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Akt-Dependent Enhanced Migratory Capacity of Th17 Cells from Children with Lupus Nephritis

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Th17 cells infiltrate the kidneys of patients with lupus nephritis (LN) and are critical for the pathogenesis of this disease. In this study, we show that enhanced activity of Stat3 in CD4⁺CD45RA⁻Foxp3⁻ and Foxp3low effector T cells from children with LN correlates with increased frequencies of IL-17–producing cells within these T cell populations. The levels of retinoic acid-related orphan receptor c and IL-17 mRNA are significantly higher in PBMCs from children with LN than in those from controls. Mammalian target of rapamycin inhibition by rapamycin reduces both Stat3 activation in effector T cells and the frequency of IL-17–producing T cells in lupus patients. Complement factor C5a slightly increases the expression of IL-17 and induces activation of Akt in anti-CD3-activated lupus effector T cells. Th17 cells from children with LN exhibit high Akt activity and enhanced migratory capacity. Inhibition of the Akt signaling pathway significantly decreases Th17 cell migration. These findings indicate that the Akt signaling pathway plays a significant role in the migratory activity of Th17 cells from children with LN and suggest that therapeutic modulation of the Akt activity may inhibit Th17 cell trafficking to sites of inflammation and thus suppress chronic inflammatory processes in children with LN. The Journal of Immunology, 2014, 193: 000–000.

Systemic lupus erythematosus (SLE) is a potentially fatal autoimmune disease that is characterized by multiorgan involvement and a high incidence of nephritis in children (1, 2). T cells contribute to the pathogenesis of SLE and infiltrate target organs, including the kidney (3–5).

Particularly, Th17 cells have recently been demonstrated to be functionally relevant in the development of lupus nephritis (LN) (6, 7). The differentiation of Th17 cells depends on Stat3. Stat3 is activated in response to cytokines such as IL-6, IL-21, and IL-23, which are highly expressed in patients with SLE (8, 9). Upon Stat3 activation, retinoic acid–related orphan receptor (ROR) γ and RORα, which are essential transcription factors for Th17 cell differentiation, are increasingly expressed.

Interestingly, mammalian target of rapamycin (mTOR)-deficient T cells fail to differentiate into Th17 cells owing to decreased activation of Stat transcription factors, including Stat3, in response to proinflammatory cytokines (10, 11). Specifically, Th17 cell differentiation requires mTOR complex 1 (mTORC1) signaling (11), which is inhibited by rapamycin. Treatment of SLE patients with rapamycin reduces disease activity (12), indicating the clinical significance. Moreover, mTOR activity is increased in lupus T cells and is involved in the enhanced production of IL-17 and especially of IL-4 in SLE patients (13, 14). mTORC1 is activated downstream of PI3K/Akt signaling, and inhibition of PI3K or Akt signaling prevents Th17 cytokine induction in memory T cells (15). Additionally, CCR2- and CCR6-mediated migration of murine Th17 cells is PI3K/Akt-dependent (16).

Th17 cells are critical for the pathogenesis of LN in animal models and in patients with SLE, but the migratory capacity of these cells and the underlying molecular mechanisms in children with LN are incompletely known.

In this study, we examine the activation status of Stat3 in CD4⁺CD45RA⁻Foxp3⁻ and Foxp3low effector T cells and the activity of Akt and mTOR in Th17 cells from children with LN. We also assess the role of Akt in Th17 cell migration. Our findings indicate that elevated levels of activated Stat3 in CD4⁺CD45RA⁻Foxp3⁻ and Foxp3low T cells from children with LN correlate with increased frequencies of IL-17–producing T cells within these T cell populations. Additionally, mTOR inhibition by rapamycin reduces both the frequency of IL-17–producing T cells and the activation of Stat3 in CD4⁺CD45RA⁻Foxp3⁻ and Foxp3low effector T cells from children with LN. Furthermore, Th17 cells from children with LN exhibit enhanced Akt activation and migration in vitro; this migration is significantly reduced by the inhibition of Akt activity.

