The Indispensable Role of CCR5 for In Vivo Suppressor Function of Tumor-Derived CD103+ Effector/Memory Regulatory T Cells

Li-Yuan Chang, Yung-Chang Lin, Chiao-Wen Kang, Chen-Yu Hsu, Yu-Yi Chu, Ching-Tai Huang, Yuan-Ji Day, Tse-Ching Chen, Chau-Ting Yeh and Chun-Yen Lin

*J Immunol* 2012; 189:567-574; Prepublished online 4 June 2012;
doi: 10.4049/jimmunol.1200266
http://www.jimmunol.org/content/189/2/567

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/06/04/jimmunol.1200266.DC1

References

This article cites 34 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/189/2/567.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Indispensable Role of CCR5 for In Vivo Suppressor Function of Tumor-Derived CD103+ Effector/Memory Regulatory T Cells

Li-Yuan Chang,† Yung-Chang Lin,‡§ Chiao-Wen Kang,*† Chen-Yu Hsu,*† Yu-Yi Chu,† Ching-Tai Huang,‡§ Yuan-Ji Day,‡‡ Tse-Ching Chen,‡§ Chau-Ting Yeh,*†§ and Chun-Yen Lin*†,‡

CD103 is a marker for identification of effector/memory regulatory T cells (Tregs). CD103+ Tregs are potent suppressors of tissue inflammation in several infectious diseases, autoimmune diseases, and cancers. However, the underlying mechanisms for this potent suppression ability remain unclear. The current study was designed to clarify this issue. Unexpectedly, we found both CD103+ and CD103− Tregs had similar suppression capacity in vitro. We then chose a murine tumor model for investigation of the in vivo behavior of these Tregs. The suppression ability in vivo against the anti-tumor ability of CD8+ T cells was restricted to CD103+ Tregs although both Tregs had equal in vitro suppression ability. In addition, CD103+ Tregs expressed significantly higher levels of CCR5 than those of CD103− Tregs and accumulated more in tumors than did CD103− Tregs. Furthermore, blockade of CCR5 signaling, either by CCR5−/−CD103+ Tregs or by CCL5 knockdown tumor, could reduce the migration of CD103+ Tregs into tumors and impair their in vivo suppression ability. In conclusion, these results indicate that the potent in vivo suppression ability of CD103+ Tregs is due to the tissue-migration ability through CCR5 expression.

The Journal of Immunology, 2012, 189: 567–574.

R egulatory T cells (Tregs) play an important role in the maintenance of immunological tolerance and in control of excessive immune responses against self-antigens (1, 2). Recently, Tregs have been shown to be phenotypically and functionally heterogeneous and composed of effector/memory Tregs and naïve Tregs (3–7). CD103 is a cell surface protein of the integrin family and is proposed as a marker for effector/memory Tregs in mice (3–6). These CD103+ Tregs are more potent in suppressing harmful inflammatory reactions compared with CD103− Tregs in different mouse models of autoimmune diseases and Leishmania infection (3, 4, 6). In contrast, we also reported that CD103+ Tregs were potent suppressors of anti-tumor immune responses in tumor-bearing mice (8). Recent studies have also shown that CD103 is a hallmark of tumor-infiltrating Tregs in TGF-β-secreting tumor (9). However, the mechanisms underlying the control of immune responses by CD103+ Tregs remain unclear.

It has been reported that a preferential migration of Tregs into inflamed tissue may be driven by differences in homing signaling in models of infection/inflammation and malignancy (10–12). The high expression levels of chemokine receptors have been demonstrated to promote such preferential migration of Tregs into tissue sites of disease (10–12). Recent studies reported that inflammation-related chemokine receptor CCR5 is highly expressed on CD103+ Tregs compared with that on CD103− Tregs (6). However, the role of CCR5 in this potent in vivo suppression ability of CD103+ Tregs remains to be identified.

