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The Importance of IL-6 in the Development of LAT-Mediated Autoimmunity

Sarah A. O’Brien, Minghua Zhu, and Weiguo Zhang

Linker for activation of T cells (LAT) is a transmembrane adaptor protein that is highly tyrosine phosphorylated upon engagement of the TCR. Phosphorylated LAT binds Grb2, Gads, and phospholipase C (PLC)γ1 to mediate T cell activation, proliferation, and cytokine production. T cells from mice harboring a mutation at the PLCγ1 binding site of LAT (Y136F) have impaired calcium flux and Erk activation. Interestingly, these T cells are highly activated, resulting in the development of a lymphoproliferative syndrome in these mice. CD4+ T cells in LATY136F mice are Th2 skewed, producing large amounts of IL-4. In this study, we showed that the LATY136F T cells could also overproduce IL-6 due to activated NF-κB, AKT, and p38 pathways. By crossing LATY136F mice with IL-6–deficient mice, we demonstrated that IL-6 is required for uncontrolled T cell expansion during the early stage of disease development. Reduced CD4+ T cell expansion was not due to a further block in thymocyte development or an increase in the number of regulatory T cells, but was caused by reduction in cell survival. In aged IL-6−/− LATY136F mice, CD4+ T cells began to hyperproliferate and induced splenomegaly; however, isotype switching and autoantibody production were diminished. Our data indicated that the LAT–PLCγ1 interaction is important for controlling IL-6 production by T cells and demonstrated a critical role of IL-6 in the development of this lymphoproliferative syndrome. The Journal of Immunology, 2015, 195: 695–705.

Cytokines play an important role in orchestrating immune responses and are vital for T cell survival, proliferation, and differentiation. aberrant cytokine production is thought to play a role in the development of inflammatory conditions, which can lead to autoimmunity. IL-6 is a cytokine that is found in high quantities in the joints and sera of patients with rheumatoid arthritis (RA). Within joint tissues, IL-6 is produced by fibroblasts, activated macrophages, and lymphocytes. It activates vascular endothelial cells to upregulate adhesion molecules, which then promote leukocyte recruitment to the site of inflammation. IL-6 is also important for B cell maturation to Ab-secreting plasma cells, leading to overproduction of autoantibodies, as seen in the patients with RA (1). Because of its many roles in instigating inflammation, IL-6 has become a promising target for the treatment of RA and other autoimmune diseases.

IL-6–mediated signaling has been well studied. Upon binding IL-6, IL-6Rα associates with gp130 and activates downstream signaling cascades. STAT1 and STAT3 are phosphorylated by JAK1/2, form homodimers, and translocate to the nucleus to activate their target genes (2). IL-6–mediated STAT3 activation induces the gene transcription of Bcl-2, Bcl-xL, cFos, and tissue inhibitor of metalloproteinase-1, all of which regulate cell survival and proliferation. Additionally, IL-6 association with IL-6Rα can induce phosphorylation of the SHP-2 binding site on gp130, resulting in ERK activation (3, 4). Finally, IL-6 signaling also activates PI3K, which is an important mediator of Akt activation and cell survival (5).

IL-6 is mainly produced by macrophages during acute inflammation; however, it is also produced by T cells during chronic inflammation (3). IL-6 plays an important role in T cell survival, differentiation, and cytokine production. It has been shown to inhibit apoptosis of naive T cells (6, 7) and to regulate the Th1/Th2 regulatory T cell (Treg) balance (8). IL-6, together with TGF-β, promotes Th17 differentiation, whereas TGF-β alone promotes induced Treg differentiation (8–10). Additionally, it has been reported that IL-6 can lead to the production of other cytokines by T cells. TCR signaling in conjuncture with IL-6 signaling can induce c-Maf expression and early IL-4 production (11). In human T cells, specifically T follicular helper cells, IL-6 can induce IL-21 production, which in turn causes plasma cell differentiation (12).

Importantly, IL-6 has been implicated in the regulation of T cell homeostasis. Microbiota within the gut have been shown to activate dendritic cells to produce IL-6 in an MyD88-dependent manner, which induces spontaneous T cell proliferation (13). Additionally, blocking Abs against IL-6R can alleviate CD8+ T cell proliferation during colitis (14). IL-6 is also important for CD4+ T cell homeostatic proliferation. In mice harboring a mutation in gp130, which results in enhanced STAT3 activation, nonhematopoietic cells increase production of IL-7, which in turn drives homeostatic proliferation of CD4+ T cells (15).

