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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
A Ligand for the Chemokine Receptor CCR7 Can Influence the Homeostatic Proliferation of CD4 T Cells and Progression of Autoimmunity

Corinne Ploix, David Lo, and Monica J. Carson

Homeostasis of T cell numbers in the periphery implies an ability of lymphocytes to sense cell numbers. Although the mechanisms are unknown, we find that the chemokine CCL21 (also known as TCA4, SLC, or 6Ckine), a ligand for the chemokine receptor CCR7, can regulate homeostasis of CD4 (but not CD8) T cells. In the absence of CCR7 ligands, transferred CD4 T cells failed to expand in lymphopenic hosts, whereas in the presence of CCL21 overexpression, homeostatic CD4 T cell proliferation occurred even in nonlymphopenic recipients. Ag-specific CD4 T cells transferred into Ag-expressing mice proliferated and induced autoimmunity only in lymphopenic recipients. Pancreatic expression of CCL21 was sufficient to replace the requirement for lymphopenia in the progression of autoimmune disease. These results suggest that CD4 T cells use local concentrations of CCR7 ligands as an index of T cell steady state numbers and that homeostatic expansion of the T cell population may be a contributing factor in the development of autoimmune disease. The Journal of Immunology, 2001, 167: 6724–6730.

In the healthy and unperturbed mammal, the numbers of T lymphocytes in circulation and the ratio of CD4/CD8 T lymphocytes appear to be determined primarily by the numbers and ratios exported from the thymus (1). However, after induction of severe lymphopenia, the numbers of CD4 and CD8 T lymphocytes return to a steady state “set point” that is species and strain specific (2–4). In aged mammals, thymic output of lymphocytes declines and peripheral mechanisms regulating circulating levels are thought to play an increased role in T cell homeostasis (5–7).

How the lymphocyte population recognizes this homeostatic set point is an unanswered question, though the persistence of naive CD4 T cells in the absence of Ag stimulation appears to depend at least in part on low-level stimulation by APCs (8–12). We hypothesized that any mechanism for sensing the steady state numbers of lymphocytes would occur primarily within lymphoid tissues and would depend on the detection of local concentrations of a stromal cell factor produced at a fixed rate. Excess amounts of this factor (representing lymphopenia) would be permissive for homeostatic expansion, whereas low levels (representing absorption by a full lymphocyte complement) would limit expansion (13). Thus, homeostatic proliferation of T cells would be driven not by a global ability to detect “space” in the immune system (14, 15), but instead by the ability to measure the local concentrations of this survival or nurturing factor. Recent evidence suggests that CD4 and CD8 T cells may depend on different factors (16). For instance, production of IL-7 and IL-15 by stromal cells has been suggested to regulate the numbers of CD8 T cells but not CD4 T cells (17).

In the context of our hypothesis, we considered the ligands for the chemokine receptor CCR7 (18–24) to be strong candidates for factors regulating homeostatic proliferation of CD4 T cells for the following reasons. Naive CD4 T cells express the CCR7 receptor (22, 23). CCR7 has two ligands, CCL21 (also known as TCA4, SLC, or 6Ckine) and CCL19 (also known as ELC), and these chemokines are expressed in sites relevant to the regulation of homeostasis. Specifically, CCL21 and CCL19 are coexpressed at high constitutive levels by the stromal reticular cells located in the lymphoid T cell compartments. CCL21 is also expressed by the high endothelial venules in lymphoid tissue (18–21). In addition, transgenic expression of the CCR7 ligand CCL21 within pancreatic islet β cells (Insulin promoter-TCA4 transgenic mice) was sufficient to induce the formation of lymphoid-like structures within the islets (24). This accumulation of T lymphocytes around CCL21-expressing islets was not associated with a decrease in T lymphocyte numbers in other peripheral lymph nodes or in spleen, suggesting that transgenic expression of excess CCL21 was able to support the survival of a larger total T lymphocyte pool (nearly 20% larger). In this study, we manipulate the levels of CCL21 expression and directly test whether CCL21 availability can regulate homeostatic proliferation and progression toward autoimmunity.

Materials and Methods

Mice

Insulin-influenza hemagglutinin (Ins-HA), TCR-SFE, and Ins-TCA4 transgenic mice were generated and characterized as previously described (24, 25). All mice were housed in the vivarium at The Scripps Research Institute (La Jolla, CA) in accordance with institutional and National Institutes of Health guidelines.

