**ABSTRACT**

Idiopathic nephrotic syndrome (INS) is the most glomerular disease that occurred in childhood with high rate morbidity. Glucocorticoid is drug of choice for INS and responsiveness to this drug determined prognosis. Glucocorticoid upregulate transcription of anti-inflammatory cytokines such as IL-4 and IL-10. IL-10 is an anti-inflammatory cytokine and has multiple role in immune response include modulate Th1/Th2 response. Vitamin D3 interact with glucocorticoid signaling. Administered active form of vitamin D3 increase dexamethasone-induced IL-10 expression by regulatory T cells in steroid resistant asthmatic patient. Here we showed increase of CD4\(^+\)IL10\(^+\) expression after treatment both prednisone only and combination prednison with vitamin D3. Both in new-onset NS or rare relaps NS, combination treatment prednisone + vitamin D3 increase CD4\(^+\)IL10\(^+\) expression significantly compared to prednisone-only treated group (p= 0.003), which first group (new-onset nephrotic syndrome + prednisone and vitamin D3 treatment) showed the most CD4\(^+\)IL10\(^+\) expression enhancement (9.53±3.89). However this study failed to show a correlation between CD4\(^+\)IL-10 expression after prednisone and vitamin D3 treatment with clinical outcome (linear regression test, p= 0.125). This study also showed that there was a no correlation between CD4\(^+\)IL-10 expression and CD3\(^+\)GR expression after prednison + vitamin D3 treatment (p= 0.088). CD4\(^+\)IL-10 expression in new-onset and rarely relapsing nephrotic syndrome patients higher in prednisone + vitamin D3 treated group than prednisone-only treated group. There is no correlation between CD4\(^+\)IL-10 expression and clinical outcome.

**Keywords:** CD3\(^+\)GR, CD4\(^+\)IL-10\(^+\), idiopathic nephrotic syndrome, prednisone, vitamin D3
Approximately 85%-90% patients with INS have a good response to glucocorticoid therapy and remit completely from proteinuria, otherwise 10%-15% have a partial or no response to glucocorticoid treatment [9, 10]. The mechanism underlying unresponsiveness to glucocorticoid treatment is complex. It is based on physiologic process that involved in glucocorticoid signaling. Glucocorticoid bind to cytoplasmic glucocorticoid receptor (GR), and then translocate to nucleus and regulate transcription process of several protein include cytokines [10-13]. Glucocorticoid downregulate transcription of pro-inflammatory cytokines such as IL-1, IL-2, IL-6, IL-8, IL-11, IL-12, TNF-α, Interferon gamma (IFN)-γ, granulocyte-macrophage colony stimulating factor (GM-CSF) and upregulate transcription of anti-inflammatory cytokines as IL-4 and IL-10 [14].

Interleukin-10 is an anti-inflammatory cytokine and has multiple role in immune response include modulate Th1/Th2 response via APC (Antigen Presenting Cells) and directly inhibit proliferation and cytokine synthesis by T cells [15-17]. In steroid resistant asthmatic patient, IL-10 increase GR expression via T CD4+ cells. Ligand--induced GR downregulation can be reserved by vitamin D3. Vitamin D3 interact with glucocorticoid signaling. Administered active form of vitamin D3 increase dexamethasone-induced IL-10 expression by regulatory T cells in steroid resistant asthmatic patient. Pre-incubation T cell with IL-10 and vitamin D3 can improve impairment IL-10 release from T cell that induced by dexamethasone [18].

Active form of vitamin D3, 1,25(OH)2-dihydroxyvitamin D3, not only has a role in calcium metabolism but also modulate immune response [19]. Terrier, et al. showed that vitamin D was safe and induced decrease of memory B cells, increase Treg and decrease Th1 and Th17 effector cells [20]. In vitro study of T CD4+ cell culture conducted by Urry, et al. showed that active form of vitamin D3 increased population of CD4+IL-10+ and CD4+Foxp3+ cells [21].

