Intrinsic Tolerance in Autologous Collagen-Induced Arthritis Is Generated by CD152-Dependent CD4⁺ Suppressor Cells

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Intrinsic Tolerance in Autologous Collagen-Induced Arthritis Is Generated by CD152-Dependent CD4\(^+\) Suppressor Cells\(^1\)

Alexandra P. Treschow, Johan Bäcklund, Rikard Holmdahl, and Shohreh Issazadeh-Navikas\(^2\)

Collagen-induced arthritis is a mouse model for rheumatoid arthritis (RA) and is commonly induced after immunization with type II collagen (CII) of a non-mouse origin. T cell recognition of heterologous CII epitopes has been shown to be critical in development of arthritis, as mice with cartilage-restricted transgenic expression of the heterologous T cell epitope (MMC mice) are partially tolerized to CII. However, the mechanism responsible for tolerance and arthritis resistance in these mice is unclear. The present study investigated the regulatory mechanisms in naturally occurring self-tolerance in MMC mice. We found that expression of heterologous rat CII sequence in the cartilage of mice positively selects autoreactive CD4\(^+\) T cells with suppressive capacity. Although CD4\(^+\)CD25\(^+\) cells did not play a prominent role in this suppression, CD152-expressing T cells played a crucial role in this tolerance. MMC CD4\(^+\) T cells were able to suppress proliferation of wild-type cells in vitro where this suppression required cell-to-cell contact. The suppressive capability of MMC cells was also demonstrated in vivo, as transfer of such cells into wild-type arthritis susceptible mice delayed arthritis onset. This study also determined that both tolerance and disease resistance were CD152-dependent as demonstrated by Ab treatment experiments. These findings could have relevance for RA because the transgenic mice used express the same CII epitope in cartilage as humans and because autoreactive T cells, specific for this epitope, are present in transgenic mice as well as in patients with RA. The Journal of Immunology, 2005, 174: 6742–6750.

Collagen-induced arthritis (CIA)\(^3\) is a commonly used animal model for rheumatoid arthritis (RA). CIA is induced in mice by injecting collagen type II (CII) in an adjuvant, leading to a disease resembling RA (1). Like RA, CIA is linked to certain MHC class II haplotypes (2), more specifically CIA has been mapped to the A\(^\beta\) molecule (3). T cells have been shown to play an important role in the pathology of CIA (4), possibly by the production of proinflammatory cytokines and by supplying B cell help. The T cell immunodominant region of heterologous CII (human, rat, bovine, and chick) has previously been located to position 256–270 in the A\(^\beta\) mouse strains (5). The heterologous immunodominant region differs from that of mouse CII by a single amino acid (glutamic acid vs aspartic acid in mouse CII at position 266) and has been shown to bind the A\(^\beta\) molecule with higher affinity than the corresponding mouse CII peptide (6). An interesting feature of this immunodominant region is that it can be postranslationally modified, where the lysine at position 264 can be hydroxylated followed by galactosylation or glucosylactosylation of hydroxylsines (5). Together with the flexibility of the glycosylated lysine side chain, this feature gives the potential for several distinct immunodominant epitopes, making tolerization decidedly complicated. However, it has been previously determined that the peptide with a galactose moiety on the hydroxylsine at position 264 is immunodominant in both mice (5) and humans (7).

CII is a possible autoantigen in RA, in which a lack of complete tolerance or interference with tolerogenic mechanisms results in an autoimmune response directed to the joint. Tolerance to CII has been studied in the CIA model by exogenous administration of CII of a non-mouse origin via several roots (8, 9); however this system is not ideal because tolerance is being studied in a heterologous system. We have therefore chosen to use a homologous system in which transgenic mice express the immunodominant epitope of rat CII as a self-Ag and hence become naturally tolerized to rat CII. We have previously shown that when the rat CII T cell epitope is expressed systemically in collagen type I, then mice are completely tolerant to rat CII and are resistant to CIA when immunized with rat CII (10). Conversely, when rat CII is expressed in a cartilage-restricted fashion, then only partial tolerance is achieved, but these mice do have an incidence of arthritis 40–50% lower than that of the nontransgenic wild-type (WT) littermates. Although incidence in mice termed mutated collagen (MMC) transgenic mice is reduced, mice that do develop arthritis have the same onset and severity as WT mice. In vitro, MMC cells have a reduced rat CII-specific proliferation, compared with WT mice, but are still able to mount a significant IFN-\(\gamma\) production (11). This result indicates that rat CII-specific T cells are not deleted but remain capable of effector functions. However, the role of these cells in explaining arthritis susceptible in MMC mice is still unclear.