CFSE labeling and T cell transfer

T cells were isolated from murine spleens as previously described (26). The resulting cell populations comprised >90% Thy-1.2+ cells, as determined by flow cytometry. For CFSE labeling, T cells were resuspended at 1–5 × 10^6/μl in RPMI 1640 supplemented with 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Ten microliters of 5 μM CFSE stock solution (Molecular Probes) was added to 5× 10^5 T cells and incubated for 10 min at 37°C. Unlabeled T cells were then added to reach the desired final concentration. After incubation for 20 min at 37°C, T cells were washed and cultured as indicated. Results were expressed as the mean ± SD of triplicate determinations.

Received for publication June 1, 2001. Accepted for publication October 5, 2001.

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1 This work was supported by National Institutes of Health Grant NS39508 (to M.J.C.), Scripps Neuro-AIDS Preclinical Studies Grant P30-MH62261 (to M.J.C.), and the Fondation pour la Recherche Médicale and the Christopher Reeve Paralysis Foundation (postdoctoral fellowships to C.P.).

2 Address correspondence and reprint requests to Dr. Monica J. Carson, Department of Molecular Biology, The Scripps Research Institute, MB 10, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: carson@scripps.edu
10^7/ml and incubated for 10 min at 37°C with 10 μM CFSE (Molecular Probes, Eugene, OR). Cells were then washed three times with 10 ml of ice-cold PBS. An aliquot from the labeled population was stored for later flow cytometric analysis to determine the parameters of the nondividing cell population. Ten million T cells were injected i.v. into recipient mice (8–10 wk old) via the retro-orbital vein. Irradiated recipients were exposed to 600 rad. Proliferation of CFSE-positive cells was analyzed in T cells recovered from spleen, peripheral, and pancreatic lymph nodes using FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA). 1, 3, 5, and 7 days post-transfer into recipient mice. In all experiments, recovered T cells were also labeled with anti-CD4 or anti-CD8 Abs (either PE- or allophycocyanin-conjugated). Therefore, selective gating on each T cell population during flow cytometric analysis allowed CD4 T cell proliferation to be examined separately from CD8 T cell proliferation.

**Histology**

Immunohistochemistry was performed on cryosections (6–10 μm) fixed in ice-cold acetone as previously described (22). Sections were stained with the purified rat anti-mouse primary Abs listed below (BD Pharmingen, San Diego, CA), followed by biotin (Fab',mouse anti-rat IgG (Jackson Immunoresearch Laboratories, Westbury, NY), and streptavidin-HRP (The Jackson Laboratory, Bar Harbor, ME). Positive cells were visualized by 3-amino 9-ethylcarbazole substrate (Sigma-Aldrich, St. Louis, MO), and tissue was counterstained with hematoxylin. The following Abs were used: anti-CD4, anti-B220, anti-mucosal addressin cell adhesion molecule-1, anti-pancreatic lymph node addressin, and anti-ER-TR7 (Accurate Chemical and Scientific, Westbury, NY).

**Flow cytometry**

PE-conjugated anti-CD4, allophycocyanin-conjugated anti-CD8, biotinylated anti-Vβ8.1/8.2, and streptavidin-CyChrome Abs (BD Pharmingen) were used to stain cell suspensions from spleen and lymph nodes. The activation state of CFSE-labeled CD4 and CD8 T cells was analyzed by flow cytometry using PE- and allophycocyanin-conjugated anti-mouse CD4, anti-mouse CD8, anti-mouse CD62L, and anti-mouse CD44 Abs. CFSE-positive and immunostained cells were analyzed using FACS Calibur and CellQuest software. In all experiments, recovered T cells were also labeled with anti-CD4 or anti-CD8 Abs (either PE- or allophycocyanin-conjugated). Therefore, selective gating on each T cell population during flow cytometric analysis allowed CD4 T cell activation to be examined separately from CD8 T cell activation.

**Serological analysis of diabetes**

Diabetes was characterized by polyuria/polydipsia, weight loss, glycosuria as assessed by urine chemistries (Bayer, Wuppertal, Germany), and persistent hyperglycemia (>250 mg/dl), as measured with blood glucose chemistries (Boehringer Mannheim, Indianapolis, IN) using an Accu-ChekII monitor (Boehringer Mannheim). Diabetes incidence curves were compared between animal groups using 2 × 2 contingency tables and χ² analysis.

**Results and Discussion**

The dependence of homeostatic T cell proliferation on the chemokine ligands of CCR7 was examined by quantitating the proliferation of CFSE-labeled CD4 and CD8 T cells after their transfer into lymphocyte-deficient mice (RAG-1<sup>−/−</sup> or irradiated BALB/c mice) in the presence or absence of wild-type levels of CCL21 and CCL19. CFSE allows both the percentage of cells proliferating and the number of cell divisions continued to increase until 5 days post-transfer, with 86.7 ± 2.9% of CD8 cells having undergone six to seven rounds of division, whereas 42 ± 10.2% of CD4 cells had undergone three to four rounds of division. Although by 7 days the numbers of cell divisions continued to increase, the percentage of dividing cells did not, with 91 ± 3.0% of CD8 T cells having undergone seven to eight rounds of division, whereas 59 ± 10.5% of CD4 T cells had undergone five to six rounds of division.

