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Cutting Edge: Self-Peptides Drive the Peripheral Expansion of CD4⁺CD25⁺ Regulatory T Cells

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CD4⁺CD25⁺ regulatory T cell selection is initiated by high-specificity interactions with self-peptides in the thymus, although how these cells respond to cytokine-derived signals and to re-exposure to self-peptide:MHC complexes in the periphery is not well understood. We have used a transgenic mouse system, in which the peptide that induces thymic selection of a clonal population of CD4⁺CD25⁺ regulatory T cells is known, to show that CD4⁺CD25⁺ T cells proliferate in response to their selecting self-peptide in vivo. Moreover, they do not proliferate in response to lymphopenia in the absence of the selecting self-peptide, reflecting a low level of expression of the high affinity receptor for IL-7 (CD127) relative to conventional CD4⁺ T cells. That their selecting self-peptide is both required for and promotes the peripheral expansion of CD4⁺CD25⁺ regulatory T cells may direct their accumulation in sites where the self-peptide is expressed. The Journal of Immunology, 2003, 171: 5678–5682.

The naive CD4⁺ T cell repertoire is primarily defined by selection events occurring in the thymus, but is also shaped by peripheral homeostatic mechanisms, such as interactions with self-peptide:MHC complexes and cytokine-derived signals, which influence the survival and lymphopenia-induced expansion of CD4⁺ T cells. In the absence of stimulation by self-peptide:MHC complexes, naive CD4⁺ T cells do not undergo lymphopenia-induced proliferation and die rapidly (1). Naïve T cells may require peripheral expression of the self-peptides that mediated their positive selection in the thymus to promote homeostatic proliferation (2–4). In addition, cytokines play a key role in the maintenance of naïve T cells (1). For conventional CD4⁺ T cells, signals received through the IL-7Rα chain (CD127) paired with the common γ-chain have been shown to promote proliferation in response to low-affinity interactions with self-peptide:MHC complexes, particularly under conditions of lymphopenia (5, 6).

However, 5–10% of the peripheral CD4⁺ T cells in normal mice are CD25⁺CD45RBlow cells that have been shown to have regulatory function (7). CD4⁺CD25⁺ regulatory T cells are generated as a result of high-affinity interactions with cognate self-peptide:MHC complexes in the thymus (8, 9). Although there is evidence that the sustained presence of CD4⁺ regulatory T cells may depend on the peripheral expression of self-Ag (10), how interactions with self-peptide:MHC complexes in the periphery contribute to maintaining or expanding the repertoire of CD4⁺CD25⁺ regulatory T cells has only begun to be examined (11–13). In this report, we have examined how CD4⁺CD25⁺ regulatory T cells respond to stimuli that could promote their expansion in the periphery using TS1 × HA28 mice. In TS1 × HA28 mice, thymocytes expressing the clonotypic 6.5 TCR undergo selection to become CD4⁺CD25⁺ regulatory T cells through interactions with the S1 peptide from influenza virus hemagglutinin expressed as a neo-self-peptide (8, 14). We show that 6.5highCD4⁺CD25⁺ regulatory T cells proliferate in response to S1 peptide in the periphery of HA28 mice. However, unlike conventional CD4⁺ T cells, CD4⁺CD25⁺ cells do not proliferate in response to lymphopenia in BALB/c mice (that lack S1 peptide). Their differing responsiveness to TCR vs lymphopenia-induced signals likely shapes the accumulation and activity of CD4⁺CD25⁺ regulatory T cells at sites where their selecting self-peptides are expressed.

Materials and Methods

Mice

TS1, TS1 × HA28, HA28, and TS1(SW) mice have been previously described (8, 14, 15). Recipient BALB/c (Harlan, Indianapolis, IN) and HA28 mice for adoptive transfers were left either nonmanipulated or were sublethally irradiated with 500 rad of gamma irradiation. All mice were maintained in sterile microisolators at The Wistar Institute animal facility and were used between 10 and 28 wk of age.

Flow cytometry

Single-cell suspensions of cells pooled from axillary, inguinal, brachial, and superficial cervical lymph nodes (LN) were stained with the following mAbs for flow-cytometric analysis: purified anti-CD16/32 (Fc block; 2.4G2; BD PharMingen), anti-CD4-allophycocyanin or anti-CD4-FITC (L3T4; BD PharMingen), anti-CD25-PE (PC61; BD PharMingen), anti-CD25-biotin or anti-CD25-FITC (7D4; BD PharMingen), anti-Vα8.3-biotin (KT50; BD PharMingen), anti-CD127-PE (SB/14; BD PharMingen; or KT50; BD PharMingen), anti-CD127-PE (SB/14; BD PharMingen; or KT50; BD PharMingen), anti-CD127-PE (SB/14; BD PharMingen; or KT50; BD PharMingen), anti-CD127-PE (SB/14; BD PharMingen; or KT50; BD PharMingen), anti-CD127-PE (SB/14; BD PharMingen; or KT50; BD PharMingen), anti-CD127-PE (SB/14; BD PharMingen; or KT50; BD PharMingen).

