Amelioration of arthritis through mobilization of peptide-specific CD8+ regulatory T cells

Jianmei W. Leavenworth,1,2 Xiaolei Tang,1,2 Hye-Jung Kim,1,2 Xiaoyang Wang,1,2,3,4 and Harvey Cantor1,2

1Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, and 2Department of Microbiology and Immunobiology, Division of Immunology, Harvard Medical School, Boston, Massachusetts, USA. 3Perinatal Center, Department of Neuroscience and Physiology, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden. 4Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, People’s Republic of China.

Current therapies to treat autoimmune disease focus mainly on downstream targets of autoimmune responses, including effector cells and cytokines. A potentially more effective approach would entail targeting autoreactive T cells that initiate the disease cascade and break self tolerance. The murine MHC class Ib molecule Qa-1 (HLA-E in humans) exhibits limited polymorphisms and binds to 2 dominant self peptides: Hsp60p216 and Qdm. We found that peptide-induced expansion of tetramer-binding CD8+ Tregs that recognize Qa-1–Hsp60p216 but not Qa-1–Qdm strongly inhibited collagen-induced arthritis, an animal model of human rheumatoid arthritis. Perforin-dependent elimination of autoreactive follicular Th (Tfh) and Th17 cells by CD8+ Tregs inhibited disease development. Infusion of in vitro–expanded CD8+ Tregs increased the efficacy of methotrexate treatment and halted disease progression after clinical onset, suggesting an alternative approach to this first-line treatment. Moreover, infusion of small numbers of Qa-1–Hsp60p216–specific CD8+ Tregs resulted in robust inhibition of autoimmune arthritis, confirming the inhibitory effects of Hsp60p216 peptide immunization. These results suggest that strategies designed to expand Qa-1–restricted (HLA-E–restricted), peptide-specific CD8+ Tregs represent a promising therapeutic approach to autoimmune disorders.

Introduction

RA is an autoimmune disorder characterized primarily by joint inflammation and erosion. The systemic nature of this disease is reflected in the involvement of multiple organ systems, including skin, lungs, and vasculature as well as diffuse tissue inflammation and increased risk of atherosclerosis (1). Although the identification of effector cytokines, including TNF, IL-1, and IL-6, that contribute to this disease has led to improved therapies, RA has remained relatively refractory to decisive intervention. More effective approaches to RA may require elimination of autoreactive T cells, which, in turn depends on a precise definition of the Tregs that may target and eliminate the pathogenic T cell subsets that induce disease.

Here we analyze the contribution of Treg and effector T cell subsets to autoimmune arthritis in the collagen-induced arthritis (CIA) animal model. This murine disease model shares several similarities with human RA, including breach of self tolerance, generation of autoantibodies, inflammatory changes in multiple joints, and erosion of bone and cartilage accompanied by pannus formation (2). Murine CIA has been used to establish the potential efficacy of several FDA-approved RA therapies, including anti-TNF Ab, IL-1 antagonists, and methotrexate (MTX) (1, 3).

The development of enlarged ectopic germinal centers (GCs) and the contribution of pathogenic autoantibodies in RA and in CIA has suggested that dysregulated follicular Th (Tfh) cell responses may contribute to ectopic GC formation and production of autoantibodies (4–6). A subset of CD8+ T cells, CD8+ Tregs, has been shown to efficiently inhibit Tfh cells through recognition of MHC class Ib molecule Qa-1 expressed by this Th subset (5). However, the potential contribution of CD8+ Treg to the pathogenesis and treatment of this autoimmune disease is not well understood.

CD8+ Tregs recognize the MHC class Ib molecule Qa-1 (HLA-E in humans) associated with peptide complexes that are upregulated by activated CD4+ T cells (5, 7). The 2 major peptides presented by Qa-1 (HLA-E) are Qdm (B7sp in humans) and Hsp60p216. The Qdm (Qa-1 determinant modifier) peptide is derived from the leader sequence of MHC class Ia, while the Hsp60p216 peptide is derived from the 60 kD heat shock protein (Hsp60, p216-224). Differential expression of these Qa-1–peptide complexes on activated cells may allow detection of abnormally stressed cells in the context of infection, autoimmunity, or cellular transformation (8–11).

