CCL5 secreted by tumor could not only attract tumor-protecting regulatory T cells but also enhance the killing ability of regulatory T cells on CD8+ T cells.

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Abstract
Effective/memory regulatory T cells (Treg cells), a subgroup of Treg cells, can protect tissue from injury by migrating to inflamed tissue and suppressing aggressive immune responses locally. Previously, we have found these effecter/memory Treg cells increased in the spheres of the tumor-bearing mice and accumulated around the tumor. In addition, these Treg cells (collectively as tumor-protecting Treg cells) were responsible for the inhibition of anti-tumor immunity in a murine colon cancer (CT26) model. However, suppression mechanisms for tumor-protecting Treg cells were still unclear. In the present study, we found a chemokine receptor, CCR5, was highly expressed in these tumor-protecting Treg cells. Furthermore, these CCR5-expressed Treg cells were also responsible for protecting tumor from tumor-specific CD8+ T cells demonstrated both in vivo assay and in vitro adoptive transfer model. Intersecting, CT26 tumor cells could also secrete CCL5, a chemokine that attracts CCR5-expressed T cells. When we knocked-down the CCL5 secretion in CT26 tumor cells (F6 tumor), these tumor cells grew slowly in wild type animal but not in nude mice when compared with wild-type CT26. The CCR5 expressed Treg cells were fewer in the spleen and tumor site in these F6 bearing mice if compared with wild type CT26. However, the ratios of Treg to CD8+ T cells in tumor sites were similar between mice with F6 tumor and mice with CT26 tumors. But, interestingly, the percentage of annexin V expressed CD8+ T cells was significantly higher in CT26 tumor sites than F6 tumor sites. Following these observations, we then showed the evidence that CCL5 could enhance the killing ability of effector/memory Treg cells on the isolated CD8+ T cells. Furthermore, this enhancement was mediated through TGF-β production, PD1L and FDL2 expression on Treg cells. Taken together, these data suggested CCL5 secreted by tumor could not only recent CCR5-expressed tumor-protecting Treg cells to tumor microenvironment but also enhance their killing ability on CD8+ T cells.

Results

Fig. 1. Splenic CCR5+Treg cells from day-28 tumor-bearing mice express effector/memory phenotype and diminish tumor-inhibition potency of CD8+ T cells. (A) Absolute number of CCR5+Treg cells in spleen of naive mice and day-28 tumor-bearing mice (B). Expression levels of CD103, CD39, CD69, CD25, and CD38 on CCR5+Treg cells in spleen of naive mice and day-28 tumor-bearing mice (C) Scheme of this experiment. CCR5+Treg cells, CCR5–Treg cells and CD8+ T cells were sorted from splenocytes from day-28 tumor-bearing mice (day-28 CT26 T cells). These CD8+ T cells (1 x 106) were transferred alone (1) or co-transferred with either CCR5+Treg cells (3 x 105) (2) or CCR5–Treg cells (3 x 106) (3) into one day tumor-bearing mouse. Control mice were inoculated with CT26 tumor cells but without adoptive transfer of T cells (1). The growths of the tumor is different groups were depicted (D). Data represent mean ± SEM of n=6 mice. Results are representative of three independent experiments. * p<0.05 for statistical analysis with Mann-Whitney U test and ** p<0.01, NS, not significant.

Fig. 2. Tumor infiltrating Treg cells that express high levels of CCR5 increased tumor progression. (A) The percentage of CD8+ T cells in total T cells was measured in different compartments of day-28 tumor-bearing mice. (B) The percentage of Treg cells in total CD8+ T cells was measured in spleen of naive mice and tumor infiltrating lymphocytes (TILs) of day-7, day-14, day-21 and day-28 tumor-bearing mice. The expression of CCR5 on CCR5+ T cells in spleen of naive mice and TILs of day-7 and day-28 tumor-bearing mice were analyzed by flow cytometry. Data represent mean ± SEM of n=6 mice. Results are representative of three independent experiments. * p<0.05 for statistical analysis with Mann-Whitney U test and ** p<0.01, NS, not significant.

Fig. 3. Knockdown of CCL5 secretion by CT26 tumor cells slow tumor growth and diminish the prevalence of tumor-protecting Treg cells in tumor microenvironment. (A) The expression of CCR5-associated chemokine genes on CT26 tumor cells and mouse fibroblasts NIH 3T3 cells were measured in vitro. (B) Secretion of CCL5 by CT26 tumor cells and mouse fibroblasts NIH 3T3 cells were measured in vitro. (C) Secretion of CCL5 by either CCR5+ or CCR5– tumor cells or CT26 tumor cells with a lentiviral vector producing shRNA to CCL5 cultured in vitro. (D) BALB/c mice were inoculated with either 1x105 wild type CT26 tumor cells (1) or 1x105 CT26 tumor cells transduced with control lentivector ((1) Nude mice (2) BALB/c mice) (2) or pre-treated with either 1x105 CCR5– tumor cells (control lentiviral vector) (1) or 1x105 CCL5 low CT26 tumor cells (2). Growth pattern of tumors are shown (E). The percentage of Treg cells in total CD4+ T cells in different compartments of day-28 tumor-bearing mice (G), and the expression of CCR5 on tumor infiltrating Treg cells from day-28 tumor bearing site (H) were determined by flow cytometry. Data represent mean ± SEM of n=6 mice. Results are representative of three independent experiments. * p=0.05 for statistical analysis with Mann-Whitney U test and ** p<0.01, NS, not significant.

Fig. 4. Knockdown of CCL5 secretion in secondary CT26 tumor cells restore concomitant tumor immunity in day-28 tumor-bearing mice through decreased apoptosis of CD8+ T cells in secondary tumor. (A) Scheme of this experiment. 1x105 CT26 tumor cells (control lentiviral vector) were inoculated on the flanks of BALB/c mice on day 0. Mice were divided into two groups, and secondary challenges with 5x104 CT26 tumor cells (control lentiviral vector) (Group B) (1) or 5x104 CCL5–/– CT26 tumor cells (Group D) (1) were made into the contra lateral flank on day 28, respectively. Growth pattern of CT26 tumor cells (control lentivector) (Group A) (1) or CCL5–/– CT26 tumor cells (Group C) (1) in mice without previous tumor inoculation as tumor growth control are shown. On day 11 after secondary tumor inoculation, secondary tumor size (B) was measured and the percentage of Treg cells in total CD4+ T cells (C), the ratio of CD8+ T cell to Treg cells (E), the expression of activated markers and effector molecules on CD8+ T cells (F) and the annexin V staining of CD8+ T cells (G) in secondary tumor mass were analyzed by flow cytometry. Each dot represents one individual sample and mean percentages in each group are shown. * p=0.05 for statistical analysis with Mann-Whitney U test and ** p<0.01, NS, not significant.

Conclusion

CCL5+ Treg cells increased with tumor progression and inhibited tumor-inhibition potency of CD8+ T cells

Tumor derived CCL5 contributed to tumor progression

To recruit CCR5+ Treg cells to tumor microenvironment

To enhance Treg cells function to increase the apoptosis of CD8+ T cells through TGF-β and PD1 signaling