Genetic Regulation of T Regulatory, CD4, and CD8 Cell Numbers by the Arthritis Severity Loci *Cia5a*, *Cia5d*, and the MHC/*Cia1* in the Rat

Max Brenner,[1,2] Teresina Laragione,[1] Nuriza C Yarlett,[1] and Pércio S Gulko[1,3,4,]*

[1]Laboratory of Experimental Rheumatology, The Robert S. Boas Center for Genomics and Human Genetics, Feinstein Institute for Medical Research, Manhasset, New York, USA; [2]North Shore-LIJ Graduate School of Molecular Medicine, Manhasset, New York, USA; [3]New York University School of Medicine, New York, New York, USA; [4]Division of Rheumatology, North Shore University Hospital, Manhasset, New York, USA

T cells have a central role in the pathogenesis of autoimmune arthritis, and several abnormalities in T cell homeostasis have been described in rheumatoid arthritis (RA). We hypothesized that T cell phenotypes, including frequencies of different subsets of T regulatory (Treg) cells and in vitro functional responses could be genetically determined. Furthermore, we considered that the genetic contribution would be accounted for by one of the arthritis regulatory quantitative trait loci (QTL), thus providing novel clues to gene mode of action. T cells were isolated from thymus, peripheral blood, and spleen from DA (arthritis-susceptible) and ACI and F344 (arthritis-resistant) strains and from F344.DA(Cia1), DA.F344(Cia5a), and DA.F344(Cia5d) rats congenic for arthritis QTL. T cell subpopulations differed significantly between DA, F344, and ACI. DA rats had an increased frequency of $CD4^+$ cells, and a reduction in $CD8^+$ and $CD4^+CD45RC^{lo}$ Treg cells, compared with F344. The differences in CD4/CD8 and $CD4^+CD45RC^{lo}$ Treg cells were accounted for by *Cia5a*. DA rats also had a reduced frequency of $CD8^+CD45RC^{lo}CD25^+$ Treg cells compared with F344, and that difference was explained by *Cia5d*. DA rats also had a significantly lower frequency of $CD4^+CD25^+$ and $CD8^+CD25^+$ thymocytes, and of peripheral blood $CD8^+CD45RC^{lo}$ Treg cells, compared with F344 rats, and that difference was accounted for by the MHC. This is the first identification of arthritis severity QTL regulating numbers of $CD4^+CD45RC^{lo}$ (*Cia5a*) and $CD8^+CD45RC^{lo}CD25^+$ (*Cia5d*) Treg cells. The MHC effect on $CD8^+$ Treg cells and $CD25^+$ thymocytes raises a novel potential explanation for its association with arthritis.

Online address: http://www.molmed.org
doi: 10.2119/2007–00003.Brenner

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting approximately 1% of the population. Available treatments induce significant clinical improvement, but rarely achieve cure or remission (1). RA has a strong genetic component, with a heritability of 60% (2), and therefore the identification of genes contributing to disease susceptibility and severity is expected to generate novel and better therapeutic targets. Indeed, several genome-wide studies have identified MHC and non-MHC susceptibility loci (3–7), but little is known about the identity and mode of action of those genes, or of those regulating arthritis severity.

Several arthritis severity quantitative trait loci (QTL) were identified in intercrosses between arthritis-susceptible DA and arthritis-resistant F344 or ACI rats (8–10), and we confirmed the arthritis regulatory effect in congenic strains (11–15). Once identified, the arthritis genes contained within those QTL congenic intervals will elucidate pathways and processes central to disease pathogenesis. We also considered the possibility that immunologic phenotypes known to be relevant to the pathogenesis of arthritis, such as T cell phenotypes, may be genetically regulated, thus generating early clues to candidate QTL genes or pathways for further characterization.

T cells are known to have a central role in the pathogenesis of pristane-induced arthritis (PIA) (16,17), collagen-induced arthritis (CIA) (18–21), and RA (22). Early synovial T cell infiltrates in rodents contain increased numbers of $CD4^+$ cells

Address correspondence and reprint requests to *Pércio S Gulko, Laboratory of Experimental Rheumatology, The Robert S Boas Center for Genomics and Human Genetics, Feinstein Institute for Medical Research, 350 Community Drive, Room 139, Manhasset, NY, 11030. Phone: (516) 562-1275; Fax: (516) 562-1153; E-mail: pgulko@nshs.edu*
Submitted January 16, 2007; Accepted for publication March 7, 2007.

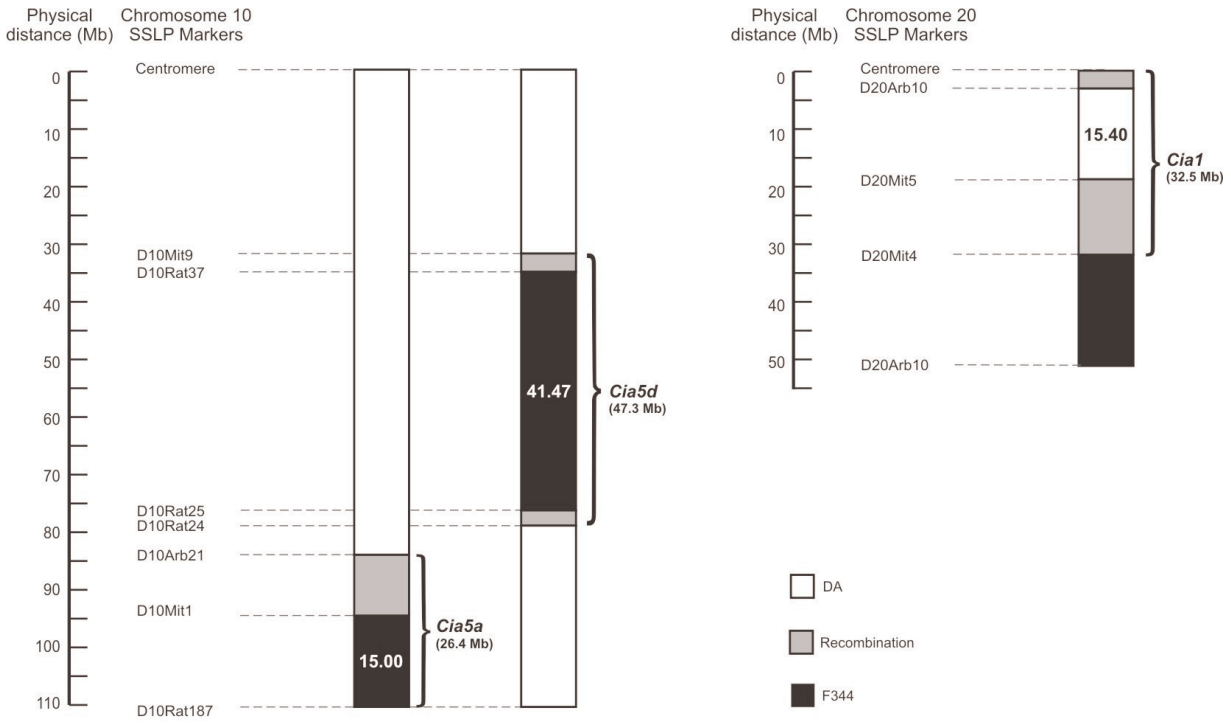

Figure 1. Rat chromosomes 10 and 20 microsatelite (SSLP) markers used in the breeding of DA.F344(Cia5a), DA.F344(Cia5d), and F344.DA(Cia1) congenic strains. Numbers indicate interval distance in megabases (Mb). White: homozygous DA alleles; grey: region of recombination; black: homozygous F344 alleles. Physical positions were retrieved from Ensembl Rat (http://www.ensembl.org/Rattus_norvegicus/) according to the Rat Genome Assembly version RGSC 3.4.

(23), and depletion of these cells prevents (24) and treats established disease (25). Additionally, $CD4^+$ T cells contribute to autoantibody production, inflammation, synovial angiogenesis, hyperplasia, and cartilage and bone destruction in PIA, CIA, and RA (26–31), and T regulatory (Treg) cells are implicated in the suppression of cellular and humoral responses in CIA (32–34) and RA (35). Furthermore, abnormalities in T cell homeostasis and in in vitro responses have been described in RA (36,37) and may be genetically determined.

We specifically hypothesized that the rat chromosome 10 QTL *Cia5a* and *Cia5d*, which determine PIA and CIA severity, may contribute to the genetic regulation of T cell phenotypes. *Cia5a* determines levels of autoantibodies against cartilage type II collagen, and similarly to *Cia5d* regulates synovial events involved in disease pathogenesis, such as infiltration with mononuclear cells, production of IL-1β, angiogenesis, hyperplasia, and bone and cartilage erosions (14), thus making both the *Cia5a* and *Cia5d* QTL obvious candidates for T cell analyses.

In the present study, we demonstrated for the first time that the arthritis-susceptible DA differs form the arthritis-resistant ACI and F344 rat strains in several T cell phenotypes, indicating genetic regulation, and that genes controlling T cell numbers, including subsets of Treg cells, co-segregate with the arthritis severity loci *Cia5a*, *Cia5d*, and the MHC (*Cia1*), providing novel insight into the mode of action of these arthritis genes.