Linker for activation of T cells (LAT) is an adaptor molecule that is highly tyrosine phosphorylated upon TCR engagement with MHC–peptide complexes. Through its phosphorylated tyrosine residues, LAT recruits Grb2, Gads, and phospholipase C phospholipase C (PLC)γ1 (16). LAT functions to link TCR engagement to downstream signaling pathways that are important for T cell activation, proliferation, survival, and cytokine production. Among the tyrosine residues in murine LAT, Y136 is responsible for binding PLCγ1. The recruitment of PLCγ1 to the LAT signalosome is important for the hydrolysis of phosphatidylinositol 4,5-biphosphate to inositol-1,4,5-triphosphate and diacylglycerol.

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; DN, double-negative; LAT, linker for activation of T cells; PLC, phospholipase C; RA, rheumatoid arthritis; Treg, regulatory T cell; WT, wild-type.

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leading to calcium mobilization and ERK activation (16, 17). In LATY136F mutant mice, thymocyte development is partially blocked at the double-negative (DN)3 stage, indicating the important role for LAT–PLCγ1 signaling at the pre-TCR checkpoint. CD4+ T cells are able to exit the thymus and hyperproliferate in secondary lymphoid organs. These CD4+ T cells have an activated phenotype (CD44+CD62L−) and low levels of surface TCR. Additionally, these T cells produce elevated amounts of IL-4. Consequently, B cells are activated, undergo isotype switching, and produce high levels of serum IgE, IgG1, and anti-dsDNA Abs (18, 19). In this study, we were interested in investigating the role of cytokines in spontaneous hyperproliferation of LATY136F T cells. Our results indicated that LATY136F T cells produced IL-6, and that IL-6 played an important role in the development of LAT-mediated autoimmune disease.

Materials and Methods

Mice

LATY136F (LATm/m) and LAT−/− mice have been previously described (18, 20). MyD88−/−, IL-6−/−, and IL-6Rα−/− mice (generated by crossing IL-6Rα−/− and CMV-Cre mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in specific pathogen-free conditions and were used in accordance with the National Institutes of Health guidelines. All experiments were approved by the Duke University Institutional Animal Care and Use Committee.

Flow cytometry and ELISA

Single-cell suspensions were prepared from spleens, lymph nodes, or thymuses and were stained with fluorescein Abs (BioLegend) in the presence of 2.4G2 (anti-FcγRII/III receptor). Intracellular staining for transcription factors was performed after cells were fixed and permeabilized (BD Biosciences). Prior to intracellular staining for cytokines, cells were stimulated in vitro for 4 h with PMA (20 ng/ml) and ionomycin (0.5 μg/ml) in the presence of monensin. For inhibitor experiments, SB203580 (30 μM), PD98059 (30 μM), LY294002 (10 μM),Akt-1/2 trifluoroacetate salt hydrate (2 μM), or QNZ (2 μM) (Sigma-Aldrich) was added to RPMI complete media supplemented with IL-2 for 48 h prior to PMA and ionomycin stimulation. For intracellular staining for p-ERK, wild-type (WT) splenocytes were incubated with different inhibitors for 48 h and then stimulated with anti-CD3 (2C11) for 30 min prior to fixation and permeabilization. 7-Aminoactinomycin D (7-AAD) distinguished live cells from dead cells. Data were acquired on the FACSCanto II (BD Biosciences) and then analyzed using FlowJo software. Anti-dsDNA and serum Ab ELISAs were performed as previously described (21).

Western blotting

T cells were purified from spleens and lymph nodes using EasySep CD4+ purification kits (Stemcell Technologies). Equal numbers of T cells from WT, LAT−/−, and IL-6−/− “LATm/m” mice were lysed, resolved on SDS-PAGE, and blotted with Abs against the following proteins: Zap70, p-Lck, Lck, p-ERK, ERK2, p-Akt (Ser473), Akt, p-p38, p38, p–NF-κB, and NF-κB (Cell Signaling Technology).

T cell proliferation

For in vitro proliferation, splenocytes were loaded with 5 μM CFSE and stimulated with plate-coated anti-CD3 (3 μg/ml 2C11) or with PMA and ionomycin overnight. After 48 h, CFSE dilution of CD4+ T cells was assessed by flow cytometry. For in vivo proliferation, 3 × 106 CD4+ T cells were sorted, loaded with CFSE, and transferred via i.v. injection to LAT−/− hosts for 6 d before CFSE dilution was assessed by FACS.

