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A CD4 CELL IS CAPABLE OF TRANSFERRING SUPPRESSION OF COLLAGEN-INDUCED ARTHRITIS

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Collagen-induced arthritis can be suppressed by i.v. injection of intact type II collagen (CII) but not type I collagen before immunization. To identify the mechanism mediating this suppression, splenocytes were obtained from mice injected with CII or OVA and administered to recipients that were subsequently immunized with CII. Mice receiving cells from donors injected with CII had a lower incidence of arthritis and lower antibody titers than those receiving cells from OVA-injected donors. Treatment of cells with 3000 rad of γ-irradiation abrogated the suppression. To determine the phenotype of these donor cells, spleen cells were fractionated by adherence to plates coated with mouse anti-IgG to enrich for Thy-1+ phenotype. Thy-1+ cells injected into naive mice could significantly suppress arthritis. Further depletion of T cell subsets by panning revealed that depletion of CD4+ cells prevented the transfer of suppression whereas removal of CD8+ cells had no effect. Isolated CD4+ cells transferred into naive mice were also suppressive. Recently the PgP-1 (Ly-24) Ag has been described to identify a unique memory subset of CD4+ cells when present on the cell surface. In CII-tolerized spleen populations, removal of the PgP-1 (Ly-24) subset of T cells abrogated suppression and transfer of isolated PgP-1+ cells suppressed arthritis. These findings indicate that the PgP-1 subset of CD4+ cells can suppress collagen-induced arthritis and suggest that a CD4+ memory cell down-regulates autoimmunity. In addition, treatment of donor animals with cyclosporin, which inhibits the development of CD4+ cells, abrogated suppression.

Joint destruction in rheumatoid arthritis results from the complex interaction of numerous factors. Immune mechanisms probably play a critical role both in initiation and regulation of the disease (for review see References 1 and 2). Autoimmunity to normal proteins present in the joint is of particular importance (2). A model for the study of autoimmunity in arthritis has been developed by immunizing susceptible animals with CII (3). Arthritis in this model is characterized by synovitis and erosive cartilage destruction, which bears similarity to some of the lesions of rheumatoid arthritis. Although the mechanisms by which this develops and is regulated are not completely understood, both humoral and cellular immunity to collagen appear to be involved (4).

We have previously found that the preinjection of CII, but not type I collagen by the i.v. route before immunization with CII induced resistance to arthritis in rats (5). In addition, investigators have reported that CIA in rodents could be suppressed by the prior injection of CII either in solution or coupled to spleen cells (5-7), or by oral feeding of native but not denatured CII (3). This suppression appeared to be Ag specific and transferable with cells to naive animals (9-11). Recent studies show abrogation of suppression of CIA by cyclophosphamide treatment (12) and suppression of CIA by a T cell hybridoma (13). This suggests that T cells mediate suppression of CIA. However, the type of T cell responsible for suppression of arthritis and the mechanism for the cell-mediated suppression of inflammation remain unclear.

The present experiments were undertaken to isolate specific regulatory cells, use them to transfer suppression of disease, and characterize the cells as to phenotype and sensitivity to cyclosporin and γ-irradiation. Identification of such cells would allow us to determine the mechanism for mediation of suppression and to provide insights into possible novel therapeutic approaches for rheumatoid arthritis.

MATERIALS AND METHODS

Animals. Female DBA/1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), maintained in groups of six in polycarbonate cages, and fed standard rodent chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. The environment was specifically virus-free and sentinel mice were tested routinely for mouse hepatitis and Sendai virus.

Preparation of CII. Native CII was solubilized from the sternums of adult chickens by limited pepsin digestion, as described earlier (14).

Tolerization. When native CII was used for tolerization, it was first dissolved at a concentration of 1 mg/ml in 0.1 M acetic acid by stirring overnight at 4°C, then dialyzed against 0.15 M NaCl and 0.02 M sodium phosphate, pH 7.4 (PBS). To tolerize mice against CII, each animal was given 1 mg CII either in one i.v. dose or as three daily i.v. doses of 0.33 mg each for 3 consecutive days.

Immunization. For routine immunization, Ag (normally chick CII) was dissolved in 0.01 N acetic acid and emulsified with an equal volume of CFA. CFA was made by grinding heat-killed Mtb (Fisheries and Food Central Veterinary Lab., Weybridge, Surrey).

