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CD56<sup>+</sup> Cells Induce Steroid Resistance in B Cells Exposed to IL-15<sup>1</sup>

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Interleukin-2 can induce steroid resistance in T cells. IL-15 shares biological activities with IL-2, as both cytokines use IL-2Rγ for signal transduction. We therefore sought to determine whether IL-15 contributes to induction of PBMC corticosteroid resistance. Surprisingly, we found that incubation of unfractionated PBMC with IL-15 for 48 h resulted in the inhibition of glucocorticoid receptor (GCR) nuclear translocation in response to dexamethasone (DEX) treatment in CD19-positive B cells significantly greater than CD19-negative non-B cells (p < 0.01). However, pure B cells incubated with IL-15 responded normally with nuclear translocation of GCR in response to steroids, but failed to translocate GCR when they were grown in the presence of CD19<sup>+</sup> cells. Coculture of B cells with CD3<sup>+</sup> (T cells), CD14<sup>+</sup> (monocytes), or CD56<sup>+</sup> (NK and NKT cells) in the presence of IL-15 revealed that only CD56<sup>+</sup> cells contributed to the steroid insensitivity of B cells. IL-15 stimulation significantly increased production of IL-4 by CD56<sup>+</sup> cells (p < 0.02). Treatment of purified B cells with combination IL-15/IL-4 resulted in abrogation of glucocorticoid receptor nuclear translocation and the inability of DEX to suppress cytokine production by B cells. In the presence of IL-4-neutralizing Ab, when B cells were cocultured with CD56<sup>+</sup> cells and IL-15, the B cells were found to be steroid sensitive, i.e., DEX induced GCR nuclear translocation. This study demonstrates that B cells develop steroid resistance in the presence of CD56<sup>+</sup> cells after IL-15 stimulation. Furthermore, IL-15 and IL-4 have the capacity to induce B cell insensitivity to steroids. *The Journal of Immunology, 2004, 172: 7110–7115.

Corticosteroids are potent suppressors of the immune response, and are therefore widely used in the therapeutic treatment of a broad range of autoimmune, inflammatory, and malignant diseases. A subset of individuals fails to respond to oral steroid treatment even at high doses of steroids. This is a major clinical concern in the management of chronic inflammatory and malignant diseases. However, the cellular and molecular mechanisms underlying corticosteroid resistance are not well understood.

Several studies (1–4) have reported that cytokine production contributes to corticosteroid resistance. In particular, the combination of IL-2 and IL-4 induces human T cells to be steroid unresponsive, suggesting that signal transduction mediated through common γ-chain of IL-2R may contribute to steroid resistance (5). IL-15 is a 14- to 15-kDa polypeptide that belongs to the 4-helix-bundle family of cytokines, including IL-2, IL-4, IL-7, IL-9, and IL-21, which use the signal-transducing βγ polypeptides of the IL-2R complex (6, 7). Thus, IL-15 shares many biological activities and may induce human PBMC to be unresponsive to steroids. In this study, we therefore examined the capacity of IL-15 to induce steroid resistance in PBMC. Interestingly, IL-15 induced steroid resistance, but its cell target was B cells, instead of T cells.

Materials and Methods

Reagents

Recombinant human IL-4-, IL-15-, and IL-4-neutralizing Abs were purchased from R&D Systems (Minneapolis, MN). Dexamethasone (DEX)<sup>6</sup> was purchased from Sigma-Aldrich (St. Louis, MO). Fluorochrome-labeled CD3 (clone SK7), CD14 (clone M5E2), CD19 (clone HB19), and CD56 (clone NCAM 16.2) Abs were purchased from BD PharMingen (San Diego, CA). Goat anti-rabbit IgG was purchased from Caltag Laboratories (Burlingame, CA), and IL-15Rα (clone H-I07) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of PBMC

Heparinized venous blood from healthy volunteers was layered on a Ficoll-Paque density gradient (Amersham Pharmacia Biotech, Bjorgatan, Sweden) and centrifuged for 25 min at 400 x g at room temperature. The PBMC layer was collected, washed, and resuspended in HBSS (Life Technologies, Grand Island, NY). The cell viability was >95%, as determined by a trypan blue exclusion assay.