Real-time PCR

Total RNAs from purified CD4+ T cells or whole lymph nodes were isolated using TRIzol reagent (Invitrogen). Lymph nodes were homogenized with 1.5-mm beads using the D1030 BeadBug homogenizer (Benchmark Scientific). cDNAs were synthesized with the SuperScript reverse transcriptase (Invitrogen). Cytokine RNAs were quantified using SYBR Green Super mix (Bio-Rad).

Immunofluorescence imaging

To determine the presence of autoantibodies, NIH3T3 cells were dropped onto 18 chamber slides, fixed with 1:1 acetone/methanol prior to incubation with serum (1:50), and stained with goat anti-mouse IgG-FITC and DAPI (Invitrogen). Cells were mounted using Fluoromount-G (SouthernBiotech) and examined using the Leica SP5 confocal microscope.

Results

LATY136F T cells overproduced IL-6

Published data indicate that CD4+ T cells from the LATY136F mice, designated as LAImm mice in this study, are Th2 skewed (18, 19). We wanted to understand the effect of aberrant LAT–PLCγ1 signaling on the production of other cytokines. RNAs were isolated from WT and LAImm/CD4+ T cells to examine cytokine production at the level of transcription by real-time PCR analysis. As shown in Fig. 1A, CD4+ T cells from LAImm mice had elevated levels of Th2 cytokines, such as IL-4, IL-5, and IL-13, as expected. Interestingly, LAImm T cells also had significantly elevated levels of IL-6 RNA compared with WT CD4+ T cells (Fig. 1A). TNF-α expression in WT and LAImm T cells was similar (Fig. 1A). Additionally, TNF-α concentration in the sera of these mice, analyzed by multiplex assay, was also similar (data not shown), suggesting that the elevated IL-6 was not a result of a systemic inflammatory response.

Because of the potential role of IL-6 in the regulation of T cell homeostasis (13–15), we chose to explore the role of IL-6 in T cell hyperproliferation and autoimmunity of LAImm mice. To determine whether the elevated IL-6 was also seen at the protein level, we isolated splenocytes from 6-wk-old mice and stimulated them in vitro with PMA and ionomycin prior to intracellular staining for IL-6. Our results showed that WT splenocytes had very little positive staining. In comparison, close to 25% of CD4+ T cells from LAImm mice produced IL-6 (Fig. 1B). Very few non-CD4+ T cells from LAImm mice were IL-6+. These data indicated that in addition to enhanced Th2 cytokine production, aberrant TCR signaling in LAImm T cells also led to overproduction of IL-6.

We next wanted to elucidate the signaling pathways downstream of the mutant LAT that were required for increased IL-6 production. We used different pharmacological inhibitors to block known pathways downstream of LAT and then determined their effect on IL-6 production. LAImm splenocytes were incubated with various inhibitors prior to stimulation with PMA and ionomycin to examine IL-6 production. Interestingly, whereas on average 23% of DMSO-treated T cells produced IL-6, treatment with both p38 and PI3K pathway inhibitors resulted in a 4-fold reduction in the percentage of IL-6–producing CD4+ T cells (6.3 and 5.6%, respectively). Downstream of PI3K, both Akt and NF-κB have been demonstrated to induce IL-6 (22, 23). In both tumor and RA model systems, IL-17 can induce Akt phosphorylation, resulting in IL-6 production (24, 25). Additionally, NF-κB binds the IL-6 promoter and induces its transcription in many cell types (26). As shown in Fig. 1C, inhibition of Akt or NF-κB resulted in a >2-fold reduction in the percentage of CD4+ T cells producing IL-6 (9.7 and 9.0%, respectively). In contrast, MEK1/2 inhibitor (PD98059) treatment caused no significant difference in IL-6 production, but it did cause a 2-fold reduction in IL-2 production (Fig. 1C). The specificities of these inhibitors were confirmed by FACS analysis of WT splenocytes activated in the presence of these inhibitors. The p38 inhibitor had very little effect on TCR-mediated Erk phosphorylation, whereas the MEK1/2 inhibitor severely reduced its phosphorylation (data not shown).

We also examined the effect of various inhibitors on IL-4 production. Among the different inhibitors, the PI3K and NF-κB inhibitors impacted IL-4 production more than the other three inhibitors (Fig. 1C). Treatment with the PI3K inhibitor resulted in a 1.7-fold reduction in the percentage of CD4+ T cells producing IL-4 (51.3% compared with 91.2% with DMSO), whereas NF-κB inhibitors severely reduced its phosphorylation (data not shown).

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inhibition resulted in a 2.5-fold reduction in the percentage of CD4+ T cells producing IL-4 (34.5%). These results indicated that both AKT and NF-κB activation were required for increased IL-6 and IL-4 production in CD4+LATm/m T cells.

