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FcRγ Presence in TCR Complex of Double-Negative T Cells Is Critical for Their Regulatory Function

Christopher W. Thomson,* Wendy A. Teft,† Wenhao Chen,* Boris P.-L. Lee,* Joaquin Madrenas,‡ and Li Zhang2*‡

TCRαβ CD4 CD8 double-negative (DN) T regulatory (Treg) cells have recently been shown to suppress Ag-specific immune responses mediated by CD8+ and CD4+ T cells in humans and mice. Our previous study using cDNA microarray analysis of global gene expression showed that FcRγ was the most highly overexpressed gene in functional DN Treg cell clones compared with nonfunctional mutant clones. In this study, we demonstrate that FcRγ-deficient DN T cells display markedly reduced suppressive activity in vitro. In addition, unlike FcRγ-sufficient DN T cells, FcRγ-deficient DN T cells were unable to prolong donor-specific allograft survival when adoptively transferred to recipient mice. Protein analyses indicate that in addition to FcRγ, DN Treg cell clones also express higher levels of TCRβ, while mutant clones expressed higher levels of Zap70 and Lck. Within DN Treg cells, we found that FcRγ associates with the TCR complex and that both FcRγ and Syk are phosphorylated in response to TCR cross-linking. Inhibition of Syk signaling and FcRγ expression were both found to reduce the suppressive function of DN Treg cells in vitro. These results indicate that FcRγ deficiency significantly impairs the ability of DN Treg cells to down-regulate allogeneic immune responses both in vitro and in vivo, and that FcRγ plays a role in mediating TCR signaling in DN Treg cells. The Journal of Immunology, 2006, 177: 2250–2257.

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Abbreviations used in this paper: Treg, T regulatory; DLI, donor lymphocyte infusion; DN, double negative; FcR, lymphoproliferative; MST, median survival time; QRT-PCR, quantitative RT-PCR; SLE, systemic lupus erythematosus.

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cells (NKT) (24), activated TCRγδ+ T cells (25), human systemic lupus erythematosus (SLE) T cells (26), and some human effector CD4+ T cells (27). Within the subset of T cells expressing FcRγ, it has been shown to take place of the conventional CD3ζ by regulating the expression of TCR complexes at the cell surface and mediating signal transduction through ITAMs upon receptor engagement (28). Whether FcRγ is expressed by DN Treg cells and is involved in their regulatory function has not been studied previously.

The goal of this study was to determine the role of the FcRγ subunit in DN T cell-mediated suppression of antidonor CD8+ T cells and to elucidate the molecular mechanism of FcRγ function in DN Treg cells. The results shown in this study demonstrate that compared with FcRγ+/− DN T cells, FcRγ−/− DN T cells have a reduced ability to down-regulate allogeneic immune responses mediated by syngeneic CD8+ T cells both in vitro and in vivo when adoptively transferred into syngeneic recipient mice. Molecular analysis showed that the FcRγ subunit is present in the TCR complex of DN Treg cells and that FcRγ and Syk are phosphorylated upon TCR cross-linking. Inhibition of the function of Syk or expression of FcRγ in DN T cells resulted in a marked reduction in DN T cell-mediated suppression. Based on these data, expression of the FcRγ subunit in DN T cells and TCR signaling through FcRγ and Syk appears to be critical in maintaining DN T cell-mediated suppression of allogeneically activated CD8+ T cells both in vitro and in vivo.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, B6 × BALB/c (CB6F1), B6.C-H2bms1/B (bm1), and BALB/c H-2dm2 (dm2, a BALB/c Ld-loss mutant, H-2Dd, Ld, Kd, I-Eβ) mice were purchased from Jackson ImmunoResearch Laboratories, and BALB/c/J292-Pr7r1ms1/J N12 (BALB/c.FcRγ−/−) and B6.129P2-Pr7r1ms1/J N12 (B6.FcRγ−/−) (29) were purchased from Taconic Farms. A breeding stock of 2C transgenic mice (on B6 background) was provided by D. Loh (St. Louis, MO) (30). The 2C (H-2b) transgenic mice were raised in our breeding colony and control C57BL/6 mice were used as controls. The mice were bred and maintained in a pathogen-free facility in our lab. The 2CF1.FcRγ−/− transgenic mice were generated by breeding mice from (2C × B6)F1 (FcRγ−/−) F1, and F2 generations via flow cytometry for 1B2 and FcRγ expression. The 2C.FcRγ−/− mice were generated by screening mice from (2C × BALB/c)F1 (FcRγ−/−) F1 and F2 generations via flow cytometry for L5 (American Type Culture Collection) and FcRγ (Upstate Biotechnology) expression. After identification of 2CFcRγ−/− and 2CD2CFcRγ−/− breeders, their identity was checked twice by the above described screening procedure before establishing a breeding colony. The 2CFcRγ−/− mice were bred with 2CD2CFcRγ−/− mice to obtain 2CF1.FcRγ−/− (H-2b, Ld, Kd, I-Eβ, FcRγ−/−) mice. All mice were maintained in the University Health Network (University of Toronto) animal colonies and conducted in accordance with guidelines set by the University Health Network Animal Care Committee.