Materials and Methods

Subjects

This study included 17 pediatric patients with definite LN (mean age ± SEM, 15.1 ± 0.9 y) who fulfilled the American College of Rheumatology revised criteria for the classification of SLE with disease onset prior to 16 y of age (17, 18), 5 patients with frequently relapsing nephrotic syndrome...
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(NS) (i.e., minimal change glomerulonephritis or focal segmental glomerulosclerosis) (mean age ± SEM, 9.3 ± 1.6 y), and 4 age-matched healthy controls (HCs) (mean age ± SEM, 13.9 ± 0.4 y). From eight patients with LN blood samples were obtained twice. Patients with LN were enrolled from the Department of Pediatrics in Innsbruck, Austria, and the Departments of Pediatrics in Heidelberg, Rostock, and Bremen in Germany. Patients with NS were enrolled from the Department of Pediatrics in Innsbruck, Austria. HCs underwent blood testing for elective surgery at the Department of Pediatric Surgery of Innsbruck Medical University in Austria.

The class of LN was assessed according to the International Society of Nephrology and the Renal Pathology Society classification (19). Three patients had class II nephritis, five patients had class III nephritis, and nine patients had class IV nephritis. Disease activity was assessed using the SLE disease activity index 2000 (SLEDAI-2K) (20, 21). SLEDAI-2K ranged from 10 to 26 (mean ± SD, 13.9 ± 5.7). From 10 to 2 median, 0) in patients with inactive LN. The treatment regimens in patients with LN at the time of sampling were as follows: mycophenolate mofetil (MMF) plus prednisone (n = 6), prednisone alone (n = 2), MMF plus prednisone plus hydroxychloroquine (n = 2), prednisone plus hydroxychloroquine (n = 1), MMF plus cyclosporine (n = 2), or MMF alone (n = 1). Three patients with active LN were untreated at the time of analysis. Patients with NS were treated with prednisone (n = 1), MMF (n = 2), or cyclosporine (n = 2).

This study was performed according to the Helsinki Declaration principles. This study was approved by local ethics committees.

Cell isolation and culture

Human PBMCs were separated by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Upppsala, Sweden) gradient centrifugation. CD4+ T cells were isolated by negative selection using a CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were cultured in 96-well round-bottom plates (BD Biosciences, San Jose, CA) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY). PBMCs were stimulated with functional-grade purified anti-CD3 (OKT3) (1 μg/ml) (eBioscience, SanDiego, CA) and functional-grade purified anti-CD28 (1 μg/ml) (eBioscience) (20). Where indicated, rapamycin (50 ng/ml) (Sigma-Aldrich, St. Louis, MO), CsA (50–100 ng/ml) (Sigma-Aldrich), or diluent was added to the cultures. After 7 d, the cells were collected, washed, counted, restimulated for 4 h with leukocyte activation cocktail (2 μM) (BD Biosciences), and analyzed by flow cytometry. For some experiments, the cells were restimulated with anti-CD3/CD28 for 24 h prior to isolation of RNA from the cell pellets or analysis by cytometry. For some experiments, the cells were restimulated with anti-CD3/CD28 (1 μg/ml) (R&D Systems) or CCL2 (50 ng/ml) (R&D Systems) or with diluent for 30 min prior to addition to the transwells. The primes for IL-17A (HS00174122_m1) and RORc (HS01076112_m1) and GAPDH (HS99999990_m1) were purchased from Applied Biosystems (Carlsbad, CA). mRNA expression levels were normalized to the amount of GAPDH.

Cell migration assays

Th17-, Th1-, or Th2-polarized CD4+ T cells were added to the upper chamber of a 5-μm pore size transwell (Corning Life Sciences, Tewksbury, MA) at 1 x 10⁵ cells/well. RPMI 1640 medium (600 μl) containing different concentrations of CCL2 (50–200 ng/ml) or CXCL10 (250 ng/ml) (R&D Systems) or CCL2 (50 ng/ml) (R&D Systems) was added to the lower wells. Where indicated, the cells were incubated with the Akt inhibitor VIII (0.2 μM; Calbiochem; EMD Millipore/Merck, Darmstadt, Germany) or with diluent for 30 min prior to addition to the transwells. The cells were allowed to migrate for 3 h at 37°C in 5% CO2. After incubation for 3 h, migrated and nonmigrated cells were collected from the lower and upper chambers, respectively.

Statistical analysis

Data are presented as the means ± SD unless stated otherwise. The Mann–Whitney U test, the Kruskal–Wallis test, and Spearman’s rank correlation test were used. The p values < 0.05 were considered significant.