Here we investigate the impact of CD8+ Tregs on the development of CIA. We find that Qa-1–restricted CD8+ Tregs efficiently eliminate pathogenic Tfh and Th17 cells and exert strong inhibitory effects on disease progression. Moreover, CD8+ Tregs that recognize complexes of Qa-1 and Hsp60p216 peptide, but not Qdm peptide, can be mobilized through peptide-based immunization to inhibit development of autoimmune arthritis. The ability to expand Qa-1–restricted CD8+ Tregs based on their TCR specificity may represent a new and effective approach to treatment of autoimmune disease.

Results

Expansion of Qa-1–restricted CD8+ Tregs inhibits arthritis. We reasoned that expansion of Qa-1–restricted CD8+ T cells in vivo might be achieved after immunization with the 2 major peptides that are bound by Qa-1–Qdm and Hsp60p216. The Qdm peptide, derived from the leader sequences of class Ia MHC proteins, is the dominant self peptide associated with Qa-1 at the cell surface. In addition, a peptide derived from the 60-kD heat shock protein (Hsp60, p216-224) replaces the Qdm peptide upon cellular activation and/or stress (12, 13). To selectively expand Qdm- or Hsp60p216–specific CD8+ cells, we immunized mice subcutaneously with peptide-pulsed Kb–/Db– DCs that do not

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2013;123(3):1382–1389. doi:10.1172/JCI66938.
express class Ia MHC, but express high levels of class Ib MHC, including Qa-1. Immunization of mice with Hsp60p216 but not Qdm-pulsed Kb−/−Db−/− DCs during the course of CIA inhibited the development of arthritis (Figure 1A).

We then generated Qa-1–peptide tetramers (tet) to determine the frequency of peptide-specific CD8+ T cells. In the case of Qa-1–Qdm tetramers, a Qa-1 protein containing a point mutation (R72A) was used to prevent binding of Qa-1–Qdm to CD94/NKG2A receptors (14) and allow specific detection of CD8+ T cells that express Qa-1–restricted TCR (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI66938DS1). We labeled cells with tetramers conjugated to 2 different fluorophores to increase specificity, followed by magnetic enrichment of cells positive for tet+ CD8+ cells. Numbers in plots before enrichment and after enrichment indicate percentage of tet+ CD8+ cells. Numbers in plots before enrichment and after enrichment (upper left quadrant) indicate percentage of tet+ CD8+ cells. (C) B6 mice were immunized with either unpulsed Kb−/−Db−/− DCs or DCs that had been loaded with the indicated peptide as described in A. CD8+ cells from dLNs from each group were analyzed at day 42 for binding to Qa-1.R72A–Qdm-tetramer or Qa-1–Hsp60p216-tetramer. Representative FACS plots are shown. Gates represent percentages of tet+ CD8+ cells. (D) The percentages and numbers of Hsp60p216-tet+ CD8+ cells that expressed the CD122+Ly49+ surface profile and CD122+Ly49− surface profile are shown. *P < 0.05.
tetramers after immunization with Hsp60p216-pulsed DCs inhibited the development of CIA (Figure 1, A and D). Although immunization with Qdm-pulsed KbDbDCs resulted in increased numbers of Qa1R72A-Qdm tet+ cells that include both CD122Ly49+ and CD122Ly49+ populations (ref. 16, Figure 1C, and Supplemental Figure 1, C and D), this immunization did not inhibit disease development (Figure 1A). Although T cells specific for several other Hsp60 epitopes have been detected in juvenile idiopathic arthritis patients along with expansion of CD4 Tregs (17), Hsp60p216 immunization did not change the frequency of Foxp3CD4+ cells (Supplemental Figure 1E). We also noted that Hsp60p216 immunization resulted in expansion of Hsp60p216-tet+ CD8+ cells within the CD122Ly49+ (16) but not the CD122Ly49+CD8 subset (Figure 1D). These results suggest that Hsp60p216-Qa1-restricted CD8+ cells belonging to the CD122Ly49+CD8 subset may inhibit autoimmune arthritis.