Abbreviations used in this paper: CII, type II collagen; CIA, collagen-induced arthritis; Mtb, Mycobacterium tuberculosis.
suppression of collagen arthritis

United Kingdom) with a mortar and pestle and then adding the ground MTB to IFA (Difco Laboratories, Detroit, MI) at a ratio of 4 mg/ml. The Ag solution was added dropwise to the CFA while continuously mixing with a Virtis 23 homogenizer (Gardner, NY). The temperature was maintained by an ice bath. The resulting suspension was injected i.d. into the base of the tail. Each mouse received a total volume of 0.05 ml containing 100 μg of MTB and 100 μg of Ag. The animals were 8 to 10 wk of age when immunized.

Measurement of the incidence of arthritis. The incidence of arthritis was determined for each mouse by examining and scoring each of the forepaws and hindpaws on a scale of 0 to 4 as described elsewhere (15). There were two separate examiners, one of whom was unaware of the identity of the treatment groups. Each mouse was scored twice a week for a total of 6 wk postimmunization and continuing 3 wk postimmunization. In some experiments the data was expressed as the percentage of arthritic limbs per group of mice. Analyzing the number of limbs involved rather than the number of mice permitted use of smaller groups. It assumed that the occurrence of arthritis in a limb was random and not related to whether other limbs in the same mouse were involved. To confirm the validity of this assumption, the incidence of arthritis (number of animals with one or more arthritic limbs) was also analyzed at one time point, usually 6 wk postimmunization.

Treatment of mice with cyclosporin A. Six mice were given 0.5 mg each of cyclosporin A (20 mg/kg) per dose in a 0.25-ml volume, given s.c. three times a week. Another six mice were treated with 0.1 ml of each of the chromophore base (the base in which cyclosporin A is mixed) daily for a total of 7 days. Both groups of mice were given 1 mg each of CII i.v. on the second day of treatment.

Measurement of serum antibody levels. Mice were bled at 4 and 8 wk postimmunization for antibodies reactive with native CII by using a modification of a previously described ELISA (16). Briefly, native CII was dissolved in 0.1 M acetic acid at 4 mg/ml and diluted to a final concentration of 5 μg/ml in 0.1 M NaHCO₃, immediately before use. Microtiter plates (Nunc, Roskilde, Denmark) were coated by adding 100-μl aliquots of collagen to the wells, incubating overnight at 4°C, and washing the plates to remove unbound collagen. Sera for analysis were serially diluted with 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, containing 0.05% Tween 20 and antibodies bound to the plates were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) were added. Plates were incubated for 4 h, washed, and developed by using 40 μg of orthophenylenediamine dissolved in 100 ml of citrate-phosphate buffer, pH 5.5, to which 40 μl of 30% H₂O₂ was added immediately before use. After 15 min, the absorbance at 490 nm was measured by using an automated reader (Vmax, Molecular Devices, Menlo Park, CA). A standard serum was added to each dilution. For these experiments, we were interested in A\textsuperscript{γ} alone and found that this was determined by computer analysis with the use of a 4-parameter logistic curve. Results are reported as units of activity, derived by comparison of test sera with the standard serum which was arbitrarily defined as having 50 U of activity.

Transfer of suppression by cells. Spleens were harvested from mice given a tolerizing injection of native CII, and separately transferred into uninununized recipients. Three days after receiving cells the recipients were immunized with CII. We found that transfer of 40 million spleen cells was capable of suppressing the development of arthritis (Table 1). By 6 wk postimmunization, 83% of the control animals, which received spleen cells or thymocytes from OVA-treated animals, had arthritis. In contrast, only 20% of the animals receiving spleen cells from mice tolerized to CII had arthritis. The numbers of arthritic limbs were also significantly reduced in mice given cells from donors tolerized with CII (p = 0.0008). In order to determine whether the transferred cells needed to be alive and actively proliferating, they were subjected to γ-irradiation. Irradiated cells from mice tolerized to CII were unable to transfer suppression (Table 1). Interestingly, three days after receiving cells the recipients were immunized with CII. We found that transfer of 40 million spleen cells was capable of suppressing the development of arthritis (Table 1). By 6 wk postimmunization, 83% of the control animals, which received spleen cells or thymocytes from OVA-treated animals, had arthritis. In contrast, only 20% of the animals receiving spleen cells from mice tolerized to CII had arthritis. The numbers of arthritic limbs were also significantly reduced in mice given cells from donors tolerized with CII (p = 0.0008). In order to determine whether the transferred cells needed to be alive and actively proliferating, they were subjected to γ-irradiation. Irradiated cells from mice tolerized to CII were unable to transfer suppression (Table 1).