In this study, we found that CD103+ Tregs had similar in vitro suppression ability as that of the CD103− Tregs. We then used a murine tumor model to study the in vivo suppression ability of CD103+ Tregs. These CD103+ Tregs from tumor-bearing mice still showed the effector/memory phenotype. The numbers of CD103+ Tregs were increased with tumor progression, and these CD103+ Tregs markedly migrated into tumors. In addition, these CD103+ Tregs showed more potent in vivo suppression ability than that of CD103− Tregs, although their in vitro suppression ability was similar. In addition, the inflammatory chemokine receptor CCR5 was highly expressed on CD103+ Tregs, especially in the tumor microenvironment. Furthermore, CCL5–CCR5 signaling was not only associated with the preferential migration of CD103+ Tregs into tumors but also required for controlling anti-tumor response of CD8+ T cells by CD103+ Tregs. These results demonstrate the potent in vivo suppression ability of the CD103+ Tregs is not due to the stronger suppression ability per cell but due to the tissue-migration ability through CCR5 expression.

Materials and Methods

Mice and cell lines

BALB/c mice were purchased from the National Laboratory Animal Center of Taiwan (Taipei, Taiwan), and CByJ.PL(B6)-Thy1a/Scrj (Thy1.1, BALB/c
background), RAG1−/− (BALB/c background), CCR3−/− (BALB/c background), and CCR5−/− (C57BL/6 background) mice were purchased from The Jackson Laboratory. CCR5−/− mice were fully back-crossed to BALB/c background for more than 10 generations. All mice were maintained in the animal house of Chang Gung Memorial Hospital and used in experiments at ages 8–10 wk. All animal breeding and experiments were in accordance with guidelines of the institutional animal ethics committee. CT26 mouse colon carcinoma cell line and NIT 3T3 mouse fibroblast cell line were purchased from the American Type Culture Collection and were maintained in our laboratory. CT26 cells and NIT 3T3 cells were regularly cultured in RPMI 1640 medium and DMEM containing 10% FBS and antibiotics.

**Semiquantitative RT-PCR**

Cell lines and tumor tissues were homogenized with 1 ml TRI Reagent to extract total RNA. The quality of isolated RNA was examined by ethidium bromide staining of RNA separated in an agarose gel, and the concentration of RNA was determined spectrophotometrically. Semiquantitative RT-PCR was performed as previously described (13, 14). Previously described primers of the targeted genes were used in the study (13, 14).

**CCL5 short hairpin RNA transfection**

Knockdown of CCL5 secretion from CT26 cells was performed as previously described (14). Knockdown of CCL5 was done by RNAi retro GIPZ lentiviral shRNAmir starter kits following the manufacturer’s instructions (Open Biosystems). Two CCL5 (NM_013653) RNA interferences were used with three RNA interference targets: GAPDH lentiviral shRNAmir, EG5 lentiviral shRNAmir, and nonsilencing control shRNAmir. After 48 h of transfection, CT26 cells were selected in 60 μg/ml puromycin to enrich the pure population. CCL5 knockdown of CT26 cells was determined by ELISA (R&D Systems) of culture supernatants.

**Flow cytometry**

For flow cytometry analysis, single-cell suspensions of the spleen and tumors were prepared. To isolate tumor-infiltrating cells, tumors were chopped into small pieces and incubated with collagenase IV (0.1%; Sigma-Aldrich) in HBSS for 30 min at 37°C. After passing through a cell strainer, single-cell suspensions were separated with Ficoll, and leukocytes were recovered from the interphase. All Abs were purchased from BD Biosciences or eBioscience, except for anti-CCR3 mAb (R&D Systems) and anti-CCR4 mAb (BioLegend). Single-cell suspensions were stained for surface or intracellular proteins as previously described (8, 14).

**Cell purification**

Donor mice (naive mice or day 28 tumor-bearing mice) were sacrificed, and their spleens were harvested under sterile conditions. Single-cell suspensions were prepared, and CD8+ T cells were sorted using magnetic microbeads conjugated with anti-mouse CD8 (Miltenyi Biotech, Bergisch-Gladbach, Germany) by AutoMACS (Miltenyi Biotech). For CD4+CD25+ effector T cell, CD4+CD25+CD103− T cell, and CD4+CD25+CD103+ T cell purification, single-cell suspensions were first enriched for CD4+ T cells via negative selection using the CD4 isolation kit (Miltenyi Biotech). Enriched CD4+ T cells were then labeled with CD25–allophycocyanin and CD103–PE and sorted using FACSaria (BD Biosciences). Cell purity (>90%) for all populations was confirmed by flow cytometry.