Cell lines

Two DN Treg clone cell lines, DN1 (CN4) and DN2 (TN12), and their associated mutant clone cell lines, MU1 (CN4.8) and MU2 (TN12.8), were used in this study. Generation of DN Treg clones was performed using previously described methods (8). To maintain the T cell clones, 5 × 10^6 cells were cultured in a 24-well plate containing 5 × 10^5 irradiated Ld+ (CB6F1) splenocytes as stimuli in α-MEM supplemented with 10% FBS, 1% 2-ME, 30 U/ml human rIL-2, and 30 U/ml rIL-4. The cells were incubated at 37°C in 5% CO₂. Cells were restimulated every 3–4 days.

Flow cytometry

CD4 (PE-CY5), CD8 (PE-CY5), TCRγδ (PETR), NK1.1 (PETR) (eBio-science), and 1B2 TCRαβ (FITC) were used to stain DN T cells. To detect FcRγ, lymphocytes were permeabilized (BD Cytofix/Cytoperm; BD Biosciences) and stained using a rabbit anti-FcRγ or rabbit IgG control primary Ab (Upstate Biotechnology), followed by a PE-conjugated anti-rabbit secondary Ab (Cedarlane Laboratories). Data were acquired and analyzed using an EPICS XL-MCL flow cytometer (Corixa).

Quantitative RT-PCR (QRT-PCR)

QRT-PCR analysis was performed with the ABI Prism 7900HT thermocycler (Applied Biosystems) using SYBR green detection. RNA from independent cultures of DN1, DN2, MU1, and MU2 clone cells was isolated using the standard TRIzol reagent protocol (InVitrogen Life Technologies) 3–4 days poststimulation, and reverse transcribed using random hexamers. Each reaction was performed in a 10 μl reaction containing 3 mM MgCl₂, 50 nM dNTP, 20 nM primers, 40 ng of cDNA, 1× Rox reference dye (InVitrogen Life Technologies), 1× SYBR green reagent, and 0.125 U/μl Jumpstart Taq polymerase (Sigma-Aldrich). The expression of β-actin was used to normalize starting cDNA concentrations. The 5′-3′ primer sequence of sense and antisense primers used for QRT-PCR were GCCGT CATTCTTTTCCACCTTT and TTCAAAACGACAGAGGTGCACC. A standard curve consisting of five 3-fold dilutions of cDNA from a pool of all four samples (1:3:9:27:81) was used for linear regression analysis of all samples.

Isolation of DN T cells

For DLI, 2CF, FcRγ+/− or 2CF, FcRγ−/− mice were injected with 4.0 × 10^7 spleen cells from (C57BL/6)F1 mice. Spleen and lymph node cells were harvested 7 days after DLI, depleted of B cells, and then passed through a nylon wool column to enrich the T cell population. To deplete CD4+ and CD8+ T cells, the cells were then incubated with murine CD4 (R172, rat IgM) (31) and CD8 (3.168.8, rat IgM) (31) depleting mAbs, followed by incubation with rabbit complement (Cedarlane Laboratories). The suspension contained <1% CD4+ and CD8+ T cells after depletion, according to flow cytometry, and was used in suppression or cytotoxicity assays. To further purify DN T cell cell suspensions, cells were stained with biotin-labeled anti-1B2 TCRαβ mAb (32) and isolated by using microbeads (Miltenyi, Biotec). The viability and purity of DN T cells was monitored by flow cytometry and were >95%.