This current study aimed to compare the effect of combination therapy of corticosteroid (prednisone) and vitamin D3 with corticosteroid-only therapy on CD4+IL-10+ expression. This study also investigate the correlation between CD4+IL-10+ and CD3+GR expression, CD4+IL-10+ expression and clinical outcome (steroid resistant or steroid sensitive nephrotic syndrome). We hypothesized that combination therapy increase CD4+IL-10+ expression more than prednisone-only treated group both in new-onset nephrotic syndrome and rare relaps nephrotic syndrome. We also hypothesized that there is a correlation between CD4+IL-10+ expression and CD3+GR expression as well as clinical outcome.

**MATERIALS AND METHODS**

**Study Design**

This experimental study was conducted in pediatric nephrology policlinic and pediatric nephrology inpatient ward at dr. Saiful Anwar Hospital, Malang, Indonesia between October 2013 and April 2014. During this period 24 children in the age group 4-12 years with new onset idiopathic nephrotic syndrome (INS) were prospectively studied. Design of this study was an experimental study with randomized clinical trial, double blind, pre and post test control group. Blood samples (GR assays and IL-10 assay) obtained two times (before and after treatment). 24 patients divided into 4 groups consist of new onset INS that treated with prednisone only as a first group, new onset of INS treated with combination of prednisone and vitamin D3 as a second group, rare relaps INS treated with prednisone only as the third group, and rare relaps INS treated with combination of prednisone and vitamin D3 as the forth group. Prednisone was given orally at dose 2 mg/kg/day for 4 weeks. Vitamin D3 (D-Vit, GraciaPharmindo) also given orally at dose 2000 IU for 4 weeks. This study had been approved by Ethical Committee of dr. Saiful Anwar Hospital Malang, Indonesia.

**Patients**

Blood samples were obtained from 24 patients (19 males, 31 females, aged 4-12 years) with nephrotic syndrome and had received treatment both in nephrology polyclinic and inpatient ward during experimental study. Inclusion criteria include: diagnosed as idiopathic nephrotic syndrome (classified as first diagnosed and intermittent relapse), age range between 1-14 years old, approved by parents to participate in this study. Exclusion criteria for this study: secondary and congenital nephrotic syndrome, frequent relapse nephrotic syndrome, and steroid resistance nephrotic syndrome. Subjects that met the inclusion criteria randomized using randomization table.

**PBMC Isolation**

Blood sample transferred from collection vacuum tubes (previously mixed with anticoagulant EDTA) to a 50 mL tube. An equal volume of PBS was added and sample was mixed by repeated pipetting. The second 50 mL tube was prepared and filled with Ficoll-
Hipaque (d=1.077 g/mL). This Ficoll-Hipaque solution carefully layer with the diluted blood with equal volume and then centrifuged at 1000 rpm for 30 minutes. PBMC layer was removed (second layer) using micropipette and transferred to a new 15 mL centrifuge tube. After washing two times using PBS 10 mL and centrifuge at 1200 rpm for 10 minutes, decant the supernatant and re-suspend the cell pellet in appropriate volume of PBS.

**Assay for CD4+ GR Expression**
Phycoerythrin (PE)-labelled antihuman CD357 (GITR) (antibody cat# 311604, Biolegend, USA) and FITC-labelled antihuman CD3 (antibody cat# 344804, Biolegend, USA) were used for intracelullar staining (flowcytometry). T cell lymphocyte added with cell staining buffer (2% fetal bovine serum in PBS). Five µl pellet from PBMC isolation was taken and ready for staining using FITC-labelled anti-human CD3. 50 µl diluted antibody was taken and mixed with T cell lymphocyte and then incubated for 20 minutes (in dark room temperature). After this incubation, samples were ready for IL-10 intracellular staining. Cells were washed with 500 µl cell staining buffer, centrifuged at 4°C, 2500 rpm for 3 minutes. Supernatant were removed, and then cells washed again, re-suspend in permeabilization buffer. Suspensions were centrifuged again at 4°C, 2500 rpm for 3 minutes. After removing the supernatant, T lymphocytes CD4+ washed with 500 µl fix buffer solution, homogenized and centrifuged at 4°C, 2500 rpm for 3 minutes. Supernatant were removed, and then cells washed again, re-suspend in permeabilization buffer. Suspensions were centrifuged again at 4°C, 2500 rpm for 3 minutes. After removing the supernatant, cells were ready for staining with PerCP/Cy5.5-labelled anti-human IL-10 antibody (1:250). Each solution stained with 50 µl PerCP/Cy5.5 anti-human IL-10 diluted antibody, incubated at dark room for 20 minutes, and then washed with 500 µl permeabilization wash buffer solution. After centrifugation at 4°C, 2500 rpm for 3 minutes, supernatant were removed and added with 350 µl cell staining buffer. Cell samples were run on FACS Calibur flow cytometer and analyzed by BD Cell Quest Pro software.