<table>
<thead>
<tr>
<th>Ag Injected prior to Transfer</th>
<th>No. of Cells Transferred</th>
<th>No. of Recipient Mice with Arthritis ( ^\ast )</th>
<th>No. of Arthritic Limbs in Recipient Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA</td>
<td>Splenocytes</td>
<td>10/12 (83%)</td>
<td>18/48 (38%)</td>
</tr>
<tr>
<td>CII</td>
<td>Splenocytes</td>
<td>2/10 (20%)( ^\ast )</td>
<td>2/40 (5%)</td>
</tr>
<tr>
<td>CII</td>
<td>Splenocytes irradiated</td>
<td>11/12 (92%)</td>
<td>20/48 (42%)</td>
</tr>
<tr>
<td>CII</td>
<td>Splenocytes given day 21</td>
<td>2/6 (33%)</td>
<td>5/24 (21%)</td>
</tr>
</tbody>
</table>

\( ^\ast \) DBA/1 mice were tolerized by injecting 1 mg of either CII or OVA i.v. Three days later their spleens and thymuses were harvested and 40 × 10⁶ cells were injected i.v. into uninununized recipients that were subsequently immunized with 100 μg CII emulsified in CFA. Treatment of CII-tolerized splenocytes with γ-irradiation removed suppression, so that these animals were no longer protected from immunized controls in numbers of arthritic mice or numbers of arthritic limbs.

\( ^\ast \) Mice were evaluated three times weekly for arthritis. Data presented are for prevalence at 6 wk postimmunization.

\( ^\ast \) A Coulter model Epics EL Flow Cytometer and laser flow cytometry.

\( ^\ast \) The χ² with Yates' correction = 11.3 (p = 0.0008).

TABLE 1

Transfer of suppression by splenocytes

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preliminary results indicate that the same cells also suppress when administered after immunization as indicated when six mice were given 50 × 10^6 CII-tolerized splenocytes 21 days after immunization. The incidence of arthritis in this group was 2/6 (33%) compared with an incidence of 83% in 10 of 12 mice given OVA-tolerized splenocytes. Five of 24 limbs or 21% were arthritic at 6 wk postimmunization compared with 18/48 (38%) arthritic limbs of mice given OVA-tolerized splenocytes. Although further work must be done to determine the ability of these cells to alter established arthritis, this preliminary finding is encouraging.

Isolation of cell populations responsible for transfer of suppression. In order to isolate the cells responsible for suppression of disease, spleens from tolerized mice were fractionated by adherence to plastic culture dishes. The nonadherent cells retained the ability to suppress disease (data not shown), suggesting that the important cell was a lymphocyte, either T or B cell. Cells were further fractionated by depleting B cells. A Thy-1" enriched, B cell-depleted population retained the ability to suppress arthritis. In addition to suppression of disease, antibody levels in CII were also significantly lower at 4 wk postimmunization in animals receiving T cells from CII-tolerized donors (Table II).

Determination of the phenotype of T cells responsible for suppression. Because a T cell appeared to be important in mediating suppression, experiments were performed to determine whether this T cell had the CD4+(helper/suppressor-inducer) phenotype or the CD8+(suppressor/cytotoxic) phenotype. Spleen cells from donors tolerized with either CII or OVA were sequentially depleted of B cells, followed by either L3T4-, Lyt-2-, or 1248A4.10-bearing cells. The resulting populations of spleen cells were injected i.v. into recipients that were then immunized with CII. Depletion of L3T4" cells abrogated the transfer of suppression, whereas depletion of Lyt-2 cells or 1248A4.10 cells did not alter it. Depletion of L3T4" cells was repeated on three separate occasions by using groups of six mice each with similar results. The data shown in Table III represent the pooled results of these experiments. Because the L3T4 Ag is specific for the CD4" (helper/suppressor-inducer) phenotype, these data suggest that a CD4" cell plays the important role in suppressing arthritis. Antibody levels to CII were also significantly lower at 4 wk postimmunization in animals receiving splenocytes depleted of Lyt-2 or 1248A4.10 cells as compared with animals receiving splenocytes depleted of L3T4 cells (Table III). Although depletion of Lyt-2" cells and 1248A4.10" cells did not abrogate suppression we cannot rule out the possibility that a Lyt-1", 1248A4.10" cell in the recipient may be the target cell through which a CD4" cell acts.