Suppression assays

Naïve 2CF, FcRγ+/− splenocytes were depleted of CD4+ T cells, used as responder cells (1000 CD8+ T cells/well), and cocultured in 96-well plates with irradiated (20 Gy) sex-matched splenocytes (1 × 10^5 cells/well) from (C57BL/6)F1 mice, α-MEM supplemented with 10% FCS, 50 U/ml rIL-2, and 30 U/ml rIL-4. Splenic DN T cells isolated as described above were used as putative suppressors in standard suppression assays. Serial dilutions of suppressor cells were added to the MLR. After a 4-day incubation, 1 μl of [3H]Tdr was added to each well. Eighteen hours later, cells were harvested and counted in a beta scintillation counter (TOPOCOUNT; Packard Instrument). Suppression was calculated using the equation: percentage of suppression = 1 − (EIR), in which E is the cpm of each well and R is the cpm of responder alone.

Inhibitors

The inhibitors of Syk family kinases, piceatannol (33) and sulfonamide-31 (3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide) (34), as well as cell-permeable irreversible inhibitors of caspase-3 (DEVD-FMK) and caspase-8 (IETD-CHO) were purchased from Calbiochem. Syk inhibitors were dissolved in DMSO (Sigma-Aldrich), and equal volumes (μl/well) were added to suppression assays, as described above. For caspase-3 and caspase-8 studies, 5 × 10^5 DN Treg cells (DN1 and DN2) were cultured in α-MEM medium supplemented with 10% FBS in the presence of equal volumes of 50 μM caspase-3 inhibitor, 50 μM caspase-8 inhibitor, or DMSO vehicle for 4 at 37°C, as described previously (35, 36). DN Treg cells were then washed three times with medium, and FcRγ expression was analyzed by flow cytometry. These treated DN T cells were assessed as putative suppressors in modified suppression assays in which DN T cells pretreated with inhibitor were then added to 2CF1 CD8+ T cells that were activated for 4 days, and cell proliferation was then assessed.

Skin grafting

The (B6 × dm2)F1, mice (Ld−) were used as recipients and were either left untreated or given 5 × 10^7 DLI-activated DN T cells isolated from syngeneic 2CF1.FcRγ+/− or 2CF1.FcRγ−/− mice, which express anti-Ld transgenic allele, as donors. One day later, recipient mice received two sex-matched skin grafts from (C56B)F1 (Ld−, donor-specific) and bm1 (Kbms1−); third-party control) mice. Grafts were monitored by visual inspection daily for the first 2 wk and twice per week thereafter. A graft was considered rejected when
>90% was necrotic. To confirm rejection, skin allografts from recipient mice were harvested, fixed in 10% buffered Formalin, embedded in paraffin, and sectioned. Sections were stained with H&E and examined under light microscopy. The accepted syngeneic skin grafts were treated in the same way and used as controls.

**Killing assays**

DN Treg clone and mutant cells were used as effector cells and plated in serial dilutions in a round-bottom 96-well plate. The 2CF1, FcRγ-deficient 2CF1 (CD8− T cells) were activated for 4 days with irradiated (20 Gy) (CB6)F1 splenocytes, labeled with 5 μCi/ml 31Cr at 37°C for 1.5 h, and washed, and 104 cells were added to each well. Each cell culture was also monitored with 50 U of rIL-2, 30 U of rIL-4 and irradiated (20 Gy) (CB6)F1 splenocytes. After coculture with effector cells at 37°C for 18 h, the cells were harvested and counted using a TOPCOUNT cell harvester and plate reader (Packard Instrument). Specific cell killing was calculated using this equation: percentage of specific killing = (SE/SD × 100), in which E (experimental) is cpm in the presence of effector cells and S (spontaneous) is cpm in the absence of effector cells.