Similarly, within 7 days after transfer into BALB/c mice, expressing wild-type levels of CCL21 and made lymphocyte deficient by sublethal irradiation, cell division could be detected in 70% of the CD4 T cells and 80% of the CD8 T cells (Fig. 1, A and B). However, the number of cell divisions undergone within 7 days by the proliferating population of transferred CD8 T cells was greater than that of the proliferating population of transferred CD4 T cells (Fig. 1 C and D). In nonirradiated mice with nondepleted lymphocyte populations, <20% of the transferred CD4 T cells and <40% of the transferred CD8 T cells had undergone proliferation within the same 7-day period (Fig. 1, A and B).

To test the dependence of homeostatic proliferation on CCR7 ligands, these same experiments were performed using irradiation-depleted plt/plt mice on the BALB/c background (Fig. 1). The plt mutation is a deletion of both the primary CCL21 gene expressed in secondary lymphoid organs and CCL19, the only other known ligand for CCR7 (22, 23, 28–30). Yet, in stark contrast to that seen in wild-type irradiation-depleted BALB/c mice, <20% of the transferred CFSE-labeled CD4 T cells underwent proliferation when transferred into lymphocyte-deficient plt/plt mice (Fig. 1, A and C). This low level of proliferation was equivalent to that seen after transfer into nonirradiated recipients. Because CFSE-labeled CD8 T cells showed normal homeostatic proliferation in the plt/plt recipients (Fig. 1B; 1 day), CD4 but not CD8 T cells appear to be strongly dependent on CCR7 ligand expression to support their homeostatic proliferation.

Two types of observations suggest that mechanisms regulating homeostatic expansion of T cells may play a significant role in the development of autoimmune disease. First, induction of lymphopenia in rodents is often used to precipitate autoimmunity; second, homeostatic expansion of CD8 T cells has been associated with the acquisition of a transient “preactivated” phenotype (31–34). Thus, to determine the relative contributions of Ag-specific and -nonspecific mechanisms toward T cell proliferation, we studied the response of normal (nontransgenic B10.D2) and TCR-transgenic CD4 T cells in lymphocyte-depleted and -nondelated recipients that expressed the target Ag for the transgenic T cells. In these mice, the HA Ag is expressed in pancreatic islet β cells (Ins-HA mice), and the TCR-transgenic T cells (TCR-SFE) are specific for the HA peptide, amino acids 110–119, presented on I-E<sup>κ</sup> (25, 26, 35, 36).

Upon transfer into normal Ins-HA recipients, nontransgenic B10.D2 T cells showed minimal homeostatic proliferation compared with those transferred into T cell-deficient Ins-HA/RAG-1<sup>−/−</sup> recipients (Fig. 1, E and F). In contrast, when HA-specific TCR-SFE cells were transferred to normal Ins-HA mice, a significant Ag-specific proliferation was evident within 5 days, especially among T cells in pancreatic lymph nodes (Fig. 1, E and F). Despite the observed T cell proliferation, diabetes did not develop in these mice. However, when HA-specific TCR-SFE cells were transferred into Ins-HA/RAG-1<sup>−/−</sup> recipients, the percentage of proliferating cells dramatically increased. Indeed, the number of divisions per cell was also greatly increased, as evidenced by the strong shift of CFSE peaks to the left, and these mice developed diabetes. Homeostatic mechanisms thus appeared to have a major influence on Ag-specific responses. Although Ag-specific T cells could proliferate in normal Ag-expressing recipients, the removal of bystander T cells released the Ag-specific T cells from significant inhibition.
We also tested whether, in the absence of other tissue sources, pancreatic expression of CCL21 by itself was sufficient to support homeostatic proliferation. For these studies, transgenic mice that expressed high levels of CCL21 (Ins-TCA4) in pancreatic islets were bred to plt/plt mice (24). Both the transgene and the plt/plt deletion were maintained on a BALB/c genetic background. Therefore, homeostatic proliferation of BALB/c T cells was examined 7 days post-transfer in mice carrying the Ins-TCA4 transgene and homozygous for the plt/plt mutation (plt × Ins-TCA4). Although CD4 T cells from BALB/c mice failed to undergo homeostatic proliferation in plt/plt mice even after irradiation (Fig. 1, A and B), pancreatic expression of CCL21 was sufficient to induce homeostatic proliferation of these same cells within the pancreatic lymph nodes of both lymphopenic and nonlymphopenic plt × Ins-TCA4 recipients (Fig. 2A). In contrast, CFSE-labeled CD4 T cells isolated from the other non-CCL21-expressing peripheral lymph nodes of these same irradiated plt × Ins-TCA4 mice failed to undergo homeostatic proliferation (data not shown).