**Infiltration of CD8 Tregs inhibits arthritis development.** The above findings indicated that expansion of Hsp60p216-specific Qa1-restricted CD8+ cells (but not control Qa1-restricted peptide) was associated with disease inhibition. Infusion of the fewer than 5% of CD8 T cells expressing the triad of surface receptors that characterize CD8+ Tregs (CD122CD44Ly49; triad) into adoptive hosts that had been given CD25 CD4+ T cells and B cells from chicken type II collagen-immune (cCII-immune) donors abolished disease progression (Figure 2A) and reduced titers of anti-CII Ab (Figure 2B). In contrast, infusion of CD122CD44Ly49+CD8+ cells failed to inhibit disease progression. The inhibitory activity of Ly49CD8 T cells depended on recognition of Qa-1 expressed by CD4 Th cells, since CD4 Th cells expressing a Qa-1 point mutation (Qa-1 D227K) that impaired recognition by CD8+ cells (5, 18) were resistant to CD8 Treg-dependent inhibition (Figure 2B). CD8 Treg activity depends on perforin and IL-15 expression. To further investigate the mechanism of suppression by Ly49CD8 Tregs, we asked whether suppression by the Ly49CD8 T cells depended on IL-15. Transfer of CD8+ cells from IL-15–/– donors failed to inhibit disease severity; indeed, an increase in disease intensity was noted (Figure 2C). Suppression also reflected perforin-dependent elimination, since purified Prf1-deficient CD8+ T cells did not reduce anti-mouse collagen Ab responses (Figure 2D). In sum, IL-15 was essential to the development of CD122CD44Ly49+CD8+ Tregs, and intracellular perforin was required for expression of suppressive activity.

**CD8 Treg cell subsets in arthritic joints.** Comparison of CD8+ T cell subsets in arthritic joints revealed that approximately 50% of CD4+ cells expressed the triad (Qa-1D227K), while another 30% expressed the triad plus ICOS (ICOSCXCR5 or ICOSBTLA) (Figure 3A). Although transfer of large numbers of CD8+ Tregs failed to inhibit disease progression, transfer of both Th subsets induced strong disease (19).
We tested the hypothesis that disease might reflect a Th17-induced autoantibody response combined with a Th17-dependent inflammatory reaction. Indeed, mice given Th17 cells and autoantibody (mainly anti-CII Ab) in place of TFH cells developed severe and sustained arthritis (Figure 3B). These data suggest that CIA initiation in intact animals may reflect a synergistic interaction in which a Th17-mediated inflammatory response enhances a Th17-dependent autoantibody production.