To confirm the inhibitor data, we next wanted to see which of these pathways were activated in LATm/m CD4+ T cells. CD4+ T cells were isolated from aged-matched 2- to 3-mo old mice, and equal numbers of cells were lysed without any stimulation. Zap70, which is upstream of LAT signaling and therefore should not be affected by the LAT mutation, was used as a loading control. As shown in Fig. 1D, the levels of p38, AKT, and NF-κB proteins were all similar, yet the phosphorylation of these proteins was increased in LATm/m T cells, indicating that these signaling pathways were activated in the absence of LAT–PLCγ1 signaling (Fig. 1D). In contrast, there was no significant difference in Erk activation. Taken together, these results suggested that the LATY136F mutation caused the activation of p38, AKT, and NF-κB pathways in CD4+ T cells, which resulted in elevated IL-6 production.

Reduced expansion of LATY136F CD4+ T cells in the absence of IL-6
LATm/m mice display splenomegaly and lymphadenopathy due to hyperproliferating CD4+ T cells as early as 4 wk of age (18, 19). To assess the contribution of IL-6 to the LATY136F-mediated autoimmune syndrome, IL-6−/−LATm/m mice were generated, and disease development was compared with that in LATm/m mice at 6 wk of age. Age-matched WT and IL-6−/− mice were used as controls. Remarkably, IL-6−/−LATm/m mice had normal spleen size and weight, similar to control WT and IL-6−/− mice (Fig. 2A, 2B). LATm/m mice had enlarged spleens and increased number of splenocytes as previously reported (data not shown). In agreement with the disparate size, spleens from LATm/m mice contained a large percentage of CD4+ T cells (∼70%). Spleens from IL-6−/−LATm/m mice, however, had dramatically reduced percentages of CD4+ T cells (2.6%), even compared with WT and IL-6−/− control mice (∼15%) (Fig. 2C). The number of CD4+ T cells in IL-6−/−LATm/m mice was also reduced compared with that in LATm/m mice (Fig. 2B). Interestingly, although LATm/m mice lack peripheral CD8+ T cells, 4- to 6-wk-old IL-6−/−LATm/m mice retained that population (Fig. 2C), although at much lower percentages than control mice (1.6% compared with 8.7% in IL-6−/− mice or 10.2% in WT mice).

In addition to being hyperproliferative, LATm/m CD4+ T cells are also characterized by their activated phenotype, low levels of surface TCR, and exaggerated Th2 cytokine production (18, 19). Whereas CD4+ T cells in IL-6−/−LATm/m mice did not expand greatly, we wanted to evaluate whether they displayed the other characteristics of LATY136F T cells. Surface staining of 4- to 6-wk-old mice revealed that the vast majority of CD4+ T cells

