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BCL-2 protects human and mouse Th17 cells from glucocorticoid-induced apoptosis

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Abstract

Background—Glucocorticoid resistance has been associated with Th17-driven inflammation, the mechanisms of which are not clear. We determined whether human and mouse Th17 cells are resistant to glucocorticoid-induced apoptosis.

Methods—Freshly isolated human blood Th17 cells and *in vitro* differentiated Th17 cells from IL-17F red fluorescent protein reporter mice were treated with dexamethasone, a potent glucocorticoid. Apoptosis was measured using annexin V and DAPI staining. Screening of apoptosis genes was performed using the apoptosis PCR array. Levels of molecules involved in apoptosis were measured using quantitative RT-PCR, flow cytometry, and Western blotting. Knockdown of BCL-2 in murine Th17 cells was performed via retroviral transduction. Cytokines were measured using ELISA. A murine Th17-driven severe asthma model was examined for Th17 glucocorticoid sensitivity *in vivo*.

Results—Human and mouse Th17 cells and mouse Th2 cells were resistant to glucocorticoid-induced apoptosis. Th17 cells had glucocorticoid receptors levels comparable to those in other T effector cells. Th17 cells had high levels of BCL-2, knockdown of which sensitized Th17 cells to dexamethasone-induced apoptosis. Production of IL-22, but not IL-17A and IL-17F, was

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Conflict of interest

All authors declare that they have no relevant conflict of interests.

Author contributions

JB, NZL, and RPS designed the study. JB, SS, YC, BSB, LMN, GRSB, LZ, SL, JX, MWL, CD, and NZL performed the experiments. JB and NZL analyzed the data. JB, NZL, BSB, and RPS wrote the manuscript.

suppressed by glucocorticoids. STAT3 phosphorylation in Th17 cells was insensitive to glucocorticoid inhibition. Lung Th17 cells in the murine severe asthma model were enhanced, rather than suppressed, by glucocorticoids.

Conclusion—Th17 cells are resistant to glucocorticoid-induced apoptosis and cytokine suppression, at least in part due to high levels of BCL-2. These findings support a role of Th17 cells in glucocorticoid-resistant inflammatory conditions such as certain endotypes of asthma.

Introduction

The presence of IL-17 producing helper T (Th17) cells has been associated with glucocorticoid-resistant severe asthma (1–3) and the mechanism of this link has not been fully examined. Glucocorticoids are potent anti-inflammatory agents that restrict helper T cell actions via induction of apoptosis and suppression of cytokine production. It has been reported that the lifespan of Th1 and Th2 cells and the level of their cytokines (e.g., IFN- γ , IL-4, and IL-5) are reduced by glucocorticoids (4). In contrast, reports on the glucocorticoid sensitivity of Th17 cells are conflicting. Two reports suggest that IL-17 may be increased by glucocorticoids (2, 5). However, the number of sub-epithelial IL-17A-expressing cells was reported to be decreased by glucocorticoids in the airways of a cohort of patients with moderate-severe asthma (6) while studies in two separate groups of moderate-to-severe asthmatic patients found no inhibition of IL-17A cytokine expression by glucocorticoids (7, 8). Although different subsets of Th17 cells (9) and diverse microenvironments at sites of inflammation (10–12) likely play a part in the conflicting reports on glucocorticoid responsiveness of Th17 cells, it has not been determined whether glucocorticoids regulate Th17 cell apoptosis.

Glucocorticoids signal through the glucocorticoid receptor (GR), a transcription factor that can inhibit the expression of some genes (e.g., IFN- γ and IL-4) while activating the expression of some other genes (e.g., BIM (BCL-2 interacting mediator) and DUSP1 (dual specificity phosphatase 1)) (13). Glucocorticoid-regulated genes coordinately mediate the anti-inflammatory benefits of glucocorticoids. For example, DUSP1 and several other glucocorticoid-induced genes act as suppressors of pro-inflammatory cytokine expression (14, 15). BIM induced by glucocorticoids mediates glucocorticoid-induced apoptosis in lymphocytes (16, 17). Recently, we have reported that different GR isoforms with distinct apoptosis-inducing capabilities are expressed in a cell type-specific manner (18–21). We, therefore, hypothesized that glucocorticoids regulate the expression of selective genes in Th17 cells, possibly via specific GR isoforms.