To confirm their importance, L3T4" cells were collected and transferred to naive animals. At 6 wk, immunized recipients had less arthritis (42%) and fewer arthritic limbs (13%) than controls. The transfer of L3T4" cells was repeated on two separate occasions by using groups of six mice each with similar results. The data shown in Table IV represent the combined data of two separate experiments.

Additional experiments were performed to determine whether the suppressive cell carried the Pgp-1 glycoprotein (Ly-24). This glycoprotein reportedly is acquired by mature murine T lymphocytes at the time of primary Ag stimulation (19). Although initially reported to be specific for memory CD8 T lymphocytes, more recently Pgp-1 has been demonstrated to distinguish Ag-specific CD4" T cells as well (20). Depletion of Pgp-1" cells abrogated the transfer of suppression and when Pgp-1" cells were isolated and transferred to naive animals they were capable of preventing the arthritis induced by subsequent immunization (Table IV).

Treating tolerized animals with cyclosporin A abrogates suppression. Cyclosporin A has been reported to inhibit TCR-mediated activation events (21). In order to determine what effect cyclosporin might have on our system. DBA/1 mice were given either cyclosporin A or the chromophore base before being tolerized with 1 mg of CII i.v. Spleens were harvested from these mice and injected into recipients that were subsequently immunized with CII. Treatment of donors with cyclosporin A

### Table I

<table>
<thead>
<tr>
<th>Ag Injected</th>
<th>Phenotype of Cells Injected</th>
<th>No. of Arthritic Mice</th>
<th>No. of Arthritic Limbs</th>
<th>Mean Antibody Levels to CII ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA Ig-</td>
<td>5/6 (83%)</td>
<td>14/24 (58%)</td>
<td>79.3 ± 31.5</td>
<td></td>
</tr>
<tr>
<td>CII Ig-</td>
<td>2/6 (33%)</td>
<td>3/24 (13%)</td>
<td>26.2 ± 13</td>
<td></td>
</tr>
<tr>
<td>CII L3T4-</td>
<td>15/18 (83%)</td>
<td>32/72 (44%)</td>
<td>97.0 ± 45.2</td>
<td></td>
</tr>
<tr>
<td>CII 1248A4.10-</td>
<td>2/6 (33%)</td>
<td>2/24 (8%)</td>
<td>48.0 ± 3.3</td>
<td></td>
</tr>
</tbody>
</table>

*These spleen from tolerized donors were depleted of Ig- cells, then various T cell subsets using monoclonal antibodies to L3T4, Lyt-2, and 1248A4.10 Ag as described in Materials and Methods. A total of 50 × 10^6 cells from each of the resulting cell populations was given to recipients who were then immunized with CII. Antibody levels were determined 6 wk after immunization and are reported as units of activity. 

Student's t-test (p < 0.005).

### Table II

<table>
<thead>
<tr>
<th>Ag Injected</th>
<th>No. of Cells Transferred</th>
<th>No. of Arthritic Mice</th>
<th>No. of Arthritic Limbs</th>
<th>Mean Antibody Levels to CII ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA Ig-</td>
<td>40 × 10^6</td>
<td>5/5 (100%)</td>
<td>76.6 ± 53.1</td>
<td></td>
</tr>
<tr>
<td>CII Ig-</td>
<td>40 × 10^6</td>
<td>4/18 (22%)</td>
<td>76.6 ± 53.1</td>
<td></td>
</tr>
</tbody>
</table>

*Donors were given 1 mg OVA or native CII i.v. and 3 days later their spleens were removed. Spleen cells were harvested and the cells fractionated as described in Materials and Methods. At 4 wk postimmunization mice were bled and antibody levels to CII determined by ELISA. Data are reported as units of activity. 

| The x^2 with Yates’ correction = 6.94; p = 0.008. |
| The x^2 with Yates’ correction = 12.85; p = 0.0003. |
at the time they received the tolerizing dose of CII abrogated the ability to transfer the suppression (Table V).

