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

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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-1b (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 Tfh subset (5). However, the potential contribution of CD8+ Treg to the pathogenesis and treatment of this autoimmune disease is not well understood.

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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 (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.

Figure 1
Selective expansion of Hsp60p216-specific Qa-1–restricted CD8+ Tregs inhibits arthritis. (A) Arthritis was induced in B6 mice as described in Methods. Irradiated LPS-activated Kb−/−Db−/− BM-derived DCs were loaded with or without Hsp60p216 or Qdm peptide and injected subcutaneously (6 × 10⁶/mouse) at the tail base at day 27, day 36, and day 49 (diamonds). Arthritis scores are shown for 5–6 mice per group. The group injected with DCs alone versus the group given Hsp60p216-loaded DCs differed significantly. *P < 0.05. (B) Flow cytometry of dLN cells from CIA-immune B6 mice injected with DC-pulsed peptides as in A, after incubation with Qa-1 tetramers labeled with phycoerythrin (Tet-PE) or allophycocyanin (Tet-APC); analysis is shown before and after enrichment by magnetic bead columns for 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 1A, C and D). Although immunization with Qdm-pulsed Kb–/–Db–/– DCs resulted in increased numbers of Qa-1 R72A-Qdm tet+ cells that include both CD122+Ly49+ and CD122+Ly49– populations (ref. 16, Figure 1C), this immunization did not impact 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 Foxp3+CD4+ cells (Supplemental Figure 1E). We also noted that Hsp60p216 immunization resulted in expansion of Hsp60p216-tet+ CD8+ T cells within the CD122+Ly49+ (16) but not the CD122-Ly49–CD8+ subset (Figure 1D). These results suggest that Hsp60p216-Qa-1–restricted CD8+ T cells belonging to the CD122+Ly49+CD8+ subset may inhibit autoimmune arthritis.

Infusion of CD8+ T cells expressing the triad of surface receptors that characterize CD8+ Tregs (CD122+CD44+Ly49–; 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 CD122+CD44+Ly49– CD8+ cells failed to inhibit disease progression. The inhibitory activity of Ly49+CD8+ 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 Ly49+CD8+ Tregs, we asked whether suppression by the Ly49+CD8+ cells depended on IL-15. Transfer of CD8+ T 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 CD122+CD44+Ly49– CD8+ Tregs, and intracellular perforin was required for expression of suppressive activity.
We tested the hypothesis that disease might reflect a TFH-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 T FH-dependent autoantibody production.

These results also suggested that inhibition of the T FH and Th17 cell response might interrupt early events in the autoimmune disease process. Indeed, mice given Th17 cells and autoantibody (mainly anti-CII Ab) in place of T FH 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 T FH-dependent autoantibody production.

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both spleen (data not shown) and inflamed joints, reflecting a broad elimination of potential effector Th activity.

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.

Transfer of CD8+ Tregs specific for Qa-1–Hsp60p216 inhibits arthritis. The IL-15 dependence of CD8+ Tregs allowed us to directly test the hypothesis raised by the results of Figure 1: Hsp60p216–Qa-1–restricted CD8+ cells mediate CD8+ Treg activity. We separated Qa-1–Hsp60p216-tet+ CD8+ cells from the tet– fraction by consecutive FACS sorting and microbead selection (Figure 5A). After in vitro expansion of enriched tet+ CD8+ cells by IL-15C, adoptive transfer of small numbers (5 × 104/mouse) of Qa-1–Hsp60p216-tet+ CD8+ cells, but not Qa-1 R72A–Qdm-tet+ CD8+ cells, into Rag2–/–Prf1–/– hosts along with cCII-immune CD4 and B cells inhibited autoantibody production and halted arthritis progression (Figure 5, B–E). Analysis of CD8+ cells in these adoptive hosts revealed expansion of Qa-1–Hsp60p216-tet+ CD8+ cells after transfer of tet+ but not tet– CD8+ cells (Figure 5E). Moreover, transfer of Qa-1 R72A–Qdm tet+ CD8+ cells did not inhibit autoantibody production (Figure 5E), consistent with the failure of Qdm immunization to suppress CIA (Figure 1).