An important cell surface protein in maintaining T cell tolerance is the CTL-associated Ag 4 (CD152). CD152 is a co-inhibitory molecule expressed on T cells that is known to suppress T cell activity. Blocking CD152 leads to exacerbation of various diseases such as experimental autoimmune encephalomyelitis and diabetes (12, 13) and mice deficient for CD152 developed fatal lymphoproliferation (14). It has also been demonstrated by us and others...
that CD152 plays an important role in inducing and maintaining tolerance, as blocking CD152 in vivo abolishes tolerance and causes autoimmune in various models (15–17). In contrast, the role of CD152 in arthritis has not been clearly defined. It has been proposed that CD152 is up-regulated in RA synovium and is involved in down-regulating the immune response (18), but the role of CD152 in tolerance to CII has not been examined in CIA. CD152 is only transiently expressed on activated T cells; however a subset of regulatory cells (CD4+CD25+) express CD152 constitutively and in this study CD152 has been reported to be important in maintaining regulatory cell function (19, 20).

It has been proposed that the naturally derived thymic CD4+CD25+ regulatory cells are important in several autoimmune diseases in which the protein associated with disease is expressed in the thymus (21). Notably there is no evidence that CII is expressed in the thymus, so the presence of thymic-derived CII-specific CD4+CD25+ regulatory cells is questionable. Furthermore, when newborn mice are depleted of CD4+CD25+ cells, a range of autoimmune diseases develop, but arthritis constitutes only a small percentage (21). Notably, CD4+CD25+ regulatory cells have also been found to be involved in artificial peripheral tolerance (22).

In the present study, using MMC mice, we had a unique opportunity to investigate regulatory mechanisms in an experimental system in which tolerance to rat CII occurs naturally. We found that expression of heterologous rat CII sequence in cartilage leads to selection of autoreactive T cells with suppressive capacity. Although it appears that CD4+CD25+ regulatory cells do not have a prominent role, CD152 does play a vital role in this tolerance. To our knowledge, this is the first report of naturally derived suppressor cells generated in the periphery against self-collagen Ags in which CD152 plays a decisive role.

**Materials and Methods**

### Mice

C3H.Q mice (H-2a) were originally received from Dr. D. C. Shreffler (Washington University, St. Louis, MO) and then kept and bred at the animal facility of Medical Immunization Research, Lund University (Lund, Sweden). Mice used were males between 8- and 16-wk-old and were age-matched in all experiments. The transgenic mouse, MMC, has previously been described (10). Briefly, the MMC transgene is a mutated mouse CII gene, where position 266 has been changed from an aspartic acid to a glutamic acid, thereby expressing the rat CII (260–270) epitope, which is expressed in the thymus (21). Notably there is no evidence that CII is expressed in the thymus, so the presence of thymic-derived CII-specific CD4+CD25+ regulatory cells is questionable. Furthermore, when newborn mice are depleted of CD4+CD25+ cells, a range of autoimmune diseases develop, but arthritis constitutes only a small percentage (21). Notably, CD4+CD25+ regulatory cells have also been found to be involved in artificial peripheral tolerance (22).

In the present study, using MMC mice, we had a unique opportunity to investigate regulatory mechanisms in an experimental system in which tolerance to rat CII occurs naturally. We found that expression of heterologous rat CII sequence in cartilage leads to selection of autoreactive T cells with suppressive capacity. Although it appears that CD4+CD25+ regulatory cells do not have a prominent role, CD152 does play a vital role in this tolerance. To our knowledge, this is the first report of naturally derived suppressor cells generated in the periphery against self-collagen Ags in which CD152 plays a decisive role.

### Antigens

In all immunization protocols pepsin digested rat CII was used. The rat CII was prepared from the SWARM (23) chondrosarcoma by pepsin digestion and further purified as described earlier (24). Lathyritic rat CII was used to restimulate rat CII primed lymphocytes to circumvent reactivity to pepsin. Antigens were prepared from the SWARM (23) chondrosarcoma by pepsin digestion and further purified as described earlier (24). Lathyritic rat CII was used to restimulate rat CII primed lymphocytes to circumvent reactivity to pepsin.