**Assay for CD4+ IL-10+ Expression**
PerCP/Cy5.5-labelled anti-human IL-10 (cat# 501418, Biolegend, USA) and FITC-labelled anti-human CD4 antibody (cat# 300506, Biogene,d,USA) intracellular staining (flowcytometry). T lymphocytes CD4+ added with cell staining buffer (2% fetal bovine serum in PBS). Five µl pellet from PBMC isolation was taken and ready for staining using FITC-labelled anti-human CD4. 50 µl diluted antibody was taken and mixed with T cell lymphocyte and then incubated for 20 minutes (in dark room temperature). After this incubation, sample was ready for IL-10 intracellular staining. Cells were washed with 500 µl cell staining buffer, centrifuged at 4°C, 2500 rpm for 3 minutes. After removing the supernatant, T lymphocytes CD4+ fixed with 500 µl fix buffer and then incubated for 20 minutes (dark, room temperature). After incubation, samples were centrifuged at 4°C, 2500 rpm for 3 minutes. Supernatant were removed, and then cells washed again, re-suspend in permeabilization buffer. Suspensions were centrifuged again at 4°C, 2500 rpm for 3 minutes. After removing the supernatant, cells were ready for staining with PerCP/Cy5.5-labelled anti-human IL-10 antibody (1:250). Each solution stained with 50 µl PerCP/Cy5.5 anti-human IL-10 diluted antibody, incubated at dark room for 20 minutes, and then washed with 500 µl permeabilization wash buffer solution. After centrifugation at 4°C, 2500 rpm for 3 minutes, supernatant were removed and added with 350 µl cell staining buffer. Cell samples were run on FACS Calibur flow cytometer and analyzed by BD Cell Quest Pro software.

**Statistical Analysis**
The normality of distribution was confirmed by the Saphiro-Wilk’sW-test (normal distribution assumed as Sig or p>0.05). The difference in IL-10 expression in particular lymphocyte subpopulations between prednisone-only and combination of prednisone and vitamin D3 treated group confirmed by one-way ANOVA (Kruskal-Wallis test as an alternative test). The difference in IL-10 expression before and after treatment confirmed by paired t-test (Wilcoxon test as an alternative test). Correlation between IL-10 expression with outcome and GR expression among patients that given vitamin D3 as adjuvant (steroid sensitive or steroid resistant nephrotic syndrome) confirmed by regression test. Data were analyzed using SPSS for Windows 17.0 (confidence interval 95%).

**RESULTS AND DISCUSSION**
This study involved 24 patients age ranged from 4-12 years with nephrotic syndrome (12 patient new-on-
Vitamin D3 Adjuvant Treatment Stimulate Interleukin-10 Expression in Children

set nephrotic syndrome and 12 patients rare relaps nephrotic syndrome. Patients divided randomized into 4 treated groups. The first and second group consists of 6 patients with new onset nephrotic syndrome. First group treated with prednisone only, and second group treated with combination of prednisone and vitamin D3. The third and fourth group consists of 6 patient with rare relapsing nephrotic syndrome. The third group treated with prednisone only, and fourth group treated with combination of prednisone and vitamin D3. Among all groups, there was no drop out. During 4 weeks treatment, patients were not showed any illness or adverse drug reactions caused by prednisone or vitamin D3.