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...
The inhibitory activity of CD8+ Tregs is exerted within the systemic Tregs found in arthritic synovia (<1% of total T cells) suggests that CD8+ Tregs inhibit TFH and Th17 CD4+ cells but do not target Th1 and Th17 cells and robust inhibition of disease. The finding that Treg activity (16), resulted in reduction of the numbers of T FH Tregs target T FH cells and Th17 cells, the major Th subsets that CD122+CD44+Ly49+ CD8+ cells, which represents less than 5% durable inhibition of this autoimmune disorder. Transfer of reported in CIA (25–27), regulatory CD4+ cells may not result in target cells requires further analysis, the low frequency of CD8+ Tregs cannot be excluded. Although definition of the precise findings (5). However, the possibility that activated DCs that induction of autoantibodies and inflammatory responses by autoimmune Th cells. Here, we describe a strategy that depends on targeting of pathogenic T FH and Th17 cells by CD8+ Tregs.

CD8+ Treg treatment was particularly effective at blunting the autoantibody response in CIA (e.g., Figures 2 and 3). Since CD8+ Tregs target T FH 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+CD44+Ly49+ CD8+ 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 FH and Th17 cells and robust inhibition of disease. The finding that CD8+ Tregs inhibit T FH and Th17 CD4+ 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 target cells requires further analysis, the low frequency of CD8+ Tregs found in arthritic synovia (<1% of total T cells) suggests that the inhibitory activity of CD8+ Tregs is exerted within the systemic lymphoid organs rather than arthritic joints.

Although defective CD4+ (Foxp3+) Treg activity has been reported in CIA (25–27), regulatory CD4+ cells may not result in sustained disease inhibition (28, 29), consistent with our finding of the relevant peptide resulted in selective expansion of CD8+ T cells specific for either Qa-1–Qdm or Qa-1–Hsp60p216, only immunization with the latter peptide efficiently inhibited the development of CIA. Moreover, transfer of a small number of Qa-1–Hsp60p216 tetramer CD8+ cells, but not Qa-1–Qdm–tetrator Qa-1–Hsp60p216–tetrator 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 induction of autoantibodies and inflammatory responses by autoimmune T cells. Further studies are required for a better understanding of the functional difference between Qa-1–Hsp60p216 and Qa-1–Qdm–specific CD8+ T cells. Possibly, differential expansion of Ly49+CD8+ T cells by the 2 self peptides may be relevant to their distinct levels of suppressive activity (Figure 1). Inhibitory signaling associated with 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 Hsp60p10–18, since a subset of CD8+ Tregs that recognizes the
Qa-1–Hsp60sp complex suppresses autoimmunity (43). Further
definition of this subset of CD8+ Tregs requires successful con-
struction 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 pheno-
type (CD122+CD44+Ly49+), they may also be expanded by anti-
gen 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,
then both Qa-1 and HLA-E MHC class Iib 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
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
Treg/Th17 pathogenic Th pair, and diminished autoantibody to
mouse collagen. Whether autoreactive Th cells that induce other
forms of autoimmunity also can be targeted by Hsp60p216–specific
CD8+ Tregs is under investigation. The development of therapeu-
tic strategies based on specific expansion and activation of pep-
tide-specific CD8+ Tregs represents a new and potentially effective
approach to the treatment of autoimmune disease.

Methods

Mice. B6, B6.Prrf1−/−, Rag2−/−, 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
tail with 150 μg of cCII emulsified in CFA (supplemented with 4 mg ml−1
Mycobacterium tuberculosis) and boosted at day 21 with 100 μg of cCII
emulsified in IFA. A cocktail of 4 monoclonal Abs (1 mg; MD Bioscience)
bounding to cCII was used to induce arthritis, as shown in Figure 3B. For
adoptive transfers, CD4+ (CD25−CD4+ Treg depleted) and B cells were
purified as described previously (5) from arthritic mice and then intra-
venously injected into Rag2−/−Prrf1−/− hosts along with FACS-sorted CD8−
or CD4+ Tregs with indicated surface phenotype before immunization at
day 0 and boosting at days 21 and 35. Clinical assessment of CIA was per-
formed every 2–3 days each week, and scoring was as follows: 0, normal; 1,
mild swelling and/or erythema confined to the mid-foot or ankle joint; 2,
moderate edematous swelling extending from the ankle to the metatarsal
joints; and 3, pronounced swelling encompassing the ankle, foot, and
digits. Each limb was graded, allowing a maximum score of 12 per mouse.