### Immunization

For arthritis experiments, mice were immunized intradermally in the tail base with 100 μg of rat CII emulsified 1:1 in CFA (Difco). Where stated, mice were given a booster injection of 50 μg of rat CII emulsified 1:1 in IFA (Difco). The effect of blocking CD152 was observed by injecting 200 μg per mouse of an anti-CD152 Ab (clone UC10, produced from our hybridoma collection) or an isotype Ab control (clone UC8, produced from our hybridoma collection) at the time of priming and boost immunization with rat CII. For in vitro lymphocyte assays, mice were immunized in the tail base and each hind footpad with 60 μg of rat CII in CFA at each location.

### Purification of cells

Purification of CD4+ cells for in vitro and in vivo experiments was achieved by first negative selection through depletion of non-CD4+ cells. Thereafter, CD4+CD25- and CD4+CD25+ subsets were further purified by negative and positive selection, respectively. Briefly, lymphocytes or pooled lymphocytes and splenocytes in MACs buffer (PBS with 0.5% FCS and 0.2 M EDTA) were incubated with biotinylated Abs specific for NK cell (clone DX5). CD11b+ cells (clone 53-67.6), CD11b+ cells (clone M1/70), and B cells (clone R3A-6B2) for 10 min at 4°C. Subsequently, streptavidin beads (Miltenyi Biotec) were added according to the manufacturer’s recommendation and labeled cells were depleted by auto MACs (Miltenyi Biotec). For further purification, CD4+ cells were incubated for 10 min at 4°C with a biotinylated anti-CD25 Ab (clone 7D4). After subsequent addition of streptavidin beads, CD4+CD25+ and CD4+CD25- cells were separated using auto MACs. All Abs were purchased from BD Pharmingen. Flow cytometry analysis of the resulting fractions routinely showed 90–95% purity. In early control experiments, the suppressive capacity of purified CD4+ cells from rat CII-immunized MMC mice was found to be identical, whether cells were purified through negative selection alone (as described above), or first purified by negative selection and subsequently by positive selection of CD4 (data not shown).

For enrichment of T cells for in vivo studies, pooled lymph node and spleen cells from individual mice were incubated with an anti-MHC class II Ab (clone M5/114, produced from our hybridoma collection) for 20 min at 4°C. Subsequently, Dynabeads (Dynal Biotech) coated with anti-rat IgG Ab were added and cells were spun (1000 rpm). Cells were resuspended and MHC class II+ cells were removed using a magnet. Flow cytometry analysis of the enriched cells showed 97% T cell purity, as determined by the percentage of CD3+ cells.

### Proliferation, coculture, and cytokine production assays

Proliferative studies of cells from inguinal and popliteal lymph nodes, 10 days after immunization, were essentially performed as described earlier (27). Briefly, bulk lymphocytes (i.e., nonfractionated cells) were cultured at a concentration of 1 × 106 cells per well, whereas purified CD4+CD25- and CD4+CD25+ cells were cultured at a concentration of 4.8 × 105 and 1.2 × 106 cells per well, respectively, together with 5 × 105 irradiated (3000 rad) APC from naive syngenic WT mice. For coculture experiments, test suppressor cells were cocultured with responder cells from immunized WT cells (5 × 105 cells per well). Test suppressor cells from WT and MMC mice included; bulk lymphocytes from either naive or preimmunized mice (2.5 × 105 or 5 × 105 cells per well); purified CD4+CD25- cells from immunized mice (1.2 × 105 or 2.4 × 105 per well); or purified CD4+CD25+ cells from immunized mice (0.3 × 105 per well). Flow cytometry analysis of the enriched cells showed 97% T cell purity, as determined by the percentage of CD3+ cells.
1 h at 25°C using 1 M HCl and then neutralized with 1 M NaOH. The ELISA was quantified using the dissociation-enhanced lanthanide fluoroimmunoassay, based on the time resolved fluoroimmunoassay technique with europium-labeled streptavidin (Wallac) as per the manufacturer’s recommendations. The plates were read using a fluorometer (Wallac).

Arthritis development and anti-rat CII Ab response

Development of clinical arthritis was monitored through visual scoring of mice, starting 2 wk after immunization and continuing until 70 days after immunization. Arthritis was evaluated using an extended scoring protocol (28) ranging from 1 to 15 for each paw with a maximum score of 60 per mouse. Each arthritic toe and knuckle was given a score of 1 resulting in a maximum score of 10 per paw. An arthritic ankle or mid-paw was given a score of 5. The anti-rat CII Ab response was determined by measuring the level of rat CII-specific Abs in serum taken at days 35 and 60. The amounts of total anti-rat CII IgG as well as the IgG1 and IgG2a isotypes were determined through quantitative ELISA (29).