These results also suggested that inhibition of the Th17 and Th17 cell response might interrupt early events in the autoimmune disease process. Indeed, reduced arthritis and autoantibody titers in recipients of CD8+ Tregs were associated with diminished numbers of Th17 and Th17 cells (Figure 3, C and D), but not IFN-γ+ (Th1) cells in adoptive hosts (Supplemental Figure 3). Elimination of Th17 and Th17 cells required perforin expression by Ly49+ CD8+ T cells (Supplemental Figure 4) and depended on Qa-1 expression (Supplemental Figure 5) after vigorous expansion: approximately 3 × 10^5 CD122+CD44+Ly49+ CD8+ cells were generated within 7 days from an initial seeding of 10^5 CD8+ cells that expressed the same triad of surface receptors. Moreover, these in vitro–expanded Th17 cells abolished anti-mouse collagen Ab responses (Figure 4A) and efficiently inhibited disease development in Rag2−/−Prf1−/− hosts given cCII-immune CD4+ T cells (Figure 4B). The inhibitory activity of IL-15–incubated CD8+ Tregs was at least as potent as that of nonincubated CD8+ Tregs (Figure 4B compared with Figure 2A), suggesting that incubation of CD8+ Tregs with low concentrations of IL-15/IL-15Rα complexes (IL-15C) (20, 21). Ly49+ CD8+ T cells incubated in vitro for 1 week in the presence of 10 ng ml−1 IL-15C maintained their surface phenotype (Supplemental Figure 5) after vigorous expansion: approximately 3 × 10^5 CD122+CD44+Ly49+ CD8+ cells were generated within 7 days from an initial seeding of 10^5 CD8+ cells that expressed the same triad of surface receptors. Moreover, these in vitro–expanded CD8+ Tregs abolished anti-mouse collagen Ab responses (Figure 4A) and efficiently inhibited disease development in Rag2−/−Prf1−/− hosts given cCII-immune CD4+ T cells (Figure 4B). The inhibitory activity of IL-15C-incubated CD8+ Tregs was at least as potent as that of nonincubated CD8+ Tregs (Figure 4B compared with Figure 2A), suggesting that incubation of in vitro IL-15C-expanded CD8+ Tregs may represent an effective therapeutic approach to CIA. Moreover, transfer of in vitro–expanded CD8+ Tregs into unirradiated C57BL/6 (B6) mice followed by immunization with cCII suppressed arthritis progression (Figure 4C and Supplemental Figure 6A) and reduced Th17 and Th17 cell numbers (Figure 4D and Supplemental Figure 6B) in...
Combination of CD8+ Tregs and MTX exerts therapeutic effects on ongoing disease. MTX, the disease-modifying anti-rheumatic drug (22, 23), is thought to prevent arthritis through inhibition of inflammation and joint destruction at the level of synoviocytes (24). In contrast, CD8+ Tregs interrupt the autoimmune pathway much earlier (e.g., see Figure 2). We therefore studied the combined effects of an MTX pulse to block joint inflammation followed by infusion of CD8+ Tregs at later time points to prevent new damage inflicted by a continuing systemic autoimmune response. Attenuation of disease activity was noted using an MTX pulse of 1.5 mg/kg over a short time period (from day 21 to day 23) (Supplemental Figure 7, A and B), while doses lower than 0.75 mg/kg had minimal effects on disease progression (Supplemental Figure 7, A–C). Coadministration of MTX at 0.75 mg/kg with CD8+ Tregs resulted in a marked reduction in disease severity (Figure 4E). The synergistic therapeutic effect of this combination therapy may depend, in part, on the failure of low doses of MTX to diminish the numbers of CD122+Ly49+ CD8+ cells (Supplemental Figure 7 and ref. 16). Together, these results suggest the potential contribution of therapy using CD8+ Tregs and MTX to ameliorate ongoing disease.

**Figure 4**
Transfer of in vitro IL-15C–expanded CD8+ Tregs inhibits CIA. (A) CD122+CD44+Ly49+ or CD122+CD44+Ly49− CD8+ cells sorted from cCII-immunized B6 mice were incubated in 10 ng ml−1 IL-15C × 1 week. Purified CD25− CD4+ and B cells from arthritic donors were transferred with or without the indicated CD8+ cells (0.6 × 10⁶) into Rag2−/−Prf1−/− mice followed by arthritis induction at day 0 and boost at days 21 and 35 (black arrows). Serum titers of anti-mouse CII IgG are shown. ***P < 0.001. (B) Arthritis scores are shown for 5–6 mice/group. Group (no CD8) vs. group (Ly49+ CD8), *P < 0.05. Data represent 3 independent experiments. (C) In vitro–expanded CD122+CD44+Ly49− CD8+ cells (2 × 10⁵) were transferred into B6 mice at days 0 and 18 (triangles). cCII immunization and boosting at days 0 and 21 (black arrows) and arthritis scores over 50 days are shown. (D) Numbers of Tfh and IL-17–expressing CD4+ cells from inflamed joints in C are shown for 5–6 mice/group. 0.75 mg/kg MTX was injected into B6 mice from day 21 to day 23 (arrows) after development of arthritis (average score of 4.5). In vitro IL-15C–expanded CD122+CD44+Ly49− CD8+ cells (2.5 × 10⁵) were transferred into mice at days 27, 30, and 38 (triangles). Arthritis scores are shown for 5–8 mice/group. Group (control) or group (MTX) versus group (CD8+ Treg + MTX) difference: *P < 0.05.