We report that human and mouse Th17 cells, in contrast to Th1 cells, are resistant to glucocorticoid-induced apoptosis. Apoptosis focused PCR-array revealed a set of anti-apoptotic molecules highly expressed in murine Th17 cells. We further confirmed that BIM protein was upregulated by glucocorticoids while BCL-2 was downregulated simultaneously in murine Th1 cells, but not in Th2 or Th17 cells. These findings suggest that differentiated T cell subtypes vary in their sensitivity to glucocorticoids based on their apoptotic machinery. Thus, the ratio of BIM over BCL-2 was significantly elevated in Th1 cells compared to Th2 or Th17 cells. The Th subset-selective regulation of apoptotic molecules

by glucocorticoids occurred despite the expression of a similar complement of GR isoforms in all T cell subsets. Lowering BCL-2 using shRNA was sufficient to render murine Th17 cells sensitive to glucocorticoid-induced apoptosis, suggesting that the mechanism of resistance involved BCL-2. Interestingly, in Th17 cell cultures, IL-17A and IL-17F, but not IL-22, were resistant to glucocorticoid inhibition, correlating to glucocorticoid insensitivity of IL-6-induced STAT3 phosphorylation. In a murine severe asthma model, lung Th17 cells were enhanced, rather than suppressed, by glucocorticoids. Our findings support the view that Th17 cells are resistant to anti-inflammatory effects of glucocorticoids and may contribute to clinically glucocorticoid resistant syndromes. Further understanding of mechanisms regulating the glucocorticoid resistance of Th17 cells may help to improve therapies for Th17-dominant inflammatory disorders.

Materials and Methods

Reagents

All recombinant cytokines were from Peprotech (Rocky Hill, NJ) or R&D Systems. Dexamethasone (DEX) was obtained from Steraloids (Newport, RI). All other reagents were obtained from Sigma (St. Louis, MO), unless otherwise specified. Antibodies and detailed protocols for cell differentiation, Western blotting, ELISA, and flow cytometry and primer sequences for realtime RT-PCR are described in the supplementary text.

Transduction of T cells

Retrovirus expressing BCL-2 short hairpin (sh) RNA (DNA/RNA delivery core at Northwestern University) and control virus using the MSCV-LTRmiR30-PIG vector (GE Dharmacon, Lafayette, CO) were generated and used to transduce murine Th17 cells as previously described (22). GFP⁺ cells were sorted and subjected to Western blot analysis to verify the knockdown of BCL-2.

Murine asthma model

Six to eight weeks old Balb/c mice were sensitized with 2 intraperitoneal injections of 50 µg ovalbumin (OVA, grade V, Sigma), alum (3 mg), Pam3CSK4 (10 µg, Invivogen, San Diego, CA), and zymosan (300 µg, Sigma) on days 0 and 7. On days 14, 16, 18, 21, 23, 25, 28, and 29 mice were challenged for 30 min with 2.5% aerosolized OVA in sterile PBS. On day 30, mice were challenged with 2 mg OVA in 100 µl PBS. Control mice received alum sensitization and PBS challenge. Six and 30 h after the last challenge, mice were treated with vehicle or DEX (2.5 mg/kg, i.p.). Bronchoalveolar lavages (BALs) and lungs were obtained 48 h after the last OVA challenge. BAL total and differential cell counts were determined by flow cytometry and SPHERO AccuCount particles (Spherotech, Inc., Lake Forest, IL). Before harvesting the lungs, pulmonary vasculature was flushed clear of blood with PBS through right ventricle. The left lung lobe was fixed for histological staining. Hematoxylin and eosin-stained lung sections were imaged on an Olympus IX71 microscope using a 10× objective at room temperature. The bottom right lobe was homogenized and supernatant was frozen at -80°C for ELISA. Top right three lung lobes were homogenized for lung T cell isolation using GentleMACS (Miltenyi) mouse lung subroutine with a 30 min digestion using collagenase (0.5 mg/ml, type I, Worthington, Lakewood, NJ) and DNase (40