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3 Abbreviations used in this paper: LN, lymph node; int, intermediate.
A/VR34; eBioscience, San Diego, CA), and 6.5-biotin (15). StreptavidinRed670 (Invitrogen, Carlsbad, CA) was used to detect biotinylated reagents. Three- or four-color analyses were performed on either FACSscan or FACSCalibur flow cytometers, respectively (BD Biosciences, San Jose, CA). Data analysis of 100,000–400,000 live events was performed using FlowJo software (Tree Star, San Carlos, CA). Cell populations were purified by FACS from pooled axillary, inguinal, brachial, and superficial cervical LN to ~95% purity at The Wistar Institute’s sorting facility using a Cytomation MoFlo and Summit software (Cytomation, Fort Collins, CO).

**CFSE labeling**

Purified CD4^+CD25^- and CD4^+CD25^+ T cells from TS1 × HA28 LN were labeled in vitro with the fluorescent dye CFSE (Molecular Probes, Eugene, OR) as previously described (16). Briefly, purified CD4^+CD25^- or CD4^+CD25^+ T cells or unfractionated TS1(SW) LN cells were washed into serum-free IMDM, incubated with 5 mM CFSE at 1 × 10^6 cells/ml for 4 min, and then incubated with 50% serum. Labeled cells were then washed into serum-free medium for adoptive transfers.

**Adoptive transfers**

A total of 1.5–2 × 10^7 purified CFSE-labeled CD4^+CD25^- or CD4^+CD25^+ cells were injected into the tail veins of recipient mice. In some cases, 3 × 10^6 CFSE-labeled TS1(SW) LN cells were coinjected with the CFSE-labeled CD4^+CD25^- or CD4^+CD25^+ T cells. Seven days later, axillary, brachial, inguinal, and superficial cervical LN were harvested from recipient mice and stained for flow cytometry. In some experiments, CFSE-labeled CD4^+CD25^- cells were purified by FACS from the LN and spleens of recipient mice and used for in vitro assays.

**In vitro proliferation assays**

Proliferation assays were performed as previously described (14). Briefly, CFSE-labeled CD4^+CD25^- or CD4^+CD25^+ cells from TS1 × HA28 mice were cultured with irradiated BALB/c splenocytes and stimulated with 1 μM S1 peptide in presence or absence of 10 ng/ml IL-2. Four days later, cells were stained for flow cytometry. For suppressor function analysis, 10^6 responder TS1 HA28 mice or from adoptive transfer recipient mice. Cells were stimulated with irradiated BALB/c spleenocytes. Bars indicate 6.5high (left panel) or CD25+ (right panel), and the frequency for each population is shown.

**Results and Discussion**

CD4^+CD25^+ regulatory T cells express low levels of CD127

TS1 mice express a transgenic TCR specific for the S1 peptide of hemagglutinin that can be detected with the mAb 6.5 (15). We previously showed that interactions with S1 peptide induce 6.5highCD4^+ single-positive thymocytes to undergo CD25^+ regulatory T cell selection in TS1 × HA28 mice, which express S1 in both the thymus and periphery (8, 14), 6.5highCD4^+CD25^+ regulatory T cells coexist in equal ratios and with equal numbers of 6.5highCD4^+CD25^- T cells in TS1 × HA28 mice (Fig. 1A; Ref. 8). Because signals received from cytokines and self-peptide/MHC complexes can each contribute to the maintenance of conventional CD4^+ T cells (1, 4), we were interested in determining how 6.5highCD4^+CD25^+ regulatory T cells respond to these signals in the periphery. 6.5highCD4^+CD25^- T cells from TS1 × HA28 mice expressed lower levels of CD127, the high-affinity receptor for IL-7, than 6.5highCD4^+CD25^- T cells (Fig. 1B). CD4^+CD25^- T cells from BALB/c mice showed similarly reduced levels of CD127 in comparison with CD4^+CD25^- T cells, excluding the possibility that the low levels of CD127 expression among 6.5highCD4^+CD25^- T cells in TS1 × HA28 mice relates to the expression of a transgene-encoded TCR (Fig. 1C). These data are consistent with other studies (12, 17) and suggested that CD4^+CD25^+ and CD4^+CD25^- cells differ in their abilities to respond to lymphopenia, where IL-7 is critical for the homeostatic proliferation of conventional CD4^+ T cells (5).