Measurement of Abs against CIA. Serum levels of anti-mouse CH IgG
were measured by ELISA after serum collection at the indicated days
after initial cCII immunization. Briefly, 96-well ELISA microplates were
coated with mouse CH (Chondrex) at 5 μg ml−1 dissolved in dilution buf-
fer (Chondrex) at 100 μl/well at 4°C overnight. 100 μl of diluted serum
sample was incubated for 2 hours at room temperature. The plates
were washed with PBST (0.05% Tween-20 in PBS) 5 times, followed by
addition of peroxidase-conjugated goat anti-mouse IgG at 1:50,000
(Sigma-Aldrich) at 100 μl/well. After 1 hour incubation at room tempera-
ture and wash, the final color development was achieved by adding TMB
substrate (BD Bioscience) to each well at 100 μl/well, and absorbance was
measured at 405 nm at the appropriate time.

Flow cytometry. Spleen and LNs were excised, and single-cell suspensions
were prepared. Draining LNs (dLN) included popliteal and inguinal LNs;
on-draining LNs (non-dLN) were axillary, cervical, and mesenteric LNs. For
isolation of intraarticular cells, paw pieces were isolated and digested with
collagenase/dispase (Roche) for 1 hour at 37°C followed by filtration to yield
single-cell suspensions. Cells were incubated with Fc block for 15 minutes
followed by staining with various Abs against surface markers or Qa-1 tetra-
mers. CD45 marker was included to gate leukocytes from joints for further
analysis. For intracellular cytokine staining, cells were restimulated with
leukocyte activation cocktail (BD Bioscience) for 5 hours, stained with sur-
face markers, fixed, and permeabilized, followed by incubation with indi-
cated Abs. Cells were acquired on a FACSCanto II using FACS Diva software
(BD Biosciences) and analyzed with FlowJo software (Tristar). Mouse-spe-
cific Abs to CD8α, TCR-β, CD44, CD45, CD62L, CD25, B220, Fas, CD3, IFN-γ,
IL-17, ICOS, IgM, Ly49Ab, Ly49G2, Ly49F, Ly49C, and NK1.1 were purchased
from BD Bioscience. Abs to Ly49C1/F4/H, CD112, BTLA, IL-21, NKG2D, 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+ cells were purified and enriched using negative selection.

2 × 106 ml−1 CD4+ cells were stimulated with 100 μg ml−1 cCII in the pres-
ence of 2 × 105 irradiated total splenocytes. For differentiation of CD4+ cells
Th17 cell phenotype, the following cytokine cocktail was added to cultures:
100 μg ml−1 TGF-β, 20 ng ml−1 IL-6, 20 ng ml−1 IL-12 p70, 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 centrifuga-
tion and used for adoptive transfer. Confirmation of Th17 phenotype was
performed using FACS analysis of IL-17 expression using or RT-PCR for
detection of transcription factor Rorc (19).

Generation of WT and R72A mutant Qa-1 tetramers. A standard tetramer gen-
eration protocol was used, as described previously (44, 45). Briefly, an R72A
mutant Qa-1 construct was generated using the QuikChange II Site-Direct-
ed Mutagenesis Kit (Stratagene) on a WT Qa-1 construct that contained
biotin-binding sites for conjugation of monomers to form tetramers. Con-
structs were then used to transform Rosetta 2 (DE3) competent cells,
and inclusion bodies were purified and stored at −80°C. For analysis of refold-
ning capacity of each individual peptide, candidates were refolded with either
WT or R72A mutant Qa-1 heavy chain in the presence of βm. The refold-
ing solution was then analyzed by size exclusion chromatography for a distinc-
tly refolded Qa-1/βm/peptide peak that was collected as a monomer and
stored at −80°C for 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 tempera-
ture followed by sorting for tetramer-positive and tetramer-negative
CD8α/β+ TCRβ− cells on a BD FACS Aria cell sorter (BD Bioscience). Sorted
cells were incubated with IL-15C (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.

Statistics. 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.

research article
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