U/ml, Worthington) at 37°C. Live cells were then washed, filtered, and isolated by Ficoll-Paque. Single cell suspensions were stimulated with PMA and ionomycin and stained for live/dead and cell surface markers. BCL-2 staining was performed overnight at 4°C. Finally, cells were washed and stained for intracellular cytokines as described above. Lung inflammation scoring and mechanics measurement are described in the supplementary text.

Statistical analysis

Prism software (GraphPad, San Diego, CA) was used. Based on the extent of glucocorticoid-induced T cell apoptosis from pilot studies, a sample size of 9 was predicted to reach a power of .80 ($\alpha = 0.05$, mean spontaneous T cell apoptosis 25%, observed glucocorticoid-induced apoptosis 40%, and SD 18%). For comparison of two groups, Student's t-test was performed. For comparison of three or more treatment groups, one-way ANOVA was performed followed by Tukey post-hoc tests. For comparisons with two or more variables, two-way ANOVA was performed followed by Bonferroni post-hoc tests. A P value < 0.05 was considered significant. Sample means and SEM are presented.

Results

Human Th17 cells are resistant to glucocorticoid-induced apoptosis

We recruited 23 subjects, 14 of whom had a history of mild upper respiratory allergies and were not on medications (Table S1). Peripheral blood CD4⁺ T cells cultured both in the absence (Th0) or presence (Th17) of Th17 conditions contained CD4⁺CCR6⁺ and CD4⁺CCR6⁻ T effector/memory cells (Fig. S1), which were sorted to $>98\%$ purity. CCR6 is the receptor for the mucosal chemokine CCL20 and CCR6 has been identified on Th17 cells by several groups (5, 23). Consistently, we found that CD4⁺CCR6⁺, but not CD4⁺CCR6⁻, are IL-17A producing Th17 cells (Fig. 1A). When freshly isolated CD4⁺ T cells were treated with DEX (100 nM), CD4⁺CCR6⁻, but not CD4⁺CCR6⁺, T cells underwent glucocorticoid-induced apoptosis (Fig. 1B and Fig. S2) as measured using flow cytometric analyses of Annexin V and DAPI staining. Comparable results were obtained using cells from allergic and non-allergic subjects. CD4⁺CCR6⁺ cells from four allergic subjects and one non-allergic subject had relatively high levels of spontaneous apoptosis, likely caused by variations among subjects as IL-17A levels did not correlate with levels of spontaneous apoptosis (Fig. S1). Overall, DEX did not increase apoptosis of CD4⁺CCR6⁺ cells at 24, 48, or 72 h of treatment. BCL-2 is a pivotal anti-apoptotic molecule and was expressed at a higher level in CD4⁺CCR6⁺ than in CD4⁺CCR6⁻ T cells (Fig. 1C). DEX did not change the level of BCL-2 protein in either subset of cells. These data demonstrate for the first time that human Th17 cells (CD4⁺CCR6⁺) are resistant to glucocorticoid-induced apoptosis. Consistent with the literature (24), we also found that Th17 cells had more BCL-2 than non-Th17 (CD4⁺CCR6⁻) cells.