**Discussion**
Clinical approaches to the treatment of arthritis have focused mainly on downstream elements of this disease process, while sparing the initiating autoimmune events including induction...
CD8+ Treg treatment was particularly effective at blunting the autoantibody response in CIA (e.g., Figures 2 and 3). Since CD8+ Tregs target T<sub>H1</sub> cells and Th17 cells, the major Th subsets that induce the humoral and inflammatory components of CIA, we asked whether infusion of CD8+ Tregs might therefore confer durable inhibition of this autoimmune disorder. Transfer of CD122<sup>DC</sup>CD44<sup>Ly49</sup>CD8<sup>+</sup> cells, which represents less than 5% of the total CD8+ cell population and carries essentially all CD8+ Treg activity (16), resulted in reduction of the numbers of T<sub>H1</sub> and Th17 cells and robust inhibition of disease. The finding that CD8+ Tregs inhibit T<sub>H1</sub> and Th17 CD4<sup>+</sup> cells but do not target Th1 cells suggests direct targeting of Th subsets, consistent with previous findings (5). However, the possibility that activated DCs that upregulate the Qa-1–peptide complexes may also be targeted by CD8+ Tregs cannot be excluded. Although definition of the precise functional difference between Qa-1–Hsp60 p216 and Hsp60 p216–specific CD8+ T cells was transferred into Rag2<sup>−/−</sup>Prf1<sup>−/−</sup> mice at day 0 along with CD4 and B cells from arthritic mice. Mice were immunized at day 0 and boosted at days 21 and 35 (black arrows) with cCII. Arthritis scores (B), numbers of splenic Hsp60<sub>216</sub>–tet<sup>−</sup> CD8+ cells at day 34 (C), and anti-mouse CII IgG titers at day 30 (D) after adoptive transfer are shown for 3–5 mice per group. Group (tet<sup>−</sup> CD8) versus group (tet<sup>+</sup> CD8), *<i>P</i> < 0.05. (E) By comparison, transfer of Qdm-tet<sup>−</sup> (or tet<sup>−</sup>) CD8+ cells had no significant effect on the response of CD4<sup>+</sup> and B cells from arthritic mice using the same conditions (D).

Transfer of Qa-1–Hsp60<sub>p216</sub> restricted CD8+ Treg inhibits arthritis. (A) Hsp60<sub>p216</sub>–tet<sup>+</sup> and Hsp60<sub>p216</sub>–tet<sup>−</sup> CD8+ cells were sorted from cCII-immune B6 mice that had been immunized 2 weeks earlier with Kb<sup>−/−</sup>Db<sup>−/−</sup> DCs loaded with Hsp60<sub>p216</sub> before incubation in IL-15C (10 ng ml<sup>−1</sup>) × 10 days. After incubation with Hsp60<sub>p216</sub> tetramer and enrichment by anti-PE microbeads, they were subjected to FACS analysis. Incubation of CD8+ cells that were initially tet<sup>+</sup> resulted in a substantial increase and enrichment of tet<sup>+</sup> CD8+ cells; identical incubation of CD8+ cells that were tet<sup>−</sup> did not result in significant levels of tet<sup>+</sup> cells after incubation in IL-15C. Hsp60<sub>p216</sub>–tet<sup>+</sup> or Hsp60<sub>p216</sub>–tet<sup>−</sup> fraction of CD8+ cells was transferred into Rag2<sup>−/−</sup>Prf1<sup>−/−</sup> mice at day 0 along with CD4 and B cells from arthritic mice. Possibly, differential expansion of Ly49<sup>+</sup>CD8+ T cells by the 2 self peptides may be relevant for a better understanding of the functional difference between Qa-1–Qdm and Qa-1–Hsp60 p216, possibly reflecting the ability of the TCR-dependent activation of CD8+ Tregs with the relevant peptide resulted in selective expansion of CD8+ T cells specific for either Qa-1–Qdm or Qa-1–Hsp60 p216, only immunization with the latter peptide efficiently inhibited the development of CIA. Moreover, transfer of a small number of Qa-1–Hsp60<sub>p216</sub> tetramer– CD8+ cells, but not Qa-1(R72A)–Qdm tetramer– CD8+ cells, inhibited disease progression in adoptive hosts. The CD8+ Treg–mediated suppression we observed following transfer of CD8+ Tregs was selectively exerted on the response to self antigens, since immune responses to foreign proteins (e.g., NP-KLH) were spared (Supplemental Figure 9).