Results

Enhanced activity of Stat3 in CD4+ CD45RA low Foxp3- and Foxp3low T cells from children with LN

Because Stat3 is a key positive regulator of IL-17 expression (22) and because IL-17 plays a central role in the pathogenesis of LN (6, 7), we first studied the activation status of Stat3 in effector T cells from children with LN and from HCs. CD4+CD45RA- memory cells that express Foxp3 at low levels (Foxp3low) have recently been described as an effector-like non-regulatory T cell subset that produces high levels of IL-17 and is increased in patients with active LN (23). Therefore, we assessed the expression...
of phosphorylated (activated) Stat3 in CD4+CD45RA−Foxp3− and Foxp3low T cells by flow cytometry.

In fresh CD4+CD45RA−Foxp3− T cells, the levels of activated Stat3 were significantly higher in patients with active LN than in patients with inactive LN or in HCs (Fig. 1A). Furthermore, upon in vitro PBMC activation using anti-CD3/CD28, we observed significantly higher expression levels of activated Stat3 in effector T cells from children with LN than in effector T cells from HCs (Fig. 1B).

Stat3 is activated by the cytokines IL-6, IL-21, and IL-23 (24–26), which are highly expressed in patients with SLE (8, 27, 28). Thus, we measured the levels of IL-6 and IL-21 in supernatants from children with LN (mean ± SD, 22.3 ± 9.4 and 26.1 ± 11.4 ng/ml, respectively) than in controls (mean ± SD, 8.6 ± 2.7 and 11.3 ± 4.7 ng/ml, respectively) (p < 0.0001). These results indicate that enhanced Stat3 activity in LN patients may be caused by increased exposure to these proinflammatory cytokines. Additionally, IL-6–induced phosphorylation of Stat3 was enhanced in CD4+CD45RA−Foxp3− T cells from lupus patients compared with controls (Fig. IC), suggesting that additional mechanisms are involved in the enhanced phosphorylation of Stat3 in lupus effector T cells. Interestingly, a role for the PI3K/Akt pathway in the phosphorylation of Stat3 has been previously described (29). Hyperactivation of Akt in CD4+CD45RA−Foxp3− and Foxp3low T cells from children with LN, as previously reported (30), might thus cause further enhanced phosphorylation of Stat3. However, a direct causal link remains to be demonstrated.

Increased frequency of IL-17–producing CD4+CD45RA−Foxp3− and Foxp3low T cells in children with LN

STAT3 upregulates the expression of the ROR/ orphan nuclear receptor RORγt, which is a key transcription factor in the differentiation of Th17 cells (31) and is encoded by the RORc gene. Fresh PBMCs were stimulated with leukocyte activation cocktail for 6 h and analyzed by flow cytometry. RORγt+IL-17–expressing cells were found in increased proportions in fresh CD4+CD45RA−Foxp3− T cells from children with LN than in PBMCs from controls (Fig. 2A). Quantitative real-time PCR was used to quantify IL-17 and RORc mRNA in PBMCs from children with LN and from controls that were activated with anti-CD3/CD28 for 7 d and restimulated with anti-CD3/CD28 for 24 h. mRNA levels of IL-17 and RORc were significantly higher in PBMCs from children with LN than in PBMCs from controls (Fig. 2B, 2C). Next, we evaluated the frequency of CD4+CD45RA−Foxp3− and Foxp3low T cells that express IL-17. PBMCs were stimulated with anti-CD3/CD28 for 7 d and then stimulated with leukocyte activation cocktail for 4 h and analyzed by flow cytometry. Children with LN displayed significantly higher percentages of IL-17–producing CD4+CD45RA−Foxp3− and Foxp3low T cells that were activated in vitro than did controls (Fig. 2D). Furthermore, the frequency of these cells correlated with the levels of activated Stat3 observed in CD4+CD45RA−Foxp3− and Foxp3low T cells as shown above, the concentrations of IL-6 and IL-21 were significantly higher in culture supernatants from children with LN than in PBMCs from controls (Fig. 2B, 2C). STAT3 upregulates the expression of the ROR/orphan nuclear receptor RORγt, which is a key transcription factor in the differentiation of Th17 cells (31) and is encoded by the RORc gene. Fresh PBMCs were stimulated with leukocyte activation cocktail for 6 h and analyzed by flow cytometry. RORγt+IL-17–expressing cells were found in increased proportions in fresh CD4+CD45RA−Foxp3− T cells from children with LN than in PBMCs from controls (Fig. 2A). Quantitative real-time PCR was used to quantify IL-17 and RORc mRNA in PBMCs from children with LN and from controls that were activated with anti-CD3/CD28 for 7 d and restimulated with anti-CD3/CD28 for 24 h. mRNA levels of IL-17 and RORc were significantly higher in PBMCs from children with LN than in PBMCs from controls (Fig. 2B, 2C). Next, we evaluated the frequency of CD4+CD45RA−Foxp3− and Foxp3low T cells that express IL-17. PBMCs were stimulated with anti-CD3/CD28 for 7 d and then stimulated with leukocyte activation cocktail for 4 h and analyzed by flow cytometry. Children with LN displayed significantly higher percentages of IL-17–producing CD4+CD45RA−Foxp3− and Foxp3low T cells that were activated in vitro than did controls (Fig. 2D). Furthermore, the frequency of these cells correlated with the levels of activated Stat3 observed in CD4+CD45RA−Foxp3− and Foxp3low T cells as shown above, the concentrations of IL-6 and IL-21 were significantly higher in culture supernatants from children with LN than

