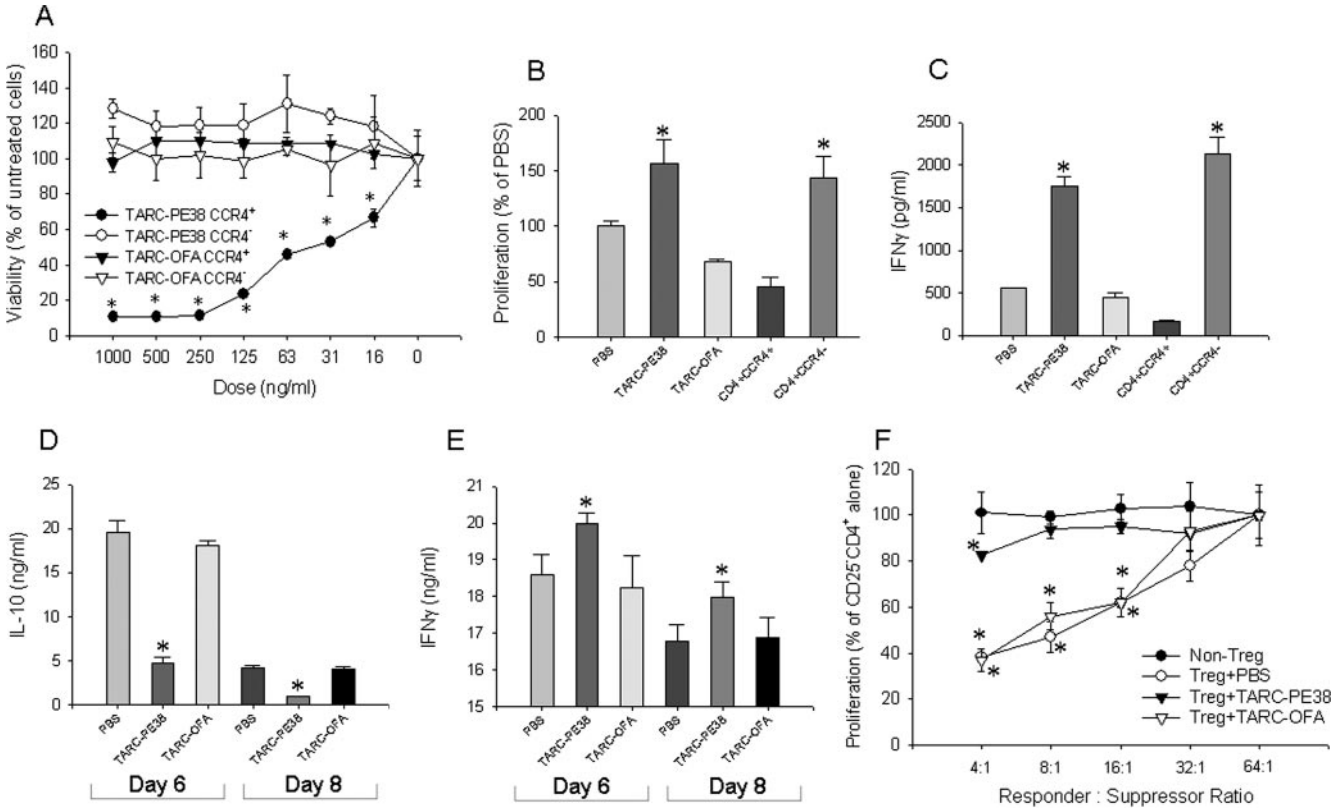

FIGURE 6. Preferential depletion of CCR4$^+$ T cells using TARC-PE38 (*A*) augments T cell proliferation (*B*) and production of IFN-γ (*C*). *A*, Purified CCR4$^+$CD4$^+$ and CCR4$^-$CD4$^+$ cells were treated for 2 days with titrated doses (ng/ml; *x*-axis) of TARC-PE38 and TARC-OFA. Cell viability was then tested using a 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (WST-1) reagent. Shown are representative data (mean ± SD triplicates) from at least three independent experiments with similar results. *, $p < 0.01$ is for comparisons with control TARC-OFA treatment. *B* and *C*, CD4$^+$ T cells were pretreated overnight with 10 μg/ml TARC-PE38, TARC-OFA, or PBS and then activated with soluble anti-CD3/CD28 Abs for 7 days to measure cell proliferation (BrdU incorporation) and the secretion of IFN-γ (pg/ml). In parallel, the proliferation and IFN-γ secretion of purified and untreated CD4$^+$CCR4$^+$ and CD4$^+$CCR4$^-$ T cells were tested after the same stimulation. *D* and *E*, Treatment with TARC-PE38, but not with TARC-OFA, promotes the Th1-type polarization of T cells, reducing IL-10 (*D*) and augmenting IFN-γ (*E*). CD4$^+$ T cells were first depleted from CD25$^+$ cells (Tregs) using anti-CD25 Ab beads. Then the cells were treated with TARC-PE38, TARC-OFA, or PBS and stimulated with plate-bound anti-CD3 Ab/CD28 Abs as described above. At the indicated time points (days), secreted cytokines were measured by ELISA. Shown are representative data (mean ± SD of triplicates) from at least two independent experiments. *, $p < 0.01$ for comparisons with PBS-treated groups. *F*, TARC-PE38 abrogates the suppressive activity of Tregs. Tregs were pretreated overnight with TARC-PE38 or TARC-OFA as in *B* and mixed with responder cells (CFSE-labeled CD25$^-$CD4$^+$ cells) at the indicated ratio. The cells were stimulated with anti-CD3 Ab (0.5 μg/ml) in the presence of irradiated autologous APCs. *, $p < 0.01$ is for comparisons with non-Tregs at the corresponding ratio.