**In vitro suppression assay**

Purified CD4+CD25+ effector T cells (5 × 10^5) and mitomycin C-treated syngeneic BALB/c T cell-depleted splenocytes (5 × 10^5) were mixed with different numbers of CD4+CD25+CD103− T cells or CD4+CD25+CD103+ T cells and incubated in round-bottom 96-well tissue culture plates with 10 μg/ml anti-CD3 mAb. The cultures were pulsed with 1 μCi [3H]thymidine for the last 16–18 h of the 72-h culture period. The cultures were harvested with a Packard Micromate cell harvester. Determination of the amount of incorporated radioactive counts was performed with a Packard Matrix 96 direct counter.Culture supernatants were harvested after 48 h after inoculation, and determination of IFN-γ in the supernatant was measured using ELISA kits (BD Biosciences).

**In vivo suppression assay**

CD8+ T cells and CD4+CD25+CD103− T cells as well as CD4+CD25+CD103+ T cells were isolated from spleen of day 28 CT26 tumor-bearing...
mice. BALB/c mice were inoculated s.c. with $1 \times 10^7$ CT26 cells. On day 1 after tumor inoculation, $1 \times 10^6$ CD8+ T cells were cotransferred with either $3 \times 10^5$ CD4+CD25+CD103+ T cells or $3 \times 10^5$ CD4+CD25- CD103- T cells into these mice. Tumor growth was evaluated for suppressive capacity of Tregs. Tumor-bearing mice without T cell adoptive transfer were used as control for tumor growth. Tumor volume was calculated according to the formula: $V = (\text{length} \times \text{width})^2/2$.

Adoptive transfer of Treg subsets for analysis of migration behavior (homing)

For in vivo homing assays, RAG1−/− mice or BALB/c mice were inoculated with CT26 tumor cells. On day 7 after tumor inoculation, Treg subsets were isolated from spleen of day 28 CT26 tumor-bearing mice and transferred into the mice. On day 14 after tumor inoculation, the percentage and absolute number of CD3+Foxp3+ cells in the spleen and tumor were evaluated for migration behavior of Tregs.

Statistical analysis

Two-tailed unpaired $t$ test was used for statistical analyses of differences between groups. The calculations were made using PRISM version 5.00 (GraphPad Software). Differences were recognized as significant at $p < 0.05$.

Results

**CD103+ Tregs express an activated/memory phenotype but with similar suppressive ability to that of CD103− Tregs in vitro**

The integrin CD103 has been identified as a marker for effector/memory Tregs (3–6). To characterize further the behavior of CD103+ Tregs, we first examined the phenotype of CD103+ Tregs from the spleen of wild-type (WT) mice. CD103+ Tregs expressed significantly higher levels of CD69, LAG-3, CD44, ICOS, CTLA-4, and GITR but significantly lower levels of CD62L compared with those of CD103− Tregs (Fig. 1A). These results indicate that CD103+ Tregs have an activated/memory phenotype in WT mice. Subsequently, we compared the suppressive capacity of CD103+ Tregs with CD103− Tregs in vitro. The purity and the expression levels of Foxp3 in sorted CD103+ Tregs were similar to sorted CD103− Tregs (Fig. 1B). Out of our expectation, CD103+ Tregs showed no difference in suppression of CD4+CD25− effector T cell proliferation and IFN-γ production in vitro compared with CD103− Tregs (Fig. 1C, 1D).