**CD4**+IL10** expression**

Table 1 showed that CD4+IL10- expression mean in 4 groups before treatment. The differences among groups was not different significantly (one-way ANOVA, p= 0.593).

There were significant differences of CD4+IL10- expression mean (t-test, p= 0.004) between new-onset nephrotic syndrome before (1.99±0.73) and after treatment (3.54±1.79) with prednisone. This finding showed that prednisone treatment increase IL-10 expression (Figure 1).

There were also significant differences of CD4+IL10- expression mean (t-test, p= 0.008) between new-onset nephrotic syndrome before (3.50±3.01) and after treatment (9.53±3.89) that treated with combination prednisone and vitamin D3. This finding showed that combination prednisone and vitamin D3 administration increase IL-10 expression (Figure 2).

There was a significant difference of CD4+IL10- expression mean (t-test, p= 0.008) between rarely relapsing nephrotic syndrome before (1.66±0.72) and af-

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**Table 1. Mean of CD4+IL10- expression**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD (Before Treatment)</th>
<th>Mean ± SD (After Treatment)</th>
<th>p-value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New-onset NS prednisone + vitamin D3</td>
<td>3.50±3.01</td>
<td>9.53±3.89</td>
<td>0.008</td>
</tr>
<tr>
<td>New-onset NS prednisone only</td>
<td>1.99±0.73</td>
<td>3.54±1.79</td>
<td>0.004</td>
</tr>
<tr>
<td>Rare relaps NS prednisone + vitamin D3</td>
<td>1.99±0.54</td>
<td>4.28±1.99</td>
<td>0.015</td>
</tr>
<tr>
<td>Rare relaps NS prednisone only</td>
<td>1.66±0.72</td>
<td>2.78±2.50</td>
<td>0.008</td>
</tr>
</tbody>
</table>

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**Figure 1.** CD4**+IL-10** expression before and after prednisone treatment. Upper figure represent before prednisone treatment: **A.** CD4+ cells number stained with FITC (red) and **B.** IL-10 expression on CD4+ cells stained with PerCP/Cy5.5 (green). Bottom figure represent after prednisone treatment: **A.** CD4+ cells number stained with FITC (red) and **B.** IL-10 expression on CD4+ cells stained with PerCP/Cy5.5 (green).
ter treatment (2.78±2.50) with prednisone. This finding showed that prednisone treatment increase IL-10 expression. There was also a significant difference of CD4‘IL10’ expression mean (t-test, p= 0.015) between rarely relapsing nephrotic syndrome before (1.99±0.64) and after treatment (4.28±1.99) with combination of prednisone and vitamin D3. This finding showed that combination of prednisone and vitamin D3 treatment increase IL-10 expression.

Figure 3 showed increase of CD4‘IL10’ expression after treatment both prednisone only or combination prednisone with vitamin D3. Before treatment, CD4‘IL10’ expression in 3 groups (new-onset nephrotic syndrome treated with prednisone only group, rarely relapsing nephrotic syndrome treated with prednisone only group, rarely relapsing nephrotic syndrome treated with combination of prednisone and vitamin D3) were almost similar. However, in new-onset nephrotic syndrome treated with prednisone and vitamin D3, CD4‘IL10’ expression was higher than other groups. It means the first group (new-onset nephrotic syndrome treated with combination of prednisone and vitamin D3) showed the most CD4‘IL10’ expression enhancement.

This study showed that CD4‘IL10’ expression after treatment in 4 groups were statistically different (one-way ANOVA, p= 0.003) Post-Hoc test showed that there was a significant different of CD4‘IL10’ expression between first group (new-onset nephrotic syndrome treated with combination of prednisone and vitamin D3) and second group (new-onset nephrotic syndrome treated with prednisone only). There was
also a significant different of CD4 IL10+ expression between first group and third group (rarely relapsing nephrotic syndrome treated with combination of prednisone and vitamin D3), and also first group and fourth group (rarely relapsing nephrotic syndrome treated with prednisone only) (Table 2).