Cell surface staining and cell sorting

Before staining, freshly isolated PBMC were washed and resuspended in PBS supplemented with 1% BSA, and subsequently incubated for 25 min at 400 x g at room temperature. The PBMC layer was collected, washed, and resuspended in HBSS (Life Technologies, Grand Island, NY). The cell viability was >95%, as determined by a trypan blue exclusion assay.

Intracellular staining

Purified CD56<sup>+</sup> or CD19<sup>+</sup> cells were initially cultured in the presence of IL-15 for 48 h. After that, the cells were stimulated for 4 h with 50 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) in 1 ml of complete medium with 10 μg/ml intracellular transport inhibitor GolgiPlug (BD PharMingen). The cells were then permeabilized with FACS-permeabilizing solution (BD PharMingen) for 10 min at room temperature, resuspended in staining buffer, and incubated for 30 min on ice with the following PE-conjugated anti-human IL-4 mAbs. Unbound Abs were removed by
two washes with staining buffer. Finally, the cells were resuspended in PBS and analyzed by flow cytometry. Cytokfluorometry was performed with a BD Biosciences FACSCalibur system (San Jose, CA). All cytokfluorometric data were subsequently analyzed and graphically displayed using BD Immunocytometry Systems CellQuest software (San Jose, CA).

Cell culture
Human PBMC, CD19- , or CD56+ cells were cultured at a concentration of 10⁶ cells/ml in complete medium: RPMI 1640 medium (Mediatech, Herndon, VA) containing 10% heat-inactivated FCS, 400 μM t-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 20 mM HEPES buffer solution (Mediatech) in the presence of 100 ng/ml human IL-15 with or without 400 U/ml human IL-4 for 48 h. In some experiments, anti-IL-4-blocking Ab (1 μg/ml) was added before IL-15 stimulation. For the studies of glucocorticoid receptor (GCR) nuclear translocation in response to steroids, charcoal-filtered glucocorticoid-free FCS (Gemini Bio-Products, Calabasas, CA) was used in the growth medium.

For the experiments that required specific separation of B cells from NK/NKT, monocytes, or T cells, B cells were put in the 24-well cell culture plates, and NK/NKT, monocytes, or T cells were put in the membrane inserts.

Measurement of cytokines in culture supernatants
CD56+ cells (10⁶ cells/well) were cultured in a 24-well plate (Corning Glass, Corning, NY) in the presence or absence of IL-15 for 48 h. B cells were grown at 10⁶ cells/ml in 24-well plates (Corning Glass) with or without IL-15/IL-4 or IL-4 in the culture medium for 48 h. Then DEX (10⁻⁶ M) was added to the culture medium for another 24 h.

After the incubation period, cell-free supernatants were collected and stored at −20°C before analysis. The amounts of IL-4 secreted by CD56+ cells or IL-6 production by CD19+ cell cultures were determined by ELISA (R&D Systems), following the manufacturer’s recommended procedure.

Immunofluorescence staining of PBMC or B cells for GCRα
GCRα nuclear translocation in response to DEX was performed using fluorescence microscopy, as previously described (5). In brief, human PBMC or purified B cell suspensions (10⁶ cells/ml) after 48 h of culture with IL-15 and IL-4 treated or not treated with DEX (10⁻⁶ M, 1 h) were settled on poly(D-lysine)-coated coverslips, fixed in 4% paraformaldehyde in PBS, permeabilized (PBS containing 0.1% BSA, 0.5% Tween 20, and 0.01% saponin (Sigma-Aldrich)), blocked (Superblock; Scytek, Logan, UT), and incubated with an Ab to GCRα (Affinity BioReagents, Golden, CO) diluted in permeabilization solution (1/250) overnight at 4°C. Purified nonimmune rabbit IgGs (Southern Biotechnology Associates, Birmingham, AL) was used as an isotype control. After the incubation period, the coverslips were washed in PBS, 0.5% Tween 20, then incubated with a donkey anti-rabbit IgG F(ab')₂-Cy3 conjugate secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) (1/2000) and 300 nM 4’,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) for 1 h at room temperature and washed again. Coverslips were mounted on slides in antifade solution and examined by fluorescence microscopy (Leica, Wetzlar, Germany) using ×63 objective.

