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Expanded Double Negative T Cells in Patients with Systemic Lupus Erythematosus Produce IL-17 and Infiltrate the Kidneys

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Double negative (DN) T cells are expanded in patients with systemic lupus erythematosus (SLE) and stimulate autoantibody production as efficiently as CD4⁺ T cells. In this study, we demonstrate that DN T cells from patients with SLE produce significant amounts of IL-17 and IFN-γ, and expand when stimulated in vitro with an anti-CD3 Ab in the presence of accessory cells. Furthermore, IL-17⁺ and DN T cells are found in kidney biopsies of patients with lupus nephritis. Our findings establish that DN T cells produce the inflammatory cytokines IL-17 and IFN-γ, and suggest that they contribute to the pathogenesis of kidney damage in patients with SLE. The Journal of Immunology, 2008, 181: 8761–8766.

Systemic lupus erythematosus (SLE) is an inflammatory disease in which an autoimmune process attacks several tissues and organs including the skin, kidneys, and joints (1). Mechanisms of tissue damage in patients with SLE involve autoantibody and immune complex deposition, as well as infiltration of tissues by lymphocytes (2). In lupus nephritis, immune complexes are deposited in the glomeruli, whereas a chronic inflammatory infiltrate is often observed in the tubule-interstitial areas (3). The pathogenic importance of the infiltrating lymphocytes is reflected by the fact that interstitial inflammation correlates with the degree of renal damage and the progression to end-stage renal failure (4, 5). The inflammatory infiltrate has been shown to be composed by activated T cells that express high levels of adhesion molecules (3, 6). However, the precise mechanism whereby T cells cause inflammation is not known.

T cells from SLE patients exhibit a number of biochemical and functional abnormalities that affect their activation process, cytokine production, and expression of costimulatory molecules (7). CD³⁺CD⁴⁻CD⁸⁻ double negative (DN) T cells are expanded in the peripheral blood of patients with SLE and have been shown to produce IL-4 (8) and induce Ig and anti-DNA Ab production as efficiently as CD4⁺ T cells (9). Nevertheless, DN T cells have not been directly implicated in causing tissue damage.

IL-17 is a proinflammatory cytokine that participates in the defense against certain pathogens, primarily extracellular bacteria and fungi (10). IL-17 is produced by several cell subsets including CD4⁺ T cells, CD8⁺ T cells, γδ-T cells, NK cells, and neutrophils (10). Th17 cells represent an effector cell type that drives inflammatory responses by virtue of producing IL-17A and IL-17F (11). Th17 cells have been implicated in the pathogenesis of autoimmune diseases including rheumatoid arthritis (12), psoriasis (13), and multiple sclerosis (14). Recent evidence suggests that IL-17-mediated inflammation might play a role in the pathogenesis of SLE. In a murine model of lupus, tolerance induction toward a histone-derived Ag resulted in improved survival and decreased frequency of IL-17-producing T cells (15). Also, abnormally high levels of IL-17 and IL-23 have been reported in human SLE sera (16). Nevertheless, direct evidence implicating IL-17-producing cells as pathogenic effector cells in patients with SLE has not been yet presented. In this study, we provide evidence that IL-17 production by T cells is increased in SLE patients. Moreover, we describe for the first time that DN (CD4⁺CD8⁻) T cells, which are expanded in the peripheral blood of patients with SLE (9), represent major producers of IL-17 and that they undergo a vigorous proliferative response following stimulation. Finally, we demonstrate the presence of DN T cells and IL-17⁺ T cells in kidney sections of patients with lupus nephritis. Taken together, our data suggest that DN T cells and IL-17 over production might play a role in the pathogenesis of SLE.

