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Tolerance to Cyclosporin A-Induced Autologous Graft-Versus-Host Disease Is Mediated by a CD4⁺CD25⁺ Subset of Recent Thymic Emigrants

Dianna Y. Wu and Irving Goldschneider

Our previous studies revealed that both the autoeffector and immunoregulatory T cells in cyclosporin A (CSA)-induced autologous graft-vs-host disease are recent thymic emigrants (RTEs). The autoeffector cells appear in and are released from the thymus during the first week of CSA treatment, whereas the immunoregulatory thymocytes appear during the second week but are not released until several days after cessation of CSA treatment. In the present study, the antigenic phenotypes of these functional T cell subsets were determined by immunomagnetic separation and flow immunocytometric analysis. During CSA wk 1, the autoeffector T cells in both the thymus and lymph node (LN) expressed a CD4⁺CD8⁺ double-positive (DP) phenotype, after which those in the LN became CD8 single positive (SP). Timed thymectomy experiments confirmed that the CD8-SP autoeffector T cells in LN originated from these DP RTEs. During CSA wk 2, the immunoregulatory thymocytes also displayed a DP phenotype. However, they were not exported to the periphery until several days after CSA treatment had been interrupted and they had acquired a CD4-SP phenotype. Cell separation and mixing experiments demonstrated that the autoeffector T cells persist in LN after cessation of CSA treatment, but their activity is not detectable in the presence of recently exported CD4⁺ T cells. Hence, the results indicate that tolerance to CSA-induced autologous graft-vs-host disease is actively mediated by CD25⁺CD4⁺ RTEs that suppress the function of CD8 autoeffector T cells. The Journal of Immunology, 2001, 166: 7158–7164.

Cyclosporine A (CSA) induces autologous graft-vs-host disease (autoGVHD) is a cell-mediated autoimmune syndrome initiated by autoeffector T cells that recognize self MHC class II Ags (1). AutoGVHD can be initiated in adult rats by the administration of CSA either before thymectomy (Tx) or after irradiation and syngeneic bone marrow transplantation (2, 3). Because the thymus is necessary for both the induction and prevention of autoGVHD, it has long been suggested that both autoeffector and immunoregulatory thymocytes are exported to the periphery during/after CSA treatment (3, 4). We have recently confirmed this hypothesis by directly demonstrating the coordinate formation in and release from the thymus of autoeffector and immunoregulatory T cells in CSA-treated rats (5). However, neither the mechanisms by which these two functional populations of recent thymic emigrants (RTEs) are generated nor their precise functions or fates in the peripheral lymphoid tissues are known. This is partially due to the lack of knowledge about their developmental histories and antigenic phenotypes.

In the present study, we traced the phenotypic development of autoeffector and immunoregulatory T cell populations in thymus and LN during and after CSA treatment. The results indicated that 1) both autoeffector and immunoregulatory thymocytes have a CD4⁺CD8⁺ (double-positive (DP)) phenotype during CSA treatment; 2) DP autoeffector thymocytes acquire a CD8-single-positive (SP) phenotype after being exported to the peripheral lymphoid tissues; 3) DP immunoregulatory thymocytes acquire a CD4-SP phenotype before being exported to the periphery; 4) immunoregulatory RTEs reside in the CD25⁺ T cell fraction; and 5) autoeffector T cells persist in lymph node (LN) after cessation of CSA treatment, but their function is suppressed by immunoregulatory T cells. Therefore, the establishment of dominant (active) immune tolerance by CD25⁺CD4-SP RTEs appears to be the major mechanism by which CSA-induced autoGVHD is prevented.

Materials and Methods

Animals and administration of CSA

Four- to six-week-old female inbred Lewis (LEW) rats, purchased from the National Cancer Institute (Frederick, MD), were used throughout this study. CSA in oral solution (generously provided by Novartis Pharmaceuticals, East Hanover, NJ) was mixed into standard rat chow in lieu of source at a concentration of 0.027% and pelleted (Dyets, Bethlehem, PA) (6). Rats were maintained ad libitum on either the CSA-containing food or the same food lacking CSA, with the former maintaining mean serum levels of CSA equivalent to those achieved by therapeutic doses administered parenterally (15 mg/kg body weight per day) (7). In some experiments, rats were fed initially with CSA-containing food for 14 days and then maintained on normal food for several weeks thereafter.