T cell proliferation. These Treg-depleted cells were subsequently pretreated with TARC-PE38, TARC-OFA, or PBS and then stimulated with anti-CD3/CD28 Abs. The cells mock treated with PBS or treated with control TARC-OFA produced significant levels of IL-10 (Fig. 6*D*) and other Th2-type cytokines such as IL-4 and IL-5 (data not shown). In contrast, the cells treated with TARC-PE38 produced dramatically reduced levels of IL-10 (Fig. 6*D*), IL-4, and IL-5 (data not shown) but significantly increased IFN-γ (Fig. 6*E*). Thus, these data suggest that non-Treg CCR4$^+$ Th2-type cells do not regulate T cell proliferation; rather, they control Th1 polarization presumably through the action of the immunomodulatory cytokines IL-10 and IL-4 (39, 42). Moreover, pretreatment with TARC-PE38, but not with TARC-OFA, specifically reversed the antiproliferative activity of purified Tregs (Fig. 6*F*), supporting our previously discussed observation that CCR4$^+$ Tregs primarily regulate T cell proliferation via cell contact and FasL-dependent processes.

Practical implications: depletion of murine CCR4$^+$ T cells augments Ag-specific CD8$^+$ T cell responses

Tregs have been reported to suppress CD8$^+$ T cell responses to tumor Ags (40), suggesting that their depletion leads to augmented antitumor cellular responses. Spleens of naive C57BL/6 mice contained up to 8% CD25$^+$ T cells (Fig. 7*A*, *upper plot*) and ~7% FoxP3$^+$ T cells (Fig. 7*A*, *lower plot*). However, only up to a quarter of these cells appear to be murine CCR4$^+$ Tregs (Fig. 7*A*, 3% CCR4$^+$CD25$^+$ and 2% CCR4$^+$FoxP3$^+$ cells of spleen T cells). For example, the activation of transgenic CD8$^+$ T cells from Pmel mice stimulated with the gp100$_{25-32}$ peptide was significantly inhibited by incubation with CD4$^+$ T cells from naive C57BL/6 mice (Fig. 7*B*). The inhibition was completely reversed and the proliferation of CD8$^+$ T cells was restored when the CD4$^+$ T cells were pretreated with TARC-PE38 but not with TARC-OFA or PBS (Fig. 7*B*). Moreover, the TARC-PE38 pretreatment may have practical benefits, because it induced significantly higher cell proliferation (Fig. 7*C*) and IFN-γ production (Fig. 7*D*) when Pmel splenocytes were pretreated with TARC-PE38 before stimulation with the gp100$_{25-32}$ peptide. Pretreatments with control TARC-OFA or PBS did not have any benefits (Fig. 7, *C* and *D*). Interestingly, the TARC-PE38-mediated depletion of CCR4$^+$ T cells appears to augment a nonspecific T cell proliferation in general, because it slightly but significantly increased proliferation (Fig. 7*C*, $p < 0.05$) but not IFN-γ production (Fig. 7*D*) of Pmel CD8$^+$