Autoreactive T<sub>H1</sub> and Th17 subsets that are chronically activated by engagement of the CD94/NKG2A receptor by the Qa-1–Qdm complex expressed by CD4+ target cells may dampen TCR-dependent activation of CD8+ Tregs (19, 42). The Hsp60-related peptides may also include the Hsp60 signal peptide (Hsp60sp) derived from the leader sequence of Hsp60<sub>p10–18</sub>, since a subset of CD8+ Tregs that recognizes the
Qa-1–Hsp60sp complex suppresses autoimmune (43). Further definition of this subset of CD8+ Tregs requires successful construction of Qa-1–Hsp60sp tetramers.

Analysis of the activity of tetramer+ CD8+ Tregs depended in part on immunization using peptide-pulsed DCs. Since CD8+ Treg are memory cells according to function and surface phenotype (CD122+CD44+Ly49+), they may also be expanded by antigen in the absence of cell-dependent costimulatory activity. For example, peptide–Qa-1–coated (HLA-E–coated) nanoparticles (NP) may represent an effective and simplified method for CD8+ Treg expansion that does not require cellular therapy. Moreover, since both Qa-1 and HLA-E MHC class Ib genes exhibit a limited polymorphism, in contrast with the extensive polymorphisms of MHC class Ia molecules (e.g., HLA-E expression is confined to 2 alleles that differ at a single amino acid), peptide–HLA-E–based immunotherapy should not require extensive individualized alleles that differ at a single amino acid), peptide–HLA-E–based immunotherapy should not require extensive individualized peptide-MHC design.

In the present studies, expansion of CD8+ cells specific for Hsp60p216 (but not a control self peptide) was associated with efficient inhibition of autoimmune arthritis, a reduction of the T12β/Th17 pathogenic Th pair, and diminished autoantibody to efficient inhibition of autoimmune arthritis, a reduction of the peptide-MHC design.

**Methods**

Mice. B6, B6.Pryt+/−, Rag2−/−, Purβ−/−, IL-15−/−, Kb−/−Db−/− (Taconic Farms), OT-1 TCR transgenic (Jackson Laboratory), and B6.Qa-1 D227K mice (back-crossed for 11 generations) (18) were housed in pathogen-free conditions. CIA induction and assessment. cCII (MD Bioscience) was dissolved in 0.01 M acetic acid at a concentration of 4 mg ml−1 by stirring overnight at 4 °C. All mice used were males between the age of 8 and 12 weeks. To induce CIA, B6 mice were injected intradermally (i.d.) at the base of the digits. Each limb was graded, allowing a maximum score of 12 per mouse. Measurement of Abs against CII. Single-cell suspensions from spleen and LNs of cCII-immune B6 mice were incubated with anti-CD8α antibody (BD Bioscience) purchased from BD Bioscience. Abs to Ly49C/I/F/H, CD122, BTLA, IL-21, and NK42β2, and Foxp3 were purchased from eBioscience.