Quantitative comparison of CFSE levels of donor cells isolated from pancreatic lymph nodes or simply stored in vitro revealed...
that nearly all CFSE-labeled cells underwent one round of division upon adoptive transfer into lymphopenic or CCL21-overexpressing mice (Fig. 2). Interestingly, the rate of proliferation was much more rapid in nonirradiated plt/H11003 Ins-TCA4 mice than in irradiated plt/H11003 Ins-TCA4. It is possible that, because irradiation depletes all lymph node compartments, adoptively transferred CD4 T cells more readily homed and were more rapidly exposed to CCL21 in the pancreatic lymph nodes in nonirradiated plt/H11003 TCA4 recipients than in the irradiated mice.

Because homeostatic proliferation of CD8 T cells has been associated with the transient acquisition of a preactivated/memory phenotype, we examined the expression of CD62L as a function of homeostatic CD4 T cell proliferation (15, 32–34). As expected, before transfer the lymph node donor CD4 T cells displayed a naive CD62L^{high} phenotype (Fig. 2B). Of the lymphocytes recovered from the pancreatic lymph nodes 7 days posttransfer (Fig. 2B), both the very small population of CD4 cells that failed to undergo a single round of division and the much larger population that underwent a single round of division displayed a similar naive phenotype (CD62L^{high}). Of the cells that had undergone between three and five rounds of division, a variable number displayed a CD62L^{low} phenotype (Fig. 2B). However, of the cells that had undergone seven rounds of division within 7 days, nearly all displayed a CD62L^{low} phenotype.

With similar kinetics, CD44 levels were found to shift from low to high as a function of cell division (data not shown), but this change was neither as dramatic nor as consistent as that of CD62L. Significantly, homeostatic proliferation induced by irradiation and/or CCL21 led to identical T cell phenotypes (Fig. 2B). Although our models and results differ slightly from those of Clarke and Rudensky (37), our results are largely consistent. In their C57BL/6 models, they detected two patterns of homeostatic CD4 T cell proliferation: a large population of CD4 T cells that proliferated very slowly and a small population that proliferated and acquired a preactivated/memory phenotype (CD44^{high}, CD62L^{low}) with similar kinetics as those examined in our studies. In both their studies and ours, the preactivated/memory phenotype induced by homeostatic proliferation was distinct from and developed more slowly than Ag-induced activation (36, 37).

Altogether our studies indicate that, depending on the degree of lymphopenia and/or the local expression of CCL21, a large population of preactivated cells potentially can be generated by homeostatic mechanisms. Because the physiological relevance of this lymphocyte population was ill defined, we investigated whether CCL21-induced homeostatic proliferation could facilitate the onset of autoimmune disease even in nonlymphopenic mice.

Because the absence of CCR7 ligands limited CD4 T cell homeostatic proliferation even in a lymphopenic environment, we
first tested whether an excess of CCL21 could overcome the inhibition in nondepleted recipients. For these experiments, transgenic mice were used that expressed high levels of either HA (Ins-HA) or CCL21 (Ins-TCA4) in pancreatic islets (24). As expected, irradiation-depleted Ins-HA recipients supported strong proliferation of transferred HA-specific TCR-SFE T cells (Fig. 3), whereas non-depleted recipients showed a lower Ag-specific response (Fig. 3). Strikingly, expression of CCL21 in the Ins-HA/Ins-TCA4 double-transgenic mice was sufficient to eliminate the need to induce lymphopenia. Strong proliferative responses (in both the percentage of dividing cells and number of cell divisions) were observed in HA-specific TCR-SFE T cells transferred into nonirradiated double-transgenic mice (Fig. 3). This was most evident in the spleen and in pancreatic lymph nodes, where responses were nearly equivalent to those found after transfer into irradiation-depleted Ins-HA mice.