**Western blotting**

DN Treg (DN1 and DN2) and mutant (MU1 and MU2) clone cells were collected, and stimulator and necrotic cells were removed using Lymphomyte M (Cedarlane Laboratories). The cells were confirmed to be >95% viable and then lysed using radioimmunoprecipitation assay buffer containing 0.1% aprotinin, 0.1% leupeptin, and 1 mM PMSF as protease inhibitors. Proteins were analyzed in whole cell lysates by previously described methods (37). A rabbit antiserum against the associated protein of 70 kDa (Zap70) was provided by J. Rojo (Centro de Investigaciones Biologicas, Madrid, Spain). The following commercially available Abs were used in these studies: rabbit anti-FcRγ, rabbit anti-Lck, and mouse anti-lymphotroponosine (4G10) (Upstate Biotechnology); rabbit anti-CD3ε and anti-TCRβ (Santa Cruz Biotechnology); anti-Syk (Cell Signaling Technology); mouse anti-β-actin (Sigma-Aldrich); goat anti-rabbit HRP-conjugated secondary Abs (Bio-Rad); and goat anti-mouse HRP-conjugated secondary Abs (Amersham Biosciences). Signal detection was performed by chemiluminescence (Boehringer Mannheim), and image acquisition and analysis were done with the Fluorchem 8000 Advanced Imaging System (Alpha Innotech) and Phoretix 1D software (NonLinear Dynamics).

**Immunoprecipitation**

TCR cross-linking of DN Treg (DN1 and DN2) and mutant cells (MU1 and MU2) was performed, as previously described (38). Briefly, DN Treg and mutant cells were plated in 24-well culture plates (1.25 × 105 cells/well), which were precoated with 1B2 mAb (10 μg/ml). All cells were supplemented with rIL-2 (50 U/ml) and rIL-4 (25 U/ml) during TCR cross-linking. Membrane protein was isolated using ProteoPrep Universal Extraction Kit (Sigma-Aldrich), and 100 μl of protein was incubated with the desired mAbs (anti-TCRβ, anti-FcRγ, or anti-Syk) in the presence of 100 μl of protein G-Sepharose 4 Fast Flow agarose beads (Amersham Biosciences) overnight at 4°C. Immunoprecipitates were then washed four times with lysis buffer before activity analysis. Proteins were analyzed in the immunoprecipitates by previously described methods (38). Hybridization signals were visualized using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer) after exposure to Kodak X-OMAT Blue x-ray film.

**Statistics**

Survival data were analyzed using the log rank test, and other data were analyzed using Student’s t test.

**Results**

FcRγ-deficient primary 2CF1, DN T cells have reduced ability to suppress allogeneically activated CD8+ T cells

Previously, we found that infusion of class I locus Ld+ splenocytes from (CB6)F1 mice (H-2b/d, Ld+) into 2CF1, anti-L-TCR transgenic (H-2b/d, Ld−) mice before transplantation activates recipient DN Treg cells, resulting in permanent acceptance of donor-specific allografts, but does not affect the normal rejection of third-party skin allografts (9, 13). To determine the importance of FcRγ expression in DN Treg cell function in vivo, FcRγ-deficient 2CF1 mice were generated, as described in Materials and Methods. First, we addressed whether primary DN T cells preferentially express FcRγ, and whether DLI can increase FcRγ expression. Naive FcRγ-deficient 2CF1 mice were either given an Ld+ DLI from (CB6)F1 mice or left untreated. The percentages of FcRγ+ cells in CD4+, CD8+, and DN T cell subsets in the spleen were determined by flow cytometry. As shown in Fig. 1A, 26% of naive DN T cells in 2CF1 mice express FcRγ protein, which was significantly increased after DLI treatment (Fig. 1A). Unlike DN T cells, neither naive nor DLI-treated CD4+ and CD8+ T cells expressed significant levels of FcRγ protein.

Next, we studied whether the FcRγ expression in primary DN T cells is important to maintain their suppressive function. DN T cells were isolated from DLI-treated FcRγ-sufficient and FcRγ-deficient 2CF1 mice, and their suppressive ability was assessed. DN T cells from FcRγ-deficient 2CF1 mice had markedly reduced ability to suppress proliferation of syngeneic CD8+ T cells activated by MHC class I Ld allo Ag compared with FcRγ-sufficient 2CF1, DN T cells (Fig. 1B). This difference in suppression was statistically significant down to 2.5:1 E:T ratio (p < 0.01). These data indicate that DLI increases the percentage of FcRγ+ DN T cells and that FcRγ is important for the ability of DLI-activated primary DN T cells to suppress CD8+ T cells.