FIGURE 1. Increased production of IL-6 by LATY136F T cells. (A) Increased IL-4, IL-5, IL-13, IL-6, and TNF-α production by real-time PCR analysis of cytokine transcripts in LATm/m CD4+ T cells compared with WT. Relative expression of cytokine RNA was normalized to β-actin. (B) Intracellular staining of IL-6. WT and LATm/m splenocytes were stimulated with PMA and ionomycin for 4 h prior to intracellular staining. (C) The effect of pharmacological inhibitors on cytokine production. LATm/m splenocytes were incubated with different inhibitors or DMSO in the presence of IL-2 for 48 h prior to stimulation with PMA and ionomycin and intracellular staining. Significance differences in cytokine production with various inhibitors (PD98059 for MEK1/2, SB203580 for p38, LY294002 for PI3K, trifluoroacetate for Akt1/2, and quinazoline for NF-κB) is compared with DMSO-treated cells. (D and E) Activation of signaling pathways in LATm/m T cells. Whole-cell lysates from WT and LATm/m T cells (D) or LATm/m and IL-6−/−LATm/m T cells (E) without activation were analyzed by Western blotting with Abs as indicated. Data are representative of three to five individual experiments. Mice used were 8–10 wk old. *p < 0.05, **p < 0.005 (two-tailed t test).
FIGURE 2. Effect of IL-6 on LATY136F T cell homeostasis and development. (A) Spleens from 6-wk-old WT, LATm/m, IL-6−/−, and IL-6−/−LATm/m mice. (B) Spleen weight and the numbers of CD4+ T cells from 4- to 6-wk-old mice. (C) Representative FACS plots of splenocytes. The expression of CD44 and CD62L was pregated on CD4+ cells. For intracellular staining of IFN-γ and IL-4, cells were stimulated with PMA and ionomycin for 4 h. (D) Analysis of thymocyte development in 4-wk-old mice. (E) The numbers of different subsets of thymocytes. (F) IL-6 deficiency had no effect on Treg cells in 6-wk-old IL-6−/−LATm/m mice. Foxp3 expression in CD4+ T cells was analyzed by intracellular staining. Data are representative of three to five individual experiments. **p < 0.005, ***p < 0.001 (two-tailed t test).
in IL-6−/−LATm/m mice had an effector/memory-like phenotype (CD44hiCD62Llo) (Fig. 2C); however, the number of naive T cells (CD44loCD62Lhi) was increased compared with that in LATm/m mice. Furthermore, staining of TCRβ revealed that both CD4+ and CD8+ T cells in IL-6−/−LATm/m mice had very low levels of TCRβ at the cell surface similar to LATm/m T cells (data not shown). To assess cytokine production, splenocytes were stimulated with PMA and ionomycin for 4 h prior to intracellular staining. Similar to LATm/m T cells, the vast majority of IL-6−/−LATm/m T cells had the capacity to produce large amounts of IL-4; therefore, they were also heavily Th2 skewed (Fig. 2C). Additionally, T cells from IL-6−/−LATm/m mice had similar phosphorilation of p38, AKT, and NF-κB as LATm/m T cells, suggesting that the activated PI3K and p38 pathways seen in LATm/m T cells were not caused by enhanced signaling through IL-6 (Fig. 1E). Taken together, these results indicated that even though IL-6 deficiency did not change the activated phenotype of LATm/m T cells, IL-6 was important in driving uncontrolled CD4+ T cell expansion in LATm/m mice.

IL-6 deficiency had no effect on the development of LATY136F T cells

Because the LATY136F mutation causes a partial block at the pre-TCR checkpoint (DN3) during thymocyte development, we next wanted to determine whether the reduced number of T cells seen in the periphery of IL-6−/−LATm/m mice was due to a further block in thymocyte development. Thymocytes from 4-wk-old IL-6−/−LATm/m, LATm/m, and control mice were analyzed. Staining for CD4 and CD8 revealed a similar block in the DN compartment. Both LATm/m and IL-6−/−LATm/m mice had 75.2 and 70.5% DN thymocytes, respectively (Fig. 2D). Further analysis of the DN compartment showed that thymocyte development was partially blocked at DN3 and the percentages of the DN1–DN4 subsets were similar between LATm/m and IL-6−/−LATm/m mice (Fig. 2D). Furthermore, the numbers of total, DN, and double-positive thymocytes were comparable in LATm/m and IL-6−/−LATm/m mice (Fig. 2E). Thus, the reduction in the number of CD4+ T cells seen in the periphery of IL-6−/−LATm/m mice was not due to a further block in thymocyte development.

No effect of IL-6 deficiency on Tregs in LATY136F mice

We next wanted to elucidate the mechanism by which IL-6 regulates T cell expansion in secondary lymphoid organs of LATm/m mice. It is known that IL-6 inhibits Treg differentiation and promotes Th17 differentiation from naive T cells (9, 10, 27). Because LATm/m mice lack functional Tregs (28, 29), it is possible that IL-6 deficiency in LATm/m mice may restore the development or function of Tregs. To detect the presence of Tregs, T cells from 6-wk-old mice were stained intracellularly for Foxp3, the master regulator of Tregs. Similar to LATm/m mice, IL-6−/−LATm/m mice were devoid of Foxp3+CD4+ T cells (Fig. 2F). This indicated that the reduced expansion of CD4+ T cells in IL-6−/−LATm/m mice was not due to the appearance of Tregs that suppressed T cell expansion. It also suggested that the absence of Tregs in LATm/m mice was not due to an overabundance of IL-6 in lymphoid tissues, but rather an intrinsic role of LAT–PLCγ1 signaling in the development or maintenance of Tregs.

MyD88 signaling is not required for LATY136F T cell proliferation

Studies have demonstrated that TLR-mediated IL-6 production can induce T cell homeostatic proliferation (13). Additionally, naïve T cells express TLRs, such as TLR1, 2, 3, 6, and 7, whereas activated CD4+ T cells express TLR4 and TLR5 (30, 31). TLR-mediated signaling may potentially intersect with aberrant TCR signaling in LATm/m T cells to induce IL-6 production. We next tested whether TLR signaling was important for IL-6 production in LATm/m mice by crossing them to MyD88−/−mice to ablate TLR signaling.