**DISCUSSION**

Our finding that cells enriched for the Pgp-1+ subset of the CD4+ phenotype can initiate suppression in the collagen-induced arthritis model is of particular importance. Recently the surface expression of Pgp-1 Ag by T cells has been shown to be acquired at the time of primary antigenic stimulation and constitutively expressed thereafter (19). The observation that Ag-specific T cells are found within the Pgp-1+ subset was first described with CD8+ cells (19, 22) and more recently with CD4+ cells (20). In addition, naive cells that acquire Pgp-1 in some experiments become high producers of IFN-γ and IL-3 (23). Our data that a CD4+ enriched cell population, isolated from spleens after i.v. injection of CII, is also enriched for cells bearing Pgp-1 implies that the cells are Ag specific and have acquired the ability to produce certain lymphokines in response to their specific Ag. Although it remains to be determined whether these CD4+ memory cells that suppress arthritis differ from Th cells that induce arthritis, other investigators have reported finding two distinct populations of memory T cells in mice (24).

Although previous investigators have demonstrated the need for CD4+ cells for the induction of arthritis (25), the ability of CD4+ to down-regulate lymphocyte has not been well established. Other investigators have reported cells capable of suppressing collagen arthritis that seem to utilize other mechanisms. Kresina and coworkers found suppression of arthritis with similar adoptive transfer experiments, but did not demonstrate the need for a CD4+ cell in their system. In vitro proliferative assays suggested a role for CD8+ cells (11) in their experiments, and the subsequent development of a T cell hybridoma capable of suppressing arthritis was negative for the CD4 surface Ag (13). Kakimoto and coworkers (26) developed a T cell line that required attenuation by irradiation to suppress arthritis. Our cells lost the ability to suppress after irradiation, suggesting a requirement for functioning, actively proliferating cells—a different mechanism of action.

Although CD8+ cells have been the classic effector cells in many suppression systems, more recently CD4+ T cells have been found to exert a potent inhibitory effect on the immune system in certain autoimmune situations. Ellerman and coworkers have isolated CD4+ cells from spleens of rats after recovery from experimental allergic encephalomyelitis that prevented the transfer of disease (27). CD4+ cells have also been found to play a role in suppression of immunity against certain immunogenic mouse tumors (28). In addition, Via and Shearer have found that the MRL-lpr/lpr mouse, which develops a disease similar to human SLE, spontaneously develops CD4+ T cells that can suppress the IL-2 production of congenic MRL+/+ L3T4+ Th cells to MHC self-restricted Ag (29). These data suggest that some CD4+ cells perform an immunoregulatory role that is a common feature of autoimmunity.

We have transferred CD4+ enriched spleen cells from tolerated to naive mice, which when used in sufficient quantities were effective in reducing the incidence of the arthritis induced by subsequent immunization. The suppression was associated with a significant decrease in mean antibody levels to CII. Animals given tolerated cells had a significant delay in onset of arthritis and differences between suppressed animals and controls were most pronounced at 6 wk postimmunization. Although the suppressive effect began to fade by 8 wk after immunization, as evidenced by an increasing frequency of arthritis that occurred in previously unaffected animals and by smaller differences in antibody levels, our preliminary data suggested that CII-tolerized spleen cells given 3 wk after immunization were capable of suppressing arthritis, even when the immune response was already established. Although extension of these observations on larger groups of arthritic mice is necessary, these preliminary findings are extremely promising.

In our system CD4+ cells may induce suppression of arthritis by several possible mechanisms. The cells may act directly on CD4+ helper cells in a regulatory feedback capacity, possibly by secreting a suppressive lymphokine similar to the suppressive system described in the MRL-lpr/lpr mouse that functions via T cells acting on other Th cells (29). Alternatively, the CD4+ cell may function as a suppressor-inducer, inducing a CD8+ cell that subsequently suppresses arthritis via a cytotoxic function. A down-regulatory phenomenon has been described in the experimental allergic encephalomyelitis model in which a CD8+ cell line is capable of lysing a CD4+ effector cell, resulting in neutralization of the encephalitogenic effect (30). Other circuits of regulatory cells which down-regulate the immune response have been described (31).

Induction of specific tolerance to CII could be an important means of determining the role of autoimmunity to this protein in human arthritis. It might also provide a new mode of therapy. Our experiments have identified what appears to be a highly specific regulatory Th cell that reduces arthritis when given before immunization. Further studies are needed to firmly establish the identity of the cells involved and of their potential to alter established immunity.

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