Materials and Methods

Patients and controls

Twenty-four SLE patients fulfilling the American College of Rheumatology revised classification criteria (17) for SLE were studied. All patients were female. Mean age was 40.22 years (range 25–57 years). SLE disease activity index scores ranged from 0 to 20 (mean 7.2). Prednisone was discontinued at least 24 h before venipuncture. Sixteen healthy volunteers served as controls. Studies and informed consent forms and processes had been approved by the involved institutions.
Cell cultures
PBMC were isolated by centrifugation against a density gradient (Lymphoprep; Nycomed). T cells along with non-T PBMC (accessory cells) were cultured for 5 days in RPMI 1640 with 10% FCS in 6-well plates that had been precoated with anti-CD3 (2–5 μg/ml; BD Pharmingen). Four hours before collection, brefeldin A (Golgi-Plug 1 μl/ml; BD Pharmingen) was added to the cell culture, along with PMA and ionomycin. Additionally, in some experiments recently isolated PBMC (1 × 10⁶ in 1 ml of RPMI 1640) were stimulated during 3 h with PMA and ionomycin in the presence of brefeldin A and stained for analysis of cytokine production.

Abs and reagents
The following reagents were used: anti-CD45RA-Pacific Blue, anti-CCR6-PE, anti-CCR6-PE-Cy5, anti-CCR4-PE-Cy7, anti-CD4-allophycocyanin, anti-TCRβ-FITC, anti-CD4-PE, anti-CD8-PerCP, anti-TNF-α-Alexa Fluor 488, anti-IFN-γ-Alexa Fluor 488, anti-IL-2-biotin, Streptavidin-PE-Cy7 (BD Pharmingen); anti-IL-17-Alexa Fluor 647, anti-human γ TCR-FITC (eBioscience), anti-CD4, anti-CD8, goat anti-mouse-Texas Red, goat anti-rabbit-Alexa Fluor 488, 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen); mouse-anti-CD3, rabbit-anti-CD3 (Abcam); anti-IL-17, anti-IL-23 (Santa Cruz Biotechnology); anti-TCR Vα24, anti-TCR Vβ24-PE (Beckman Coulter); and anti-human CD3-PE (R&D Systems).

Flow cytometry
Intracellular staining was performed with the BD Cytofix/Cytoperm Kit, according to the instructions of the manufacturer. Samples were acquired in a LSRII flow cytometer (BD Biosciences). Analysis was performed with FlowJo v. 7.2.2 (Tree Star). For the analysis of cell populations and cytokine production, a first gate that included live lymphocytes (according to forward scatter and side scatter characteristics) was used. Next, T cells (gated according to CD3 expression) were plotted in a CD4 vs CD8 graphic that allowed the identification of discrete CD4 and CD8 populations. Subsequent analysis was performed on these cell populations. Thirty-thousand T cells were acquired for analysis. For some of the experiments, cells were stained with anti-TCRβ-FITC, anti-CD4-PE, and anti-CD8 APC-Cy7 and sorted in a FACSaria flow cytometer (BD Biosciences). Postsorting cell purity was always >98%.

Immunofluorescence and confocal microscopy
Kidney samples from five patients with lupus nephritis were obtained from biopsy material obtained for diagnostic purposes. Two patients had membranous (type V) glomerulonephritis and three patients had proliferative glomerulonephritis (Types III or IV). Tissue was snap-frozen to −70°C, and sections were cut with a cryostat and fixed in acetone. Samples were served in DN T cells (DN T cells 52.85 ± 5.6, p < 0.001) more common among DN than CD4⁺ T cells, which cell subpopulation was responsible for the production of IL-17. When we performed experiments with control subjects (control 42.36 ± 4.4 vs SLE 56.73 ± 2.91, p = 0.03). To investigate which cell subpopulation was responsible for the production of IL-17, we performed multicolor flow cytometry experiments. In recently isolated T cells, as well as in cells cultured in the absence of plate-bound anti-CD3, production of IL-17 was detectable only in a small proportion of CD4⁺ T cells (Fig. 1, A and B). In sharp contrast, a proportionally larger fraction of DN T cells expressed IL-17 in both basal conditions (control DN T cells 2.63 ± 0.9; SLE DN T cells 3.5 ± 1.0, p = 0.29) and after 5-day culture in the absence of anti-CD3 (control DN T cells 19.41 ± 2.8; SLE DN T cells 25.2 ± 6.4, p = 0.38). IL-17-producing cells were significantly (p = 0.001) more common among DN than CD4⁺ cells, both in normal individuals and SLE patients. CD8⁺ T cells produced minimal amounts of IL-17 and at levels comparable to those produced by CD4⁺ cells (data not shown). After in vitro stimulation with plate-bound anti-CD3 Ab in the presence of monocytes, the production of IL-17 increased in both CD4⁺ and DN T cells (Fig. 1C). In normal individuals, the production of IL-17 was still significantly higher in DN T cells than in CD4⁺ T cells (DN T cells 50.84 ± 5.6; CD4⁺ T cells 32.92 ± 2.3, p = 0.01). In contrast, in patients with SLE, IL-17-producing cells were significantly (p = 0.033) more common among DN than CD4⁺ cells, both in normal individuals and SLE patients. DN T cells represent a T cell subset capable of producing IL-17 without in vitro stimulation. After CD3 stimulation, the IL-17-producing capacity of DN T cells increases vigorously conferring them a marked proinflammatory capacity. The expansion of this T cell subset is partially responsible for the amplified IL-17 production observed in SLE patients. Although DN T cells from SLE patients do not produce more IL-17 than normal DN T cells, they are abnormally expanded in patients with SLE and thus represent a higher proportion of the total T cells (9). The second component of the amplified IL-17 response observed in SLE patients is explained by the fact that a larger proportion of CD4⁺ T cells produce IL-17 after stimulation (Fig. 1C). In summary, IL-17 production in SLE T cells is augmented because they have an expanded DN population and because a larger fraction of the CD4⁺ T cells produce IL-17 upon CD3 stimulation.