Neonatal thymectomy

MMC and WT mice were thymectomized or sham operated at 3–5 days of age. Mice were anesthetized by hypothermia and the thymus was removed using forceps and surgical glue was used to close the incision. When the mice were 7 wk of age a blood specimen from the tail was taken and flow cytometry analysis was performed, as described below. These mice were then immunized at 8 wk of age with the methods previously described.

Transfer experiments

WT and MMC mice were immunized as described above, at day 35 mice were given a boost injection of rat CII in IFA and the arthritis was monitored until day 40. For transfer of bulk lymphocytes, draining lymph nodes were removed from immunized donors individually and a single cell suspension was made in PBS. The cell suspension (20–30 × 10^6 cells) from each donor mouse was then injected into a naive WT mouse and 24 h later the recipients were immunized for arthritis. Recipients were also given a boost injection of rat CII 35 days later. For transfer of enriched T lymphocytes (prepared as stated in the section; purification of cells), the cell suspension (from individual donors) was injected into naive WT mice and 24 h later the recipients were immunized for arthritis. These mice were not given a booster injection.

For transfer of fractionated cells from MMC mice, splens and lymph nodes were prepared and pooled from seven nonarthritic MMC mice, which had been immunized with rat CII 40 days prior as described. Cells were purified into three fractions: CD4 depleted, CD4+CD25+, and CD4+CD25−, as described earlier. CD4-depleted cells (2.8 × 10^6), CD4+CD25− purified cells (1.48 × 10^6), and CD4+CD25+ purified cells (4 × 10^5), i.e., 2.7% of CD4+CD25+ cells that corresponded to their representation in pooled bulk spleen and lymph nodes (as determined by flow cytometry) were then injected i.p. into naive WT mice (five recipients per fraction). Recipient mice were then immunized with rat CII 24 h later along with nontransferred WT and MMC control mice. These mice were not given a booster injection.

Flow cytometry

To determine the percentage of CD4+CD25+ cells, FcRs of the investigated cells were blocked, using an anti-FcRII/III Ab (clone 24G2, from our hybridoma collection). Cells were then stained with the following Abs: allophycocyanin-conjugated anti-Thy1.2 (clone 53-2-1; BD Pharmingen) or cytochrome anti-CD3ε chain (clone 145-2C11; BD Pharmingen); PE-conjugated anti-CD4 (clone H129.19; BD Pharmingen); and cytochrome-conjugated anti-CD8 (clone 53-6.7; BD Pharmingen). However, the latter Ab was omitted if cytochrome anti-CD3 was used. The cells were then analyzed by flow cytometry (FACSort; BD Biosciences). Cells were gated as Thy1.2+ (or CD3+), and the percentage of CD4+CD25+ cells in MMC or WT mice was compared.

Statistics

Frequency of arthritis was analyzed by the χ² test or Fisher’s exact test, whereas Ab levels, in vitro lymphocyte assays and arthritis severity were analyzed with Mann-Whitney U test.

Results

Transgenic expression of the rat CII epitope in MMC mice leads to positive selection of CD152-dependent CD4+ suppressor cells

The suppressive capability of MMC cells was investigated in vitro by coculture of either bulk or purified test suppressor cells from MMC mice with responder cells from rat CII-primed WT mice. Responder cells cocultured with control suppressor cells from WT mice was used for comparison. Interestingly, bulk lymphocytes from both naive and rat CII-immunized MMC mice could significantly suppress the proliferation of the responder cells (Fig. 1A). An aliquot of the bulk lymphocytes from rat CII-immunized MMC and WT mice in Fig. 1A were purified into CD4+CD25+ and CD4+CD25− T cells and tested for their suppressive capacity. Cell numbers of the purified populations were adjusted as described in Materials and Methods. In agreement with earlier studies (19, 30–35) CD4+CD25+ T cells from both immunized WT and MMC mice could suppress the proliferation of responder cells (Fig. 1A). However, only CD4+CD25+ T cells from rat CII-immunized MMC and not WT mice could suppress the proliferation of CII-primed WT responder cells (Fig. 1A).