Murine Th17 cells are resistant to glucocorticoid-induced apoptosis

To determine whether Th17 cells are resistant to glucocorticoid-induced apoptosis in another species, we examined Th17 cells from IL17F/RFP (red fluorescent protein) reporter mice. Splenic CD4⁺ T cells were cultured under Th1, Th2, or Th17 polarizing conditions. Intracellular cytokine staining was used to confirm cell differentiation (Fig. S3). Th0

(freshly isolated undifferentiated cells) and Th1 cells were more sensitive to DEX than Th2 and Th17 cells (Fig. 2A). Importantly, RFP+ Th17 cells were resistant to glucocorticoid-induced apoptosis (Fig. 2B). To further confirm the Th subset-specific sensitivity to glucocorticoid-induced apoptosis, active pan caspase activity (another indicator of apoptosis) was evaluated. We found caspase activity to be significantly lower in DEX-treated Th2 and Th17 cells than in Th1 cells (Fig. 2C). Thus, in vitro differentiated murine Th2 and Th17, but not Th1 cells, were resistant to DEX-induced apoptosis.

GR levels and activities were not different among Th subsets

The GR gene produces several GR isoforms, which we have previously shown to have distinct abilities to induce apoptosis (19, 20, 25). To determine whether GR isoforms play a role in the distinct glucocorticoid sensitivity of Th subsets, GR levels were determined in differentiated murine Th cells. Western blot analyses indicate that there were no differences in total GR protein levels among the three Th subsets (Fig. 3A, 3B). GR α isoforms were also comparable among all three Th subsets. GR α -A and -B were the predominant isoforms in Th subsets while GR α -D was detected at low levels. GR β was undetectable by real-time RT-PCR in any of the Th subsets. To determine whether GR signaling pathways (including ligand binding, translocation, and gene regulation) are functional in Th17 cells, differentiated Th cells were treated with vehicle or DEX (100 nM, 6 h) and the level of glucocorticoid-induced leucine zipper (GILZ), a known GR target gene, was measured using real-time RT-PCR. GILZ was induced to comparable levels in all Th subsets by DEX (Fig. 3C). These observations indicate that the distinct glucocorticoid sensitivity of Th subsets was not due to altered expression of GR isoforms and is downstream of GR activation.

Selective regulation of apoptosis genes by glucocorticoids in Th subsets

A published cDNA microarray study has indicated that human Th17 cells have 32 glucocorticoid gene targets whereas Th1 cells have 57, few of which are involved in apoptosis (5). We used an apoptosis-focused PCR array and examined the expression of 84 apoptosis genes in murine Th1 and Th17 cells before and after treatment with vehicle or DEX (100 nM, 6 h). Of the 84 apoptosis-related genes on the PCR array, a subset of genes were differentially expressed in Th1 and Th17 cells at baseline and after DEX treatment (Fig. 4). Consistent with the literature, Th1 cells had significantly higher Fas ligand (FasL) than Th17 cells (26). Neutralizing FasL, however, did not inhibit DEX-induced apoptosis of Th1 cells (data not shown). Reflecting the observations in humans, we found that murine Th17 cells had higher BCL-2 expression than Th1 cells. In total, Th1 cells had 9 pro-apoptotic genes expressed at higher levels than Th17 cells. In addition, DEX induced BIM and decreased anti-apoptotic BCL-XL in Th1 cells. To confirm the findings from the PCR array analysis, real-time RT-PCR revealed that BCL-2 mRNA, although not regulated by DEX, was higher in Th17 than in Th2 cells (Fig. S4A). Survivin (BIRC5), another anti-apoptotic gene, was significantly higher in Th17 cells than in Th1 cells. BCL-XL mRNA was higher in Th2 than in Th17 cells. In contrast, BIM mRNA at baseline was higher in Th1 than in Th2 or Th17 cells and DEX significantly increased the expression of BIM in Th1 but not in Th2 or Th17 cells. Using Western blot analyses, we further confirmed that BCL-2 protein was downregulated (Fig. 5A, 5B) while BIM protein was upregulated by glucocorticoids in Th1 cells, but not in Th2 or Th17 cells (Fig. S4B). The ratio of BIM over

BCL-2 was significantly elevated in Th1 cells but not in Th2 or Th17 cells (Fig. S5). These observations suggest that glucocorticoids shift the balance of pro- and anti-apoptotic proteins towards increased expression of pro-apoptotic genes in a Th subset-specific manner.