MATERIALS AND METHODS

Rats and Construction of the QTL Congenic and Subcongenic Lines

Specific pathogen-free 8- to 12-week-old female DA/Hsd (DA, arthritis-susceptible) and F344 and ACI (both arthritis-resistant) rats were purchased from Harlan-Sprague Dawley (Indianapolis, IN, USA), and DA/BklArb rats were purchased from Bentin & Kingman (Freemont, CA, USA). The rats were maintained at the Arthritis and Rheumatism Branch at the National Institutes of Health in Bethesda (Arb, NIAMS, NIH) and subsequently transferred to the Feinstein Institute for Medical Research (FIMR). Construction of the DA.F344(Cia5a) and DA.F344(Cia5d) subcongenic and F344.DA(Cia1) congenic lines was previously described (11,12,14). Briefly, an 89.7-Mb interval containing the 2-LOD support interval comprising *Cia5* was introgressed from F344 into DA/BklArb rats through eight genotype-guided backcrosses, followed by intercrossing to generate homozygous DA.F344(Cia5) congenic rats. These congenics were further backcrossed twice to

generate recombinants at the *Cia5a* (26.4 Mb) and *Cia5d* (47.3 Mb) intervals. Identical recombinants were intercrossed to generate DA.F344(Cia5a) and DA.F344(Cia5d) homozygous subcongenic lines (Figure 1). Similarly, the 32.6-Mb 2-LOD interval containing *Cia1*, which includes the rat MHC, was introgressed from DA/BklArb into F344 rats through eight genotype-guided backcrosses followed by at least five intercrosses to generate homozygous F344.DA(Cia1) congenic rats.

All rats were housed in cages with two to three animals, in 12-h light cycles, and received water and regular chow *ad libitum*. All animal experiments were reviewed and approved by the FIMR Institutional Animal Care and Use Committee.

T cell isolation. Peripheral blood, spleen, and thymus were obtained from 8- to 12-week-old nonimmunized rats. Spleen tissue was teased and digested for 30 min with collagenase D (Roche, Indianapolis, IN, USA), and DNase I (Sigma-Aldrich, St. Louis, MO, USA). Mononuclear cell suspensions were isolated through density gradient separation with Lymphocyte Separation Medium (Mediatech, Herndon, VA, USA), and depleted of residual erythrocytes with Gey's lysis buffer. Peripheral blood, thymus, and spleen mononuclear cell suspensions were selectively depleted of B, NK, and myeloid cells with magnetic beads coated with antibodies against OX43, CD172a (SIRP), CD161, and CD45RA (StemCell Technologies, Vancouver, BC, Canada), resulting in a purified suspension of negatively selected T cells (99% $CD3^+$ in blood and spleen) and thymocytes (99% $CD90^+$ in thymus). T cells were stained with Turk's solution and counted. T cells and thymocytes were 93% to 97% viable based on trypan blue (Cambrex, Walkersville, MD, USA) staining.

Flow-cytometric characterization of T cell subsets. Freshly obtained T cells were suspended in PBS with 0.02% azide (Sigma-Aldrich) and 1% bovine serum albumin (P Biomedicals, Aurora, OH, USA), then incubated with anti-CD32 (BD-Pharmingen, San Diego, CA, USA) to block FcγII receptors, and four-color stained with saturating concentrations of different combinations of FITC, PE, PerCP, APC, or biotin-conjugated monoclonal antibodies to CD4 (Serotec, Raleigh, NC, USA), CD3, CD8, CD90, CD45RC, and CD25 (BD-Pharmingen), or the respective isotype controls. Stained cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry within 72 h in a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), using the BD Cell-Quest Pro version 4.0.1 software (Becton Dickinson).

In vitro T cell stimulation, activation, proliferation, and activation-induced cell death (AICD). T cells were plated in 96-well plates at 5×10^5 cells per well (200 µL/well), stimulated with plate-bound anti-CD3 (0.75 µg/well, BD-Pharmingen) plus anti-CD28 (2 µg/mL, BD-Pharmingen), and cultured in RPMI 1640 (Cellgro, Herndon, VA, USA) with 10% fetal bovine serum (Mediatech), 2 mM glutamine, 20 µg/mL gentamicin, 0.5 µg/mL amphotericin B (Invitrogen, Carlsbad, CA, USA), and 50 µM β2-mercaptoethanol (Sigma-Aldrich) at 37°C and 5% CO_2.

T cell activation. Cell surface expression of CD25 (flow cytometry) was used to determine the frequency of activated cells before and 24 h after in vitro stimulation.

T cell proliferation. T cells were labeled with 1 µM carboxy-succinimidyl-fluorescein-ester (CFSE, BD-Pharmingen) and washed with PBS. CFSE incorporation was confirmed by flow cytometry prior to plating. CFSE intensity was measured again 72 h after in vitro stimulation, and the proliferative index was calculated with ModFit LT version 2.0 (Verity Software House, Topsham, ME, USA). Specifically, the proliferative index indicates the average number of cell divisions that the original population underwent, and it is determined by the sum of cells in all generations divided by the calculated number of total precursor cells in the original population (38).

AICD. AICD at 24, 72, and 96 h of in vitro stimulation was quantified by flow cytometry, and cells were considered viable when stained negative for both annexin-V and 7-aminoactinomycin (7-AAD, BD-Pharmingen).

Quantification of cytokines. Supernatant aliquots were collected from splenic T cell cultures after 72 h of stimulation and stored at –80° C until used. Commercially available ELISA kits were used for the determination of IFNγ, TNFα, IL-4, and IL-10 (R&D, Minneapolis, MN, USA). All assays were run in duplicates.

Statistical Analysis

Medians were compared with the Mann-Whitney U test. Differences in cytokine absorbance values were normally distributed, and were compared with the Student t test. SigmaStat version 3.0 (SPSS) was used for statistical analyses. A P value of 0.05 was considered significant.

RESULTS

***Cia5a* accounts for the difference in percentage of CD4 and CD8 T cells seen in DA and F344 rats.** The frequency of mature peripheral blood and spleen CD4 T cells ($CD4^+CD90^-$) was significantly higher in DA compared with F344 rats ($P \leq 0.001$, Figure 2A; and $P = .008$, Figure 2B, respectively), suggesting that numbers of $CD4^+$ cells are genetically regulated. That difference was not explained by the MHC, as F344.DA(Cia1) congenics had $CD4^+$ cell numbers similar to F344. Instead, the higher number of $CD4^+$ cells in DA was accounted for by the non-MHC locus *Cia5a*, as the number of $CD4^+$ $CD90^-$ cells in DA.F344(Cia5a) rats was similar to F344 ($P = .002$, Figure 2A; $P = .033$, Figure 2B).

Peripheral blood and spleen $CD4^+$ recent thymic emigrants (RTE, $CD4^+CD90^+$) followed a distribution similar to $CD4^+$ mature T cells, with increased percentages in DA compared with F344 ($P = .004$, Figure 2C; and $P = .013$, Figure 2D, respectively), and this difference was not explained by the MHC. The difference in