Suppression was found to require cell-to-cell-contact as no suppression was observed in a transwell system (Fig. 1B). To investigate the specificity of the observed suppression, responder cells were also stimulated with PPD (the antigenic component of CFA, used for immunization) and cocultured with bulk lymphocytes and purified T cells from rat CII-immunized MMC and WT mice. Again, purified CD4+CD25+ T cells from both rat CII-immunized MMC and WT mice could suppress proliferation of responder cells to PPD, but no suppression was observed with nonpurified bulk lymphocytes (Fig. 1C). Importantly, in contrast to the suppression observed for CD4+CD25− T cells from MMC mice against rat CII (Fig. 1A), no suppression was observed against PPD, indicating that suppressive properties of CD4+CD25− T cells in MMC mice was restricted to rat CII.

To investigate the role of CD152 in the observed MMC cell-mediated suppression against rat CII, a CD152-blocking Ab was used in the coculture system. Strikingly, blocking CD152 completely reversed the suppressive ability of both bulk lymphocytes and purified CD4+CD25+ T cells from rat CII-immunized MMC mice (Fig. 1D). Finally, bulk lymphocytes as well as the CD4+CD25+ and CD4+CD25− T cell subsets used earlier as test suppressor cells were also investigated for their proliferative response to rat CII (Fig. 1E). Confirming earlier reports of the tolerized phenotype of MMC mice due to their transgenic expression of the immunodominant rat epitope in the cartilage (10), MMC mice had a significantly reduced proliferative response, compared with WT mice. However, the difference in rat CII-specific immunity between WT and MMC mice, was found to be restricted within the CD4+CD25− population whereas the CD4+CD25+ subsets were found to be equally nonresponsive in both mice.

We also investigated whether the degree of suppression could be correlated with the level of cytokine production. However, we did not detect an increased production of TGF-β, IL-10, or IFN-γ that could be correlated to the degree of suppression (Fig. 2). We also did not detect any increased production of IL-4, irrespective of cell type tested (purified or bulk cells) or in vitro stimuli condition (24, 48, and 72 h of stimulation, data not shown).

Neonatal thymectomy does not alter arthritis susceptibility in MMC mice

CD4+CD25+ cells have been shown to possess potent regulatory properties both in vitro and in vivo (19, 32, 35) and the frequency of CD4+CD25+ regulatory T cells have been found to increase rapidly following induction of oral tolerance (36). As CD4+CD25+ T cells constitutively express CD152, it was plausible that they could be involved in the arthritis resistant characteristic for MMC mice. Consequently, we also wished to determine whether there was any difference in the frequency of CD4+CD25+ T cells between MMC and WT mice in lymph nodes 10 days
postimmunization with rat CII. However, MMC and WT mice were found to harbor comparable numbers of CD4^+CD25^+ T cells (Fig. 3A).

CD4^+CD25^+ cells develop in the thymus of mice after 7 days of age, and thus, neonatally thymectomized mice are deficient of such cells (37). To investigate the role of these cells in rat CII-specific tolerance in MMC mice, 3- to 5-day-old mice were

![FIGURE 1. MMC cells inhibit proliferation of nontransgenic WT littermate cells.](image)

![FIGURE 2. MMC does not suppress nontransgenic littermate cell proliferation through cytokine production.](image)
thymectomized mice. The depletion of CD4⁺CD25⁺ cells following thymectomy was then confirmed at 7 wk of age by flow cytometry analysis (Fig. 3, B and C). Although the frequency of CD4⁺CD25⁺ T cells was decreased, compared with sham-operated animals, there was no difference in total T cell numbers or CD4 to CD8 ratios.

To investigate whether neonatal thymectomy would alter rat CII-specific tolerance and CIA resistance in MMC mice, thymectomized mice were immunized with rat CII at 8 wk of age and monitored for development of clinical arthritis. Forty days after immunization, lymph node cells from nonarthritic MMC mice as well as from arthritic and nonarthritic WT mice were transferred directly into naive WT mice without any Ag stimulation in vitro. Recipient mice were then immunized with rat CII and observed for any alteration in disease susceptibility.

In line with the in vitro data, recipients of lymph node cells from nonarthritic MMC donors had a significantly reduced incidence of arthritis when compared with recipients of WT cells (Fig. 4A). Incidence was also significantly reduced when compared with WT mice that did not receive any cells. In contrast, transfer of lymph node cells from arthritic MMC mice did not offer protection to the WT recipient mice (data not shown). This suggests that once the suppressor cells have lost their regulatory capacity (i.e., arthritis develops in the MMC mouse), they cannot suppress development of disease in arthritis-challenged WT recipients. However, transfer of cells from either arthritic- or nonarthritic WT donors into WT recipient control mice, resulted in comparable arthritis (data not shown).