Similar to IL-6−/−LATm/m mice, we analyzed MyD88−/−LATm/m mice at 4–6 wk of age for IL-6 production and the development of autoimmunity. We first analyzed the ability of MyD88−/−LATm/m T cells to produce IL-6. As shown in Fig. 3A, 10.9% of MyD88−/−LATm/m T cells produced IL-6, which was comparable to LATm/m T cells (8.7%). To assess autoimmunity, we first analyzed spleen weight and the number of CD4+ T cells, which were similar in MyD88−/− and WT mice (Fig. 3B). MyD88−/−LATm/m mice had significantly larger spleens and increased numbers of CD4+ T cells compared with MyD88−/− and WT control mice; however, the splenomegaly was not as drastic as that in LATm/m mice (Fig. 3B). The spleens of MyD88−/−LATm/m mice contained ~34% CD4+ T cells with virtually no CD8+ T cells (Fig. 3C). Whereas the percentage of CD4+ T cells varied, the average number of CD4+ T cells in the spleen was not significantly different between MyD88−/−LATm/m mice and LATm/m mice. Additionally, MyD88 deficiency did not rescue the activation status of LATm/m T cells, similar to IL-6 deficiency (Fig. 3C). T cell hyperproliferation in MyD88−/−LATm/m mice was similar to that seen in LATm/m mice, and it did not recapitulate the phenotype seen in IL-6−/−LATm/m mice. These data indicated that TLR signaling through MyD88 did not play a major role in IL-6 production and T cell expansion in LATm/m mice.

Defect in T cell survival in the absence of IL-6

In addition to its role in T cell differentiation, IL-6 is also important for both T cell survival and proliferation (6, 7, 13, 15). To examine whether IL-6 deficiency blocked proliferation in IL-6−/−LATm/m T cells, we examined T cell proliferation by Ki67 staining, which marks cells that are in active phase of cell cycle, not resting cells. Although the percentage of CD4+ T cells in IL-6−/−LATm/m mice was relatively small (~5%), almost half of those T cells were in active proliferating phase (Fig. 4A). Even though LATm/m mice had enlarged spleens by 6 wk of age owing to uncontrolled expansion of CD4+ T cells, only 11% of them were actively proliferating, similar to WT and IL-6−/− T cells. This large Ki67+ population in IL-6−/−LATm/m mice may be a factor of the lymphoproliferative environment. This result suggested that there was not a defect in T cell proliferation in the absence of IL-6.

To further confirm that T cell proliferation was not affected by IL-6 deficiency in IL-6−/−LATm/m T cells, we examined their proliferation by CFSE dilution both in vitro and in vivo. To assess T cell proliferation in vitro, splenocytes were labeled with CFSE and then stimulated through the TCR using plate-bound anti-CD3 or by PMA and ionomycin for 24 h. CFSE dilution was analyzed 48 h later. As seen in Fig. 4B, neither LATm/m T cells nor IL-6−/−LATm/m T cells proliferated in response to TCR engagement, likely due to low surface TCR levels, and both cells diluted CFSE similarly in response to PMA and ionomycin stimulation. To access in vivo proliferation, 3 × 106 CD4+ T cells were sorted from LATm/m or IL-6−/−LATm/m mice, stained with CFSE, and transferred into IL-6−/− hosts, which are deficient in T cells. CFSE dilution was analyzed 6 d later. Similar slow homeostatic proliferation was detected for both LATm/m and IL-6−/−LATm/m T cells (Fig. 4C).

To examine the role of IL-6 in LATm/m T cell survival, we examined cell death by staining with annexin V and 7-AAD. CD4+ T cells were pregated to remove doublets, and then the percentage of live cells (annexin V−7-AAD−) was determined. WT and IL-6−/− spleens contained on average 82.2 and 84.4% live CD4+
T cells, respectively (Fig. 5A). LATm/m mice had an average of 81.1% live CD4+ T cells in their spleens, which was similar to WT and IL-6−/− mice. In contrast, 72.6% of CD4+ T cells in IL-6−/−LATm/m spleens were live, which was significantly reduced compared with WT, IL-6−/−, and LATm/m T cells (Fig. 5A). Previous studies have demonstrated that IL-6 signaling induces the expression of the antiapoptotic molecules, Bcl-2 and Bcl-xL, to rescue T cells from cell death (3, 6, 32). Intracellular staining of T cells from 6-wk-old mice showed that IL-6−/−LATm/m CD4+ T cells expressed low levels of Bcl-2 similar to LATm/m CD4+ T cells (Fig. 5B). Because Bcl-2 expression was not further reduced in IL-6−/−LATm/m CD4+ T cells compared with LATm/m CD4+ T cells, we were able to conclude from these data that the effect of IL-6 on the survival of CD4+ T cells in LATm/m mice is less likely through the regulation of Bcl-2 expression.