FcRγ is important for primary 2CF1, DN T cell-induced donor-specific skin allograft survival

We have previously shown that the adoptive transfer of DLI-activated primary DN T cells can significantly prolong donor-specific skin graft survival (15). To determine the in vivo effect of...
FcRγ deficiency on DN T cell function, Lδ+ (B6 × dm2)F₁ mice were infused with syngeneic 2CF₁,FcRγ+/- or 2CF₁,FcRγ-/- DN T cells that had been preactivated by DLI treatment, as described above. One day after DN T cell infusion, each mouse was given a donor-specific Lδ+ (CB6F₁) and a third-party Kbm1+ (bml) skin allograft, and graft survival was monitored (Fig. 2). Lδ+ allograft survival was significantly prolonged (p < 0.0001) after treatment with 2CF₁,FcRγ+/- DN Treg cells (mean survival time (MST) = 24.7) compared with no treatment (MST = 12.6). However, 2CF₁,FcRγ-/- DN T cell infusion did not significantly improve Lδ+ skin allograft survival (MST = 14.3) when compared with untreated animals. All third-party bml skin grafts were rejected in a similar rate in all treatment groups, indicating that the enhancement of skin graft survival was donor specific. Therefore, FcRγ expression in DN T cells is important for maintenance of their ability to prolong donor-specific skin allograft survival.

FcRγ expression correlates with regulatory function of DN Treg cell clones

After confirming the importance of FcRγ for DN T cell-mediated regulatory function, we sought to elucidate the molecular basis of its function. Due to the fact FcRγ is only detectable intracellularly in DN T cells, it is not possible to sort out viable DN T cells from 2CF₁,FcRγ-/- mice based on their FcRγ expression. We thus analyzed two DN Treg and two mutant cell clones that were derived from 2CF₁,FcRγ-/- mice whose function has been described previously in vitro and in vivo (8, 16, 17). First, we confirmed that DN Treg cell clones express high levels of FcRγ mRNA and protein compared with mutant cell lines (Fig. 3, A and B). We then assessed the regulatory function of FcRγ+/- DN Treg cell clones and FcRγ+/- nonmutant cell clones (Fig. 3C) to confirm our previously reported function of these cell lines (8). Regulatory function was assessed using the killing assay instead of the suppression assay to allow for functional testing on the day of protein isolation. These data indicate a strong correlation of FcRγ expression with regulatory function of DN Treg clones.

**FIGURE 2.** Adoptively transferred DN T cells from FcRγ-sufficient, but not FcRγ-deficient, mice are able to prolong allograft survival. (B6 × dm2)F₁ recipients were either left untreated or infused with 5 × 10⁶ DLI-activated DN T cells from syngeneic 2CF₁,FcRγ+/- or 2CF₁,FcRγ-/- mice. On the next day, all recipient mice were given a (CB6F₁) (donor-specific, open symbols) and a bml (third-party, closed symbols) skin allograft. A, Survival rates were compared among 2CF₁,FcRγ+/- DN T cell-treated (circles), 2CF₁,FcRγ-/- DN T cell-treated (squares), and untreated (triangles) animals (n = 10). Lδ+ (CB6F₁) grafts on 2CF₁,FcRγ+/- mice (left), 2CF₁,FcRγ-/- mice (middle), and untreated mice (right). All groups except 2CF₁,FcRγ-/- treated Lδ+ grafts show >90% necrosis and growth of nascent skin are visible underneath the dearly staining necrotic skin graft. Filled arrows on histology indicate hair follicles in the accepted skin graft, while few hair follicles were observed on rejected grafts.

**DN Treg cell clones contain FcRγ, but lack Lck and Zap70**

Our microarray study of DN Treg clone cells found that in addition to FcRγ, several other TCR-related genes, including Lck and Zap70, were differentially expressed at the mRNA level (16). To further understand the molecular role of FcRγ in the TCR complex, we sought to determine the protein expression level of TCR-related molecules. Western blotting analysis indicated that DN Treg clone cell lines express high levels of TCRβ and FcRγ, but lack Zap70 and Lck expression. In contrast, mutant clone lines express high levels of Zap70 and Lck, a low level of TCRβ, and an absence of FcRγ (Fig. 4A). Several other TCR-related proteins were also assessed, including linker for activation of T cells, AKT-1, and Csk, but no significant differences were observed (data not shown). Furthermore, we demonstrate that DN Treg TCRβ coprecipitates with FcRγ subunits instead of CD3ζ (Fig. 4B). In addition, we found that both FcRγ and Syk were phosphorylated after TCR cross-linking of DN Treg cells, suggesting that FcRγ and Syk are functional components of the TCR complex (Fig. 4C). This molecular analysis of TCR components of DN Treg cells suggests that FcRγ and Syk are part of the DN Treg TCR complex in place of the conventional CD3ζ and Zap70 subunits.