Thymectomy

Tx was performed under ether anesthesia. The sternum was divided in its superior portion, the pericardial soft tissue including the thymus was removed by gentle suction, and the thorax and skin were closed with stainless steel wound clips (BD Biosciences, Sparks, MD). Sham-Tx rats were treated in a similar fashion, but the thymus was not removed.
Cell preparation

Single-cell suspensions of thymus (freed of adherent LN) and peripheral LN (pooled cervical, axillary, and inguinal) were made by gently pressing the lymphoid tissues through a 50-mesh stainless steel tissue into cold RPMI 1640 (Life Technologies, Grand Island, NY). Single-cell suspensions were then subjected to immunomagnetic separation (IMS) followed by flow cytometric analysis as described below.

Immunomagnetic cell separation

To obtain T cell subsets based on surface expression of CD4, CD8, or CD25 molecules, total thymocytes or B cell-depleted LN lymphocytes were reacted with mouse-anti-rat TCR-αβ (clone R73), CD4 (clone W3/25), CD8 (clone OKT8), or CD25 (clone OKT-39) mAbs (Harlan Bioproducts, Indianapolis, IN) at 4°C for 20 min, washed, suspended in staining buffer (PBS with 5 mM EDTA and 0.01% sodium azide;10^7 cells/80 μl), and incubated with goat-anti-mouse IgG microbeads (Miltenyi Biotec, Sunnyvale, CA) at 6–12°C for 20 min (10^7 cells/20 μl). The cells were then washed twice, suspended in separation buffer (PBS with 5 mM EDTA, 0.5% BSA, 0.01% sodium azide; 10^6 cells/500 μl), and applied to the top of a separation column in a magnetic field (Vario MACS; Miltenyi Biotec). B lymphocytes were removed from LN cell suspensions by treating with titrated mouse mAb to rat IgM (clone G53-238, biotin-conjugated; BD Pharmingen, San Diego, CA), for 20 min at 4°C, washed twice, suspended in staining buffer (10^6 cells/90 μl), and treated with streptavidin-conjugated microbeads (Miltenyi Biotec) at 6–12°C for 20 min (10^7 cells/20 μl). After several washes and backflushes with separation buffer, the cells in the elute contained ≥95% T cells as determined by FCM for TCR-αβ.

FCM analysis

One- to three-color labeling for TCR-αβ, CD4, CD8, and/or CD25 was performed on IMS-purified fractions of thymocytes and LN cells by incubating 10^6 cells with FITC-conjugated, PE-conjugated, and/or biotin-conjugated Abs (8). Isotype-matched Abs were used as negative controls. All incubations were for 20 min at 4°C, after which the cells were washed with buffer (PBS containing 0.5% BSA and 0.1% sodium azide). Cells that were stained with biotin-conjugated mAb were then incubated with streptavidin-conjugated microbeads and analyzed on a FACScan flow cytometer (BD Biosciences) equipped with an argon ion laser (488 nm), and data from immunofluorescence samples were analyzed using FACSscan Research Software (BD Biosciences). Lymphocytes were gated on the basis of forward and side scatter, and the percentage of thymocytes and LN T cells belonging to each subset was determined based on 10^6 events.

Quantitative local syngeneic graft-vs-host reaction (synGVHR) assay

Induction. The optimal dose- and time-response kinetics for this assay have been determined previously (5). Briefly, 3 × 10^6 thymocytes or LN T cells from CSA-treated and control LEW rats were injected s.c. with 0.1 ml into the right (experimental) and left (control) hind footpads of normal syngeneic recipients. Alternatively, CD4- and/or CD8-depleted or -depleted subsets of thymocytes and LN T cells isolated by IMS, were injected in numbers equal to those originally present among 3 × 10^6 total cells. Seven days after injection, the total number of T cells in the draining popliteal LN (PLN) was determined by FCM analysis, and the degree of local synGVHR was calculated according to the following formula: local synGVHR index = number of T cells in right PLN/number of T cells in left PLN.

Inhibition. As described previously (5), 3 × 10^6 unfractionated thymocytes or LN T cells from CSA-treated and control LEW rats (or the proportionately lower numbers of cells from CD4- and/or CD8-depleted or -depleted subsets isolated therefrom) were analyzed for immunoregulatory activity after being mixed with 3 × 10^6 LEW LN cells from day 5 of CSA treatment as a standardized source of autoeffector cells. The respective cell mixtures were injected in 0.1 ml into the right (experimental) and the left (control) footpad of normal syngeneic recipients, and the PLNs were harvested 7 days later. The percentage of inhibition of local synGVHR was calculated according to the following formula: percentage of inhibition of local synGVHR = [(number of T cells in left PLN – number of T cells in right PLN) × 100]/number of T cells in left PLN.