Knockdown of BCL-2 sensitized Th17 cells to glucocorticoid-induced apoptosis

To determine whether altering the balance of pro- and anti-apoptotic proteins changes the susceptibility of Th17 cells to glucocorticoid-induced apoptosis, knockdown of BCL-2 was performed using retrovirus carrying a specific BCL-2 shRNA in RFP+ Th17 cells and knockdown efficiency was confirmed by Western blot analyses (Fig. 5C, 5D). BCL-2 knockdown in Th17 cells increased spontaneous apoptosis and restored glucocorticoid-induced apoptosis whereas cells transduced with shRNA vector controls were still resistant to DEX killing (Fig. 5E). Thus, reducing BCL-2 sensitized Th17 cells to glucocorticoid-induced apoptosis.

Glucocorticoid regulation of cytokine expression in Th1, Th2, and Th17 cells

To determine the ability of glucocorticoids to regulate expression of pro-inflammatory cytokines, we measured the levels of IFN- γ from Th1, IL-4 from Th2, and IL-17A, IL-17F, and IL-22 from Th17 murine cell cultures by ELISA. DEX effectively suppressed IFN- γ production from Th1 cells (Fig. 6A) and IL-4 production from Th2 cells (Fig. 6B). IL-17A and IL-17F, but not IL-22, were resistant to glucocorticoid suppression in Th17 cell cultures (Fig. 6C). These findings suggest that Th17 cells are selectively resistant to certain aspects of glucocorticoid signaling including glucocorticoid-induced apoptosis and cytokine suppression. Since STAT3 is a key transcription factor for Th17 cells, we examined the effects of glucocorticoids on STAT3 activation. Dexamethasone (100 nM, 2 h prior to IL-6) did not inhibit IL-6 (50 ng/ml) induced STAT3 phosphorylation in Th17 cells (Fig. 6D), providing a possible mechanism underlying insensitivity of Th17 cells to glucocorticoid repression.

Lung Th17 cells were resistant to glucocorticoid suppression in a murine asthma model

To determine whether Th17 cells are resistant to glucocorticoid suppression *in vivo* and whether BCL-2 levels are different among lung Th subsets, we adopted a murine severe asthma model (27–29). After two sensitizations with OVA using zymosan and Pam3CSK4 as adjuvants and repeated OVA challenges, a predominant Th17/neutrophilic inflammation was observed (Fig. 7, S6). We identified lung Th1, Th2, and Th17 cells via cell surface markers CD4+CXCR3+, CD4+CCR3+, and CD4+CCR6+ and intracellular staining of IFN γ , IL-4, and IL-17, respectively (Fig. 7A, 7B). Lung Th17 cell numbers were more than 20-fold higher than that of Th1 or Th2 cells and highest in the OVA+DEX group. BAL neutrophil numbers were over 100-fold higher than that of eosinophils (Fig. S6B). Reflective of the Th17-driven inflammation, BAL IgG2a but not IgE and lung G-CSF and KC were significantly elevated (Fig. S6D). While *in vivo* DEX treatment decreased BAL eosinophils, total lymphocytes, KC, and IgG2a, it did not reduce lung Th17, BAL neutrophil, monocyte/macrophages, lung G-CSF, or airway hyper responsiveness to methacholine (Fig. S6D, S6E). BCL-2 levels were comparable among lung Th17 and the few Th1 and Th2 cells (Fig. 7C). Dexamethasone did not change the BCL-2 levels in any of the lung Th subsets, suggesting repeated OVA stimulation may have blocked glucocorticoid inhibition of BCL-2 in lung Th1

cells. Corresponding to these observations in lung T cells, we found that in the presence of PMA and ionomycin activation, glucocorticoids did not reduce BCL-2 in *in vitro* differentiated Th1 cells (Fig. S7), further supporting that T cell activation may decrease Th1 glucocorticoid sensitivity.