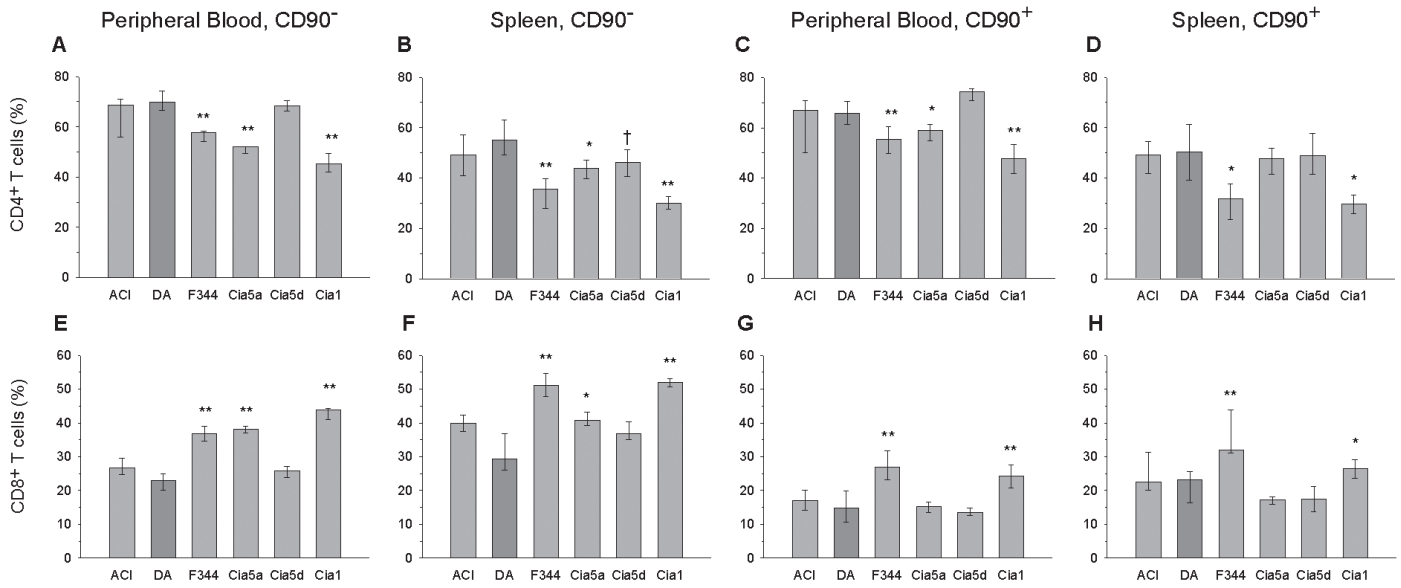

Figure 2. Frequency of $CD4^+$ and $CD8^+$ mature T cells and recent thymic emigrants (RTE). Peripheral blood and spleen of F344 and F344.DA(Cia1) rats had decreased frequency of $CD4^+$ mature T cells (A-B) and $CD4^+$ RTEs (C-D). *Cia5a* accounted for the circulating levels of $CD4^+$ T cells in blood (A) and spleen (B), and of $CD4^+$ RTE in blood. Peripheral blood and spleen of F344 and F344.DA(Cia1) rats had increased frequency of $CD8^+$ T cells (E-F) and RTE (G-H). *Cia5a* accounted for the difference in mature $CD8^+$ T cells (E-F), but not RTE (G-H). Results are expressed as medians ± 25-75 percentiles for ACI (n = 9), DA (n = 9), F344 (n = 9), DA.F344(Cia5a) (n = 5), DA.F344(Cia5d) (n = 6) and F344.DA(Cia1) (n = 6) rats. All results were compared with DA. $^{**}P \leq 0.01$; $^{*}P \leq 0.05$; † $P = .052$; Mann-Whitney U test.

the frequency of $CD4^+CD90^+$ RTE cells was explained by *Cia5a* in peripheral blood ($P = .018$, Figure 2C), but not in the spleen (Figure 2D). These observations indicate that *Cia5a* accounts for the variation in frequency of mature and RTE $CD4^+$ T cells, with the exception of $CD4^+$ RTE in spleen, suggesting that early migration of RTE to the spleen is regulated by genes outside the *Cia5a* interval.

Conversely, peripheral blood and spleen from DA rats had significantly lower frequencies of both $CD8^+$ $CD90^-$ ($P \leq 0.001$, Figure 2E; $P = .006$, Figure 2F, respectively) and $CD8^+$ $CD90^+$ T cells compared with F344 rats ($P \leq 0.001$, Figure 2G; $P = .003$, Figure 2H). The non-MHC locus *Cia5a* accounted for the difference in the percentage of mature $CD8^+$ $CD90^-$ cells ($P = .002$, Figure 2E; $P = .016$, Figure 2F), but not the difference of $CD8^+$ $CD90^+$ RTE (Figure 2G-H), detected in peripheral blood and spleen of DA and F344 rats. These observations implicate *Cia5a* in the regulation of levels of $CD8^+$ $CD90^-$ T cells and suggest that a non-MHC gene other than *Cia5a* or *Cia5d* regulates levels of $CD8^+$ $CD90^+$ cells.

The number of mature splenic $CD4^+$ and $CD8^+$ T cells in DA.F344(Cia5d) were similar to F344 but different from DA rats [DA.F344(Cia5d) versus DA: CD4, $P = .052$, Figure 2B; CD8, $P = .068$, Figure 2F], suggesting that this locus may also regulate differences between DA and F344. There was no significant difference in the number of $CD4^+$ and $CD8^+$ T cells between DA and ACI rats.

The MHC regulates the frequency of thymocytes with a $CD25^+$ regulatory phenotype. F344 rats had a 2.4-fold increased frequency of $CD4^+$ $CD25^+$ thymocytes ($P = .034$, Figure 3A), and a fourfold increased frequency of $CD8^+$ $CD25^+$ thymocytes ($P = .004$, Figure 3B) compared with DA. F344.DA(Cia1) and DA rats had similar frequencies of $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ thymocytes, demonstrating that the MHC accounts for the difference between DA and F344 rats. The numbers of $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ thymocytes were also similar in ACI and DA rats, which have the same MHC haplotype, further supporting the MHC contribution. The frequency of $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ T cells in peripheral blood and spleen of DA, F344, and ACI rats was not significantly different (data not shown).

***Cia5a* regulates the frequency of $CD4^+$ $CD45RC^{lo}$ regulatory cells.** The frequency of $CD4^+$ $CD45RC^{lo}$ cells in peripheral blood and in the spleen was increased in F344 rats ($P \leq 0.001$, $P = .021$, respectively) and F344.DA(Cia1) rats ($P = .001$, $P = .058$, respectively) compared with DA rats (Figure 4A-B), indicating that it is predominantly influenced by genes outside the MHC. The role of non-MHC genes in the regulation of the number of $CD4^+$ $CD45RC^{lo}$ T cells was further demonstrated by the significant differences between ACI and DA rats (peripheral blood: $P = .002$, Figure 4A; spleen: $P = .038$, Figure 4B). The percentage of $CD4^+$ $CD45RC^{lo}$ T cells in peripheral blood of DA.F344(Cia5a) rats was similar to F344 and different from DA ($P = .006$,

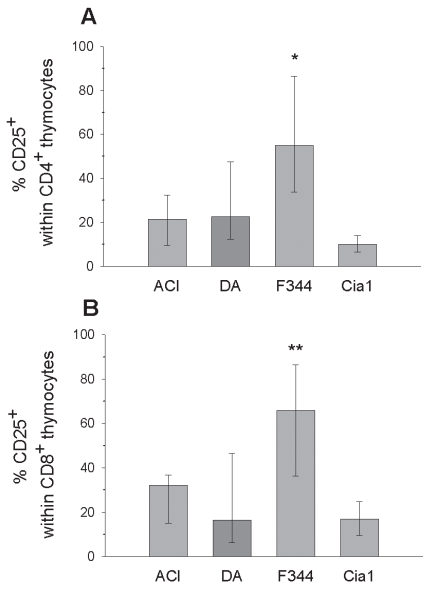

Figure 3. Frequency of CD25 surface expression in thymocytes. F344 rats had increased frequency of (A) $CD4^+$ $CD25^+$ and (B) $CD8^+$ $CD25^+$ thymocytes. DA and F344.DA(Cia1) rats had similar frequencies of $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ thymocytes. Results are expressed as medians ± 25-75 percentiles for ACI (n = 9), DA (n = 9), F344 (n = 9), and F344.DA(Cia1) (n = 6) rats. All results were compared with DA. ** $P \leq 0.01$; * $P \leq 0.05$; Mann-Whitney U test.

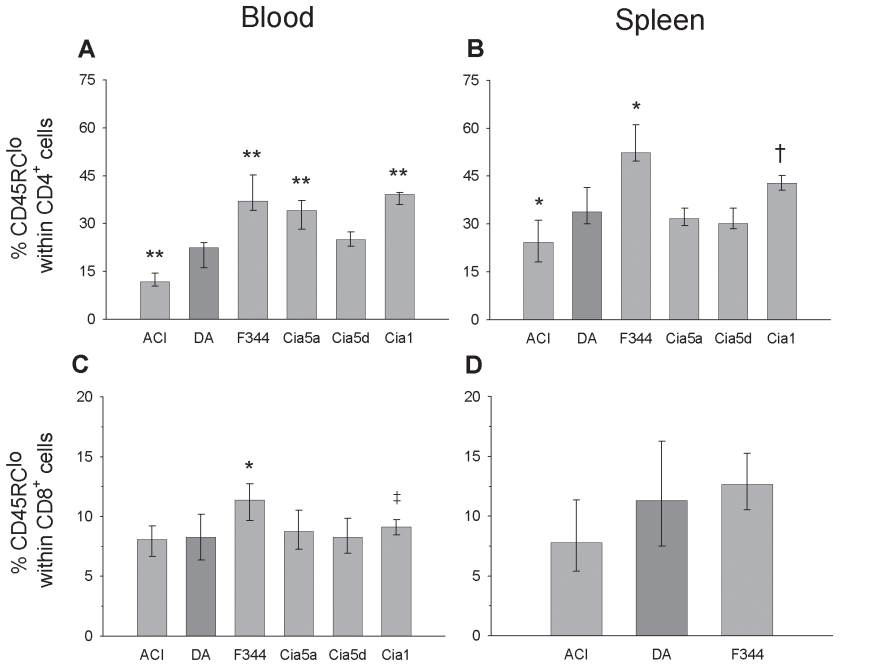

Figure 4. Frequency of $CD45RC^{lo}$ T cells. The frequency of $CD4^+$ $CD45RC^{lo}$ T cells was increased in F344 and F344.DA(Cia1) in both peripheral blood (A) and spleen (B). *Cia5a* accounted for the difference of $CD4^+$ $CD45RC^{lo}$ T cells in peripheral blood (A). ACI rats had reduced frequency of $CD4^+$ $CD45RC^{lo}$ (A-B). $CD8^+$ $CD45RC^{lo}$ T cells were also increased in F344 peripheral blood (C), but not in spleen (D), which was not observed in F344.DA(Cia1) rats (C). Results are expressed as medians ± 25-75 percentiles for ACI (n = 10), DA (n = 10), F344 (n = 10), DA.F344(Cia5a) (n = 5), DA.F344(Cia5d) (n = 9) and F344.DA(Cia1) (n = 6) rats. Compared with DA: ** $P \leq 0.01$, * $P \leq 0.05$, † $P = .058$. Compared with F344: ‡ $P = .039$. Mann-Whitney U test.