Statistical analysis

All experiments were conducted with pooled cells from cohorts of four to eight donors injected into groups of four recipients and were repeated at least twice. The significance of the differences in means between the number of T cells in right (experimental) and left (control) PLN was determined by the paired Student t test.

Results

Phenotypes of autoeffector and immunoregulatory T cells in the thymus during and after CSA treatment

Thymocytes were positively and negatively sorted by IMS according to their CD4 and/or CD8 phenotypes. The degree of enrichment or depletion (≥95%) and the proportion of total cells represented by each fraction was determined by FCM analysis. The autoeffector and immunoregulatory activity of each cell fraction was then tested by local synGVHR.

Results in Fig. 1 show that, on day 4 of CSA treatment, autoeffector activity was recovered in the CD4-, CD8-, and CD4/CD8-enriched thymocyte fractions, which contained both SP and DP cells. In contrast, no autoeffector activity was detected in the CD4-, CD8-, or CD4/CD8-depleted fractions, which contained only SP and/or double-negative cells. In control experiments, autoeffector activity was associated with the TCR-αβ-enriched fraction but was not detected in mixtures of CD4-depleted and CD8-depleted thymocytes (data not shown). Hence, by exclusion, the predominant phenotype of the autoeffector T cells in thymus on day 4 of CSA treatment was CD4^+ CD8^-.

Similar, albeit quantitatively reduced, results were obtained among thymocyte subsets obtained on day 7 of CSA treatment. However, as anticipated (5), no autoeffector activity was detected among thymocytes from day 10 of CSA treatment or day 4 after CSA treatment.

In contrast to autoeffector T cell activity, immunoregulatory T cell activity was not detected in the thymus until the second week of CSA treatment, even after separation of thymocytes into CD4- and/or CD8-positive or -negative subsets. As shown in Fig. 2, the immunoregulatory thymocytes present during wk 2 also expressed a DP phenotype, as did those at wk 3 and 4 of CSA treatment (data not shown). However, within 4 days after cessation of CSA treatment, most of the immunoregulatory thymocytes displayed a CD4-SP phenotype, as evidenced by significantly increased activity in the CD8-depleted but not the CD8-enriched fractions. The immunoregulatory cells then disappeared from the thymus and appeared in LN by day 10 after CSA treatment (see Results below). At all stages, the immunoregulatory activity in the thymus was associated with the TCR-αβ-enriched cell fraction (data not shown).

Phenotypes of autoeffector and immunoregulatory T cells in peripheral LN during and after CSA treatment

As in the thymus, most autoeffector T cells in LN of rats on day 4 of CSA treatment displayed a DP phenotype (Fig. 3). Therefore, they resembled the bulk of RTEs that are released during the first week of CSA treatment (9, 10). However, by day 10 of CSA treatment, some of the autoeffector T cells appeared to have acquired a CD4-SP phenotype, as evidenced by significantly increased activity among thymocytes from day 10 of CSA treatment or day 4 after CSA treatment.

To demonstrate that the CD8-SP autoeffector T cells in LN are the descendants of the DP autoeffector thymocytes generated during wk 1 of CSA treatment, rats were Tx on CSA treatment days 0 or 5 and fed CSA until day 14. Autoeffector activity was then tested among the CD8-SP LN T cells present on day 14 after CSA

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The Journal of Immunology 7159
treatment. As shown in Fig. 4, the appearance of autoeffector T cells in LN was dependent upon the presence of the thymus during, but not after, the first 5 days of CSA treatment.

It was of special interest that, by day 10 after CSA treatment, autoeffector T cell activity in LN continued to be detectable in the CD4-depleted and CD8-enriched fractions but not in the unfractionated or CD4/CD8-enriched fractions (Fig. 3). The explanation for this phenomenon appeared to be the arrival in LN of CD4-SP immunoregulatory T cells between days 4 and 10 after CSA treatment (Fig. 5). As shown in Fig. 6, most of these immunoregulatory cells expressed the CD25 marker characteristic of anergic/suppressor T cells (11–13).

The preceding experiments were done using B cell-depleted LN cells as the source of autoeffector and immunoregulatory cells, thereby suggesting that they were T cells. This was verified by coisolating the reactive LN cells in the TCR-αβ fraction (data not shown).