FIGURE 1. Stat3 activity is enhanced in effector T cells from children with LN. The phosphorylation of Stat3 was analyzed by flow cytometry. The cells were pregated on CD4+ cells and subsequently gated based on the expression of CD45RA and Foxp3. (A) Representative histograms and bar graphs show p-Stat3 fluorescence intensity (mean and SD) in fresh CD4+CD45RA−Foxp3− T cells from children with active LN (n = 5), inactive LN (n = 5), and HCs (n = 5). (B) PBMCs from children with LN (n = 17), children with NS (n = 5), and HCs (n = 19) were activated with anti-CD3/CD28 for 7 d. Representative histograms show the expression of p-Stat3 in the indicated T cell populations (left). The data are summarized in box-and-whisker plots (right) (MFI). (C) The cells were stimulated with recombinant human IL-6 for the indicated time periods. Histograms show the p-Stat3 fluorescence intensity in CD4+CD45RA−Foxp3− T cells and are representative of n = 4 for each group. Data are the MFI of specific Ab minus the MFI of matched isotype control.
in culture supernatants from controls. The observed TFG-β1 concentrations in culture supernatants from children with LN and from HCs were 2.7 ± 0.8 and 4.4 ± 0.8 ng/ml (mean ± SD), respectively. These data suggest a pathogenic significance of Th17 cells in childhood-onset LN, which is consistent with previous reports from experimental models and adults with SLE (6, 7).

Rapamycin decreases the frequency of IL-17–producing CD4+CD45RA−Foxp3− and Foxp3low T cells and the activity of Stat3 in CD4+CD45RA−Foxp3− and Foxp3low T cells from children with LN

Akt activation is an important upstream inducer of mTORC1 that is sensitive to rapamycin (32). mTOR activity is increased in lupus T cells (13). Moreover, treatment with rapamycin improves disease activity in SLE patients (12) and reduces necrosis and IL-4 production of double-negative lupus T cells (14). Additionally, mTOR signaling is required for Th17 cell differentiation (11) and IL-17 is critically involved in the pathogenesis of LN (6, 7). However, the influence of mTORC1 inhibition on Th17 differentiation in children with LN remains unknown. Therefore, we tested the effect of rapamycin on IL-17–producing T cells from children with LN. PBMCs were stimulated with anti-CD3/CD28 for 7 d in the presence or absence of rapamycin. Rapamycin significantly reduced the frequency of IL-17–producing CD4+CD45RA−Foxp3− and Foxp3low T cells in children with LN (fold decrease mean ± SD, 4.4 ± 1.6 and 2.5 ± 0.6, respectively, n = 17) and from HCs (n = 14). These results are in accordance with recent findings of rapamycin-induced suppression of IL-17 expression in CD4+ T cells from adults with SLE (33). Because Stat3 is essential for Th17 differentiation (22, 34), we assessed the effect of rapamycin on Stat3 activity in T cells from children with LN. Treatment with rapamycin considerably reduced Stat3 phosphorylation and the expression of phosphorylated S6 ribosomal protein S6RP (p-S6RP; downstream of mTOR) in CD4+CD45RA−Foxp3− T cells from children with LN (Fig. 3B and 3C, respectively), indicating that rapamycin may impair the differentiation of CD4+CD45RA−Foxp3− T cells from children with LN into IL-17–producing cells, also via downregulation of Stat3. This finding has previously been described in a mouse model in which the mTOR gene is deleted in CD4+ T cells (10). However, rapamycin blocks cell cycle progression during T cell activation in general (35).