Figure 4A), indicating regulation by *Cia5a*. The percentage of $CD4^+$ $CD45RC^{lo}$ T cells in DA.F344(Cia5d) congenics was similar to DA (Figure 4A-B), demonstrating that F344 alleles at the *Cia5d* locus are not involved in the regulation of the number of these cells.

The MHC regulates the frequency of $CD8^+$ $CD45RC^{lo}$ regulatory cells. The frequency of peripheral blood $CD8^+$ $CD45RC^{lo}$ T cells was also higher in F344 compared with DA rats ($P = .031$, Figure 4C), but F344.DA(Cia1) had a frequency similar to DA, indicating that genes within the MHC account for this difference. Accordingly, there was no difference in the number of $CD8^+$ $CD45RC^{lo}$ T cells between DA and ACI rats. *Cia5a* and *Cia5d* did not influence the numbers of $CD8^+$ $CD45RC^{lo}$ T cells in peripheral blood. No significant difference was observed in splenic $CD8^+$ $CD45RC^{lo}$ T cells between DA, F344, and ACI rats (Figure 4D).

***Cia5d* regulates levels of the $CD8^+$ $CD45RC^{lo}CD25^+$ cells.** In peripheral blood, most $CD8^+$ $CD45RC^{lo}$ and mature $CD4^+$ $CD45RC^{lo}$ T cells were $CD25^{+,}$ and there were no significant differences between the parental strains (Figure 5A and Figure 5B). Although ACI rats had fewer splenic $CD4^+$ $CD45RC^{lo}$ T cells than DA rats ($P = .038$, Figure 4B), ACI rats expressed $CD25^+$ more frequently than DA rats ($P = .031$, Figure 5C). The percentage of splenic $CD8^+$ $CD45RC^{lo}CD25^+$ cells was also higher in F344 and DA.F344(Cia5d) rats compared with DA rats ($P = .066$ and $P = .005$, respectively, Figure 5D). These results suggest that F344 rats are enriched for the $CD8^+$ T regulatory cell subpopulation expressing both $CD45RC^{lo}$ and CD25, and that *Cia5d* accounts for this difference.

ACI thymocytes are enriched for double-negative 2 (DN2) cells. We considered that thymocyte development could be genetically regulated, and differences in thymocytes could significantly affect peripheral blood and tissue numbers of T cells. Thymic cells from ACI, DA, and F344 rats were analyzed by flow cytometry. The frequency of thymocytes in the maturation stage DN2 was increased in ACI ($P = .021$), but not in F344 rats, compared with DA (Table 1) rats. The frequency of DN3 and DN4, double positive (DP), single positive (SP) CD4, and SP CD8 thymocytes was not significantly different between DA and F344 or ACI rats (Table 1). These observations suggest

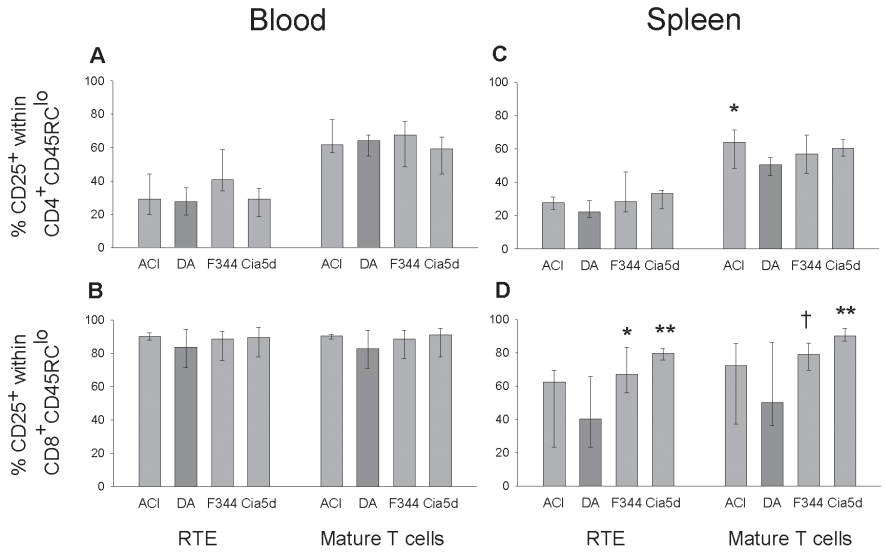

Figure 5. Frequency of CD25 cell surface expression within $CD45RC^{lo}$ T cells. The frequency of $CD4^+$ $CD45RC^{lo}CD25^+$ and $CD8^+$ $CD45RC^{lo}CD25^+$ RTE and mature T cells in peripheral blood was similar in all strains (A-B). ACI rats had a higher percentage of $CD25^+$ cells in splenic $CD4^+$ $CD45RC^{lo}$ mature T cells (C). The frequency of $CD8^+$ $CD45RC^{lo}CD25^+$ splenic cells was increased in F344 rats, and *Cia5d* accounted for this difference (D). Results are expressed as medians ± 25-75 percentiles for ACI (n = 10), DA (n = 10), F344 (n = 10), and DA.F344(Cia5d) (n = 9) rats. All results were compared with DA. $^{**}P \leq 0.01$; $^{*}P \leq 0.05$; †$P = .066$; Mann-Whitney U test.

a unique intrathymic development homeostasis in ACI rats, with yet unclear peripheral consequences.

Total numbers of circulating RTE in DA rats are similar to ACI and F344 rats. The overall frequency of RTE, defined as $CD3^+$ $CD90^+$ cells, did not differ between the strains tested (Table 1), and therefore we could not detect in rats the abnormalities in numbers of RTE previously reported in patients with RA.

DA, ACI, and F344 rats have similar T cells activation, proliferation, cytokine production, and AICD in response to in vitro stimulation. After 24 h of in vitro stimulation, the percentage of spleen and peripheral blood $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ T cells increased similarly in all three parental strains, reflecting similar activation thresholds (Figure 6). After 72 h of stimulation, T cell culture supernatants had similar levels of IFNγ and TNFα (Table 1). Levels of IL-10 were higher in F344 than in DA rats, but this difference did not reach statistical significance (Table 1). Levels of IL-4 were below the detection level (data not shown).

All three parental strains had similar proliferative responses of $CD4^+$ and $CD8^+$

Table 1. Frequencies of different thymocyte maturation stages, recent thymic emigrants, and in vitro cytokine production by stimulated T cells from DA, F344, and ACI rats[a]

		DA	F344	ACI
Subset	*Immunophenotype*			
DN2[b]	CD3-$CD90^+$	39.6 (37.7-46.8)	33.4 (29.9-37.9)	55.9 (51.2-56.5)[c]
DN3 plus DN4[d]	$CD3^+$ $CD90^+$ $CD4^-CD8^-$	0.8 (0.2-1.1)	0.8 (0.2-0.4)	0.5 (0.4-0.8)
DP[d]	$CD3^+$ $CD90^+$ $CD4^+$ $CD8^+$	62.4 (40.7-81.7)	68.0 (47.0-83.3)	68.5 (40.7-79.2)
SP CD4[d]	$CD3^+$ $CD90^+$ $CD4^+$ $CD8^-$	4.3 (2.9-6.3)	3.3 (2.3-4.7)	5.4 (3.3-7.3)
SP CD8[d]	$CD3^+$ $CD90^+$ $CD4^-CD8^+$	29.0 (15.4-52.6)	28.9 (10.6-50.2)	25.6 (15.2-51.2)
RTE blood[e]	$CD3^+$ $CD90^+$	32.8 (30.6-39.7)	33.3 (28.2-44.2)	36.1 (28.8-45.6)
RTE spleen[e]	$CD3^+$ $CD90^+$	37.3 (33.5-41.6)	37.2 (25.3-43.3)	33.9 (27.3-37.1)
Cytokine[f]	*concentration*			
Interferon-γ	ng/mL	46.3 (39-65)	54.4 (47-62)	48.56 (48-48)
IL-10	pg/mL	344 (183-601)	671 (363-908)	232.5 (167-354)
TNFα	pg/mL	311 (207-353)	236 (164-324)	352 (296-366)

[a]Medians (25-75 percentile).
[b]Percentage of total thymocytes, DN = double negative.
[c]$P = .021$, compared with DA (Mann-Whitney U test).
[d]Percentage of $CD3^+$ thymocytes cells; DP = double positive, SP = single positive.
[e]Percentage of T cells; RTE = recent thymic emigrant.
[f]Measured in the supernatant of cultures stimulated with anti-CD3 and anti-CD28 antibodies for 72h.

thymocytes, peripheral blood and splenic RTE, and mature T cells (Figure 7). AICD of peripheral blood, thymic, and splenic T cells increased similarly over time in all three strains (Figure 8). Taken together, these results show no significant differences in in vitro responses of stimulated naïve T cells, suggesting that intrinsic abnormalities in TCR-signaling in the presence of costimulation do not play a significant role in determining the arthritic phenotypes of these parental strains.

