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## Evaluation of the Effect of IL-22 on Human Cord Blood CD4<sup>+</sup> T Cells

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### ABSTRACT

IL-22 is a member of IL-10 cytokine family which is believed to play an important role in inflammatory responses. IL-22 has similarities with IL-10 including conserved sequences with IL-10. IL-22 receptor is also comprised of two chains known as L-22R1 and L-10R2; supporting the speculation that the two cytokines may have similar effects.

The aim of this study was to shed some light on the biological activity of IL-22 upon the cord blood CD4<sup>+</sup>CD25<sup>-</sup> T cells. In this research, cord blood T CD4<sup>+</sup>CD25<sup>-</sup> cells were cultured in presence of anti CD2/CD3/CD28 coated beads, IL-2 and IL-22 for two weeks at 37 °C and 5% CO<sub>2</sub>. Flow cytometry analysis showed that IL-22 has no effect upon CD25 and Foxp3 expression.

Also, the results indicated that IL-22 is not involved in CD4<sup>+</sup> T cell proliferation. Moreover, the results of suppression assay did not show any suppression effect on the cultured T cells. Thus, it seems that umbilical cord blood T cells probably do not express IL-22R1 on their surface.

**Key words:** Cord blood; Foxp3; IL-22; Regulatory T cells

### INTRODUCTION

During the last decade, cord blood has been regarded as an alternative source to recover hematopoietic cells for allogenic transplantation. Cord blood has the advantage of easy access and presence of naïve T cells in it. This is considered property as a reason for decreased

GVHD in transplant recipients.<sup>1-3</sup> At present, novel strategies for increasing engraftment, decreasing GVHD, and transplant related mortality and an increase in graft versus leukemia (GVL) in cord blood transplantation are being studied especially in adults. Among these strategies, simultaneous injection of cord blood stem cells and T regulatory cells may be considered, in which cell-cell signaling and cytokine production regulate immune responses and are effective in increasing engraftment, decreasing neutrophil regeneration time and decreasing GVHD.<sup>4</sup>

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IL-22, also known as IL-TIF (IL-10 related T cell derived inducible factor), is a member of IL-10 cytokine family. It is an inflammatory cytokine secreted by T cells (especially Th1 cells), mast cells, thymus, brain, increasing the production of acute phase proteins.<sup>5,6</sup>

Two molecular chains have been recognized as IL-22 receptors, including CRF2-9 (IL-22R1) and CRF2-4 (IL-10RB or IL-10R2). The former is specific for IL-22 and has homology with the second IL-10 receptor, while the latter is shared by both IL-10 and IL-22 and is required for signaling. IL-22R1 has limited expression with the highest expression in acinar pancreatic cells.

It is also expressed in lower amounts (but higher efficiency) in skin, colon, liver and kidney. The effect of IL-22 is similar to IL-6 and therefore it is known as an inflammatory cytokine. IL-22 and IL-10 have different binding affinity for CRF2-4. CRF2-4 alone is sufficient for binding to IL-22 but the presence of the second receptor is necessary for effective IL-10 binding. CRF2-9 and CRF2-4 are also very similar to IFN $\gamma$  receptor (IFN $\gamma$ R $\alpha$ ).<sup>7-9</sup>

Moreover, we know that IL-10 is a multi functional cytokine formerly known as cytokine synthesis inhibitory factor. Recently, the suppressive effect of IL-10 on T cells has been shown in blocking the CD28 signaling pathway and consecutive function of PI-3 kinase. In this study we intended to evaluate the effect of IL-22 on cord blood T cells in induction of T regulatory cells considering the similar structure of this cytokine and its receptor with IL-10.<sup>10,11</sup>

## MATERIALS AND METHODS

### Cord Blood Sample

All cord blood samples were recovered by gynecologist in sterile conditions after normal full term delivery from mothers with a history of normal pregnancy. Each cord blood sample was collected in 50 ml Falcon tubes containing EDTA. The cord blood sample had each a volume between 20 to 50 ml and was transferred to cell culture laboratory in 4 °C container.

### Isolation of CD4<sup>+</sup> T Cells from Mononuclear Cord Blood by MACS Column

After cord blood sampling, the nucleated RBC (nRBC) were removed with hydroxyethyl starch (Fresenius, Bach Homburg, Germany) and then the mononuclear cells were isolated using Ficol

(Cedarlane, Ontario, Canada). Then, CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T cell Isolation kit (Biotec Miltenyi GmbH, Gladbach, Germany) was used to isolate TCD4<sup>+</sup> cells. The cells were isolated in two stages. In the first step, non CD4 cells were isolated using a set of biotin conjugated antibodies and anti biotin beads by LD column. In the second step, CD4<sup>+</sup>CD25<sup>+</sup> cells were isolated using CD25 microbeads using MS columns.