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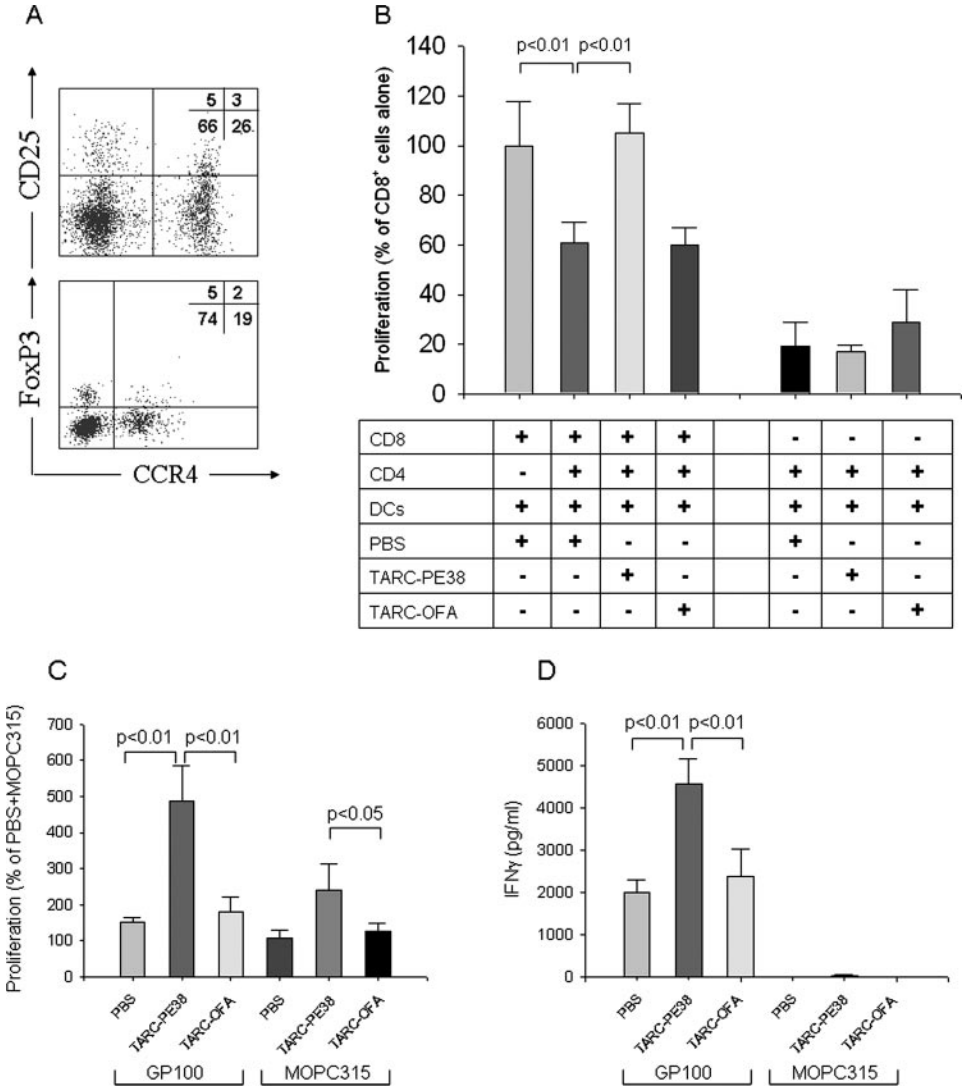

FIGURE 7. *A*, Murine spleen CD4$^+$ T cells have a significant proportion of CCR4$^+$ and FoxP$^+$ cells. CD4$^+$ T cells were isolated from spleens of C57BL/6 mice and stained with anti-CD25 and CCR4 (*upper dot plot*) or anti-FoxP3 and CCR4 Abs (*lower dot plot*). Numbers indicate the percentage of cells in the corresponding quadrant. *B*, Depletion of murine CCR4$^+$ T cells renders them unable to suppress Ag-specific CD8$^+$ T cell proliferation. C57BL/6 mouse CD4$^+$ T cells were pretreated with TARC-PE38 or controls (PBS or TAR-OFA) as in Fig. 6*B* and mixed with Pmel CD8$^+$ T cells at 1:1 ratio. *C* and *D*, Pmel CD8$^+$ cells were stimulated for 5 days with irradiated autologous DCs pulsed with 1 μg/ml gp100$_{25-32}$ peptide. Pmel splenocytes depleted from CCR4$^+$ cells proliferate better (*C*) and produce more IFN-γ (*D*) in response to Ag-specific stimulation. Splenocytes were pretreated with TARC-PE38 or controls as in Fig. 6*B* and cultured for 3 days with gp100$_{25-32}$ or control MOPC315 peptides. Representative data (mean $\pm$ SD of triplicates) from at least three independent experiments.

T cells stimulated with the control MOPC315 peptide. These results suggest that murine spleen-derived CCR4$^+$ Tregs behave similarly to human Tregs and suppress Ag-specific CD8$^+$T cell responses. These data also indicate that the suppressive state of CD4$^+$T cells can be completely reversed by the depletion of CCR4$^+$ T cells using TARC-PE38, an important consideration for clinical applications.