**To directly examine their abilities to respond to lymphopenia, CD4^+CD25^- and CD4^+CD25^+ T cells were purified from LN of TS1 × HA28 mice by cell sorting, labeled with the intracellular dye CFSE, and injected into normal or sublethally irradiated BALB/c mice. We also coinfected a second population of CFSE-labeled LN cells from TS1(SW) mice, which express a TCR specific for an analog of the S1 determinant (termed S1(SW)); Ref. 8). Seven days later, LN cells were harvested from recipient mice and stained with 6.5 to identify cells from TS1 × HA28 mice or with anti-Va0,3 to identify cells from TS1(SW) mice (8), and the division of the transferred cells was determined based on decreasing CFSE intensity. As expected (2, 4), none of the transferred cell populations divided in nonirradiated BALB/c mice (Fig. 2A). When transferred into lymphopenic BALB/c mice, both the 6.5highCD4^+CD25^- T cells from TS1 × HA28 mice (which express CD127) and the CD4^+Va0,8+ T cells from TS1(SW) mice underwent one to two rounds of division. However, the 6.5highCD4^+CD25^- T cells from TS1 × HA28 mice failed to proliferate in lymphopenic BALB/c mice. The recipient mice were indeed lymphopenic, because the CD4^+Va0,8+ cells that were coinfected with the 6.5highCD4^+CD25^- cells went through one to two rounds of homeostatic division. Therefore, 6.5highCD4^+CD25^- T cells, which express low levels of CD127, do not appear to respond to signals derived from IL-7.
or to any other stimuli presented by lymphopenic environments lacking S1 peptide. 

**S1 peptide drives the peripheral expansion of 6.5highCD4+ CD25+ T cells**

We next transferred CFSE-labeled CD4+CD25+ or CD4+CD25− cells from TS1 × HA28 mice into normal or sublethally irradiated HA28 mice, and as in BALB/c mice, CFSE-labeled LN cells from TS1(SW) mice were also coinfected. In this case, coinfection of TS1(SW) cells, which have a ~100-fold lower affinity for the S1 peptide than 6.5+ cells (8), allowed the specificity of proliferative responses to S1 peptide to be assessed. In contrast to BALB/c mice in which they failed to divide, the 6.5highCD4+CD25+ cells divided up to three times in nonirradiated HA28 mice (Fig. 2A). By contrast, CD4+Vα8+ T cells from TS1(SW) mice did not divide in nonirradiated HA28 mice; this, and the failure of 6.5high CD4+CD25+ T cells to divide in BALB/c mice, indicates that the proliferation of 6.5highCD4+CD25+ T cells in HA28 mice is a specific response to S1 peptide (Fig. 2A).

6.5highCD4+CD25+ T cells also induced 6.5highCD4+CD25+ T cells to proliferate in lymphopenic HA28 mice (Fig. 2A). In this case, 6.5highCD4+CD25+ T cells divided approximately four to five times, whereas CD4+Vα8+ T cells went through one to two rounds of division (similar to their division in lymphopenic BALB/c mice). Interestingly, the expansion of 6.5highCD4+CD25+ T cells in lymphopenic HA28 mice was greater than in nonirradiated HA28 mice, suggesting that their proliferative response to S1 peptide may be enhanced by an increase in space provided by lymphopenia. It is possible that IL-7 signals synergize with TCR signals to enhance proliferation of CD4+CD25+ T cells in lymphopenic HA28 mice, despite the low levels of CD127 that are expressed. However, if this is the case, the ability to respond to IL-7 is nevertheless dependent on interactions with S1 peptide, because these cells did not proliferate in response to lymphopenia alone in BALB/c mice. It is also noteworthy that 6.5highCD4+CD25+ T cells did not suppress the homeostatic proliferation of the cotransferred CD4+Vα8+ T cells, as has previously been reported (18, 19). Together with the observations in BALB/c mice, these findings indicate that the peripheral expansion of 6.5highCD4+CD25+ regulatory T cells in HA28 mice is both promoted by and dependent on interactions with the S1 peptide.