Intensity of GCRα staining was assessed using image analysis software (Slidebook; Intelligent Imaging Innovations, Denver, CO) and expressed as mean fluorescence intensity (MFI). In brief, a series of 1-μm z-frames were taken through groups of cells in both the DAPI (nuclear) and Cy3 (GCRα) channels. Each stack of images then underwent nearest neighbors’ deconvolution. This allowed for exact localization of the nucleus within the cell and removed background fluorescence from above and below the z-frame that could affect the reliability of the results. A single computer-generated mask was then defined based on the DAPI staining. The intensity of Cy3 staining (GCRα) was assessed by the analysis software purely within the DAPI mask and, hence, the nucleus. In this way, increases in GCRα within the nucleus could be detected.

Statistical analysis
Immunofluorescence and cytokine production data were analyzed using paired Student’s t test. A value of p < 0.05 was considered statistically significant.

Results
IL-15 induces B cell resistance to steroids
To study the effects of IL-15 on steroid response, we examined nuclear translocation of GCRα in PBMC from healthy donors after incubation in the presence and absence of IL-15 for 48 h and then treatment with 10⁻⁶ M DEX for 1 h. Using image analysis, we were able to quantify the relative intensity of GCRα staining by MFI. In the absence of IL-15, GCRα translocation was always associated with increased MFI of Cy3 (GCRα) in the nuclear region of cells that was defined by the nuclear staining dye, DAPI. We examined GCR intracellular distribution in different PBMC populations and found that IL-15 had a selective effect on GCR nuclear translocation in B cells (Fig. 1). After DEX stimulation, GCRα translocated normally to the cell nuclei in CD19− non-B, but not CD19+ B cells when PBMC were incubated with IL-15. After DEX treatment, the MFI of nuclear GCRα were 185.18 ± 10.89 vs 405.14 ± 33.54 (p < 0.01, n = 4), respectively, for B and non-B cells.

IL-15-induced B cell resistance to steroids requires non-B cells
Because IL-15 induced B cells to be unresponsive to DEX when whole populations of PBMC were incubated with IL-15, it raised the question as to whether IL-15 worked on the B cells directly or through collaboration with other cell types. Therefore, B and non-B cells were separated by cell sorting, and B cells were incubated with IL-15 in the presence or absence of non-B cells. Fig. 2 shows that GCR nuclear translocation of B cells cultured with non-B cells was lower than B cells that were cultured alone in the presence of IL-15 for 48 h. For B cells with non-B cells vs B cells alone, the nuclear MFI of GCRα were as follows: 180.23 ± 16.8 vs 424.08 ± 34.64 (p < 0.01, n = 4). Therefore, we concluded that IL-15-induced steroid resistance of B cells required the presence of non-B cells.

![FIGURE 1](http://www.jimmunol.org/DownloadedFrom.jpg)
Non-B cells induce B cell steroid resistance via indirect contact in the presence of IL-15

To determine whether non-B cells affect the responsiveness of B cells to DEX through direct contact or through the production of cytokines, non-B cells were physically separated from B cells by membrane inserts and cultured in the presence of IL-15 for 48 h. B cells alone responded normally to DEX after incubation with IL-15. However, when B cells were incubated with non-B cells in the insert, in the presence of IL-15, this resulted in decreased GCR nuclear translocation in response to 1-h DEX treatment. For B cells with non-B cells in the insert vs B cells alone, the MFI of GCR in nucleus was 231.42 ± 12.9 vs 421.5 ± 13.8 (p < 0.01, n = 4), respectively (Fig. 3).