Although it has been shown that IL-6 can rescue Ag-stimulated T cells from cell death (33), it was also reported that the effect of IL-6 on T cell survival is restricted to naive T cells, and there is little effect on activated T cells due to differential signaling and expression of IL-6Rα (7). LATm/m T cells spontaneously develop an activated phenotype. Therefore, these cells may not be able to signal through the IL-6Rα. Indeed, when we examined IL-6Rα expression, LATm/m CD4+ T cells expressed lower levels of surface IL-6Rα compared with WT T cells (Fig. 5C). Whereas some cytokine receptors become internalized upon engagement with their ligands, this is likely not the case in this study, as IL-6−/−LATm/m CD4+ T cells also expressed low surface IL-6Rα, despite the absence of IL-6.

To test whether low IL-6Rα expression could still allow for STAT3 activation, splenocytes were left unstimulated or stimu-
lated in vitro with IL-6 for 20 min, prior to intracellular staining for pSTAT3. As shown in Fig. 5D, STAT3 phosphorylation was induced in WT T cells upon IL-6 stimulation whereas STAT3 phosphorylation was unchanged in LATm/m T cells. Quantitation of fold changes of STAT3 phosphorylation from unstimulated to stimulated showed that WT T cells had a ∼2.1-fold increase in pSTAT3 (Fig. 5E). STAT3 phosphorylation was not increased in LATm/m T cells. Additionally, there was no detectable pSTAT3 in LATm/m T cells at 5, 10, or 30 min after IL-6 stimulation either (data not shown). As controls, IL-6Rα2/2 and IL-6Rα2/2 LATm/m splenocytes were also stimulated with IL-6, and those T cells showed a lack of STAT3 phosphorylation (0.87- and 0.98-fold change in mean fluorescence intensity, respectively), similar to LATm/m T cells (Fig. 5E). Taken together, these data suggested that it is unlikely that IL-6 acts directly on T cells in LATm/m mice to promote cell survival.

IL-6 in LATY136F-mediated autoimmunity

Finally, we wanted to determine whether this regulation of T cell expansion by IL-6 persisted in aged mice. To examine this, 3-mo-old IL-6−/− LATm/m mice were analyzed for T cell expansion and development of autoimmunity. Again, IL-6−/− mice had normal spleen sizes and similar T cell populations as did WT mice. In contrast to young mice, 3-mo-old IL-6−/− LATm/m mice now displayed severe splenomegaly, with large percentages of CD4+ T cells (49.7% compared with young mice with ∼3%) (Fig. 6A, 6B). Aged IL-6−/− LATm/m mice had a T cell profile similar to LATm/m mice, with close to half of the splenocytes being CD4+ T cells and very few CD8+ T cells (Fig. 6B). From these data, we were able to conclude that over time, there is a compensatory mechanism that promotes expansion and survival of CD4+ T cells in the absence of IL-6.

The other aspect of LATY136F-mediated disease is the effect of CD4+ T cells on B cells. B cells become activated and express high levels of MHC class II, CD80, and CD86. They undergo isotype switching, resulting in high concentrations of serum IgG1 and IgE, as well as autoantibodies (18, 19). The number of B cells in IL-6−/− LATm/m mice was not significantly different compared with LATm/m mice (data not shown). B cells from 3-mo-old IL-6−/− LATm/m mice also had similar upregulation of MHC class II; however, whereas 26.6% of LATm/m B cells had undergone class switching, only 9.4% of IL-6−/− LATm/m B cells were IgD−IgM+ (Fig. 6C). Furthermore, analysis of serum Abs from 3-mo-old mice demonstrated significantly less serum IgG1 and IgE in IL-6−/− LATm/m mice compared with LATm/m mice, although still more than WT and IL-6−/− mice (Fig. 6D). Interestingly, quantitation of anti-dsDNA Abs by ELISA revealed that IL-6−/− LATm/m had lower levels of autoantibodies than did LATm/m mice. This result was also confirmed by immunofluorescence staining of NIH3T3 cells with the sera from these mice. Staining with the LATm/m sera revealed marked nuclear and cytoplasmic staining (Fig. 6E). Although detectable, staining of NIH3T3 cells with IL-6−/− LATm/m sera was much weaker.