Rapamycin suppressed the activation of mTOR signaling assessed by measuring the fluorescence intensity of p-S6RP (Fig. 3C) and the frequency of p-S6RP+CD4+CD45RA−Foxp3− T cells (86.1 ± 9.2% in the absence of rapamycin and 11.4 ± 2.7% in control cultures. These findings indicate that rapamycin reduces the frequency of IL-17–producing and Stat3 active CD4+CD45RA−Foxp3− T cells in children with LN by blocking the activation of mTOR signaling.

FIGURE 2. IL-17–producing CD4+CD45RA−Foxp3− and Foxp3low T cells are increased in children with LN. (A) RORγyt and IL-17 expression by gated CD4+CD45RA−Foxp3− T cells was determined by flow cytometry after stimulation of fresh PBMCs with leukocyte activation cocktail. Representative dot plots for one patient with LN and one HC. Data show mean and SD of four children with active LN, four children with inactive LN, and five HCs. (B and C) Quantitative real-time PCR was used to assess the expression of (B) IL-17 mRNA and (C) RORc mRNA relative to GAPDH mRNA in anti-CD3/CD28–activated PBMCs from children with LN (n = 15) and from HCs (n = 15). Data are represented as the mean and SD. (D) PBMCs were activated with anti-CD3/CD28 for 7 d, restimulated with leukocyte activation cocktail, and analyzed by flow cytometry. Representative dot plots of IL-17 expression in the indicated T cell populations are presented. The numbers indicate the percentages of IL-17+ cells. Scatter plots depict the data obtained from children with LN (n = 17) and from HCs (n = 17).
The presence or absence of rapamycin (14). Foxp3 expression was enhanced and IL-4 production was reduced in lupus T cells that were cultured in the presence of rapamycin compared with lupus T cells that were cultured in its absence (Fig. 3E and 3F, respectively). Rapamycin did not affect the expression of RORγt in CD4+CD45RA−Foxp3− T cells (Fig. 3G). These results are consistent with previous studies (36, 37), additionally illustrating impaired nuclear translocation of RORγt in the presence of rapamycin that alters Th17 differentiation (37).

C5a slightly increases the frequency of CD4+CD45RA−Foxp3− IL-17+ T cells in pediatric patients with LN

The binding of immune cell–produced complement C5a, which is secreted following the interaction of T cells with APCs, to C5a receptor on T cells is involved in effector T cell expansion and immunity (38) and costimulates T cell alloimmunity (39). Additionally, C5a levels are significantly elevated in patients with SLE (40, 41) and play a critical role in the pathogenesis of LN (42). Furthermore, C5 can be therapeutically targeted (43). Thus, we assessed the effect of increasing concentrations of C5a on Th17 cell differentiation.

Unactivated or anti-CD3/CD28–activated PBMCs were incubated in the presence or absence of up to 100 ng/ml C5a, or CD4+ T cells were differentiated into Th17 cells via the addition of skewing cytokines and increasing concentrations of C5a. The addition of C5a to unactivated PBMC cultures from children with LN slightly increased the frequency of IL-17–producing CD4+CD45RA−Foxp3− T cells (1.7-fold increase in the presence of C5a, Supplemental Fig. 1A). Also, plasma C3 levels, which are decreased in patients with active LN, inversely correlated with the frequency of IL-17–producing CD4+CD45RA−Foxp3− T cells in children with LN (r = -0.86, p = 0.012). However, we failed to observe a further enhanced differentiation of lupus T cells into IL-17–producing cells in cell cultures that were activated with anti-CD3/CD28 in the presence of C5a (14.7 ± 2.2 and 15.2 ± 3.3% in the absence and presence of C5a, respectively, Supplemental Fig. 1B). Additionally, the IL-17 concentrations observed in culture supernatants from anti-CD3/CD28–activated PBMCs were similar in the presence and absence of additional C5a (data not shown). However, consistent with previous reports that C5a binds to the C5a receptor on the responding T cells and activates the Akt signaling pathway (38, 39), C5a-induced phosphorylation of Akt was observed in anti-CD3–activated CD4+CD45RA−Foxp3− effector T cells from lupus patients (Supplemental Fig. 1C). C5a also induced phosphorylation of S6RP, a downstream target of mTOR (Supplemental Fig. 1D). These data suggest that in certain circumstances, the high levels of C5a that are observed in children with active LN might contribute to enhanced Th17 cell immune responses in these patients.