CD56+ cells change the responsiveness of B cells to DEX

Because non-B cells include monocytes, T cells, and CD56+ (NK/NKT) cells, we sought to determine which cell type contributes to the IL-15-induced steroid resistance in B cells. Therefore, NK/NKT, T cells, and monocytes were separated and isolated by cell sorting, put in the inserts, and cultured individually with B cells in the cell culture wells in the presence of IL-15 for 48 h. After DEX stimulation, GCR nuclear translocation in B cells incubated with NK/NKT cells in the inserts was significantly lower than in B cells incubated with monocytes or T cells (Fig. 4). For the B cells with NK/NKT and B cells alone, the MFI of nuclear GCRα was 234.7 ± 9.89 vs 429.26 ± 16.44 (p < 0.005, n = 4). In contrast, B cells cocultured with T cells or monocytes responded normally to steroids, as indicated by GCR nuclear translocation in the presence of DEX (Fig. 4).

IL-15 increases the production of IL-4 in CD56+ cells

IL-4 has been reported to be involved in the induction of steroid resistance induced by IL-2 (1). We therefore examined whether the production of IL-4 in NK/NKT cells can be increased by IL-15. To accomplish this, intracellular staining was performed with PE-conjugated anti-IL-4 mAb and then analyzed by flow cytometry. As shown in Fig. 5, treatment of purified CD56+ cells (they include NK and NKT cells) with IL-15 for 48 h significantly increased the percentage of IL-4-producing CD56+ cells as compared with CD56+ cells that were cultured in the medium alone (47.25 ± 9.03% vs 11.11 ± 3.03%, p < 0.01, n = 5). Amounts of IL-4 in cell culture supernatants were significantly increased as well when CD56+ cells were cultured in presence of IL-15, as compared with medium alone (1664.0 ± 365.4 pg/ml vs 237.4 ± 12.3 pg/ml, p < 0.02, n = 5, respectively). We also did intracellular IL-4 staining for B cells to examine whether the production of IL-4 in B cell can be increased by IL-15. We did not find any increase in IL-4 intracytoplasmic staining of B cells after IL-15 treatment (data not shown).

IL-4 collaborates with IL-15 to induce B cell resistance to steroids

The observation that IL-15 induces the production of IL-4 in CD56+ cells and that CD56+ cells changed the responsiveness of B cells to DEX raised the question as to whether IL-4 induces B cells to become insensitive to DEX in the presence of IL-15. Therefore, purified B cells were incubated with IL-4 and IL-15, or with IL-4 alone, for 48 h. Indeed, nuclear translocation of B cells treated with IL-4 and IL-15 was significantly reduced as compared with B cells treated with IL-4 (228.65 ± 12.6 vs 423.37 ± 37.1, p < 0.02, n = 4) (Fig. 6).

Because CD56+ (NK/NKT) cells contribute to IL-15-induced steroid resistance in B cells and IL-15 increases the production of IL-4 in CD56+ (NK/NKT) cells, we examined the selectivity of this IL-4 effect by determining whether IL-4-blocking Ab reverses this effect. In contrast to IL-15 alone, coculture of B cells and CD56+ cells in the presence of IL-4-blocking Ab with IL-15 for 48 h maintains a steroid-sensitive state, i.e., DEX can increase nuclear translocation of B cell GRα (MFI, 295.01 ± 11.31 vs 220.50 ± 9.59, p < 0.015, n = 3).

The effect of IL-4 on IL-15R expression was also studied. In these experiments, we stimulated B cells with IL-4 for 48 h and measured IL-15R expression on B cells using flow cytometry. After IL-4 stimulation, B cells increased the expression of IL-15R.
IL-15R expression after IL-4 vs 5.72 ± 0.48% IL-15R expression without IL-4, p < 0.016, n = 6

**Discussion**

Previous studies have reported that cytokines are one of the major factors that contribute to the induction of corticosteroid resistance. It has been shown that treatment of human T cells with combination IL-2/IL-4 decreases GCR-binding affinity to its ligand and inhibits GCR nuclear translocation following stimulation with steroids (1, 5). This leads to the inability of steroids to suppress T cell proliferation and cytokine generation. In the current study, we analyzed the effects of IL-15 on cellular responses to steroids. Both IL-15 and IL-2 bind to a heterodimeric receptor complex, which shares the IL-2Rα and IL-2Rγ chains (6, 10, 11). Therefore, IL-15 shares many biological activities with IL-2 (11–14) and could possibly contribute to the induction of corticosteroid resistance as well.