Discussion

Our data show that human peripheral blood contains several distinct populations of suppressive CD4$^+$ T cells: two discrete populations of Tregs that regulate T cell proliferation differentiated by the expression of CCR4 and non-Treg CCR4$^+$ T cells that control Th1 cell polarization. In concordance with published data (39, 42), Th2-type non-Treg CCR4$^+$CD4$^+$ cells express IL-10, which is presumably a primary suppressor of Th1 polarization. In support, the capability to express IFN-γ was drastically augmented by the depletion of these IL-10-expressing cells from the CD4$^+$ T cells (Fig. 6, *B* and *C*). Moreover, T cell proliferation is primarily regulated by two phenotypically distinct subsets of Tregs (CCR4$^+$ Tregs and CCR4$^-$ Tregs) that express a common marker of regulatory T cells, FoxP3 (15). The two subsets appear to have different homing and functional properties. Similarly as the Tregs described by others (22, 41), CCR4$^+$ Tregs have a memory phenotype (CD45RA$^-$/RO$^+$) and presumably circulate between the periphery (6, 21, 37) (as they exclusively express a skin-homing marker CLA$^+$; Ref. 22) and the secondary lymphoid organs (as they also express LN homing markers CD62L and CCR7) (19). In contrast, CCR4$^-$ Tregs exhibit a naive phenotype (CD45RA$^+$) and do not express cutaneous lymphocyte Ag but do express CCR7 and CD62L. Thus, the majority of CCR4$^-$ Tregs may represent recently reported nnTregs that home to the lymph node (13). Similarly, these Tregs presumably are the CD45RA$^+$ naive subpopulation of the CD62L- and CCR7-expressing Tregs that were reported while this manuscript was in submission (43). Importantly, the CD45RA$^+$ naive Tregs maintained suppressive activity after expansion with high doses of IL-2 in the presence of anti-CD3/CD28 Abs. It is tempting to speculate that the nnTregs (13) or the CD45RA$^+$ naive Tregs (43) as CCR4$^-$ Tregs would not be suppressive if the authors omitted strong stimulations or expansions. Although CCR4$^+$ Tregs are readily suppressive and require no additional stimulations, freshly isolated CCR4$^-$ Tregs cannot inhibit T cell proliferation unless they are stimulated with anti-CD3 Abs. In contrast, CCR4$^-$ Tregs seems to be in a presensitized regulatory state, as a brief pretreatment with suboptimal amounts of anti-CD3 Abs was sufficient to render them suppressive. Moreover, as others observed for nnTregs (13), stimulation with anti-CD3/CD28 Abs up-regulated the expression of CCR4 on CCR4$^-$ Tregs. Thus, we propose that CCR4$^-$ Tregs presumably migrate to

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lymphoid organs, as reported by others for CCR7$^+$ naive and central memory T cells (19), to acquire the TCR-dependent activation signals from Ag-loaded APCs and to differentiate into specialized circulating Tregs such as primed memory-type CCR4$^+$ Tregs (natural/peripheral Tregs). The CCR4$^+$ Tregs, in turn, home and regulate immune responses in the skin (CCR4$^+$CLA$^+$CCR7$^-$ Tregs) or in secondary lymphoid organs (CCR4$^+$CCR7$^+$CD62L$^+$ Tregs). In fact, activated CCR4$^+$ T cells were shown to migrate both toward MDC/CCL22 produced by mature DCs (44) in lymph nodes and inflamed skin (6, 21, 45, 46).