To increase the number of cells recovered from individual donors, both spleen and lymph nodes from immunized donors were used for subsequent transfer experiment. However, transfer of

Activated MMC T cells are able to suppress arthritis when transferred to WT mice

We next wished to determine whether the observed suppression mediated by cells from MMC mice in vitro was also valid in vivo in a disease-relevant situation. To accomplish this, MMC and WT mice were immunized with rat CII and monitored for development of clinical arthritis. Forty days after immunization, lymph node cells from nonarthritic MMC mice as well as from arthritic and nonarthritic WT mice were transferred directly into naive WT mice without any Ag stimulation in vitro. Recipient mice were then immunized with rat CII and observed for any alteration in disease susceptibility.

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To increase the number of cells recovered from individual donors, both spleen and lymph nodes from immunized donors were used for subsequent transfer experiment. However, transfer of
pooled bulk spleen and lymph node cells from preimmunized MMC mice did not suppress arthritis susceptibility in recipient WT mice (Fig. 4B). This is most likely explained by the fact that pooled bulk splenocytes and lymphocytes from immunized mice contain more activated rat CII-specific B cells than lymph node cells alone. As a result, transfer of activated rat CII-specific B cells would enhance arthritis in recipient mice, as Abs directed toward CII are of critical importance in the effector phase of arthritis development (27). In accordance with this, depletion of APC from pooled bulk spleen and lymph node cells, before transfer, resulted in a significant suppression of arthritis development in rat CII-immunized recipients (Fig. 4B).

After having determined that APC were not involved in the MMC-mediated suppression, we fractionated the non-APC population of MMC donor cells further to identify the suppressive cell population. The in vitro data suggested that the suppressive capabilities reside among CD4⁺ population. Therefore, splenocytes and lymphocytes from preimmunized MMC mice were pooled and purified into three fractions: 1) depleted for CD4⁺, 2) purified CD4⁺CD25⁻, and 3) purified CD4⁺CD25⁺ cells. These fractions were then transferred directly into naive WT mice (again without in vitro stimulation), and recipient mice were then immunized with rat CII in CFA 24 h later and monitored for development of arthritis. Recipients of CD4⁺-depleted cells did not have a suppressed arthritis incidence compared with WT control mice, but rather showed a tendency for an earlier onset of disease (Fig. 4C).

In contrast to the observed in vitro suppression (Fig. 1A), the purified CD4⁺CD25⁻ cells from preimmunized MMC mice did not suppress the arthritis incidence of WT recipients (Fig. 4C). In contrast, a suppressive effect in vivo was suggested for the CD4⁺CD25⁺ fractions. Although the difference in incidence of arthritis did not reach significance (p = 0.168), recipients of CD4⁺CD25⁻ cells from preimmunized MMC mice displayed significantly reduced arthritis severity, compared with WT controls (p = 0.047; Fig. 4C).

Lymph node cells from both naive and rat CII-immunized MMC mice were found to be able to suppress the proliferation of rat CII-primed WT cells in vitro (Fig. 1A). However, when tested in vivo, neither naive MMC bulk spleen cells nor T cell-enriched lymph node cells could suppress arthritis in WT recipients (data not shown). This result suggests that the suppressive population needs to be activated to have an effect in vivo.

**Blockade of CD152 breaks tolerance in MMC mice**

Because in vitro suppression by MMC cells could be abolished by blocking CD152 signaling (Fig. 1D), we also wished to investigate the role of CD152 in vivo. Analysis of naive lymphocytes from MMC and WT mice by flow cytometry showed comparable levels in CD152 expression on CD4⁺ cells (5.9 ± 1.47% compared with 5.4 ± 1.46%, respectively). Expression levels were also comparable when investigated 10 days after immunization with rat CII (5.09 ± 0.63% and 5.03 ± 0.69% for MMC and WT mice, respectively).

To address the role of CD152 in the arthritis resistance of MMC mice, WT and MMC mice were immunized with rat CII and at the same time, an anti-CD152 blocking Ab was administered and arthritis development was monitored. Strikingly, low dose administration of the blocking anti-CD152 Ab resulted in loss of arthritis resistance in MMC mice (Fig. 5A). Interestingly, blocking CD152 raised incidence but did not significantly affect severity of arthritis (Fig. 5B). This observation is in accordance with the fact that MMC mice have a reduced incidence but when disease occurs, it develops with the same severity as WT mice (10). However, the reversal of arthritis resistance by CD152 blockade was not complete, as 36% of anti-CD152 treated MMC mice still remained healthy, compared with 11% of the WT control mice. This result suggests that additional mechanisms are operating or that the treatment was not fully effective in MMC mice. In contrast to MMC mice, blocking CD152 had no significant effect on arthritis in WT mice, most probably due to the low dose used. The common protocol is 200 μg per mouse every second day (i.e., a total of 35 injections), but we chose to inject mice only twice.