Discussion

There has been an increase in the literature of evidence supporting a role of the IL-17/neutrophil axis in more severe and glucocorticoid-resistant subsets of patients (1, 3, 30, 31). We report here that human primary Th17 cells and mouse *in vitro* differentiated Th17 cells do not undergo glucocorticoid-induced apoptosis. In a murine severe asthma model, lung Th17 cells were enhanced, rather than suppressed, by glucocorticoids. Our findings are in line with the concept that Th17 cells play a role in glucocorticoid resistance in certain inflammatory conditions such as severe asthma (2, 7, 8). Complementary to our findings, it has been reported that the ability of Th17 cells to proliferate is not inhibited by glucocorticoids (5). Furthermore, glucocorticoids have also been found to stimulate inflammasome activation and the release of IL-1 β (32), a potent Th17 stimulus. Together with these other reports, our finding of a resistance of Th17 cells to glucocorticoid-induced apoptosis provides a potential underlying mechanism for the glucocorticoid-resistance of Th17 cells.

A cohort of patients with moderate-severe asthma respond to glucocorticoids by reducing the number of sub-epithelial IL-17A-expressing cells (6). It is unclear, however, whether the cells examined in those patients were Th17 cells. In addition to Th17 cells, various innate cells such as natural killer T cells, $\gamma\delta$ T cells, type 3 innate lymphoid cells, natural killer cells, and neutrophils have been reported to produce IL-17A (33, 34). These additional cell types are potential targets for glucocorticoid regulation. The reduced number of IL-17A-expressing cells in certain patients and reduced IL-17A levels in several animal models (35, 36) by glucocorticoids could also be due to glucocorticoid inhibition of trafficking and recruitment of inflammatory cells (37, 38). In contrast to suppression of the recruitment of most leukocytes, a recent report suggests that glucocorticoids actually promote Th17 attraction and retention via upregulation of the airway epithelial production of CCL20 (23), the ligand for CCR6 on Th17 cells. Our findings support that glucocorticoids could selectively promote the accumulation of Th17 cells in inflamed lungs.

We found that both human and mouse Th17 cells were resistant to glucocorticoid-induced apoptosis, likely due to the high levels of BCL-2 proteins. BCL-2 protein was downregulated by glucocorticoids in mouse Th1, but not Th2 or Th17 cells, the mechanisms of which are likely post-transcriptional since glucocorticoids did not change BCL-2 mRNA levels in any of the T cell subsets. One such post-transcriptional mechanism could be through glucocorticoid regulation of micro RNAs. Glucocorticoids have been reported to downregulate mir-16 (39), a repressor of BCL-2 at the post-transcriptional level (40). If this pathway is active in Th17 cells, BCL-2 protein levels will be maintained by glucocorticoids. Alternatively, BCL-2 protein turn over (40) could be a target for GR signaling in a Th subset-specific manner. We were also able to measure BCL-2 levels in the few lung Th1 cells in the asthma model and there were no differences between OVA and OVA+DEX

groups. This is in contrast to *in vitro* differentiated mouse Th1 cells that lost BCL-2 in response to glucocorticoids. There are several possible reasons for the discrepancy between *in vitro* and *in vivo* observations on BCL-2 levels. Glucocorticoid treatment *in vivo* coincides with repeated TCR stimulation that is absent in *in vitro* experiments. We have evidence indicating that T cell activation blocks glucocorticoid inhibition of BCL-2 in Th1 cells. In addition, apoptotic cells are rapidly phagocytized and cleared out of the lungs *in vivo* whereas BCL-2 levels measured *in vitro* include both healthy and cells undergoing apoptosis. In addition, differences in experimental protocols could have contributed to the discrepancy. We used Western blot analyses to measure the BCL-2 levels in *in vitro* differentiated T cells whereas to measure BCL-2 levels in lung T cells, we treated isolated lung T cells with PMA and ionomycin in order to determine the Th identity by cytokine, e.g., IFN γ for Th1 cells, before measuring BCL-2 levels using flow cytometry. PMA treatment may have altered BCL-2 levels in lung T cells. We found that PMA altered BCL-2 and in the presence of PMA, DEX did not inhibit BCL-2 in *in vitro* differentiated Th1 cells. Furthermore, the discrepancy we observed could be reflective of the heterogeneity of Th1/17 cells (41).