**CD103+ Tregs are more potent than CD103− Tregs in suppressing the anti-tumor ability of CD8+ T cells in vivo**

CD103+ Tregs had been shown to be more potent suppressors in vivo than CD103− Tregs in different mouse models of autoimmune diseases (3, 4, 6). It was then interesting to understand this discrepancy between the in vivo and in vitro behavior of CD103+ Tregs. We therefore chose the mouse CT26 colon tumor model to study this issue. As shown in Fig. 2A, both the frequencies and absolute numbers of CD103+ Tregs in the spleen were increased with tumor progression. These CD103+ Tregs still had an activated/memory phenotype (Fig. 2B). In addition, the in vitro suppression abilities of CD103+ Tregs were also similar to those of CD103− Tregs in tumor-bearing mice (Fig. 2C, 2D). In contrast, the in vivo suppressive capacity of CD103+ Tregs was evaluated by cotransfer of CD8+ T cells with CD103+ Tregs or
CD103+ Tregs into the mice 1 d after CT26 tumor inoculation. Notably, CD103+ Tregs were more potent than CD103- Tregs in suppressing CD8+ T cell tumor inhibition potency (Fig. 2E). Taken together, these results indicate that although the in vitro suppression abilities are similar between CD103+ Tregs and CD103- Tregs, these CD103+ Tregs are more potent than CD103- Tregs in suppressing the tumor inhibition abilities of CD8+ T cells in vivo.

CD103+ Tregs accumulate in the tumor microenvironment and express high levels of CCR5 in tumor-bearing mice

It was reported that CD103+ Tregs were enriched within lymphoid tissues draining an inflammatory site or an inflammatory site (4, 5). We next examined whether CD103+ Tregs could accumulate in the tumor-draining lymph node or in tumors. As shown in Fig. 3A, CD4+Foxp3+ Tregs markedly accumulated in tumors compared with that in the spleen and tumor-draining lymph node. The majority of CD4+Foxp3+ Tregs in tumors, but not those from the spleen and tumor-draining lymph node, expressed high levels of CD103 (Fig. 3A). These results indicate that CD103+ Tregs, similar to the accumulation in the inflammation site, are enriched within the tumor microenvironment.

It has been shown that chemokine receptors CCR3, CCR4, and CCR5 could mediate trafficking of Tregs to tumors (8, 14–16). We next examined the expression of these chemokine receptors on CD103+ Tregs in the spleen and tumors of mice bearing CT26 tumors. As show in Fig. 3B, CD103+ Tregs in the spleen and tumors expressed high levels of CCR3 and CCR5 compared with those of CD103- Tregs. However, CCR4 was highly expressed on CD103+ Tregs in the spleen, but not in the tumor microenvironment (Supplemental Fig. 1A). In contrast, only CCR5 expression but not CCR3 or CCR4 expression on CD103+ Tregs in the tumor microenvironment was significantly increased (Fig. 3B and Supplemental Fig. 1A). These results indicate that CCR5 is highly expressed on CD103+ Tregs especially in the tumor microenvironment.

CCR5 expression is associated with migration of CD103+ Tregs into tumors

To evaluate the migrating ability of CD103+ Tregs to tumor sites, we sorted CD103+ Tregs and CD103- Tregs from the spleen of mice bearing CT26 tumors and transferred them into RAG1-/- mice 7 d after CT26 tumor inoculation (Fig. 4A). We observed that both the transferred CD103+ Tregs and CD103- Tregs proliferated in the spleen and tumors at similar rates (Supplemental Fig. 2). In contrast, the transferred CD103+ Tregs migrated into tumors more efficiently than CD103- Tregs (Fig. 4B, 4C). In addition, there was no difference between the transferred CD103+ Treg and CD103- Treg numbers in the spleen. These results indicate that CD103+ Tregs migrate preferentially to tumors but not the spleen. To investigate whether CCR5 or CCR3 expression affects the migration of CD103+ Tregs into tumors, we also sorted