**(Persistence of autoeffector T cells in LN after the appearance of immunoregulatory T cells)**

To demonstrate formally that CD8-SP autoeffector T cells persist in peripheral lymphoid tissues after cessation of CSA treatment but that their activity is suppressed by CD4-SP immunoregulatory T cells (see Fig. 3, post-CSA-day 10), LN T cells on day 12 after CSA treatment were separated according to their CD4/CD8 phenotypes. As shown in Fig. 7, autoeffector activity was detectable in the CD8-SP fraction but not in the unfractionated or CD4-SP fraction. Furthermore, autoeffector T cell activity was completely suppressed when the two fractions were mixed.

**Discussion**

We have previously demonstrated that the autoeffector and immunoregulatory T cells involved in autoGVHD are sequentially exported from the thymus of rats during and after CSA treatment,

**FIGURE 1.** Phenotype of autoeffector thymocytes during and after CSA treatment. Experimental rats were fed CSA for up to 10 days or for 14 days followed by normal food for an additional 4 days. Control rats were fed normal food only. Total thymocytes ($3 \times 10^6$) or the respective numbers of cells in the CD4- and/or CD8-enriched/depleted subsets therefrom were obtained at the indicated times and injected into the right (experimental) and left (control) footpads of syngeneic recipients. The relative levels of local syn-GVHR were determined 7 days later. The arrow on the y-axis indicates the level above control values at which significant responses occurred ($p < 0.05$).

**FIGURE 2.** Phenotype of immunoregulatory thymocytes during and after CSA treatment. Experimental rats were fed CSA for up to 12 days or for 14 days followed by normal food for up to an additional 10 days. Control rats were fed normal food only. Total thymocytes ($3 \times 10^6$) or the respective numbers of cells in the CD4- and/or CD8-enriched/depleted subsets therefrom were obtained at the indicated times, mixed with $3 \times 10^6$ LN cells from day 5 of CSA treatment as a source of autoeffector cells, and injected into the right (experimental) and left (control) footpads of syngeneic recipients. The relative levels of inhibition of local synGVHR were determined 7 days later. The arrow on the y-axis indicates the level above control values at which significant inhibitory responses occurred ($p < 0.05$).
respectively (5). Here, the antigenic phenotypes of these functionally disparate subsets of thymocytes and T cells are defined by IMS and FCM analysis. As summarized in Fig. 8, autoeffector T cells, generated in the thymus and released to the periphery during the first week of CSA treatment, have a DP phenotype characteristic of cortical thymocytes. Furthermore, the exported T cells express Thy1 (5), which is characteristic of RTEs in the rat (8). These results are consistent with our earlier observations (9, 10) that the vast majority of RTEs that are released during wk 1 of CSA treatment have a DP phenotype. By the second week of CSA treatment, these autoeffector T cells acquire a CD8-SP phenotype and persist in the periphery for at least 2 wk after cessation of CSA treatment. Again, these results are consistent with our previous demonstration of continued maturation of RTEs in the peripheral lymphoid tissues (8).

Immunoregulatory thymocytes, in contrast, first appear in the thymus during wk 2 of CSA treatment. Although they too display a DP phenotype, they are not exported from the thymus until several days after cessation of CSA treatment, during which time they acquire a CD4-SP phenotype. We do not yet know whether CD25 is expressed by these CD4-SP immunoregulatory thymocytes, although this seems likely (14). However, its presence on their descendants in LN is significant, as CD4\(^+\)CD25\(^+\) immunoregulatory T cells have been demonstrated to suppress T cell activation by inhibiting IL-2 production (15–17). In addition, CD4\(^+\)CD25\(^+\) immunoregulatory RTEs have been postulated to prevent the induction of auto-GVHD and organ-specific autoimmune disorders in neonatal mice (11, 16, 17).

The sequential appearance of autoeffector and immunoregulatory activity in the thymus during CSA treatment raises the possibilities that the responsible cell subsets are lineally related or that the latter is induced in response to the former. However, because autoeffector and immunoregulatory thymocytes both reside in the DP population at this time, it will be necessary to study additional markers and TCR\(\alpha\)\(\beta\) usage to document their origins and precise kinetics of generation. Such studies should also provide useful insights into the mechanisms underlying the physiological development of dominant tolerance during normal ontogeny (11).