Enhanced activation of Akt in CD4+CD45RA−Foxp3− IL-17+ T cells from pediatric patients with LN

The PI3K/Akt/mTORC1 pathway controls the differentiation of Th17 cells by regulating the nuclear translocation of RORγt and the presence of rapamycin, Fig. 3D). Furthermore, in accordance with previous findings in adults with SLE who were treated with rapamycin (14), Foxp3 expression was enhanced and IL-4 production was reduced in lupus T cells that were cultured in the presence of rapamycin compared with lupus T cells that were cultured in its absence (Fig. 3E and 3F, respectively). Rapamycin did not affect the expression of RORγt in CD4+CD45RA−Foxp3− T cells (Fig. 3G). These results are consistent with previous studies (36, 37), additionally illustrating impaired nuclear translocation of RORγt in the presence of rapamycin that alters Th17 differentiation (37).
other factors (37). We previously demonstrated that Akt is overactivated in CD4+CD45RA−Foxp3− and Foxp3low T cells from children with LN (30). In this study, we further assessed the activation status and function of Akt in IL-17-producing CD4+CD45RA−Foxp3− and Foxp3low T cells from children with LN.

The frequency of Th17 cells was increased in children with LN. Additionally, the activity of Akt was significantly higher in fresh IL-17–producing T cells from patients with active LN (MFI mean ± SD, 15.8 ± 8.9, n = 5) than in those from patients with inactive LN (MFI mean ± SD, 7.1 ± 1.8, n = 5) or controls (MFI mean ± SD, 2.8 ± 0.6, n = 5) (Fig. 4A). The fluorescence intensity of Akt correlated with the disease activity, as assessed by the SLEDAI-2K (r = 0.81, p = 0.005).

Upon short-term in vitro stimulation using anti-CD3/CD28, the activation of Akt was enhanced and prolonged in IL-17–producing CD4+CD45RA−Foxp3− T cells from children with LN compared with those from HCs (Fig. 4B). Additionally, after long-term activation with anti-CD3/CD28, Akt phosphorylation was enhanced in IL-17–producing CD4+CD45RA−Foxp3− T cells from children with LN compared with those from HCs (Fig. 4C). These data indicate that the activity of Akt signaling is increased in IL-17–producing T cells from children with LN.

Also, activation of mTOR signaling (p-S6RP) was increased in IL-17–producing CD4+CD45RA−Foxp3− T cells from children with LN compared with HCs (percentage p-S6RPCD4+CD45RA−Foxp3−IL-17+ cells: LN, 93.8 ± 6.1; HCs, 85.2 ± 4.3; MFI p-S6RP: LN, 7.1 ± 2.3; HCs, 3.1 ± 0.9). These results are in line with earlier studies demonstrating enhanced mTOR activation in lupus T cells (13, 14).

**Akt-dependent enhanced migratory capacity of Th17 cells from children with LN**

As demonstrated above, Th17 cells from lupus patients display enhanced Akt activity compared with those from HCs. We studied the role of Akt in the migratory capacity of Th17 cells from children with LN. Th17 cells express high levels of the chemokine receptor CCR6 (44, 45), and CCR6 mediates renal recruitment of Th17 cells (46). Thus, we used the corresponding chemokine CCL20 in the migration assays using Th17-polarized CD4+ T cells. We observed a concentration-dependent migration of Th17-polarized cells from patients with LN and from HCs toward CCL20 (Fig. 5A). Additionally, the percentages of migrated total CD4+ T cells and migrated Th17-polarized cells were significantly higher in patients with LN than in HCs (migrated total CD4+ T cells [mean ± SD], 47.4 ± 7.4 and 19.4 ± 3.8%, respectively, Fig. 5A).