---

**FIGURE 3.** CD103+ Tregs accumulate in the tumor microenvironment and express high levels of CCR3 and CCR5. (A) Flow cytometric analysis of splenocytes, tumor-draining lymph node cells (DLN), and tumor-infiltrating cells from day 28 tumor-bearing mice show the frequency of CD103+CD4+Foxp3+ T cells. The graphs summarize data showing the frequency of CD4+Foxp3+ T cells and CD103+CD4+Foxp3+ T cells from different compartments of analyzed animals. (B) The expression levels of CCR3 and CCR5 on CD103+CD4+Foxp3+ T cells and CD103-CD4+Foxp3+ T cells from the spleen and tumors of day 28 tumor-bearing mice were determined by flow cytometry. (C) Flow cytometric analysis of splenocytes and tumor-infiltrating cells from day 28 tumor-bearing mice show the expression levels of CCR3 and CCR5 on CD4+Foxp3+ T cells. Data show mean ± SEM of n = 5 and are representative of three independent experiments. **p < 0.01.
CD103+ Tregs from the spleen of CCR5^{−/−} and CCR3^{−/−} mice bearing CT26 tumors and transferred them into RAG1^{−/−} mice 7 d after CT26 tumor inoculation. The transferred CCR5^{−/−}-CD103+ Tregs but not the transferred CCR3^{−/−}-CD103+ Tregs had a decreased ability to migrate into tumors (Fig. 4B, 4C). Thus, these results indicate that CCR5 expression is associated with the preferential migration of CD103+ Tregs into the tumor microenvironment.

**Tumor-derived CCL5 promotes the migration of CD103+ Tregs into tumors**

We next examined the gene expression of CCR5 and CCR3-associated chemokines in CT26 tumor cells. As shown in Fig. 5A, CCL5 mRNA, but not other chemokines, was highly expressed in CT26 cells compared with that in NIH 3T3 fibroblast cells. Secretion of CCL5 from CT26 cells was confirmed by ELISA performed on culture supernatants (Supplemental Fig. 3). To investigate whether secretion of CCL5 from CT26 cells could mediate trafficking of CD103+ Tregs to tumors, we then knocked down CCL5 secretion from CT26 cells by using lentiviral short hairpin RNA (CCL5 knockdown CT26 cells) (Supplemental Fig. 3). As shown in Fig. 5B and Supplemental Fig. 4, we sorted CD103+ Tregs from the spleen of mice bearing CT26 tumors and transferred them into RAG1^{−/−} or BALB/c mice 7 d after control CT26 tumor inoculation or CCL5 knockdown CT26 tumor inoculation. In the mice with CCL5 knockdown CT26 tumor inoculation, the number of the transferred CD103+ Tregs was fewer in tumors but was similar in the spleen compared with that in mice with control
CT26 tumor inoculation (Fig. 5C and Supplemental Fig. 4). These results indicate that secretion of CCL5 from tumors could be responsible for the recruitment of CD103+ Tregs into tumors.

CCR5<sup>−/−</sup> CD103<sup>+</sup> Tregs fail to suppress anti-tumor ability of CD8<sup>+</sup> T cells in vivo

To examine whether the CCR5 or CCR3 expression affects in vitro and in vivo suppression capacity of CD103<sup>+</sup> Tregs, we sorted CD103<sup>+</sup> Tregs from the spleen of WT, CCR5<sup>−/−</sup>, or CCR3<sup>−/−</sup> mice bearing CT26 tumor. As shown in Fig. 6A and 6B, lack of CCR5 or CCR3 expression on CD103<sup>+</sup> Tregs did not affect their ability to suppress proliferation and IFN-γ release of CD4<sup>+</sup> effector T cells in vitro. In contrast, in the adoptive transfer study, only CCR5<sup>−/−</sup>-CD103<sup>+</sup> Tregs failed to suppress CD8<sup>+</sup> T cell tumor inhibition potency in vivo (Fig. 6C). Thus, these results clearly indicate that CCR5 expression is required for the in vivo suppression capacity of CD103<sup>+</sup> Tregs in tumor-bearing mice.