Polymersase chain reaction
RNA purification was performed with the use of the RNeasy Mini kit (Qiagen) from recently isolated or sorted cells. cDNA was produced using oligo dT primers, from an equal amount of RNA. The following primers were designed using Primer3 software (18): RORC2 (homologous to murine RORγt): 5′-AGGCCTCGTAGTGGAGACCAC-3′, 5′-AAGGTTGATACGTA-3′; IL17A: 5′-CGAAAATCCAGAGGCCC-3′, 5′-CAG AGTTCTGAGTGGATCC-3′; IL17F: 5′-CATGACAGTGAAGACCC-3′; GAGTACGGTGAGTTCC-3′; and IL23R: 5′-CTCTGAGTGGATGAGAC-3′.

Statistical analysis
Paired and nonpaired Student two-tailed t tests were used. Results are expressed as the mean ± SEM, unless noted otherwise.

Results
IL-17 production is increased in SLE
T cells with aberrant biochemical profile have been reported in the peripheral blood of patients with SLE and cells with similar features have been found in inflamed tissues (3, 6). However, the exact mechanisms whereby pathogenic T cells instigate inflammation in lupus are not known. Therefore, we sought to investigate whether IL-17 is involved in the autoimmune response of patients with SLE. Initially, we measured CCR4⁺CCR6⁺ and CCR3⁺ CCR6⁺ subsets of memory (CD45RA⁻) CD4⁺ T cells in SLE patients and normal individuals because they have been found to be enriched in cells that produce IL-17 or both IL-17 and IFN-γ, respectively (19). We did not find any difference in the relative expression of different cell subsets in the peripheral blood lymphocytes from patients with SLE and healthy controls (CCR4⁺CCR6⁺ 20.1% ± 2.6 vs 16.4% ± 3.5; CCR3⁺CCR6⁺ 8.11% ± 2.6 vs 7.0% ± 0.8).