Despite the production of IL-10, the Th2-type non-Treg cells (CD25$^-$CCR4$^+$CD4$^+$) do not affect T cell proliferation. However, they were reported by others to be capable of generating Tregs such as Tr1 (adaptive/inducible Tregs) with cytokine-dependent, anti-proliferative activities (14). For example, IL-10 - producing Tr1 cells were produced from non-Treg CD4$^+$ T cells stimulated with anti-CD3 Ab and other stimuli (18, 47, 48), and IL-10 was implicated in the Treg-mediated protection from inflammatory bowel disease in mice (25, 49). In our hands, both CCR4$^+$ Tregs and CCR4$^-$ Tregs produced IL-10, though the former expressed it at significantly higher levels (Fig. 3*A*). However, similarly as the Tregs described by others (27, 28, 50), these Tregs required cell contact to inhibit T cell proliferation. Conditioned medium from the activated Tregs, which contained significant amounts of IL-10, IL-5, and IFN-γ, failed to inhibit the proliferation of CD8$^+$ T cells. Although a precise mechanism is not yet elucidated, the Tregs characterized here appear to use distinct suppression pathways. It was reported by others that activated Tregs could kill target cells, including CD4$^+$ T cells, CD8$^+$ T cells, using both GZ-A and GZ-B (28). However, we did not detect GZ-A expression by either Treg subsets regardless of anti-CD3/CD28 or IL-2 stimulation. This discrepancy may be due to the nature of the Tregs used or to differences in the type and strength of activation; for example, GZ-A expression may require a prolonged stimulation with the complement regulator CD46 (48). In support, others have reported that unfractionated but activated, CD25$^+$CD4$^+$ Tregs do not express GZ-A but instead use perforin-independent and GZ-B-dependent suppression (27). In our hands, GZ-B was not detected in nonactivated T cells. However, upon activation GZ-B was exclusively expressed in CCR4$^-$ T cells (both Tregs and non-Treg cells), but not in CCR4$^+$ Tregs (Fig. 3*C*), and did not correlate with their suppressive activities. Thus, GZ-B may not be a primary mechanism of regulation, although we cannot rule out its use by activated CCR4$^-$ Tregs, for example, when used at significantly higher cell concentrations (data not shown).

Fas/FasL (CD95/CD95L) signaling plays a major role in the physiological regulation of T cell homeostasis (38), and Fas deficiency on T cells has been associated with the development of long-term allergic airway disease in mice (51). However, FasL/Fas has never been associated with the function of Tregs, including the GZ-A- and GZ-B-expressing Tr1 (28). Our data indicate that FasL is expressed at significant levels on the surface of CCR4$^+$ Tregs even upon brief in vitro culture without any additional stimulation. In contrast, CCR4$^-$ Tregs only expressed FasL after stimulation with anti-CD3/CD28 Abs. Importantly, neutralizing anti-CD95L Ab significantly reversed the inhibitory effects of Tregs on the proliferation of CD8$^+$ T cells. Moreover, although CD8$^+$ T cells can express FasL and modulate suppression, it is unlikely that it played an important role in the suppressions described because FasL (at significantly low levels, data not shown) only started being detected on the surface of CD8$^+$ T cells at the end of the suppression assays. It appears that Tregs use FasL specifically for inhibition of the proliferation of naive T cells without the induction of cell death despite the fact that they were capable of inducing FasL/Fas-mediated apoptosis in model Jurkat cells. Taken together, these data indicate that both CCR4$^+$ Tregs and CCR4$^-$ Tregs use the FasL/Fas signaling pathway, although the latter require additional stimulation via TCR to express FasL and exert regulation. We cannot rule out the involvement of additional suppressive mechanisms, because treatment with neutralizing anti-FasL Ab only partially reversed the Treg-mediated inhibition. At present, it remains unknown whether CCR4$^-$ Tregs differentiate into different Tregs that use alternative suppressive signaling pathways, such as FasL (CCR4$^+$ Tregs) and GZ-B$^+$ (other Tregs).

Similar subsets of Tregs may also exist in mice, though we have only circumstantial evidence due to the lack of proper Abs and model systems. A small proportion (up to 2%) of T cells isolated from the spleens of naive mice coexpressed CCR4$^+$ and FoxP3$^+$ and can be considered CCR4$^+$ Tregs. However, we are unable to determine the exact proportion of murine FoxP3$^+$CCR4$^+$ T cells, because the FoxP3 intracellular staining consistently reduced surface staining for CCR4. Moreover, it remains unknown whether murine CCR4$^+$ Tregs use the same regulatory mechanism as human Tregs, although others reported that unfractionated murine Tregs express GZ-B but not GZ-A (27). We have demonstrated that murine splenic CCR4$^+$CD4$^+$ T cells can inhibit Th1 polarization and T cell proliferation that can be reversed if these cells are specifically depleted. The fact that the depletion leads to a significant increase in tumor Ag-specific CD8$^+$ T cell responses suggests that this strategy may be also used to improve adaptive cellular responses to vaccines. The chemotoxins we developed may have significant clinical value for treatment of autoimmune diseases, tumors, and chronic infections that are controlled by Th2-type cytokine skewed cells and Tregs (7–9). TARC chemotoxins may efficiently shift responses toward Th1 polarization by killing both Th2-type non-Treg CD4$^+$T cells and, at the same time, may deplete CCR4$^+$ Tregs, thereby promoting the activation and proliferation of Ag-specific CD8$^+$ T cells, an essential requirement for the induction of potent therapeutic responses against tumors and chronic diseases.

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Disclosures

The authors have no financial conflict of interest.

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