It is interesting that selective Th17 cytokines were sensitive to glucocorticoids. IL-22, but not IL-17A or IL-17F, was inhibited by glucocorticoids. A negative glucocorticoid response element that can mediate the suppressive activity of GR has been identified in the promoter of the IL-17F gene (42), suggesting that glucocorticoid-activated GR is capable of suppressing IL-17F gene expression. Indeed, we have reported that glucocorticoids are able to inhibit IL-17A in a murine thymoma cell line (43). Thus, the loss of glucocorticoid inhibition of IL-17 expression in primary Th17 cells suggests that additional factors modulate GR regulation of IL-17. It has been reported that STAT3 binding to the same promoter as GR can interfere with GR activity (44). Since STAT3 is highly active in Th17 cells and STAT3 phosphorylation is resistant to glucocorticoid inhibition, STAT3 interference of the GR may be a candidate mechanism underlying the loss of glucocorticoid regulation of IL-17 gene expression.

One caveat of our studies is that we focused on glucocorticoid sensitivity of fully differentiated Th17 cells. Further studies are needed to clarify whether glucocorticoids regulate how Th17 cells are generated. It will also be interesting to determine glucocorticoid sensitivity of Th17 cells at different developmental and pathogenic stages (41). Another area that requires further investigation is how glucocorticoids other than DEX regulate Th17 responses. In particular, some of the newly developed GR modulators seem to have improved anti-inflammatory benefits and reduced side effects (45). Moreover, the mechanisms of Th2 resistance to glucocorticoid-induced apoptosis need to be clarified. In summary, our findings support the concept that diseases mediated by Th17 cells may have features of glucocorticoid resistance. Further understanding of the mechanisms regulating the resistance of Th17 cells to glucocorticoid-induced apoptosis, e.g., identifying triggers that elevate and maintain BCL-2 in Th17 cells, may help to improve therapies for Th17-driven inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BIM	BCL-2 interacting mediator (BCL-2-like 11)
BIRC5	baculoviral IAP repeat containing 5
DEX	dexamethasone
DUSP1	dual specificity phosphatase 1
FasL	Fas ligand
GILZ	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
RFP	red fluorescent protein
shRNA	short hairpin RNA

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Figure 1.

Human Th17 (CD4+CCR6+) cells are resistant to glucocorticoid-induced apoptosis. (A) CD4+CCR6+, but not CD4+CCR6-, were positive for intracellular IL-17A. (B) Human Th17 (CD4+CCR6+), but not non-Th17 (CD4+CCR6-) cells, from allergic (N = 14) and non-allergic donors (N = 9) were resistant to dexamethasone (DEX, 100 nM)-induced apoptosis. **, $P < 0.01$, ***, $P < 0.001$; Two-Way ANOVA with Bonferroni post-hoc tests. (C) BCL-2 MFI (mean fluorescence intensity) was higher in Th17 cells than in non-Th17 cells. *, $P < 0.05$; Two-Way ANOVA with Bonferroni post-hoc tests (N = 12, 7 allergic and 5 non-allergic).

Figure 2.

Mouse Th17 cells are resistant to glucocorticoid-induced apoptosis. (A) Th17 and Th2 cultures were resistant to DEX (24 h)-induced apoptosis. Δ , $P < 0.05$ vs Th1, Th2, and Th17 cells; *, $P < 0.05$ vs Th2; +, $P < 0.05$ vs Th17; Two-Way ANOVA with Bonferroni post-hoc tests (N = 3–6). (B) Th17 (RFP+) cells were resistant to DEX killing. *, $P < 0.05$ vs RFP+ cells; Two-Way ANOVA with Bonferroni post-hoc tests (N = 3–6). C, DEX (100 nM) increased caspase activity in Th1 but not Th2 or Th17 cells. *, $P < 0.05$ vs Th1 cells; One-Way ANOVA with Tukey post-hoc tests (N = 6).