We also examined the proliferation of 6.5highCD4+CD25− T cells from TS1 × HA28 mice following transfer into HA28 mice. 6.5highCD4+CD25− T cells proliferated extensively in response to S1 peptide in both nonirradiated and irradiated HA28 mice, in each case going through as many as five divisions (Fig. 2A). Although they express high levels of CD127 and produce high levels of IL-7 (8), these T cells did not appear to significantly enhance 6.5highCD4+CD25− cell proliferation in lymphopenic BALB/c mice. The proliferation of CD4+CD25− T cells did not appear to limit the proliferation of the cotransferred CD4+Vα8+ T cells in the time period examined, in contrast with recent studies in another system (20). Importantly, the different proliferative capacities of 6.5highCD4+CD25− and 6.5highCD4+CD25− T cells in response to S1 peptide in HA28 mice correlated well with the abilities of these cells to respond to S1 peptide in vitro where, in the absence of IL-2, 6.5highCD4+CD25− T cells divided one to two times, whereas 6.5highCD4+CD25− T cells divided four to five times (Ref. 14 and Fig. 2B).

Peripheral expansion does not abrogate regulatory function of CD4+CD25+ T cells

To determine whether they retain regulatory function following proliferation in vivo, CD4+CD25+ cells from TS1 × HA28 mice that had undergone division in response to S1 peptide in HA28 mice were reisolated based on CFSE intensity (Fig. 3A). These cells were then examined for their ability to suppress S1-induced in vitro proliferation of TS1 LN cells. CD4+CD25+ cells that had undergone division in vivo were as potent suppressors of proliferation as undivided cells (Fig. 3B). CD4+CD25+ cells were also as potent suppressors as freshly isolated CD4+CD25+ T cells from TS1 × HA28 mice, whereas freshly isolated CD4+CD25− cells from TS1 × HA28 mice did not suppress proliferation of TS1 LN cells. This indicates that CD4+CD25+ T cells retain their regulatory properties following in vivo proliferation, as previously described (13). These data further show that the regulatory CD4+CD25+ cells themselves (and not some other population of CD4+CD25+ cells) undergo division in response to S1 peptide in HA28 mice.

![Diagram](http://www.jimmunol.org/Downloaded_from)
The peripheral expansion of 6.5 highCD4 cell populations is dependent on interactions with S1 peptide, because unlike conventional CD4+ T cells (and also 6.5 highCD4+ CD25+ T cells), the 6.5 highCD4+ CD25+ T cells did not proliferate in response to lymphopenia in BALB/c mice. Several studies have previously demonstrated the proliferation of polyclonal CD4+ CD25+ T cells upon transfer into lymphopenic hosts, and some have shown that CD4+ CD25+ T cells proliferate in response to Ag administered by immunization or expressed as a self-Ag (12, 13, 17, 18, 20, 21). Our data establish that the ability to proliferate in response to selecting self-peptide:MHC complex, rather than cytokine-derived signals, directs the expansion of CD4+ CD25+ T cells in the periphery.

The failure of CD4+ CD25+ T cells to proliferate in response to lymphopenia likely reflects the down-modulation of CD127, which is an important cytokine in directing division of conventional CD4+ T cells under lymphopenic conditions. In this respect, it is noteworthy that conventional CD4+ T cells also down-modulate CD127 upon activation, and that CD4+ CD25+ regulatory T cells exhibit other phenotypic characteristics, such as increased levels of CTLA-4 and glucocorticoid-induced thymocyte receptor, that they share with activated conventional CD4+ T cells (Refs. 22–26 and data not shown). Whether CD4+ CD25+ regulatory T cells exhibit characteristics in common with activated CD4+ T cells as a consequence of high-affinity interactions with self-peptides during their selection in the thymus and/or as a result of ongoing interactions with self-peptides in the periphery remains to be determined. Nevertheless, down-modulation of CD127 on CD4+ CD25+ regulatory T cells, coupled with the ability to proliferate in response to their selecting peptide, likely significantly affects how CD4+ CD25+ regulatory T cells accumulate in the periphery. 6.5 highCD4+ CD25+ T cells accumulate in large numbers systemically in TS1 × HA28 mice, because S1 peptide is expressed in a wide variety of tissues (data not shown), and CD4+ CD25+ regulatory T cells directed toward ubiquitously expressed bona fide self-Ags are likely to be similarly represented in high numbers and to be systemically distributed. In other cases, CD4+ CD25+ T cells that are specific for tissue-restricted Ags proliferated and accumulated selectively in LN draining sites of Ag expression (11, 12, 27). Their differing responsiveness to TCR- vs cytokine-mediated signals provides a mechanism by which CD4+ CD25+ T cells specific for tissue-restricted self-Ags (and that may undergo thymic selection via promiscuous expression in thymic epithelial cells (28)) can accumulate selectively at sites of Ag expression, even in lymphopenia.

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