### Cell Culture

After isolating T CD4<sup>+</sup>CD25<sup>+</sup> cells, they were divided into 4 groups. In each group, T cells were treated with different combinations of RPMI 1640 culture medium containing 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin (All from GIBCO, Life Technologies, Merelbeke, Belgium) as follows:

Group 1: T cells + IL-2

Group 2: T cells + Anti CD2/CD3/CD28 coated bead

Group 3: T cells + IL-2+ Anti CD2/CD3/CD28 coated bead

Group 4: T cells + IL-2+ Anti CD2/CD3/CD28 coated bead + IL-22

In each group 10<sup>6</sup> cells were used. 20 ng/ml IL-2 (R&D systems, Minneapolis, MN, USA) and 100 ng/ml IL-22 (R&D systems, Minneapolis, MN, USA) were added to culture medium for 10<sup>6</sup> cells in respective groups. 5 $\mu$ g/ml Anti-Biotin MACSiBead particle (Miltenyi Biotec GmbH, Gladbach, Germany) was also added to each group. For proliferation of T cells, a Bead/cell ratio of 1:2 was selected. Then, the cells were incubated for two weeks at 37 °C and 5% CO<sub>2</sub>. Every three to four days, IL-2 and IL-22 were added to each group of cells.

### Evaluation of Surface Markers Using Flow Cytometry

CD4 T cells were evaluated for CD4 and CD25 markers using mouse anti-human CD4 FITC-conjugated and anti-human CD25 PE-conjugated antibodies before and after cultures. Mouse IgG1 FITC-conjugated and Mouse IgG2b PE-conjugated antibodies were used for isotype control (All antibodies were purchased from Miltenyi Biotec GmbH, Gladbach, Germany). For this reason, nearly 5 $\times$ 10<sup>5</sup> cells from each group were suspended in 100  $\mu$ l buffer containing 0.5% BSA and 2mM EDTA; then, 10  $\mu$ l of the above antibodies was also added to cells and incubated in dark for 10 minutes

at 4-8 °C. 10 µl isotype control was also added to control group. Then the cells were washed using the buffer and suspended in 1 ml buffer to perform flow cytometry.

### Evaluation of Foxp3 Gene Expression Using Flow Cytometry

CD4 T cells were evaluated for Foxp3 marker by mouse anti-human Foxp3 APC-conjugated antibody and FoxP3 Staining Buffer Set (both from Miltenyi Biotec, GmbH, Gladbach, Germany) before and after cultures. Mouse IgG1 APC-conjugated antibody (Miltenyi Biotec, GmbH, Gladbach, Germany) was used for isotype control.

For intracellular staining of Foxp3, 1 ml freshly prepared Fixation/Permeabilization (1:4) working solution (Miltenyi Biotec, GmbH, Gladbach, Germany) was added to each sample, followed by twice washings with 1-2 ml 1X Permeabilization Buffer (Miltenyi Biotec, GmbH, Gladbach, Germany).

The samples were then incubated with 10 µl Anti Foxp3 APC in the dark for at least 30 min at 4 °C. Thereafter, cells were washed twice with 1-2 ml 1X Permeabilization Buffer, and then resuspended in suitable amount of buffer for analysis by flow cytometry.

### Evaluation of T Cell Proliferation

After isolating CD4<sup>+</sup>CD25<sup>-</sup> T cells, they were divided in 4 groups, and 2 × 10<sup>4</sup> T cells with different combinations as mentioned previously were treated in 96 well plates. The cells were incubated for 72 hours at 37 °C and 5% CO<sub>2</sub>. During this period Cell Proliferation ELISA, BrdU kit (Roche Diagnostics, Mannheim, Germany) was used to assay T cell proliferation.

### Reverse Transcriptase PCR (RT-PCR)

To evaluate the expression of Foxp3 gene using RT-PCR, at first total cell RNA was extracted from cells before and after cultures using RNA extraction kit (Isogene Lab.ltd, Moscow, Russia). Then, reverse transcription reaction was performed and the cDNA produced was used as template for PCR reaction. Extracted RNA from T regulatory cells was also used as positive control. To do PCR, at first master mix (containing 10X buffer, MgCl<sub>2</sub> buffer, dNTP and water) was added in required quantities to each vial, and then other components including cDNA, Forward (5'-AGTGCTTTGTCTGGGGTGGAGAG-3') and reverse

(TCTGTGTGGCTGGTTGTGAAGGC-3') primers and Taq polymerase enzyme were added. The PCR schedule for Foxp3 was as follows: 95°C for 10 min, 40 cycles of 94°C for 1min, 59°C for 1 min and 72°C for 1 min and then 72°C for 10 min. After PCR, to assure amplification of desired region, PCR products were electrophoresed on 1% agarose gel and stained using ethidium bromide. In this experiment a 421 bp band was observed.