Next, we analyzed SLE T cell IL-17 production. When we studied recently isolated cells, the fraction of IL-17-expressing T cells was significantly higher in SLE patients than in normal controls (0.84 ± 0.4 vs 1.83 ± 0.1, p = 0.02) (Fig. 1, A and D). The same trend was observed at the mRNA level (data not shown). Likewise, T cell stimulation with autologous accessory cells and plate-bound anti-CD3 Ab induced IL-17 expression in a higher proportion of T cells from patients with SLE compared with control subjects (control 42.36 ± 4.4 vs SLE 56.73 ± 2.91, p = 0.03). To investigate which cell subpopulation is responsible for the production of IL-17, we performed multicolor flow cytometry experiments. In recently isolated T cells, cells cultured in the absence of plate-bound anti-CD3, production of IL-17 was detectable only in a small proportion of CD4⁺ T cells (Fig. 1, A and B). In sharp contrast, a proportionally larger fraction of DN T cells expressed IL-17 in both basal conditions (control DN T cells 2.63 ± 0.9; SLE DN T cells 3.5 ± 1.0, p = 0.29) and after 5-day culture in the absence of anti-CD3 (control DN T cells 19.41 ± 2.8; SLE DN T cells 25.2 ± 6.4, p = 0.38). IL-17-producing cells were significantly (p = 0.001) more common among DN than CD4⁺ cells, both in normal individuals and SLE patients. CD8⁺ T cells produced minimal amounts of IL-17 and at levels comparable to those produced by CD4⁺ cells (data not shown). After in vitro stimulation with plate-bound anti-CD3 Ab in the presence of monocytes, the production of IL-17 increased in both CD4⁺ and DN T cells (Fig. 1C). In normal individuals, the production of IL-17 was still significantly higher in DN T cells than in CD4⁺ T cells (DN T cells 50.84 ± 5.6; CD4⁺ T cells 32.92 ± 2.3, p = 0.01). In contrast, in patients with SLE, IL-17 production by stimulated CD4⁺ T cells was heightened and reached levels similar to those observed in DN T cells (DN T cells 52.85 ± 4.0; CD4⁺ T cells 49.13 ± 5.6, p = 0.53). Thus, our results indicate that both normal individuals and SLE patients, DN T cells represent a T cell subset capable of producing IL-17 without in vitro stimulation. After CD3 stimulation, the IL-17-producing capacity of DN T cells increases vigorously conferring them a marked proinflammatory capacity. The expansion of this T cell subset is partially responsible for the amplified IL-17 production observed in SLE patients. Although DN T cells from SLE patients do not produce more IL-17 than normal DN T cells, they are abnormally expanded in patients with SLE and thus represent a higher proportion of the total T cells (9). The second component of the amplified IL-17 response observed in SLE patients is explained by the fact that a larger proportion of CD4⁺ T cells produce IL-17 after stimulation (Fig. 1C). In summary, IL-17 production in SLE T cells is augmented because they have an expanded DN population and because a larger fraction of the CD4⁺ T cells produce IL-17 upon CD3 stimulation.

T cell stimulation causes expansion of the DN T cell subset
In peripheral blood, DN T cells represent a minor population which normally constitutes <5% of peripheral blood T cells. However, in patients with SLE, and in certain mice with lupus, they are expanded (9, 20). In SLE patients, DN cells have been shown to
promote the production of pathogenic anti-DNA Abs (9). We evaluated DN T cell numbers and, as expected, found them to be increased in patients compared with healthy controls ($p < 0.05$; Fig. 2). DN cells proliferated following anti-CD3 stimulation, and their relative frequency among T cells (defined as either CD3$^+$ or TCR$^+$) increased to $\approx 20\%$ by the end of the 5 day stimulation period (Fig. 2). These results indicate that DN cells represent a T cell subset capable of undergoing significant expansion following stimulation.

**DN T cells produce IFN-$$\gamma$$ and TNF-$$\alpha$$**

To define thoroughly the phenotype of DN T cells, we compared the cytokine production profile with that of CD4 T cells in patients with SLE and normal individuals. As shown in Fig. 3, DN cells produced significantly less IL-2 than CD4 T cells, both in normal subjects and in patients with SLE. Conversely, their ability to produce IFN-$$\gamma$$ was increased compared with CD4$^+$ T cells. Among normal cells, DN and CD4$^+$ T cells produced comparable amounts of TNF-$$\alpha$$, CD4$^+$ T cells from SLE patients produced less TNF-$$\alpha$$ compared with normal CD4$^+$ T cells, whereas DN T cells from SLE patients produced negligible amounts of TNF-$$\alpha$$ (Fig. 3). T cells from SLE patients are known to produce decreased amounts of IL-2 (21), IFN-$$\gamma$$ (22), and TNF-$$\alpha$$ (23). Our experiments demonstrate that DN cells, which are expanded in the peripheral blood of SLE patients, also fail to produce these cytokines.