Discussion

The integrin CD103 is helpful to distinguish distinct lineages or differentiation stages of Tregs. CD103<sup>+</sup> Tregs express an activated/memory phenotype with low expression levels of CD62L and high expression levels of CD44 (4, 6). These cells display potent in vivo suppression capacity in suppressing inflammatory diseases in mouse models of autoimmune diseases, such as colitis, chronic graft-versus-host disease, and arthritis (3, 4, 6). In the current study, we found there was no difference in vitro suppression capacity between CD103<sup>+</sup> and CD103<sup>−</sup> Tregs. We then chose the murine tumor model to explore the possible mechanisms of in vivo suppression ability of CD103<sup>+</sup> Tregs. We found that CD103<sup>+</sup> Tregs accumulated in the tumor microenvironment. In addition, similar to CD103<sup>+</sup> Tregs from WT mice, CD103<sup>+</sup> Tregs from tumor-bearing mice also displayed an activated/memory phenotype. In addition, these CD103<sup>+</sup> Tregs had more potent in vivo suppressive ability in inhibiting CD8<sup>+</sup> T cell-mediated antitumor immune responses than that of CD103<sup>−</sup> Tregs, although both Tregs held similar in vitro suppression capacity. Furthermore, the expression of CCR5 on the CD103<sup>+</sup> Tregs contributed significantly to the in vivo tumor-protection ability mainly through the migration into the tumor microenvironment. Thus, these observations suggest the potent in vivo suppression ability of the CD103<sup>+</sup> Tregs is not due to the stronger suppression ability per cell but possibly due to the tissue-migration ability through CCR5 expression.

Recent studies have shown that the percentage of CD103<sup>+</sup> Tregs increased in the tumor microenvironment in four different murine models of cancer (9). It was suggested that tumor-derived TGF-β could induce the expression of CD103 on tumor-infiltrating Tregs directly in the tumor microenvironment (9). However, blockade of
FIGURE 6. CCR5<sup>−/−</sup>CD103<sup>+</sup> Tregs fail to inhibit anti-tumor responses of CD8<sup>+</sup> T cells. CD8<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup>CD103<sup>+</sup> T cells as well as CD4<sup>+</sup>CD25<sup>+</sup> effector T cells were purified from splenocytes of day 28 tumor-bearing BALB/c mice. CCR3<sup>−/−</sup>CD4<sup>+</sup>CD25<sup>+</sup>CD103<sup>+</sup> T cells and CCR5<sup>−/−</sup>CD4<sup>+</sup>CD25<sup>+</sup>CD103<sup>+</sup> T cells were purified from splenocytes of day 28 tumor-bearing CCR3<sup>−/−</sup> and CCR5<sup>−/−</sup> mice. (A) Effector T cells were cocultured with different CD103<sup>+</sup> Tregs in the presence of soluble anti-CD3 (10 μg/ml) along with mitomycin C-treated T cell-depleted splenocytes. Cultures were pulsed with [3H]thymidine for the last 16–18 h of a 72-h culture period. (B) Forty-eight hours after cultures, supernatants were collected and assayed for IFN-γ production by ELISA. (C) BALB/c mice were inoculated with 1 × 10<sup>6</sup> CT26 cells, and 1 d later, CD8<sup>+</sup> T cells alone (1 × 10<sup>6</sup>; ▼) or together with WT CD4<sup>+</sup>CD25<sup>+</sup> CD103<sup>+</sup> T cells (3 × 10<sup>5</sup>; △), CCR3<sup>−/−</sup>CD4<sup>+</sup>CD25<sup>+</sup> CD103<sup>+</sup> T cells (3 × 10<sup>5</sup>; ▽), or CCR5<sup>−/−</sup>CD4<sup>+</sup> CD25<sup>+</sup>CD103<sup>+</sup> T cells (3 × 10<sup>5</sup>; ○) were cotransferred into these mice. The control group of mice was inoculated with tumors but without cell transfer (●). Data show mean ± SEM of n = 5 and are representative of three independent experiments. *p < 0.05.
study, our findings showed CCR5 signaling was important for CD103+ effector/memory Tregs in control of anti-tumor immune responses. In contrast, CCR5 antagonists are available for treatment of HIV infection in several clinical trials (34). Thus, CCR5 antagonists may be another choice for targeting effector/memory Tregs in cancer immunotherapy.

In conclusion, this study demonstrates that CD103+ effector/memory Tregs had similar suppression ability as that of CD103+ Tregs in vitro. However, the tissue-migration ability through CCR5 expression on CD103+ effector/memory Tregs determined their potent in vivo suppression ability on tissue inflammation, as demonstrated in this study by a murine tumor model.

Disclosures
The authors have no financial conflicts of interest.

References