**Reduction of FcRγ expression via caspase-3 inhibition reduces suppression mediated by DN Treg clone cells**

Recent studies have found that increased caspase-3 expression in T cells from SLE patients causes reduced CD3ζ expression and a reciprocal increase in FcRγ expression (36). Furthermore, we previously showed increased caspase-3 mRNA in DN Treg cells vs nonfunctional mutants in a cDNA microarray study (16). Because no known inhibitors of FcRγ are currently available, we used commercially available inhibitors of caspase-3 to further confirm the requirement of FcRγ expression for robust DN Treg cell function. DN Treg cells were incubated with a caspase-3 inhibitor (DEVD-FMK), caspase-8 inhibitor (IETD-CHO), or DMSO control. Only caspase-3 inhibitor treatment caused a significant reduction in FcRγ expression, while caspase-8 and DMSO had little effect (Fig. 5A). In addition, caspase-3 inhibitor-treated DN T cells that express low levels of FcRγ were tested for their ability to suppress activated CD8+ T cells. The suppression assay was modified to allow for testing of suppression function within 24 h of inhibitor incubation, and allow the inhibitors to only act on the DN T cells. We found that the caspase-3-treated DN T cells had significantly reduced suppressive function vs caspase-8 inhibitor-treated or DMSO-treated DN Treg cells (5.1 DNT:CD8; p = 0.02) (Fig. 5B). Therefore, these data further suggest that FcRγ expression is required to maintain the suppressive capacity of DN Treg cells.
inhibitor sulfonamide-31 (34) that functions at much lower concentrations than piceatannol. When increasing concentrations of Syk inhibitors were added to suppression assays with primary 2CF1 DN T cell effectors, the suppression was reduced as inhibitor concentration was increased (Fig. 6). This evidence suggests that signaling through Syk and the associated FcRγ pathway may be important to maintain the suppressive function of primary DN Treg cells.

**Discussion**

Treg cells have been shown to increase graft survival in various models of transplantation (5–7). We have shown previously that DLI-activated DN Treg cells can down-regulate CD8+ and CD4+ T cell-mediated immune responses and prolong allo- and xenograft survival in a donor-specific manner (8, 9, 12). The molecular mechanisms by which DLI activates DN Treg cells and prolongs graft survival remain unclear. In this study, we provide evidence indicating that the FcRγ subunit plays an important role in DN Treg cell-mediated suppression of antidonor immune responses. First, a significant portion of primary naive DN T cells expresses FcRγ, while FcRγ protein is barely detectable in the peripheral CD4+ and CD8+ T cells (Fig. 1A). Second, the percentage of FcRγ+ 2CF1 DN T cells was found to be significantly up-regulated upon DLI activation, whereas FcRγ levels remained unchanged in CD4+ and CD8+ T cells. Third, DN Treg clones that express FcRγ can suppress syngeneic CD8+ T cells in vitro (Fig. 3) and prolong cardiac allograft survival when adoptively transferred into naive mice, whereas their FcRγ-negative mutants fail to do so (16, 17). Most importantly, DLI-activated DN T cells from FcRγ+/− 2CF1 mice can suppress syngeneic CD8+ T cell proliferation dose dependently in vitro and significantly prolong allogeneic skin allograft survival in a donor-specific manner upon transfer into syngeneic recipients (Figs. 1B and 2). However, these functions are lost in DN T cells obtained from DLI-treated FcRγ−/− mice. Collectively, these data demonstrate that FcRγ plays an important role in DN Treg cell-mediated suppression in vitro and in vivo, and suggest that DLI may preferentially activate FcRγ-expressing DN Treg cells.