Adoptive transfer of HA-specific SFE T cells into nondepleted Ins-HA mice was sufficient to induce limited Ag-specific proliferation (26) (Fig. 3). However, progression toward end-stage autoimmune diabetes could only be induced in lymphopenic Ins-HA mice (Fig. 1, E and F). Therefore, we tested whether, in the presence of CCL21 overexpression, the host lymphocyte population still needed to be depleted, perhaps to deplete CD4 regulatory cells. We find that transfer of HA-specific TCR-SFE T cells into Ins-HA/Ins-TCA4 recipients caused autoimmune diabetes with the same kinetics (Fig. 4) as that seen in lymphopenic recipients expressing wild-type levels of CCL21. Together, these data suggest that the excess CCL21 was able to replace the requirement for T cell depletion of the recipient. Thus, the augmentation of HA-specific responses by the transgene CCL21 appeared to include the boosting of effector responses.

Simple overexpression of CCL21 in the absence of other inducing factors is insufficient to induce the development of autoimmune diabetes. In previous studies, we have found that the expression of excess CCL21 alone in Ins-TCA4 transgenic mice was insufficient to induce autoimmune islet responses (24). However, lymphocyte recruitment induced by the chemokine did support the development of lymphoid tissue organization that may increase the efficiency of islet Ag-specific T cell responses (Fig. 4, A–D). As described in models of spontaneous autoimmune diabetes such as the nonobese diabetic (NOD) mouse (26, 38) and the Ins-HA/TCR-transgenic mouse model (25, 26, 35, 36), islet lymphoid infiltrates show a characteristic organization strikingly similar to normal lymphoid tissue, and the Ins-HA/Ins-TCA4 mice fit this pattern. Thus, not only did T cells segregate spontaneously from B cells, but the development of lymphoid-like stroma (identified by induced expression of ER-TR7 on islet stromal cells) was induced to support these compartments (Fig. 4, A–D).

These studies link two important phenomena, CD4 T cell homeostasis and autoimmunity, by suggesting that a CCR7 chemokine ligand can have a major influence on CD4 T cell homeostatic proliferation and induction of T cell effector responses in vivo. Homeostatic proliferation failed to occur in the absence of both CCR7 ligands (CCL21 and CCL19), even in lymphopenic mice. However, transgenic expression of one CCR7 ligand (CCL21) was sufficient to restore homeostatic proliferation within lymph nodes.

**FIGURE 3.** Homeostatic CD4 T cell proliferation is not dependent on lymphopenia in the presence of CCL21 overexpression. CFSE-labeled HA-specific donor T cells (SFE-TCR) were recovered from the spleens (left panels), pancreatic lymph nodes (center panels), and peripheral lymph nodes (right panels) of recipient mice 5 days after their transfer into irradiated Ins-HA (open bars), nonirradiated Ins-HA × Ins-TCA4 (filled bars), and nonirradiated Ins-HA (gray bars) mice. A, Mean percentage of proliferating donor cells isolated from three mice in one of three representative experiments. B, Irradiated Ins-HA. C, Nonirradiated Ins-HA × Ins-TCA4. D, Nonirradiated Ins-HA. B–D, Number of cycles of cell division induced in each recipient. In all panels, only the data from the CD4$^+$ gate are displayed.
in close proximity to CCL21 expression. Similarly, in nonlymphopenic mice with undisrupted CCL21 and CCL19 genes, transgenic overexpression of CCL21 was sufficient to induce a 20% increase in the T lymphocyte pool and to promote autoimmunity in the proximity of transgene expression (24). Because CCL19 and CCL21 share the same receptor (CCR7), we would predict that similar effects would have been observed if a CCL19 transgene and not a CCL21 transgene had been expressed.

These effects may help explain a number of well-established observations on the relationship between lymphopenia and susceptibility to autoimmune disease. For example, it has been found that in models of adoptively transferred disease, including autoimmune diabetes in NOD mice (39, 40), inflammatory bowel disease (31), and a number of transgenic mouse models (25, 39), recipients had to be depleted of T cells by irradiation or genetic deficiency (e.g., SCID, RAG-1/−/−) (31). Similarly, susceptibility to spontaneous autoimmunity in the BB rat model of autoimmune diabetes (41) can depend on lymphopenia induced by neonatal thymectomy (41, 42) or a genetic mutation (e.g., the lyp gene in the BB rat model of autoimmune diabetes) (43). Strikingly, spontaneous development of autoimmune diabetes in nonlymphopenic NOD mice is associated with expression of CCL21 in prediabetic mice (38). In most of these cases, CD4 T cells mediate and/or regulate the onset of autoimmune disease. Thus, although lymphoid stromal cell-derived chemokines such as CCL21 may have a primary function in T cell homeostasis, perturbations in their expression can also alter susceptibility to autoimmunity.

Acknowledgments
We thank Dr. Akio Matsuzawa for his generous gift of the plt/plt mutant mice.

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