**DN T cells express ROR-$$\gamma$$t and IL23R**

Th17 cells represent a recently described effector subset of CD4$^+$ T cells (10). They produce large amounts of IL-17 and have been linked to autoimmune processes in humans and animals (10). Th17 cells differentiate through a distinct pathway that involves the transcription factor orphan nuclear receptor ROR-$$\gamma$$t and express IL-23

**FIGURE 1.** An abnormally high fraction of T cells from patients with SLE produce IL-17. IL-17 expression was quantified in T cells, either immediately after isolation (A), or after 5-day culture in the absence (B) or presence (C) of plate-bound anti-CD3. At day 6, intracellular staining was performed. Cumulative data from 14 patients with SLE (black bars) and 12 healthy controls (white bars) is presented. *, $p < 0.05$. D. Freshly isolated T cells were treated with brefeldin A and stimulated for 3 h with PMA and ionomycin before staining for flow cytometry. Representative dot plots (IL-17-Alexa Fluor 647 vs FSC; Total T cells, CD4$^+$ T cells, DN T cells) from a healthy control and a patient with SLE are depicted. The gate was set according to an isotype control Ab. E. T cells stimulated during 5 days with plate-bound CD3 in the presence of accessory cells were harvested and stained for flow cytometry analysis. Representative histograms of a control and a SLE patient are shown. Dotted line represents isotype control staining.

**FIGURE 2.** Double negative cells expand significantly following anti-CD3 stimulation. T cells were incubated for 5 days with autologous accessory cells. TCR$^+$ cells were gated and single positive (CD4$^+$CD8$^-$; CD4$^-$CD8$^+$) and DN (CD4$^-$CD8$^-$) cells were quantified in eight patients with SLE and seven healthy controls. The percentage of DN (A) and CD4 (B) T cells from normal subjects and SLE patients in the absence (white bars) and presence (black bars) of plate-bound anti-CD3. Representative samples from a control and a patient are presented (C). *, $p < 0.05$. 

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one patient with SLE. Cells from freshly isolated T cells of two normal individuals and CD4+ patients and six controls is presented as mean area considered positive. On the right panels, cumulative data from six lower panels.

CD4+ TCR IL-17 production by SLE DN T cells is increased in the cells. This suggests that functional similarities exist between them and Th17 cells enriched in cells bearing the alternative γδ TCR (25). To determine whether the DN cells responsible for IL-17 production in SLE patients were indeed TCR αβ+, we analyzed TCR and IL-17 expression in DN cells from patients and controls (Fig. 4, A and B).

There was no difference in the frequency of TCR γδ+ cells among DN T cells from SLE patients and healthy controls (controls 43.44 ± 9.9 vs SLE 43.74 ± 12.8, p = 0.98). IL-17 production could be detected in both TCR αβ+ and TCR γδ+ DN T cells from patients and controls. IL-17+ TCR γδ+ DN T cells, though, were not different between patients and controls (Fig. 4, A and B). However, IL-17 expression in TCR αβ+ DN T cells was significantly higher in SLE patients compared with healthy individuals (Control 1.6 ± 0.6 vs SLE 4.2 ± 1.5, p = 0.05).

To rule out the presence of NKT cells within the DN T cell population, we stained T cells with an anti-TCR Vα24. As shown in Fig. 4D, TCR Vα24+ cells were virtually confined to the CD4+ T cell compartment in both SLE patients and controls. The number of Vα24+ DN T cells was negligible.

IL-17 and IL-23 are present in the kidneys of patients with lupus nephritis

IL-17 acts on a broad range of cells and induces multiple proinflammatory effects including the production of cytokines and chemokines (IL-6, IL-8, GM-CSF, G-CSF, CXCL1, CXCL10). In addition, it plays a role in the local recruitment and activation of neutrophils (26). IL-23 is a cytokine produced by APCs which increases IL-17 production by IL-23R-bearing cells. To investigate the involvement of IL-17 and IL-23 in an organ affected by the autoimmune process in SLE patients, we stained kidney sections from biopsies obtained from patients with lupus nephritis. IL-17+ and IL-23+ cells were observed. Shown is a representative field in which two NKT cells are marked with white circles.
double-stained tissues. This finding was observed in all the analyzed biopsies.