Despite the importance of DLI treatment to activate DN Treg cells, we found that naive animals do have a quantity of FcRγ+ DN T cells (Fig. 1A), suggesting that DLI may not necessarily be required to induce all functional DN Treg cells, but some may be naturally induced. This is also supported by the fact that DN Treg clone cells generated from both DLI-treated and naive mice are FcRγ+ and function in a similar fashion (8) (Figs. 3 and 4). The increase in FcRγ+ DN T cells after DLI might be due to an expansion of the naturally present subset of FcRγ+ suppressive cells or, alternatively, may be due to a transition of a subset of naive/non-suppressive FcRγ− DN T cells to a suppressive FcRγ+ phenotype, similar to what has been suggested for CD4+ and CD8+ Ag-induced Treg cells (40). This concept of transition of signaling machinery is supported by finding that FcRγ is up-regulated in human effector CD4+ T cells, but not detected in naive or memory CD4+ T cells (27, 41). Further studies will be required to determine at which developmental stage DN Treg cells begin to express FcRγ.

We observed a high amount of FcRγ and no detectable amounts of CD3ζ protein expression in DN Treg clones (Fig. 4). Reduced levels of CD3ζ and increased FcRγ have been observed in human T cells from a wide array of chronic diseases such as chronic infections, autoimmune diseases (including SLE), and cancer (41). Despite the fact that this alteration may occur in pathogenic T cells, it is possible that DN Treg cells may also be serving to suppress these pathogenic cells. A large expansion of DN T cells

**FIGURE 3.** FcRγ is up-regulated in DN Treg clone cells compared with nonfunctional mutants. DN Treg (DN1 and DN2) and mutant (MU1 and MU2) clone cells were analyzed for their FcRγ expression. A. Quantitative real-time PCR analysis of FcRγ mRNA expression in DN1 vs MU1 and DN2 vs MU2 cells. B. DN1 DN Treg cells and MU1 mutant cells are stained with rabbit anti-FcRγ or rabbit IgG control primary Ab (Upstate Biotechnology) and a PE-conjugated secondary Ab (Cedarlane Laboratories). FcRγ log fluorescence is shown. C. On the day of protein extraction, regulatory function was assessed. DN Treg clone and mutant cells were used as effectors cells, and activated 2CF1, FcRγ−/− CD8+ T cells were used as targets in a killing assay, as described in Materials and Methods. Percentage of specific cell killing is shown. This experiment was repeated at least three times to ensure reproducibility of the trend, and each data point was taken as the average of triplicate samples.

**Inhibition of Syk significantly reduces primary 2CF1 DN T cell-mediated suppression in vitro**

After establishing that the presence of FcRγ in DN Treg cells strongly correlated with suppressive function, we wanted to determine whether signaling through the FcRγ pathway was also required for suppression mediated by primary DN T cells. Syk is a kinase that has been established to bind to phosphorylated FcRγ molecules and mediate downstream signal transduction (39). We used the Syk family kinase inhibitor piceatannol, which has been described to preferentially inhibit the tyrosine kinase activity of Syk in a dose-dependent manner (33), and a newly described Syk

![Graph A](image1.png)

**A**

**B**

**C**

**Fold Increase in mRNA Ratio (FcRγ/GAPDH)**

**DN1 vs. MU1**

**DN2 vs. MU2**

**Effector:CD8**

**Specific Lysis**

**10:1 5:1 2.5:1 1:2.5 1:6.1**

**% Specific Lysis**

**DN1**

**DN2**

**MU1**

**MU2**

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has been observed in Fas mutant autoimmune lymphoproliferative syndrome patients as well as the Fas mutant lymphoproliferative (lpr) mouse model. We previously found that the DN T cells isolated from lpr mice still maintain regulatory capacity, but are less effective due to the Fas mutation on autoimmune effector T cells (15). Our recent data suggest that FcRγ may also play a role in mediating the suppressive function of lpr DN T cells and their ability to control lymphoproliferative disease (C. W. Thomson, W. Chen, J. R. Torrealba, and L. Zhang, submitted for publication).