To analyze whether anti-CD152 treatment enhanced the autoimmune response against rat CII, blood samples were collected and lymph nodes isolated at the end of the arthritis experiment (day 76 postimmunization). Anti-CD152 treatment resulted in raised anti-rat CII Ab levels in MMC mice (Fig. 6A); however, this increase did not reach statistical significance. In agreement with the increase in arthritis susceptibility of anti-CD152-treated MMC mice, lymph node cells from these mice showed a significantly increased IFN-γ production when stimulated with either the non-modified or glycosylated rat CII peptide compared with control Ab-treated MMC mice (Fig. 6B). A similar increase was also seen in WT mice, although this did not correlate with an increase in arthritis susceptibility (Fig. 6B). The effect of anti-CD152 treatment was also investigated as early as day 10 after immunization with rat CII. However, although anti-CD152 treated MMC and WT mice displayed a somewhat stronger immune response compared with control-treated mice, differences did not reach statistical significance (data not shown), possibly as a result of the single dose of anti-CD152 Ab administered.

**Discussion**

In the present report, we have investigated the mechanism of naturally occurring tolerance to CIA in a homologous model in which transgenic mice express the heterologous immunodominant T cell epitope of rat CII as a self-Ag in a cartilage restricted fashion. This
shown to play a critical role, as blocking signaling through CD152 abolished the suppressive effect of MMC T cells in vitro.

The suppressive capacity of MMC cells was also confirmed in vivo, as transfer of bulk lymph node cells from arthritis-resistant MMC mice resulted in a suppressed arthritis in WT recipient mice. It could be argued that the reduced arthritis seen in recipients of bulk MMC cells, compared with recipients of cells from preimmunized WT mice, was due to the fact that MMC cells contain a lower number of rat CII-specific B cells than WT donor cells. However, this scenario is doubtful as WT mice transferred with MMC cells even had a significantly reduced arthritis incidence compared with nontransferred WT mice (i.e., they did not receive any CII-specific B cells before immunization). Instead, the above result indicates that MMC-derived cells had an active role in protecting from disease. In addition, suppression was also seen when APC-depleted MMC cells as well as CD4⁺CD25⁻ purified MMC cells were transferred. Furthermore, no suppression was seen with MMC-derived cells that had been depleted for CD4⁺ cells.

Thymus-derived CD4⁺CD25⁺ T cells have been shown to have a potent regulatory effect in several models of autoimmune diseases (32). A role of CD4⁺CD25⁻ cells has also been documented in murine CIA (39), although in this case the results are somewhat controversial. Controversy in that report has arisen due to the findings being based on the use of an Ab directed against CD25 that only gives a transient effect on CD25⁺ T cells. The effect of this anti-CD25 Ab in vivo is not fully clarified, and may result in down-regulation of the IL-2R, modulated function of the target cells, or depletion of the CD25⁺ cells in general. Whatever the mechanism of action of this anti-CD25 Ab might be, the fact that it only gives a transient effect questions the findings obtained in the report as the Ab was only administered before immunization. Moreover, a role for naturally occurring CD25⁺ regulatory T cells in CIA is difficult to explain in light of the possibility that regulatory CD25⁺ T cells are selected in the thymus (40), an organ in which we have no evidence for expression of CII (10, 41). Furthermore, it is unknown whether the enzymes that are responsible for the glycosylation of CII are expressed in the thymus. Glycosylation of CII is determined by chondrocytes and an interesting property of rat CII is that APCs have difficulties in processing the O-linked galactose in the immunodominant peptide (42). These two properties may provide an explanation for the unique tissue dependency of the control of tolerance to rat CII.