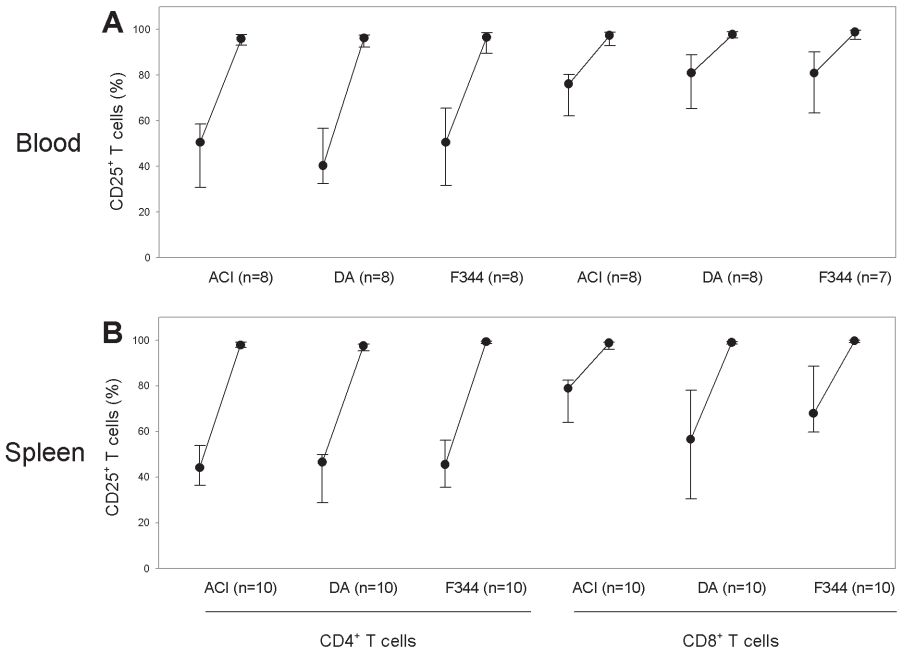

Figure 6. In vitro T cell activation. Mature T cells were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 for 24h. $CD4^+$ and $CD8^+$ T cells from DA and F344 had similar frequency of $CD25^+$ cells at baseline and 24h after stimulation in (A) peripheral blood and (B) spleen. Results are expressed as medians ± 25-75 percentiles. Results were compared with DA using the Mann-Whitney U test, and no significant differences were found.

DISCUSSION

In the process of identifying arthritis severity and susceptibility genes, we hypothesized that at least some of these genes could regulate T cell phenotypes, including T cell development, homeostasis, and function. We included in our studies cell surface marker characteristics and in vitro responses previously described to be abnormal in peripheral blood of RA patients, such as numbers of RTE, CD4/CD8 numbers or ratios, and proliferative responses (35–37, 39–41). Additionally, several T cell characteristics including thymocyte maturation subsets, spleen and peripheral blood subpopulations, and T regulatory cells were analyzed because of their relevance to immune responses. A strong genetic influence on the regulation of several of these T cell characteristics was detected, where arthritis-susceptible and arthritis-resistant strains differed. We also observed for the first time that genetic loci (QTL) implicated in the regulation of arthritis severity also regulate numbers of $CD4^+$ Treg (*Cia5a*) and $CD8^+$ Treg (*Cia5d*) cells.

Arthritis-prone DA rats had increased $CD4^+$ and decreased $CD8^+$ T cell numbers compared with F344 rats, resulting in an increased CD4/CD8 ratio, similar to that reported in synovial and circulating lymphocytes of patients with RA (41). The fact that the CD4/CD8 ratio difference was also detected in RTE suggests that it derives from strain-specific variation in thymic output. Abnormalities in CD4/CD8 thymic lineage commitment are unlikely, because DA and F344 rats had similar frequencies of $CD4^+$ and $CD8^+$ single-positive thymocytes, suggesting differences in post-thymic homeostasis. Genes within the MHC have been shown to affect CD4/CD8 T cell ratio in the rat (42), but F344 and F344.DA(Cia1) rats (congenic for the DA MHC) had similar frequencies of $CD4^+$ and $CD8^+$ cells in peripheral blood and spleen, both of which were different from frequencies in DA rats, pointing to the effect of genes outside the MHC. Indeed, the frequencies of peripheral blood and splenic $CD4^+$ and $CD8^+$ T cells were accounted for by *Cia5a*. The overall lack of effect of *Cia5a* on numbers of $CD4^+$ and $CD8^+$ RTEs suggests that this gene regulates the number of mature T cells by modulating pathways related to post-thymic T cell homeostasis, such as peripheral expansion and/or cellular longevity. Intrinsic abnormalities in T cell proliferative responses and susceptibility to apoptosis were not supported by our in vitro studies. Hence, it is possible that the *Cia5a* effect on T cell turnover is indirect, for example operating via Treg cells, a hypothesis supported by data showing that Treg cells do regulate the numbers of $CD4^+$ T cells (43) .

Results of several studies indicate that Treg cells are able to ameliorate autoimmune diseases in rodents (44), including arthritis (32,33,45,46). Rat natural Treg cells were originally described as $CD45RC^{lo}$TCR α/β $CD4^+$ $CD8^-CD90^-$ thoracic duct lymphocytes (47), and most of these cells were later recognized to be $CD25^+$ (48,49), in agreement with Treg characteristics in mice (50) and humans (51). $CD4^+$ $CD45RC^{lo}CD25^-$ cells can also suppress the development of autoimmunity in vivo (48,49,52). These cells express PD-1, but not FoxP3 (52), and it remains unclear whether they are related to the $CD4^+$ $CD25^-$–inducible Treg cells (Th3 and Tr1) described in the mouse (53). We hypothesized that the number of Treg cells may be genetically regulated

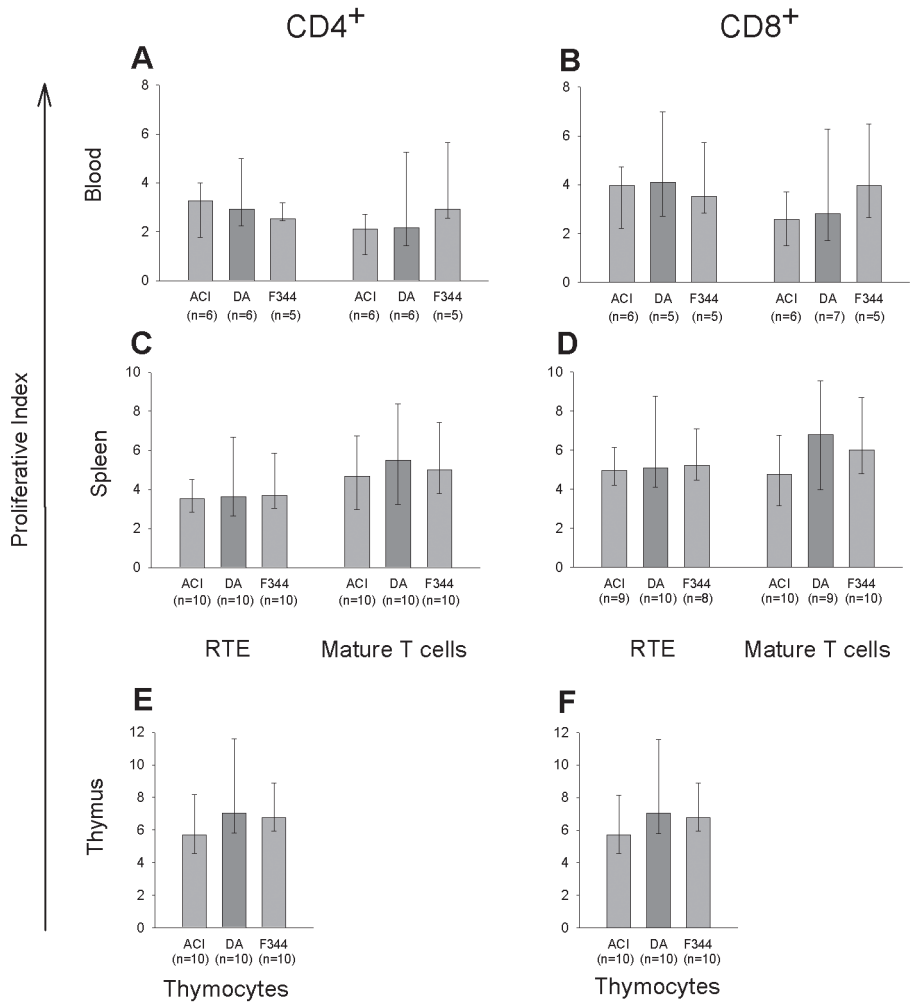

Figure 7. In vitro T cell proliferation. T cells labeled with CFSE were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 for 72 h, and then analyzed by flow cytometry. The proliferative index is the average of divisions undergone by CFSE-labeled cells. Results are expressed as medians ± 25-75 percentiles. Results were compared with DA using the Mann-Whitney U test, and no significant differences were found.

and thus would differ between DA and F344 or ACI rats, and that genetic control could be regulated by *Cia5a* or *Cia5d*.