Corticosteroids mediate their anti-inflammatory effects through the GCR that acts as a transcription factor. After association with its ligand, the GCR translocates into the cell nucleus, where it induces transcription of anti-inflammatory mediators through direct interaction with glucocorticoid response elements (*trans* activation) or inhibits the transcription of various proinflammatory proteins due to its interactions with other transcriptional factors (*trans* repression) (15). It has been suggested that deficient GCR nuclear translocation in response to steroids is associated with reduced corticosteroid responses (15, 16).

**FIGURE 4.** Coculture of CD19⁺ (B) cells with CD56⁺ (NK/NKT) cells, physically separated by membrane inserts, in the presence of IL-15 results in inability of GCRα to undergo nuclear translocation in response to DEX. GCRα intracellular distribution was assessed in IL-15-stimulated CD19⁺ (B) cells that were cocultured with CD4⁺ (T) (A), CD14⁺ (Mo) (B), or CD56⁺ (NK/NKT) (C). Cells were physically separated by membrane inserts. The images are representative of four different donor experiments. MFI of Cy3 in the nucleus were analyzed (D). Mean ± SEM are shown. The cell type that was put into membrane insert is indicated in parentheses.

**FIGURE 5.** IL-15 treatment increased the percentage of IL-4-positive CD56⁺ cells. Purified CD56⁺ cells were cultured with or without IL-15 for 48 h. IL-4 production by CD56⁺ cells was analyzed by intracellular staining.

**FIGURE 6.** Treatment of purified CD19⁺ (B) cells with combination IL-15/IL-4 leads to abrogation of GCRα nuclear translocation in response to DEX. MFI of Cy3 in nucleus were analyzed (four donors). Mean ± SEM are shown.

**FIGURE 7.** DEX does not inhibit production of IL-6 by CD19⁺ (B) cells if the cells are pretreated with IL-15/IL-4 combination, but not IL-4 alone. Mean ± SEM are shown.
The present study demonstrates that incubation of human PBMC with IL-15 induces the insensitivity of B cells to steroids, while non-B cells continue to respond normally to steroids, as shown by GCR nuclear translocation in the presence of DEX. The α-chain of the IL-2R and IL-15R recognizes only its cognate cytokine. IL-15Rα chain can transduce a signal even in the absence of the β- and/or γ-chain (17, 18). Therefore, it is possible that the IL-15Rα subunit is responsible for the differential effects of IL-15 and IL-2 on many cell types (19). Bulanova et al. (20) reported that Syk kinase physically and functionally associates with IL-15Rα chain in B cells and that Syk plays a key role in mediating IL-15-induced signal transduction. This may lead to the distinct functional consequences of IL-15 vs IL-2 binding to B cells.

IL-15 is mainly produced by activated monocytes, muscle cells, and human epidermal keratinocytes (21–23). Its cellular targets include T and B lymphocytes, NK cells, monocytes, eosinophils, and neutrophils (24–29). Various studies have implicated its role in many kinds of diseases, including atopy. Studies in our lab showed that decreased IL-15 levels may contribute to acute inflammation in atopic dermatitis (30). It has also been shown as well that overexpression of IL-15 inhibits allergic inflammation in a murine model of asthma (31).