In vitro differentiation of Th17 subsets and adoptive transfer. Cells from spleen and LNs were collected from B6 mice immunized with 150 μg cCII/CFA, and CD4+CD25−CD4+ cells were purified and enriched using negative selection. 2 × 10^6 ml−1 CD4+ cells were stimulated with 100 μg ml−1 cCII in the presence of 2 × 10^5 irradiated total splenocytes. For differentiation of CD4+ cells to Th17 cell phenotype, the following cytokine cocktail was added to cultures: 3 ng ml−1 TGF-β, 20 ng ml−1 rIL-6, 20 ng ml−1 rIL-23, 10 μg ml−1 anti-IL-12 Ab, 10 μg ml−1 anti-IFN-γ Ab, 10 μg ml−1 anti-IL-4 Ab. At day 5, live CD4+ cells were harvested from cultures by Percoll gradient centrifugation and used for adoptive transfer. Confirmation of Th17 phenotype was performed using FACS analysis of IL-17 expression or using RT-PCR for detection of transcription factor Rorc (19).

Generation of WT and R72A mutant Qa-1 tetramers. A standard tetramer generation protocol was used, as described previously (44, 45). Briefly, an R72A mutant Qa-1 construct was generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) on a WT Qa-1 construct that contained biotin-binding sites for conjugation of monomers to form tetramers. Constructs were then used to transform Rosetta 2 (DE3) competent cells, and inclusion bodies were purified and stored at −80 °C. For analysis of refolding capacity of each individual peptide, candidates were refolded with either WT or R72A mutant Qa-1 heavy chain in the presence of βM. The refolding solution was then analyzed by size exclusion chromatography for a distinctly refolded Qa-1/βM/peptide peak that was collected as a monomer and stored at −80°C before conjugation with streptavidin-phycocerythrin or streptavidin-allophycocyanin to form tetramers prior to each use.

Enrichment for tetramer-positive cells. Single-cell suspensions from spleen and LNs of cCII-immune B6 mice were incubated with anti-CD8α, anti–TCR-β, and PE-labeled Qa-1 tetramers for 30 minutes at room temperature followed by sorting for tetramer-positive and tetramer-negative CD8α+ TCRβ+ cells on a BD FACSaria cell sorter (BD Bioscience). Sorted cells were incubated with IL-15 (10 ng ml−1) for 10 days before further enrichment of tetramer-positive cells as described (10). Briefly, cultured cells were washed and incubated with PE-labeled Qa-1 tetramers for 30 minutes at room temperature followed by washing and resuspension in sorter buffer. Anti-PE microbeads (Miltenyi Biotec) were added to each sample and incubated for 15 minutes at 4°C. Cells were washed, and PE-labeled cells were isolated by passage over an LS magnetic column (Miltenyi Biotec) followed by reanalysis of tetramer staining and adoptive transfer.

**Statistical**. Statistical analyses were performed using 2-tailed Student’s t test or Mann-Whitney test for comparison of 2 conditions. Error bars denote mean ± SEM. A P value of < 0.05 was considered to be statistically significant.
Stud approval. All experiments involving vertebrates were performed in compliance with federal laws and institutional guidelines and approved by the Dana-Farber Cancer Institute Animal Care and Use Committee.

Acknowledgments

This work was supported in part by NIH research grant AI037562, a collaborative research agreement with NovoNordisk A/S, and a gift from the LeRoy Schechter Research Foundation to H. Cantor; and a Swedish Research Council Award and VINNMER—Marie Curie international collaboration (VINNOVA, 2011-03458) to X. Wang. J.W. Leavenworth is an National Research Service Award fellow (T32 CA070083); X. Tang is an NRSA fellow (T32 CA 009382); H.-J. Kim is a scholar of the Arthritis National Research Foundation. We thank C. Schellack Wenander for critical reading and insightful comments and A. Angel for manuscript and figure preparation.

Received for publication September 18, 2012, and accepted in revised form December 13, 2012.

Address correspondence to: Harvey Cantor, Dana-Farber Cancer Institute, 450 Brookline Ave., Boston, Massachusetts 02215 USA.
Phone: 617.632.3348; Fax: 617.632.4630; E-mail: Harvey_Cantor@dfci.harvard.edu.