### Suppression Assay of Cultured T Cells

This method was used to assess the suppressive potential of T cells. CD4<sup>+</sup>CD25<sup>-</sup> T cells were divided in to 4 groups and in each group 10<sup>6</sup> T cells were treated with different combinations as mentioned previously in 24 well plates. The cells were incubated for two weeks at 37 °C and 5% CO<sub>2</sub>. Then, Anti-Biotin MACSiBead particles were removed from culture medium using MACSiMAG separator (Miltenyi Biotec, GmbH, Gladbach, Germany).

After removing the particles from culture medium, 10<sup>5</sup> cultured T cells from each group were cultured together with autologous CD4<sup>+</sup>CD25<sup>-</sup> T cells in 1:1 ratio in 96 well plates for three days at 37 °C in 5% CO<sub>2</sub> incubator. Then, the Cell Proliferation ELISA, BrdU kit (Roche Diagnostics, Mannheim, Germany) was used to assay T cell proliferation.

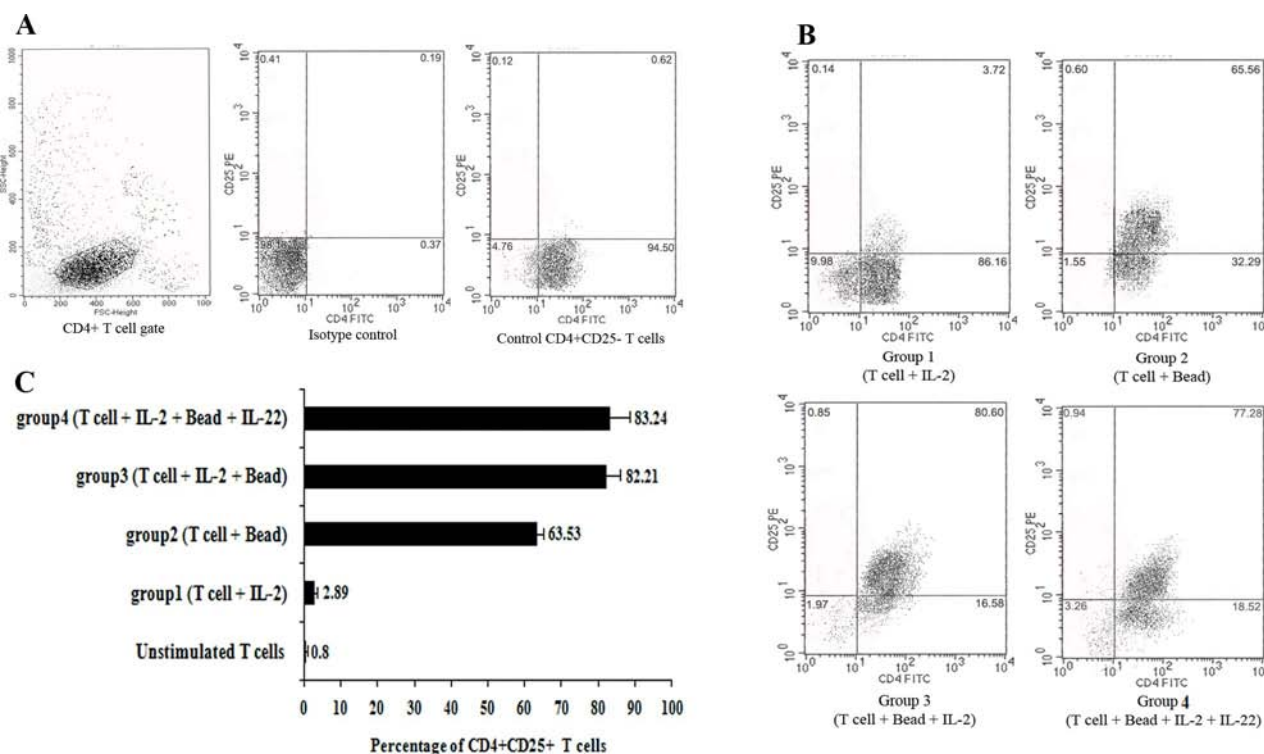
### Statistical Analysis

To evaluate mean variation for data analysis for significance, Student T test was used. In all cases significance level was regarded as p<0.05.