Surprisingly, when purified human B cells were cultured in the presence of IL-15, they responded normally to steroids. Addition of non-B cells to the cell culture subverted this response, suggesting that non-B cells were required to provide a signal to B cells (either by direct cell-cell interaction or through some mediators) that induced their steroid resistance. To examine the mechanism for this phenomenon, B cells were physically separated from non-B cells by the membrane inserts, cultured with IL-15 for 48 h, and then exposed to DEX. The coculture experiments revealed that B cell steroid resistance was induced by non-B cells. In this way, we assumed that factor/factors secreted by non-B cells were responsible for the induction of steroid resistance.

To find out which cell type provided such a factor, B cells were cocultured with CD3+, CD14+, or CD56+ cells from the same donor for 48 h in the presence of IL-15. As before, the cells were physically separated by the membrane, so only exchange with soluble factors was possible between the chambers. CD56+ cells were the only cell type that changed the responsiveness of B cells to DEX. After incubation of B cells with other cell types in the presence of IL-15, they remained steroid sensitive.

Although CD56+ population includes NK and CD56+ T cells (32), in the current study, we did not further separate NK and NKT cells, because it has been reported that IL-15 promotes development and survival of NK and NKT cells (32). Furthermore, both cell populations are known to produce IL-4 (33–35). NK cells are capable of immunoregulation and may also play a role in determining the response to allergens. They may in part determine development of allergic airway inflammation (36). It has been demonstrated that patients with asthma have an increased number of NK cells and show stronger than normal NK activity (37). NK cells contribute to IgE-mediated allergic responses (38). Human NK cells have been divided up into NK1 and NK2 subsets: NK1 cells that predominantly express IFN-γ and IFN-γ-nonsecreting NK2 cells that secrete IL-4, IL-5, and IL-13 (33).

Because addition of IL-4 to IL-2 was required for induction of steroid resistance in human T cells (1), we decided to evaluate whether combination IL-4/IL-15 was required for the induction of steroid resistance in B cells as well. Indeed, we found that IL-15 stimulation induced secretion of IL-4 by NK cells. Therefore, we assumed that IL-4 secreted by NK/NKT cells is the cytokine that collaborates with IL-15 to induce B cell resistance to corticosteroids. NK cells express IL-15Rα (39), and IL-15 is able to activate NK cells via IL-2Rβ and common γ-chain and its own α-chain (40). Culture of purified B cells with IL-4 and IL-15 as well inhibited GCR nuclear translocation in response to DEX. Furthermore, IL-4-blocking Ab prevented the steroid resistance of B cells induced by CD56+ cells in the presence of IL-15. These results indicate that both IL-4 and IL-15 contribute to the induction of steroid resistance in B cells.

Activated B cells are capable of secreting immunomodulatory factors such as cytokines (41). IL-6 is one of the major cytokines derived from B cells (42, 43). To further confirm that IL-15 and IL-4 act together to induce functional B cell steroid resistance, IL-6 production was measured in the supernatants from B cells prestimulated with IL-15/IL-4 as compared with IL-4 alone for 48 h and then treated with DEX for an additional 24 h. IL-6 production was significantly inhibited by DEX when the cells were treated with IL-4, but remained almost unaffected when B cells were prestimulated with the combination of IL-15 and IL-4. This observation is consistent with our data revealing reduced GCR nuclear translocation in B cells exposed to IL-15/IL-4.

Various inflammatory diseases, including asthma, are often associated with frequent viral infections (44, 45). Corticosteroids are widely used to control these inflammatory conditions. It has been reported that recurrent respiratory virus infections (respiratory syncytial virus and rhinoviruses) can contribute to allergic sensitization and increase the risk of atopy (46, 47). Because NK and NKT cells represent the first line of defense against viral infections (48, 49), our current study suggests a previously unrecognized role for these cells in the control of B cell steroid response and suggests that IL-15 and CD56+ cells can contribute to sustained B cell activation and steroid resistance. This may alter B cell function under conditions in which viral infections are treated with corticosteroids. Although such studies are beyond the scope of the present investigation, it suggests that more work should be done to evaluate the possible role of activated CD56+ cells in the control of B cell responses during clinical situations that are triggered by viral infection, and corticosteroids are used for control of immune activation.

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References