Recent studies reported that treatment of T cells derived from SLE patients with caspase-3 inhibitors reduces the expression of FcRγ (36). Interestingly, we found that DN Treg cells express higher levels of both FcRγ and caspase-3 mRNA compared with nonfunctional cells (16), suggesting that these proteins may be similarly regulated in murine DN Treg cells. As no FcRγ inhibitor was available, we used a caspase-3 inhibitor reagent (DEVD-FMK) that effectively reduces both FcRγ expression and suppressive function of DN Treg cells (Fig. 5). This study further confirmed the need for FcRγ expression to achieve robust DN T cell-mediated suppression. Reports have suggested that caspase-3 does not directly act on FcRγ molecules, but rather functions by cleaving CD3ζ molecules, and the reduced levels of CD3ζ protein lead to increased FcRγ expression (36, 42). In other T cells, such as human effector CD4+ T cells and tumor-infiltrating lymphocytes, up-regulation of FcRγ is associated with down-regulation of CD3ζ expression (27, 43). Based on this model, we would expect that the FcRγ-low mutant cells would have increased levels of CD3ζ protein. However, we observed that both suppressive and mutant clones had undetectable levels of CD3ζ protein in the TCR complex (Fig. 4B). This suggests that the regulation of FcRγ and CD3ζ expression may be more complex than originally thought or perhaps the changes that resulted in the mutant clone phenotype may have disrupted the normal processes that regulate FcRγ and CD3ζ proteins. In addition, the low TCR levels observed in mutant cells (Fig. 4A) may also be related to the absence of either FcRγ or CD3ζ, because they are required to form stable TCR complexes (23).

FIGURE 4. DN Treg clone cells contain FcRγ and lack Lck and Zap70. DN Treg (DN1 and DN2) and mutant (MU1 and MU2) cells were collected and protein was isolated, as described in Materials and Methods. A, Western blot analysis was used to detect protein expression of TCRβ, FcRγ, Zap70, and Lck in cell lysates. B, TCR complexes were immunoprecipitated from membrane fractions with TCRβ and probed with FcRγ and CD3ζ. Membrane protein (without immunoprecipitation) from B6.FcRγ+/+ and B6.FcRγ−/− splenocytes was also run as Ab controls. C, TCR complexes were cross-linked by 10 μg/ml plate-bound anti-2C TCR Ab (1B2), and phosphorylation status of FcRγ and Syk was observed. Membrane protein was isolated before and 15 min after TCR cross-linking. FcRγ and Syk were immunoprecipitated and probed with antiphosphotyrosine (4G10).

FIGURE 5. Reduction of FcRγ expression via caspase-3 inhibition reduces DN Treg clone cell-mediated suppression. A, DN T clone cells (DN1) were cultured for 4 h at 37°C in the presence of equal volumes of either: 50 μM caspase-3 inhibitor, 50 μM caspase-8 inhibitor, or DMSO vehicle. After incubation, cells were washed three times with medium and the cells were analyzed for FcRγ expression by flow cytometry. B, The ability of DN T cells treated with caspase inhibitors to maintain their regulatory function was analyzed in a modified suppression assay in which only DN T cells are exposed to caspase inhibitors. DN T cells treated as above were added as putative suppressor cells to 2CF1 CD8+ T cells activated for 4 days with irradiated (CB6)F1 splenocytes. Cell proliferation was measured, as described in Materials and Methods. This experiment was repeated at least three times to ensure reproducibility of the trend, and each data point was taken as the average of triplicate samples (*, p = 0.01, and **, p = 0.02).
Syk kinase may act to promote survival and increase activation and/or other functions that affect the regulatory ability of DN Treg cells. FcRγ-associated signaling in monocytes has been shown to increase levels of the survival proteins Bcl-2 and Bcl-xL (49), suggesting that FcRγ may promote the survival of DN T cells that are otherwise prone to apoptosis. Consistent with this possibility, DN Treg cells have been found to express Bcl-xL constitutively and up-regulate Bcl-2 expression after TCR cross-linking, leading to a significant resistance to TCR cross-linking-induced apoptosis (50). Aside from increasing survival, FcRγ-associated signaling may serve to promote the activation state of naïve DN T cells, allowing them to make the transition into functional regulatory cells.

Our findings suggest that FcRγ is required for efficient regulatory function of DN T cells. Others have reported recently that human DN Treg cells function very similarly to murine DN T cells in their ability to suppress CD8+ T cells in an Ag-specific manner (51). If it is confirmed that human DN Treg cells also use FcRγ in their TCR complex, we can potentially exploit this biomarker in therapeutic regimens. We propose that DN T cell-associated FcRγ expression could serve to assess the presence of functional DN Treg cells for predicting therapeutic responses. Considering their function in suppressing immune responses, FcRγ-expressing DN T cells could be considered as potential targets of various pharmaceutical agents such as antirejection or chemotherapeutic drugs.

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References