In the current study, there was no observed difference between MMC and WT cells with regard to the frequency of CD4⁺CD25⁺ T cells. However, this does not necessarily exclude the possibility of an important role of CD4⁺CD25⁺ regulatory cells in MMC mice. Because the MMC mouse has a fully diverse T cell repertoire, it might be difficult to identify such a small population by flow cytometry analysis. Theoretically, a rat CII-specific TCR transgenic MMC mouse would be ideal for such studies. However, the rat CII peptide expressed in the MMC mice is subject to post-translational modifications, thereby generating several epitopes, which are each recognized by distinct T cell clones (5, 26). However in support of the flow cytometry analysis, neonatal thymectomy did not alter the disease profile of MMC mice. Still, this does not exclude a role for peripherally derived CD4⁺CD25⁺ regulatory cells in mediating arthritis resistance in MMC mice. Nevertheless, we show that transfer of CD4⁺CD25⁺ from MMC mice did not protect WT recipient mice from arthritis, indicating that CD4⁺CD25⁺ regulatory cells do not play a major role in this model. It is possible that suppression of arthritis would have been
observed had a larger number of CD4+CD25+ cells been transferred. However, to resemble the natural situation of MMC mice, the number of CD4+CD25+ and CD4+CD25− cells transferred was set to correspond to their representation within the CD4+ T cell compartment. Hence, although the transfer result does not exclude a potential role for CD4+CD25+ cells, the CD4+CD25− cell fraction appeared to be more potent in mediating suppression.

It is plausible that C3H.Q mice are less dependent on CD4+CD25+ regulatory cells compared with other strains, as they did not develop a severe wasting disease (a disease known to be regulated by CD4+CD25+ cells) when neonatally thymectomized. It is conceivable that the genetic background may alter the relative importance of various regulatory populations in acquiring tolerance to self-Ags. In fact, we have recently shown that rat CIA-specific tolerance in MMC mice is differently regulated in mice of different genetic background (43), where MMC mice on the B10.Q background or on the F1 background of B10.Q × C3H.Q appear completely resistant to CIA. Therefore, it would be of interest to investigate whether the rat CIA-specific tolerance in MMC mice on the B10.Q background is more dependent on CD4+CD25+ regulatory cells.

There are several different types of suppressive/regulatory T cells described in the literature. T regulatory type 1 cells have been described as being able to suppress diseases such as arthritis and colitis (44, 45). These cells were found to mediate their effect by producing large quantities of IL-10, as well as TGF-β. However, we found no statistical difference in IL-10 and TGF-β production when MMC- or WT-derived cells were used as test suppressor cells, indicating that the suppressive MMC T cells are not sharing this phenotype. In addition, the protection from arthritis seen in MMC B10.Q mice is not reversed if the IL-10 gene is deleted (46). In contrast, it has been shown recently that orally induced T cell tolerance in a colitis model are dependent on CD152 but not IL-10 (47), which is similar to the findings of the present study. The precise mechanism of the suppressor cells in MMC mice remains to be examined, i.e., whether the suppression is via T cell-T cell interaction or whether the suppression is due to alteration of the rat CIA-presenting APC.

We showed that blocking CD152 in vivo abolished MMC resistance to arthritis and increased the T cell activation to a level comparable to WT mice. There is a general consensus in literature that CD152 engagement leads to a higher activation threshold of T cells. This mechanism is believed to be 3-fold: reduction of IL-2 production (48), a direct effect on cell replication (49), and through interference with TCR signal by dephosphorylation of CD3 (50). However, there is considerable dispute on how CD152 can regulate other T cells or APCs. CD152 has been reported to mediate suppression through enhancement of TGF-β production in some (51), but not all studies (52). It has also been proposed that CD152 mediates suppression by competing for costimulatory molecules on the APC, as the affinity of CD152 for B7 is higher than that of CD28 for B7 (53). Furthermore, it is believed that CD152 engagement of B7 on T cells, when APC-B7 is limiting or absent, leads to unresponsiveness (54). In contrast, it is possible that CD152 mediates T cell suppression directly through T cell-T cell signaling; as activated murine T cells express B7.2. Another interesting possibility is that expression of CD152 on memory T cells protects them from apoptosis and thereby increases their longevity and prolongs their functional role as regulatory T cells (55).

In conclusion, for the first time we report that transgenic expression of heterologous Ag (rat CIA) in mouse cartilage leads to positive selection of autoreactive T cells with regulatory potential. In addition, these intrinsic suppressive T cells provide one way to protect against autoimmune arthritis. In view of the fact that MMC mice share several similarities to patients with RA (express the same CIA epitope, contain CIA-specific autoreactive T cells, and have incomplete tolerance to CIA), this model is valuable in deciphering the mechanism of tolerance directed toward self-Ag.

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Disclosures

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References