**FIGURE 4.** Enhanced Akt activity in IL-17–producing T cells from lupus patients. (A) Freshly isolated PBMCs from children with active (a) LN (n = 5), inactive (i) LN (n = 5), and HCs (n = 5) were stimulated with PMA and ionomycin for 4 h in the presence of GolgiPlug, and the phosphorylation of Akt in gated CD4+CD45RA−Foxp3−IL-17+ T cells was analyzed by flow cytometry. Data are the mean and SD. (B) PBMCs were stimulated with anti-CD3/CD28 for 7 d, restimulated with PMA and ionomycin, rested for 2 h in serum-free medium, and then activated with anti-CD3/CD28 for the indicated times. The histograms of p-Akt expression in gated CD4+CD45RA−Foxp3−IL-17+ T cells are representative of four children with LN and four HCs. (C) PBMCs were stimulated with anti-CD3/CD28 for 7 d and restimulated with PMA and ionomycin in the presence of GolgiPlug. (C) p-Akt expression in the indicated T cell populations from children with LN (n = 17), children with NS (n = 5), and HCs (n = 19) was analyzed by flow cytometry (MFI). Data are the MFI of specific Ab minus the MFI of matched isotype control.
To assess the role of Akt, we performed migration assays in the presence of different concentrations of the Akt inhibitor VIII. Treatment with the Akt inhibitor dose-dependently decreased Akt activation (Supplemental Fig. 2). As a result, the migratory response of Th17 cells from children with LN to CCL20 was inhibited by the Akt inhibitor in a dose-dependent manner (Fig. 5B), indicating that Akt overactivation in Th17 cells from children with LN causes enhanced migration of these cells. Also, Th1- and Th2-polarized cells from children with LN showed enhanced migration compared with cells from HCs (Fig. 5C), and migration was blocked by the Akt inhibitor (Fig. 5D and 5E, respectively).

Discussion

Th17 cells infiltrate the inflamed kidneys in patients with LN (7) and are critically involved in the pathogenesis (6). In this study, we investigated the molecular mechanisms that underlie Th17 immune responses in children with LN. We demonstrate that enhanced activation of Stat3 in CD4+CD45RA^−Foxp3^− and Foxp3^low^ effector T cells from children with LN correlates with increased frequencies of IL-17–producing cells within these populations. Additionally, the expression of RORc and IL-17 mRNA in PBMCs from children with LN is significantly higher than in PBMCs from HCs. Furthermore, inhibition of mTOR by rapamycin decreases both the frequency of IL-17–producing T cells and Stat3 activity in CD4^+^CD45RA^-^Foxp3^-^ and Foxp3^low^ effector T cells from lupus patients. Complement factor C5a slightly increases IL-17 expression and induces the activation of Akt and mTOR in effector T cells from lupus patients. Importantly, IL-17–producing T cells from children with LN exhibit enhanced activation of the Akt/mTOR signaling pathway and an enhanced migratory capacity that is significantly reduced by the inhibition of Akt activity.

Stat3 is activated by various cytokines, including IL-6, IL-21, and IL-23. In this study, we observed increased levels of IL-6 and IL-21 in supernatants of PBMCs from children with LN. This finding is consistent with previous reports from adults with SLE (8, 47). Additionally, as recently demonstrated, serum levels of IL-23 are significantly higher in children with active LN than in those with inactive LN or in controls (48). These findings suggest that the enhanced concentrations of these proinflammatory cytokines mediate upregulation of Stat3 activity in CD4^+^CD45RA^-^Foxp3^-^ and Foxp3^low^ effector T cells in children with LN. Previous studies also indicated that total and phosphorylated Stat3 levels are increased in T cells from patients with SLE (49). Moreover, we found that when the same concentration of IL-6 was used in T cell activation experiments, the phosphorylation of Stat3 was higher in effector T cells from children with LN than in those...
from controls. These results indicate that in addition to high levels of proinflammatory cytokines, additional mechanisms mediate Stat3 phosphorylation in lupus effector T cells. Interestingly, the PI3K/Akt pathway has been reported to be involved in the phosphorylation of Stat3 (29, 50). We previously demonstrated that Akt is hyperactivated in effector T cells from lupus patients (30). Hyperactivation of Akt may thus further enhance Stat3 activity. However, a direct causal link remains to be demonstrated.

Stat3 regulates the expression of the RORγt transcription factor, which is essential for Th17 cell differentiation (51). Consistent with these earlier studies, we observed that in addition to enhanced Stat3 activity, increased RORc mRNA expression and increased differentiation of effector T cells into IL-17–producing cells occur in children with LN.