This study showed that there was no correlation between CD4 IL10+ expression after prednisone and vitamin D3 treatment with clinical outcome (linear regression test, p= 0.125).

**Correlation of CD4 IL10+ expression and clinical outcome**

Increase of CD4‘IL-10’ expression after glucocorticoid treatment consistent with previous study conducted by Barrat, et al. (2002) that showed glucocorticoid not only increase IL-10 production in T lymphocyte, but also decrease production of IL-4, IL-5 and IL-13 in human T CD4+ cells [22]. Other study showed that renal T CD4+ cells was the source of endogenous IL-10 [23]. Glucocorticoid treatment not only up-regulate IL-10 secretion by macrophage/ dendritic cells and Th2 cells, but also involved in Treg proliferation, major immunoregulatory T lymphocyte subtype that can produce IL-10 abundantly [23]. Glucocorticoid can also trigger IL-10 secretion by T CD4+ cells directly [24]. Other cells that can be induced to produce IL-10 is monocyte. Mozo, et al. (2004) showed that monocyte, not T or B cells, up-regulate IL-10 production (IL-10 protein and mRNA) after treatment with physiologic dose of dexamethasone (after 12 hour). This study suggest that dexamethasone treatment can induce IL-10 production at transcriptional level [25].

CD4‘IL10+’ expression increased significantly in patients with new-onset nephrotic syndrome and rare relapsing nephrotic syndrome treated with prednisone and vitamin D3). This finding means that treatment with prednisone or prednisone combined with adjuvant vitamin D3 has a beneficial effect to increase CD4‘IL-10+’ expression, but addition of vitamin D3 increase CD4‘IL-10+’ expression more than prednisone-only treated group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Differences</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>New-onset NS prednisone-vitamin D3 vs New-onset NS prednisone only</td>
<td>0.424</td>
<td>0.142 - 0.706</td>
<td>0.005*</td>
</tr>
<tr>
<td>New-onset NS prednisone-vitamin D3 vs Rare relaps NS prednisone only</td>
<td>0.588</td>
<td>0.306 - 0.870</td>
<td>0.000*</td>
</tr>
<tr>
<td>New-onset NS prednisone-vitamin D3 vs Rare relaps NS prednisone + vitamin D3</td>
<td>0.338</td>
<td>0.056 - 0.620</td>
<td>0.021*</td>
</tr>
<tr>
<td>New-onset NS prednisone+vitamin D3 vs Rare relaps NS prednisone only</td>
<td>0.338</td>
<td>0.056 - 0.620</td>
<td>0.021*</td>
</tr>
<tr>
<td>New-onset NS prednisone only vs Rare relaps NS prednisone+vitamin D3</td>
<td>-0.086</td>
<td>-0.368 - 0.196</td>
<td>0.333</td>
</tr>
<tr>
<td>New-onset NS prednisone only vs Rare relaps NS prednisone only</td>
<td>0.164</td>
<td>-0.118 - 0.446</td>
<td>0.238</td>
</tr>
<tr>
<td>Rare relaps NS prednisone+vitamin D3 vs Rare relaps NS prednisone only</td>
<td>0.250</td>
<td>-0.032 - 0.532</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Significant differences when p-value < 0.05
relapses nephrotic syndrome that treated using combination of prednisone and vitamin D3 (compared with prednisone-only treated group). This finding showed that addition of vitamin D3 has beneficial effect in inducing IL-10 expression. Xytraxis et al. (2006) showed that active form of vitamin D3 increase level of IL-10 and Foxp3 cells in human peripheral blood culture [18].

Heine, et al. (2008) demonstrated that calcitriol (an active form of vitamin D3, 1,25(OH)2D3) treatment increase both cell number that express IL-10 and the amount of IL-10 expression each cells [26]. Kickler et al. (2012) showed calcitriol induce T cell population that secrete IL-10. Active metabolite of vitamin D influence T cell activation mediated by CD46 by controlling the CD46 expression and T CD4+ cell and T CD8+ cell function. Calcitriol promote switching not only by reduction of IFN-γ production, but also promote IL-10 production via CD46 co-stimulation. This study also showed that additional calcitriol treatment can reverse IL-10:IFN-γ ratio (increase IL-10 secretion, decrease IFN-γ) in multiple sclerotic patient with decrease of IL-10 production [27].