The overall frequencies of $CD25^+$ T cells in peripheral blood and spleen were similar in DA, F344, and ACI rats. However, the number of $CD4^+$ $CD45RC^{lo}$ cells in peripheral blood and spleen was increased in F344 rats compared with DA rats. In peripheral blood, but not in spleen, $CD4^+$ $CD45RC^{lo}$ cell numbers were regulated by *Cia5a*. We have previously shown that *Cia5a* regulates levels of pathogenic autoantibodies against type II collagen along with arthritis severity (14). Considering that $CD4^+$ $CD45RC^{lo}$ cells are capable of reducing levels of pathogenic antithyroglobulin antibodies along with the incidence of autoimmune thyroiditis in the rat (54), it is very possible that the arthritis and autoantibody regulatory effect of *Cia5a* is mediated via the regulation of $CD4^+$ $CD45RC^{lo}$ Treg cell numbers. Therefore, identifying the *Cia5a* gene may provide a novel way of increasing the generation of Treg cells to treat arthritis and perhaps other autoimmune diseases as well.

The number of $CD4^+$ $CD45RC^{lo}$ cells in arthritis-resistant ACI rats was consistently lower than in DA, and the significance of this finding remains to be determined. We also observed an increased number of $CD4^+$ $CD45RC^{lo}CD25^+$ cells in ACI, raising the possibility of a compensatory increase of this cell population, thus enabling adequate suppression of autoreactive cells and maintenance of the arthritis-resistant phenotype.

$CD8^+$ Treg cells have been implicated in the induction of oral tolerance to various antigens (55), as well as in the regulation of oil-induced arthritis (56), experimental autoimmune encephalomyelitis (57), experimental autoimmune uveitis (58), allograft rejection (59), and graft-versus-host disease (60). $CD8^+$ $CD45RC^{lo}CD25^+$ Treg cells express FoxP3 and CTLA4 and suppress activity of $CD4^+$ T cell responses by cell-to-cell contact (60–62). Our results revealed that F344 rats have increased numbers of splenic $CD8^+$ $CD45RC^{lo}CD25^+$ T cells compared with DA, and that this difference was accounted for by *Cia5d*. Therefore, *Cia5d* may control processes leading to the accumulation of $CD8^+$ $CD45RC^{lo}CD25^+$ cells in the spleen, such as preferential homing or increased local generation. Our data also revealed that numbers of peripheral blood $CD8^+$ $CD45RC^{lo}$ cells, which are a more heterogeneous population, were regulated by the MHC, in agreement with a previous study (63).

F344 rats had a markedly increased frequency of $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ thymocytes compared with DA rats. This difference in $CD25^+$ thymocytes was accounted for by the MHC. These cells are thought to give rise to natural Treg cells and are capable of modulating immune responses in the rat (48,64,65) and in humans (66,67). MHC genes have a major role in the regulation of autoimmune arthritis in rodents (8) as well as in humans (68); nevertheless, nearly 30 years after the first report of the association of

MHC with RA (69), it remains unclear how the MHC regulates disease susceptibility and severity. Our observations suggest a potentially novel model whereby arthritis-favoring alleles at the MHC could interfere with the thymic development of Treg cells.

Surface staining of thymocytes revealed that the DN2 compartment in ACI rats contained nearly 50% more cells than DA, whereas other thymic subpopulations were similar. Although the significance of this finding remains uncertain, DN2 cells are known to give origin to regulatory γ/δ T cells (70–72). Therefore, our observation raises the possibility that ACI DN2 thymocytes might have a reduced rate of apoptosis in vivo, or perhaps differentiate more frequently into regulatory γ/δ T cells. Because ACI and DA rats share the same MHC haplotype, this difference cannot be regulated by the MHC. Instead, numbers of DN2 cells may be regulated by non-MHC arthritis regulatory loci identified in DAxACI F2 studies (9,10,73).

Reduced numbers of RTE have been reported in RA, suggesting decreased thymic output (36,37). However, the similar percentage of $CD90^+$ cells in peripheral blood and spleen indicates that baseline differences in thymic output are not genetically regulated in the DA, F344 and ACI parental strains and do not associate with the arthritic phenotype in these strains.

Increased T lymphocyte proliferative response (36,37) and resistance to AICD (74) were previously reported in RA. Additionally, mouse chromosome 11 loci syntenic to *Cia5d* have been implicated in the regulation of thymocyte proliferation (75), and T cell activation (76) and proliferation (77). While the relevance of these in vitro T cell responses for RA susceptibility and severity remains unclear, they were the basis for some of our experiments. We considered that, similarly to RA, arthritis-susceptible DA rats would have increased in vitro proliferative responses compared with arthritis-resistant ACI or F344 rats, and that *Cia5d* would account for differences between DA and

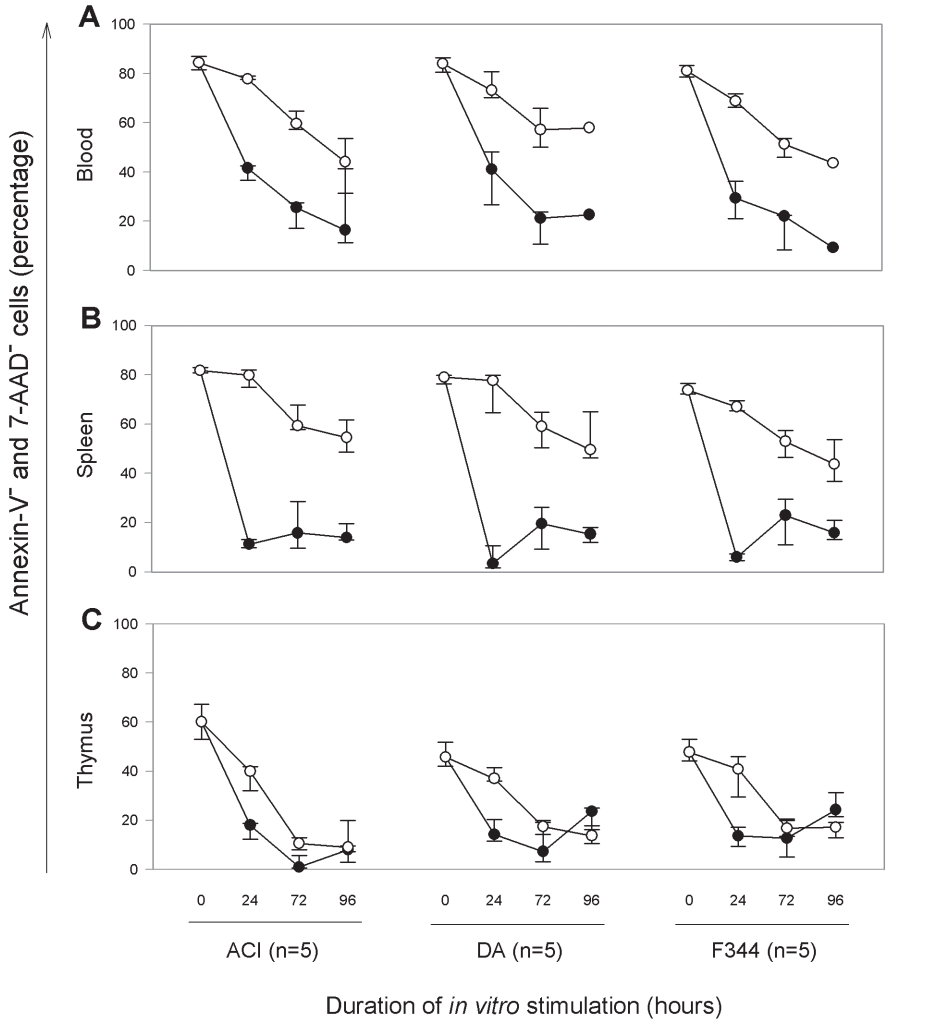

Figure 8. T cell susceptibility to activation-induced cell death. (A) Peripheral blood T cells, (B) spleen T cells, and (C) thymocytes were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 for 24, 72, or 96 h, then labeled with annexin-V and 7-AAD and quantified by flow cytometry. Double-negative cells were considered viable. Results are expressed as medians ± 25-75 percentiles. Results were compared with DA using the Mann-Whitney U test.