Intriguingly, immune cell–derived complement factor C5a is involved in effector T cell expansion and immunity (38) and costimulates T cell alloimmunity (39). Additionally, C5a is enhanced in patients with LN (40) and is critical for the pathogenesis of this disease (42). Thus, we hypothesized that increasing concentrations of C5a might enhance the Th17 immune response in children with LN. However, we failed to observe a further increase in IL-17–producing CD4+CD45RA−Foxp3+ T cells in anti-CD3/CD28–activated PBMC cultures from children with LN or in CD4+ T cells cultured under Th17-polarizing conditions in the presence of increasing concentrations of C5a. However, the addition of C5a to unactivated PBMC cultures from children with LN slightly increased the frequency of IL-17–producing CD4+ CD45RA−Foxp3+ T cells. This finding suggests that the abundant levels of C5a observed in children with active LN might be involved in Th17 immune responses in children with LN under certain conditions. A role of C5a in Th17 immune responses was also recently described in murine autoimmune arthritis (52) and in age-related macular degeneration (53).

mTORC1 is located downstream of Akt, and mTOR activity is enhanced in lupus T cells (13). Moreover, the inhibition of mTOR by rapamycin reduces disease activity in patients with SLE (12). Previous studies also demonstrated that double-negative T cells from rapamycin-treated SLE patients display reduced necrosis and IL-4 production (14). Additionally, the PI3K/Akt/mTORC1 pathway controls the differentiation of Th17 cells by regulating the nuclear translocation of RORγt and other factors (37). T cell–specific deletion of mTORC1 activity is associated with a decrease in Stat3 activation in response to the skewing cytokines and with a failure to differentiate into Th17 cells (10, 11). Very recently, rapamycin has been shown to suppress IL-17 expression by CD4+ T cells from adults with SLE (33). In this work, we found that treatment of cell cultures with rapamycin considerably reduced both Stat3 activation in CD4+CD45RA−Foxp3+ and Foxp3low T cells from children with LN and the frequency of IL-17–producing cells within these T cell populations. These findings may contribute to our understanding of the molecular mechanisms that underlie the therapeutic efficacy of rapamycin in patients with SLE.

Forced activation of Akt in transgenic T cells increased the percentages of cells highly expressing IL-17 (37). Additionally, the Akt signaling pathway also mediates Th17 cytokine production (15). Our results show that the activity of Akt/mTOR signaling is enhanced in IL-17–producing effector T cells from children with LN. Accumulating evidence indicates that Akt regulates cell motility in physiological and pathological settings, including cancer metastasis (54). Additionally, Akt plays a pivotal role in cytotoxic T cell migration (55). Our observation that the inhibition of Akt in Th17 cells from children with LN significantly reduces their migratory activity suggests a key role for the Akt signaling in Th17 cell trafficking to sites of inflammation in children with LN. However, a major limitation to our study is the relatively small number of patients, and further studies are needed.

In the future, therapies that target the Akt signaling pathway and thus Th17 cell differentiation and trafficking may suppress chronic inflammatory processes and improve outcomes in children with LN.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1

**C5a slightly increases the expression of IL-17 in CD4⁺CD45RA⁻Foxp3⁻ T cells from children with LN.** (A, B) PBMCs from children with LN were cultured (A) in the presence or absence of recombinant human C5a (100 ng/ml) alone for 7 days or (B) in the presence or absence of 100 ng/ml of recombinant human C5a plus anti-CD3/CD28 and were reactivated with Leukocyte Activation Cocktail. Box-and-whisker plots show the frequencies of IL-17-expressing CD4⁺CD45RA⁻Foxp3⁻ T cells from 7 children with LN. (C) CD4⁺ T cells from children with LN (n=5) were stimulated with plate-bound anti-CD3 in the presence or absence of C5a (100 ng/ml) for 24 hours. Representative histogram and box-and-whisker plots show the fluorescence intensity of pAkt in gated CD4⁺CD45RA⁻Foxp3⁻ T cells (MFI, median fluorescence intensity). (D) CD4⁺ T cells from children with LN (n=5) were stimulated with plate-bound anti-CD3 in the presence or absence of C5a (100 ng/ml) for 24 hours. Cumulative data of pS6RP expression in gated CD4⁺CD45RA⁻Foxp3⁻ T cells. Percent positive cells were calculated in relation to isotype matched control antibodies. The MFI was calculated as the MFI of specific antibody minus the MFI of the corresponding isotype control.
Supplemental Figure 2. **Treatment with Akt inhibitor dose-dependently reduces phosphorylation of Akt.** Th17-polarized cells were stimulated with PMA and ionomycin in the presence of GolgiPlug and then were treated with the Akt inhibitor VIII (Akt Inh) at the indicated concentrations or with diluent. Representative histogram of pAkt expression in Th17-polarized cells from children with LN (n=4).