It has been suggested that many self MHC-reactive thymocytes escape negative selection in CSA-treated animals due to reduced MHC class II expression in the thymus medulla (1, 5). CSA can also affect positive selection by interfering with calcineurin activation (18, 19). However, due to its variable absorption from the gut (20), it is possible that positive selection may not be blocked completely during the first few days of CSA treatment, when the bulk of autoeffector T cells are exported. This is important, as intrathymic positive selection unopposed by negative selection has been shown to favor the production of autoreactive T cells (21). A similar scenario has been suggested in anti-H-Y Ag-specific TCR-transgenic mice, in which DP autoeffector T cells are detected in the peripheral lymphoid tissues of male, but not female, mice and subsequently become SP T cells (22). It is also likely that the autoeffector T cells that cause GVHD in rats treated with CSA after irradiation and syngeneic bone marrow transplantation have a similar origin (2, 23).

**FIGURE 3.** Phenotype of autoeffector T cells in LN during and after CSA treatment. Experimental rats were fed CSA for up to 10 days or for 14 days followed by normal food for up to an additional 10 days. Control rats were fed normal food only. Total LN T cells (3 × 10\(^6\)) or the respective numbers of cells in the CD4- and/or CD8-enriched/depleted subsets therefrom were obtained at the indicated times and injected into the right (experimental) and left (control) footpads of syngeneic recipients. The relative levels of local synGVHR were determined 7 days later. The arrow on the y-axis indicates the level above control values at which significant responses occurred (\(p < 0.05\)).

**FIGURE 4.** Effect ofTx on the appearance of CD8-SP autoeffector T cells in LN on day 14 after CSA treatment. Experimental rats were fed CSA for 14 days followed by normal food for 14 days. Control rats were fed normal food only. Rats were Tx or sham Tx either before CSA treatment (CSA-day 0) or on day 5 of CSA treatment. On day 14 after CSA treatment, the numbers of CD8-SP lymphocytes originally present among 3 × 10\(^6\) LN T cells were injected into the right (experimental) and left (control) footpads of syngeneic recipients. *, Significant induction of local synGVHR (\(p < 0.05\)).
During the second week of CSA treatment, the concentration of intrathymic CSA appears to reach peak levels, as judged by cessation of thymocyte export and accumulation of double-negative and TCR\textsuperscript{low} DP thymocytes (8, 9, 24). At this point, it is likely that positive, as well as negative, selection is blocked. This inference is supported by the presence of a markedly increased proportion of DP thymocytes that express TdT (10, 25) and CD53 (our unpublished observation), a marker that is down-regulated during positive selection (26). Therefore, it seems probable that autoeffector thymocytes are no longer formed after the first week of CSA treatment. It also is possible that the DP thymocytes that ultimately generate immunoregulatory T cells need to undergo maturation and selection after cessation of CSA treatment before they become functionally competent. This seems likely, as CSA-induced immunoregulatory thymocytes attain a TCR\textsuperscript{high} CD4-SP phenotype during reconstruction of the thymic medulla after CSA treatment (1). Nonetheless, it is apparent that any necessary maturation/selection also can occur in the periphery (27–29), as DP thymocytes from wk 2 of CSA treatment express immunoregulatory activity after being injected into the footpad.

Although the generation of immunoregulatory thymocytes was first observed more than three decades ago (30–32), only indirect evidence has since been provided for their export to the peripheral lymphoid tissues (33–37). Although highly suggestive, this evidence could not preclude the possibility of expanded and/or induced immunoregulatory activity among previously released peripheral T cells, possibly under indirect thymic influence. Neither could it preclude the possibility that the main targets of immunoregulatory thymocytes were autoeffector T cells that had back-migrated to the thymus (38). Similarly, although induction of immune tolerance by intrathymic injection of alloantigens has been used to prevent allograft rejection (39–42), the underlying mechanisms have not been well defined, especially as deletional and nondeletional systems may coexist. Hence, the present data and those of a companion paper (5) are significant because they 1)
directly demonstrate the immediate intrathymic origin of peripheral CD4\(^+\)CD25\(^+\) immunoregulatory T cells in dominant tolerance; and 2) indicate that the mechanism of action involves reversible suppression of CD8-SP autoeffector T cells.

References


FIGURE 8. Schematic representation of the intrathymic origins, stages of phenotypic maturation, and kinetics of export of autoeffector and immunoregulatory T cells in CSA-treated rats. See Discussion for details.