F344. However, we observed no significant difference on T cell in vitro proliferative responses between strains. Levels of cytokines in the supernatant were also similar in all parental strains, although F344 T cells tended to produce more IL-10 than DA. $CD4^+$ $CD45RC^{lo}$ and $CD8^+$ $CD45RC^{lo}$ cells are known to release IL-10 upon stimulation (60,78, 9), and it is thus conceivable that the enrichment for these cells may have contributed to the moderately increased levels of IL-10.

The identification and characterization of the *Cia5a* and *Cia5d* arthritis severity genes will significantly increase our understanding of suppressive mechanisms operating in the pathogenesis of autoimmune arthritis. It will also provide strong candidate genes and pathways for case-control association studies in RA. Most importantly, the identification of these two genes will generate new biomarkers for prognostication, and novel targets for therapeutic interventions. Furthermore, *Cia5a* and *Cia5d* may identify a novel way of modulating Treg cells' function or numbers, which will have direct relevance not only for arthritis and other au-

toimmune diseases, but also for cancer, transplantation and infectious diseases. In conclusion, we have identified for the first time arthritis QTL that regulate $CD4^+ CD45RC^{lo}$ (*Cia5a*) and $CD8^+ CD45RC^{lo} CD25^+$ (*Cia5d*) subsets of T regulatory cells. Additionally, we have determined that MHC genes significantly influence the frequency of the more heterogeneous $CD8^+ CD45RC^{lo}$ cell population in peripheral blood, and the frequency of $CD25^+$ thymocytes, raising a novel potential explanation for the long-known MHC association with RA and other forms of autoimmune arthritis.

ACKNOWLEDGMENTS

Funded by National Institutes of Health grants R01-AR46213, R01-AR052439 (NIAMS) and R01-AI54348 (NIAID) to Dr. P Gulko. The authors thank Franak Batliwalla and Houman Kahlili for helpful suggestions with T cell in vitro experiments.

REFERENCES

1. Weinblatt ME, Keystone EC, Furst DE, Kavanaugh AF, Chartash EK, Segurado OG. (2006) Long-term efficacy and safety of adalimumab plus methotrexate in patients with rheumatoid arthritis: ARMADA 4-year extended study. *Ann. Rheum. Dis.* 65:753-9.
2. MacGregor AJ et al. (2000) Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum.* 43:30-7.
3. Cornelis F et al. (1998) New susceptibility locus for rheumatoid arthritis suggested by a genome-wide linkage study. *Proc. Natl. Acad. Sci. U. S. A.* 95:10746-50.
4. Jawaheer D et al. (2001) A genomewide screen in multiplex rheumatoid arthritis families suggests genetic overlap with other autoimmune diseases. *Am. J. Hum. Genet.* 68:927-36.
5. MacKay K et al. (2002) Whole-genome linkage analysis of rheumatoid arthritis susceptibility loci in 252 affected sibling pairs in the United Kingdom. *Arthritis Rheum.* 46:632-9.
6. Shiozawa S et al. (1998) Identification of the gene loci that predispose to rheumatoid arthritis. *Int. Immunol.* 10:1891-5.
7. John S et al. (2004) Whole-genome scan, in a complex disease, using 11,245 single-nucleotide polymorphisms: comparison with microsatellites. *Am. J. Hum. Genet.* 75:54-64.
8. Remmers EF et al. (1996) A genome scan localizes five non-MHC loci controlling collagen-induced arthritis in rats. *Nat. Genet.* 14:82-5.
9. Gulko PS. et al. (1998) Identification of a new non-major histocompatibility complex genetic locus on chromosome 2 that controls disease severity in collagen- induced arthritis in rats. *Arthritis Rheum.* 41:2122-31.
10. Meng HC et al. (2004) Identification of two novel female-specific non-major histocompatibility complex loci regulating collagen-induced arthritis severity and chronicity, and evidence of epistasis. *Arthritis Rheum.* 50:2695-2705.
11. Joe BEF et al. (2000) Genetic dissection of collagen-induced arthritis in Chromosome 10 quantitative trait locus speed congenic rats: evidence for more than one regulatory locus and sex influences. *Immunogenetics* 51:930-44.
12. Remmers EF et al. (2002) Modulation of multiple experimental arthritis models by collagen-induced arthritis quantitative trait loci isolated in congenic rat lines: different effects of non-major histocompatibility complex quantitative trait loci in males and females. *Arthritis Rheum.* 46:2225-34.
13. Brenner M et al. (2005) The non-major histocompatibility complex quantitative trait locus Cia10 contains a major arthritis gene and regulates disease severity, pannus formation, and joint damage. *Arthritis Rheum.* 52:322-32.
14. Brenner M et al. (2005) The non-MHC quantitative trait locus Cia5 contains 3 major arthritis genes that differentially regulate disease severity, pannus formation, and joint damage in collagen- and pristane-induced arthritis. *J. Immunol.* 174:7894-903.
15. Joe B et al. (2002) Evaluation of quantitative trait loci regulating severity of mycobacterial adjuvant-induced arthritis in monocongenic and polycongenic rats: identification of a new regulatory locus on rat chromosome 10 and evidence of overlap with rheumatoid arthritis susceptibility loci. *Arthritis Rheum.* 46:1075-85.
16. Holmberg J, Tuncel J, Yamada H, Lu S, Olofsson P, Holmdahl R. (2006) Pristane, a non-antigenic adjuvant, induces MHC class II-restricted, arthritogenic T cells in the rat. *J. Immunol.* 176:1172-9.
17. Vingsbo C, Sahlstrand P, Brun JG, Jonsson R, Saxne T, Holmdahl R. (1996) Pristane-induced arthritis in rats: a new model for rheumatoid arthritis with a chronic disease course influenced by both major histocompatibility complex and non-major histocompatibility complex genes. *Am. J. Pathol.* 149:1675-83.
18. Klareskog L, Holmdahl R, Larsson E, Wigzell H. (1983) Role of T lymphocytes in collagen II induced arthritis in rats. *Clin. Exp. Immunol.* 51:117-25.
19. Yoshino S, Cleland LG. (1992) Depletion of alpha/beta T cells by a monoclonal antibody against the alpha/beta T cell receptor suppresses established adjuvant arthritis, but not established collagen-induced arthritis in rats. *J. Exp. Med.* 175:907-15.
20. Durie FH, Fava RA, Foy TM, Aruffo A, Ledbetter JA, Noelle RJ. (1993) Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. *Science* 261:1328-30.
21. Knoerzer DB, Karr RW, Schwartz BD, Mengle-Gaw LJ. (1995) Collagen-induced arthritis in the BB rat. Prevention of disease by treatment with CTLA-4-Ig. *J. Clin. Invest.* 96:987-93.
22. Kremer, J. M., R et al. (2003) Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N. Eng.l J. Med.* 349:1907-15.
23. Holmdahl R, Jonsson R, Larsson P, Klareskog L. (1988) Early appearance of activated $CD4^+$ T lymphocytes and class II antigen- expressing cells in joints of DBA/1 mice immunized with type II collagen. *Lab. Invest.* 58:53-60.
24. Moder KG, Luthra HS, Griffiths M, David CS. (1993) Prevention of collagen induced arthritis in mice by deletion of T cell receptor V β 8 bearing T cells with monoclonal antibodies. *Br. J. Rheumatol.* 32:26-30.
25. Yoshino S, Cleland LG, Mayrhofer G. (1991) Treatment of collagen-induced arthritis in rats with a monoclonal antibody against the alpha/beta T cell antigen receptor. *Arthritis Rheum.* 34:1039-47.
26. Brennan FM, Hayes AL, Ciesielski CJ, Green P, Foxwell BM, Feldmann M. (2002) Evidence that rheumatoid arthritis synovial T cells are similar to cytokine-activated T cells: involvement of phosphatidylinositol 3- kinase and nuclear factor kappaB pathways in tumor necrosis factor α production in rheumatoid arthritis. *Arthritis Rheum.* 46:31-41.
27. Burger D. (2000) Cell contact-mediated signaling of monocytes by stimulated T cells: a major pathway for cytokine induction. *Eur. Cytokine Netw.* 11:346-53.
28. Cho ML et al. (2004) Effector function of type II collagen-stimulated T cells from rheumatoid arthritis patients: cross-talk between T cells and synovial fibroblasts. *Arthritis Rheum.* 50:776-84.
29. Koenders MI et al. (2005) Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall-induced arthritis. *Arthritis Rheum.* 52:3239-47.
30. Chabaud M, Garnero P, Dayer JM, Guerne PA, Fossiez F, Miossec P. (2000) Contribution of interleukin 17 to synovium matrix destruction in rheumatoid arthritis. *Cytokine* 12:1092-9.
31. Kotake S et al. (1999) IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J. Clin. Invest.* 103:1345-52.
32. Morgan ME et al. (2003) $CD25^+$ cell depletion hastens the onset of severe disease in collagen-induced arthritis. *Arthritis Rheum.* 48:1452-60.
33. Morgan, M. E et al. (2005) Effective treatment of collagen-induced arthritis by adoptive transfer of $CD25^+$ regulatory T cells. *Arthritis Rheum.* 52:2212-21.
34. Loughry A, Fairchild S, Athanasou N, Edwards J, Hall FC. (2005) Inflammatory arthritis and dermatitis in thymectomized, $CD25^+$ cell-de-