Figure 3.

GR isoforms are comparable among murine Th1, Th2, and Th17 cells. (A) A representative Western blot of GR levels in Th0, Th1, Th2, and Th17 cells. (B) Densitometric analyses of GR levels among Th subsets. $P > 0.05$; One-Way ANOVA with Tukey's post-hoc test ($N = 3$). (C) GILZ (glucocorticoid-induced leucine zipper) mRNA levels among Th1, Th2, and Th17 cells treated with vehicle or DEX (100 nM, 6 h). *, $P < 0.05$ vs CON; One-Way ANOVA with Tukey's post-hoc tests ($N = 3-4$).

Figure 4.

Apoptosis PCR Array of murine Th1 and Th17 cells. (A) Genes associated with apoptosis and differentially expressed (> 1.5 fold) at baseline in Th1 and Th17 cells. (B) Genes regulated by DEX in Th1 or Th17 cells.

Figure 5.

BCL-2 mediates glucocorticoid resistance of Th17 cells. (A–B) Glucocorticoids regulate BCL-2 differently in murine Th1, Th2, and Th17 cells. Th1, Th2, and Th17 cells were treated with DEX (0–1000 nM, 24 h) and BCL-2 protein was analyzed using Western blots and densitometry. Western blots of three independent experiments (A) and averages ($\pm$ SEM) of five experiments (B) are shown. *, $P < 0.05$ vs CON treatment; One-Way ANOVA with Tukey's post-hoc tests. (C–E) BCL-2 knockdown restores sensitivity to glucocorticoid-induced apoptosis in murine Th17 cells. (C) Representative flow plots of transduced IL-17F/RFP+GFP+ cells. (D) A representative Western blot demonstrating efficient BCL-2 knockdown in purified GFP+ cells. (E) Averages ($\pm$ SEM) of annexin V and DAPI staining results of IL-17F/RFP+GFP– and IL-17F/RFP+GFP+ cells treated with vehicle or DEX (100 nM, 24 h). ***, $P < 0.001$; One-Way ANOVA with Tukey's post-hoc tests ($N = 6$).

Figure 6.

Selective glucocorticoid sensitivity of murine Th17 cytokines. Th1, Th2, and Th17 cell cultures were treated with vehicle or DEX (100 nM, 24 h) in the presence of PMA (20 ng/ml) and ionomycin (250 ng/ml). (A) IFN- γ levels in Th1 culture supernatants. (B) IL-4 in Th2 cultures. (C) IL-17A, IL-17F, and IL-22 levels in Th17 cultures. Values were normalized to total protein and Sample means $\pm$ SEM are shown. *, $P < 0.05$; **, $P < 0.001$; Student's t-test ($N = 4-7$). (D) DEX (100 nM, 2 h prior to IL-6) did not inhibit IL-6 (50 ng/ml) induced STAT3 phosphorylation in Th17 cell culture ($N = 3$).

Figure 7.

Glucocorticoid resistance of lung Th17 cells *in vivo*. Lung single cell suspension was obtained from control (CON), dexamethasone (DEX), OVA sensitized and challenged (OVA), and OVA+DEX mice. (A) Lung Th cell counts using cell surface markers and flow cytometry. *, $P < 0.05$; One-Way ANOVA with Tukey's post-hoc tests ($n=3-8$). (B) Lung Th subset percentages obtained using intracellular cytokine staining. *, $P < 0.05$; One-Way ANOVA with Tukey's post-hoc tests ($n=3-8$). (C) Lung Th subset BCL-2 levels. $P > 0.05$; One-Way ANOVA with Tukey's post-hoc tests ($n=3-8$).