It has previously been demonstrated that the role of IL-6 on Ab production is indirectly mediated through induction of IL-21 by T cells (34). Therefore, we wanted to examine IL-21 levels in LATm/m mice with and without IL-6. Because the phenotype seen...
in the spleen was mirrored in the lymph nodes, we homogenized whole lymph nodes for preparation of total RNAs for real-time PCR analysis of IL-21. There was no difference in IL-21 mRNA levels in LAT–/– and IL-6–/–LAT–/– lymph nodes (Fig. 6F). We also examined whether reduced early T cell survival and less autoantibody production in IL-6–/– LAT–/– mice had any effect on the survival of these mice. On average, LAT–/– mice lived for 4.7 mo (Fig. 6G). IL-6–/–LAT–/– mice appeared to have better long-term survival and lived an average of 6.1 mo, although this difference was not statistically significant. Taken together, our data showed that in LAT–/– mice, IL-6, which is produced mainly by T cells, plays an important role in early T cell survival and B cell class switching and autoantibody production.

**Discussion**

Published studies show that the LAT–PLCγ1 interaction is essential in regulating T cell homeostasis and controlling production of Th2 cytokines. Our results in this study demonstrated that this interaction is also important for modulating IL-6 production. IL-6 is normally produced by innate cells such as monocytes and macrophages at the site of acute inflammation, and also by T cells during chronic inflammation (3). Its expression is induced upon activation of IL-1, TNF-α, or TLR signaling pathways, all of which depend on the transcription factor NF-κB. Our results showed that basal phosphorylation of both NF-κB and AKT was enhanced in T cells with the LAT136F mutation. Moreover, inhibition of these proteins reduced the ability of these T cells to produce IL-6. Interestingly, p38 activation was also enhanced in LAT–/– T cells, and inhibition of p38 also prevented LAT–/– T cells from producing IL-6. It has been demonstrated that in cardiac myocytes, MKK6 activates NF-κB–mediated IL-6 production in a p38-dependent manner (35). Thus, it is possible that in LAT–/– T cells, p38 is enhancing NF-κB–mediated IL-6 production.

A published study demonstrated that β-catenin is constitutively degraded in peripheral T cells. Interestingly, a nondegradable form of β-catenin can act as a negative regulator of the LAT–PLCγ1 signaling cascade (36). Because WNT and β-catenin signaling can induce an inflammatory response and IL-6 production, we analyzed β-catenin expression in these CD4+ T cells by Western blotting. β-catenin protein levels were slightly elevated in LAT–/– T cells compared with WT peripheral T cells (data not shown). Thus, it is possible that the LAT136F mutation may cause sustained β-catenin expression in addition to increased IL-6 production in these T cells.

Signaling through TLRs is a major mechanism for IL-6 induction. Our data showed that LAT136F CD4+ T cells were able to produce IL-6 in an MyD88-independent manner. Additionally, MyD88–/–LAT–/– mice had CD4+ T cells that were hyperproliferative and activated. These results suggested that TLR-mediated MyD88 activation is neither required for T cell IL-6 production or for IL-6–induced expansion of T cells in LAT–/– mice. Our intracellular staining of IL-6 suggested that macrophages and B cells were not the main producers of IL-6 in LAT–/– mice. Although our data indicated that CD4+ LAT–/– T cells increased IL-6 production, we cannot rule out the possibility that nonhematopoietic cells may contribute to IL-6 production in these mice. The liver is able to produce IL-6 as part of an acute phase response, and muscle cells can also produce IL-6. Because our multiplex assay for different cytokines did not detect high levels of IL-6 in the serum of LAT136F mice (unpublished data), it is possible that IL-6 is acting locally in secondary lymphoid organs and is not part of a systemic inflammatory response. Additionally, endothelial cells and fibroblasts can produce IL-6. These cells may also contribute to local IL-6 production in the spleens and lymph nodes of LAT mutant mice.

Ki67 intracellular staining indicated that IL-6–/–LAT–/– T cells were not defective in proliferation compared with LAT–/– T cells. Additionally, when we transferred IL-6–/–LAT–/– or LAT–/– T cells
T cells into LAT−/− mice (T cell deficient) to examine homeostatic proliferation by CFSE labeling, we did not observe a difference in CFSE dilution. Previous studies have shown that IL-6 signaling plays an important role in IL-7 production in vivo. In mice with a mutation in gp130 (a subunit of the IL-6R) that causes enhanced STAT3 signaling, IL-7 production is enhanced.
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The authors have no financial conflicts of interest.

References