pleted adult mice. *Rheumatology (Oxford)* 44:299-308.
35. Ehrenstein MR et al. (2004) Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. *J. Exp. Med.* 200:277-85.
36. Ponchel F et al. (2002) Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis. *Blood* 100:4550-6.
37. Koetz K, Bryl E, Spickschen K, O'Fallon WM, Goronzy JJ, Weyand CM. (2000) T cell homeostasis in patients with rheumatoid arthritis. *Proc. Natl. Acad. Sci. U. S. A.* 97:9203-8.
38. Xystrakis E et al. (2006) Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. *J. Clin. Invest.* 116:146-55.
39. Schonland SO et al. (2003) Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. *Proc. Natl. Acad. Sci. U. S. A.* 100:13471-6.
40. Ravlic-Gulan J, Gulan G, Novak S, Duletic-Nacinovic A, Matovinovic D, Rukavina D. (2005) A comparison of lymphocyte subpopulations simultaneously on local and systemic levels in acute rheumatoid arthritis patients. *Coll. Antropol.* 29:661-9.
41. Cantagrel A et al. (1988) The transsynovial lymphocytic ratio. Characterization of blood and synovial fluid lymphocytes from patients with arthritic diseases. *J. Rheumatol* 15:899-904.
42. Damoiseaux JG et al. (1999) A dominant role for the thymus and MHC genes in determining the peripheral CD4/CD8 T cell ratio in the rat. *J. Immunol.* 163:2983-9.
43. Khattri R, Cox T, Yasayko SA, Ramsdell F. (2003) An essential role for Scurfin in $CD4^+$ $CD25^+$ T regulatory cells. *Nat. Immunol.* 4:337-42.
44. Shih FF, Mandik-Nayak L, Wipke BT, Allen PM. (2004) Massive thymic deletion results in systemic autoimmunity through elimination of $CD4^+$ $CD25^+$ T regulatory cells. *J. Exp. Med.* 199:323-35.
45. Lu S, Holmdahl R. (1999) Different therapeutic and bystander effects by intranasal administration of homologous type II and type IX collagens on the collagen-induced arthritis and pristane-induced arthritis in rats. *Clin. Immunol.* 90:119-27.
46. Thompson SJ et al. (1993) Prevention of pristane-induced arthritis by the oral administration of type II collagen. *Immunology* 79:152-7.
47. Fowell D, Mason D. (1993) Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes: characterization of the $CD4^+$ T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* 177:627-36.
48. Stephens LA, Mason D. (2000) CD25 is a marker for $CD4^+$ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both $CD25^+$ and CD25- subpopulations. *J. Immunol.* 165:3105-10.
49. Stephens LA, Barclay AN, Mason D. (2004) Phenotypic characterization of regulatory $CD4^+$ $CD25^+$ T cells in rats. *Int. Immunol.* 16:365-75.
50. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155:1151-64.
51. Stephens LA, Mottet C, Mason D, Powrie F. (2001) Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J. Immunol.* 31:1247-54.
52. Hillebrands JL et al. (2006) A regulatory $CD4^+$ T cell subset in the BB rat model of autoimmune diabetes expresses neither CD25 nor Foxp3. *J. Immunol.* 177:7820-32.
53. Liu H, Leung BP. (2006) $CD4^+$ $CD25^+$ regulatory T cells in health and disease. *Clin. Exp. Pharmacol. Physiol.* 33:519-24.
54. Seddon B, Mason D. (1999) Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. *J. Exp. Med.* 189:877-82.
55. Lider O, Santos LM, Lee CS, Higgins PJ, Weiner HL. (1989) Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. II. Suppression of disease and in vitro immune responses is mediated by antigen-specific $CD8^+$ T lymphocytes. *J. Immunol.* 142:748-52.
56. Jansson AM, Lorentzen JC, Bucht A. (2000) $CD8^+$ cells suppress oil-induced arthritis. *Clin. Exp. Immunol.* 120:532-6.
57. Jiang H, Zhang SI, Pernis B. (1992) Role of $CD8^+$ T cells in murine experimental allergic encephalomyelitis. *Science* 256:1213-5.
58. Zhang X, Jiang S, Manczak M, Sugden B, Adamus G. (2002) Phenotypes of T cells infiltrating the eyes in autoimmune anterior uveitis associated with EAE. *Invest. Ophthalmol. Vis. Sci.* 43:1499-508.
59. Yamaguchi YN et al. (1998) Infiltrating CD45RC- T cells are associated with immunologic unresponsiveness induced by donor class I major histocompatibility complex antigens in rats. *Hepatology* 28:450-8.
60. Xystrakis E et al. (2004) Identification of a novel natural regulatory CD8 T-cell subset and analysis of its mechanism of regulation. *Blood* 104:3294-301.
61. Vukmanovic-Stejic M, Vyas B, Gorak-Stolinska P, Noble A, Kemeny DM. (2000) Human Tc1 and Tc2/Tc0 CD8 T-cell clones display distinct cell surface and functional phenotypes. *Blood* 95:231-40.
62. Han G et al. (2007) Suppressor role of rat CD8(+) $CD45RC^{lo}$ T cells in experimental autoimmune uveitis (EAU). *J. Neuroimmunol.* 183:81-8.
63. Xystrakis E et al. (2004) Functional and genetic analysis of two CD8 T cell subsets defined by the level of CD45RC expression in the rat. *J. Immunol.* 173:3140-7.
64. Saoudi A, Seddon B, Fowell D, Mason D. (1996) The thymus contains a high frequency of cells that prevent autoimmune diabetes on transfer into prediabetic recipients. *J. Exp. Med.* 184:2393-8.
65. Seddon B, Saoudi A, Nicholson M, Mason D. (1996) $CD4^+$ $CD8^-$ thymocytes that express L-selectin protect rats from diabetes upon adoptive transfer. *Eur. J. Immunol.* 26:2702-8.
66. Annunziato F et al. (2002) Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. *J. Exp. Med.* 196:379-87.
67. Bienvenu B, Martin B, Auffray C, Cordier C, Becourt C, Lucas B. (2005) Peripheral $CD8^+$ $CD25^+$ T lymphocytes from MHC class II-deficient mice exhibit regulatory activity. *J. Immunol.* 175:246-53.
68. Gregersen PK, J. Silver J, Winchester RJ. (1987) The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum.* 30:1205-13.
69. Stastny P. (1978) Association of the B-cell alloantigen DRw4 with rheumatoid arthritis. *N. Eng.l J. Med.* 298:869-71.
70. Rothenberg EV, Taghon T. (2005) Molecular genetics of T cell development. *Ann. Rev. Immunol.* 23:601-49.
71. Corthay A, Johansson A, Vestberg M, Holmdahl R. (1999) Collagen-induced arthritis development requires alpha/beta T cells but not gammadelta T cells: studies with T cell-deficient (TCR mutant) mice. *Int. Immunol.* 11:1065-73.
72. Skelsey ME, Mellon J, Niederkorn JY. (2001) gama/delta T cells are needed for ocular immune privilege and corneal graft survival. *J. Immunol.* 166:4327-33.
73. Brenner M, Laragione T, Yarlett NC, Li W, Mello A, Gulko PS. 2006. Cia27 is a novel non-MHC arthritis severity locus on rat chromosome 10 syntenic to the rheumatoid arthritis 17q22-q25 locus. *Genes Immun.* 7:335-41.
74. Salmon M et al. (1997) Inhibition of T cell apoptosis in the rheumatoid synovium. *J. Clin. Invest.* 99:439-46.
75. Hsu HC et al. (2002) Age-related change in thymic T-cell development is associated with genetic loci on mouse chromosomes 1, 3, and 11. *Mech. Ageing Dev.* 123:1145-58.
76. Havelkova H, Krulova M, Kosarova M, Holan V, Hart AA, Demant P, Lipoldova M. (1996) Genetic control of T-cell proliferative response in mice linked to chromosomes 11 and 15. *Immunogenetics* 44:475-477.
77. Krulova MH et al. (1997) IL-2-induced proliferative response is controlled by loci Cinda1 and Cinda2 on mouse chromosomes 11 and 12: a distinct control of the response induced by different IL-2 concentrations. *Genomics* 42:11-15.
78. Subra JF et al. (2001) The balance between CD45RChigh and CD45RClow CD4 T cells in rats is intrinsic to bone marrow-derived cells and is genetically controlled. *J. Immunol.* 166:2944-52.
79. Nagai Y, Inobe M, Kikuchi K, Uede T. (1993) Functional and phenotypical analysis of subsets of rat $CD4^+$ T cells. *Microbiol. Immunol.* 37:623-32.