**DN T cells represent a component of the cellular infiltrates in SLE kidneys**

To investigate whether DN T cells are directly involved in the autoimmune tissue damage in patients with SLE, we stained kidney biopsies with rabbit anti-human-CD3 and a combination of mouse anti-human-CD4 and mouse anti-human-CD8. Alexa Fluor 488-labeled goat anti-rabbit and Texas Red-labeled goat anti-mouse were used as secondary Abs. As shown in Fig. 6, this approach allowed the distinction between CD4\(^+\)/H11001 or CD8\(^+\)/H11001 T cells (yellow) and DN (CD4\(^-\)/CD8\(^-\)) T cells (green) within an inflammatory infiltrate. We found that the T cell infiltrates are composed of an admixture of both populations. This notion was observed in all the analyzed samples.

Kidney sections were also stained with anti-TCR V\(^{\alpha}24\) and anti-TCR \(\gamma\delta\) to exclude the presence of TCR \(\gamma\delta\) cells and NKT cells within the kidney infiltrates. As shown in Fig. 4, C and E, the presence of both types of cells could be observed in very small amounts interspersed within other T cells. Interestingly, TCR \(\gamma\delta\) T cells were mostly negative for IL-17.

**Discussion**

The evidence presented in this communication indicates that DN T cells represent a proinflammatory T cell subset able to produce IL-17 and proliferate vigorously following anti-CD3 stimulation. Further, it demonstrates that IL-17 production is increased in SLE patients due to the expansion of the proinflammatory DN T cell subset and to the excessive IL-17 production of CD4\(^+\) T cells. The pathological relevance of these findings is confirmed by the presence of DN and IL-17\(^+\) T cells in kidneys of patients with lupus nephritis.

DN T cells represent a major component of thymocytes and a small proportion of peripheral blood T cells. An expanded DN T cell subset has been reported in patients with SLE and in some lupus-prone animals (9, 20). In this study, we demonstrate that TCR\(\alpha\beta\) TCR V\(^{\alpha}24\)/CD3\(^+\)CD4\(^-\)/CD8\(^-\) T cells exist in peripheral blood of normal individuals (Fig. 2), have a distinct cytokine production profile (Fig. 3), and expand following anti-CD3 in vitro stimulation (Fig. 2). Our results imply that DN T cells comprise a physiological component of the immune system able to rapidly proliferate and produce proinflammatory cytokines following Ag stimulation. Further, our findings suggest that the expanded...
DN T cell subset found in SLE patients represents a pathologically expanded T cell subset that maintains its proliferation and cytokine production characteristics. Although their frequency is significantly increased in patients with SLE, DN T cells behave similarly to DN T cells from normal individuals in terms of proliferation and cytokine production (Fig. 2 and 3). The signals that drive DN T cell expansion in SLE patients will need to be investigated in future work.

The second major finding of this work is the demonstration that IL-17 production is increased in T cells from SLE patients. As mentioned earlier, this phenomenon is partially due to the expansion of the IL-17-producing DN T cell subset. However, IL-17 production is increased in T cells from SLE patients. As future work. The demonstration of DN T cells and IL-17+ T cells within T cell infiltrates in kidneys from patients with lupus nephritis (Fig. 5) has a number of conceptual implications and underscores the importance of T cells as effector cells in tissue injury. Previous work from our laboratory has provided evidence of the presence of T cells in kidneys of patients with lupus nephritis (3, 6), but the fact that infiltrating T cells produce IL-17 is a strong argument that implies proinflammatory activity and suggests that T cells play a direct pathogenic role in kidney inflammation. Further, the presence of DN T cells in inflamed tissue strongly suggests that they are key mediators of the autoimmune response in lupus.

In summary, this study provides evidence which demonstrates that DN cells represent a T cell subset that produces large amounts of inflammatory cytokines and proliferates vigorously upon CD3 stimulation. In addition, our data shows that the IL-17 effector response is augmented in patients with SLE due to an increased production by CD4+ T cells, along with an expansion of the proinflammatory DN compartment. The pathogenic importance of this finding is illustrated by the fact that IL-17 and IL-23 are found in kidneys affected by lupus nephritis, along with infiltrates of IL-17-producing CD4+ and DN T cells.

Disclosures

The authors have no financial conflict of interest.

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