In vitro study conducted by Allen et al. (2012) demonstrated that there was an increase of IL-10 production by PBMC after vitamin D treatment, but T CD4+ cells frequency that produced IL-10 was not change significantly. Increase of IL-10 production may be caused by vitamin D effect on monocyte, dendritic cells, or B cells [28].

Glucocorticoid treatment not only increase IL-10 production in T lymphocyte, but also trigger Treg cells proliferation if administered in combination with active form of vitamin D3, 1,25(OH)2D3. Overall, research data showed that glucocorticoid-only or combination glucocorticoid + 1,25(OH)2D3 potentiate Treg immunosuppressive effect by increase IL-10 production and/or Foxp3 expression [29].

Colin et al. (2010) demonstrated 1,25(OH)2D3 inhibit production of TNF-α, IL-17 and IFN-γ, and induce expression of IL-4, IL-5, and IL-10 in PBMC or T CD4+ cells. Furthermore, 1,25(OH)2D3 increase differentiation of T CD4+ cells which produce IL-10 in patients with multiple sclerosis and Crohn disease [30].

1,25(OH)2D3 play an important role for T lymphocyte proliferation and differentiation. T lymphocyte treatment using calcitriol or its analogs inhibit secretion of various pro-inflammatory cytokines from Th1, Th9 and Th22 cells but induce anti-inflammatory cytokines production from Th2 cells. Calcitropic action of vitamin D was suggestion for vitamin D treatment in clinical nephrology. 1,25(OH)2D3 proven has anti-proteinuria effect and inhibit renin angiotensin aldosterone system (RAAS). 1,25(OH)2D3 has a beneficial effect in preserve podocyte structural integrity and functional and also suppress renin structural expression directly at transcriptional level [31].

T lymphocyte treatment with 1,25(OH)2D3 cause reduction of IL-17, IFN-γ and IL-21 level and significantly increase gene expression in Treg cells. Treg cell has anti-inflammatory role and control autoimmune disease by IL-10 and TGFβ secretion. Besides that, Treg cell can be induced and stimulated by 1,25(OH)2D3 through indirect pathway via APC and dendritic cells or direct pathway via endocrine effect or intracrine conversion from 25(OH)D become 1,25(OH)2D3 by Treg cells itself. 1,25(OH)2D3 has a various effect on inflammation and autoimmune disease by decrease Th17 cell population. 1,25(OH)2D3 can also influence T cell behavior, promote tolerance through Th2 and Treg activity induction and Th1 and Th17 cell activity suppression [31].

In this study, there was no correlation between CD4+IL10+ expression and clinical outcome. This finding is not consistent with other study that showed short term IL-10 expression could improve glomerulonephritis and glomerulosclerosis. Potential mechanism underlying renoprotective effect of IL-10 maybe complex because IL-10 has a dual action. Major effects of IL-10 are inhibit activation and effector function of T cells, monocytes, and macrophages. IL-10 bind to its receptor (IL-10R), which this binding can activate several signaling pathway depend on cell type, include pathway that depend on STAT-3, NF-kB and AP-1. IL-10 also potentially inhibit several cytokine production such as IL-1 and TNF-α, chemokine such as MCP-1 and RANTES in activated monocyte/macrophage, and also suppress MCP-1 in inflammatory cell population (this effect can delay monocyte/macrophage recruitment in renal interstitium). IL-10 also inhibits dendritic cell function and T cell activation [32]. However, correlation between IL-10 expression and clinical outcome need further investigation.

CONCLUSIONS

We conclude that CD4+IL10+ expression in nephrotic syndrome patient (new-onset and rare relapses) were higher in prednisone + vitamin D3 treated group than prednisone-only treated group. This study also concluded that there is no correlation between CD4+IL10+ expression and CD3+GR expression nor CD4+IL10+ expression and clinical outcome.
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