## RESULTS

### The Effect of IL-22 on Percentage of T CD4<sup>+</sup>CD25<sup>+</sup> Cells

The results of flow cytometry using CD4 marker indicated that more than 90 percent of cord blood T CD4<sup>+</sup>CD25<sup>-</sup> cells express CD4 marker on their surface (Figure 1A). After two weeks of culture, the percentage of T CD4<sup>+</sup>CD25<sup>+</sup> cells in culture cell groups was compared with control group (unstimulated T CD4<sup>+</sup>CD25<sup>-</sup> cells) Fig 1B and 1C. Concerning the percentage of T CD4<sup>+</sup>CD25<sup>+</sup> cells, comparison of different groups with control group showed that except for group 1, other groups showed significant increase relative to control group (P<0.05).



**Figure 1.** Evaluation of the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells in different groups. After isolating CD4<sup>+</sup>CD25<sup>+</sup> T cells, they were divided into four groups already mentioned, and in each group, the T cells were treated with different combinations of IL-2 and anti-CD2/CD3/CD28 coated beads and IL-22 in culture medium. A) Flow cytometry analysis before culture indicating more than 90 percent of cord blood CD4<sup>+</sup>CD25<sup>+</sup> T cells, express CD4 marker. B) After two weeks of culture, the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells in cultured cell groups was compared with control group (unstimulated CD4<sup>+</sup>CD25<sup>+</sup> T cells). C) The diagram shows the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells in various cell groups after a two weeks culture.

Also, in comparison of anti CD2/CD3/CD28 coated bead containing groups (groups 2, 3 and 4) with group lacking anti CD2/CD3/CD28 coated bead (group 1), there was a significant increase in percentage of T CD4<sup>+</sup>CD25<sup>+</sup> cells ( $P < 0.05$ ).

Comparison of anti CD2/CD3/CD28 coated bead and IL-2 containing groups (groups 3 and 4) with group containing only anti CD2/CD3/CD28 coated beads (group 2) also displayed this significant increase. Comparison between anti CD2/CD3/CD28 coated bead, IL-2 and IL-22 containing group (group 4) with group containing only anti CD2/CD3/CD28 coated bead and IL-2 (group 3) did not show a significant increase in the percentage of T CD4<sup>+</sup>CD25<sup>+</sup> cells.

Thus, the results indicate that presence of anti CD2/CD3/CD28 coated bead alone or together with IL-2 in culture medium can increase the percentage of T CD4<sup>+</sup>CD25<sup>+</sup> cells, but the results for IL-22 showed that this cytokine has no effect upon increasing percentage of T CD4<sup>+</sup>CD25<sup>+</sup> cells.

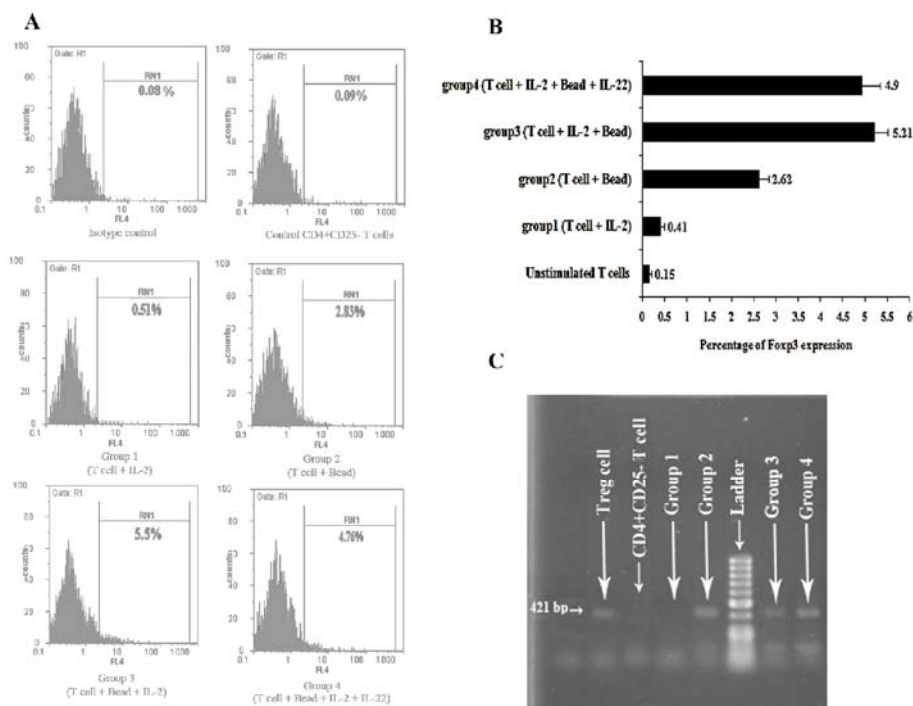
#### Analysis of CD4<sup>+</sup> T Cells for Foxp3 Expression

Figure 2A and 2B show the flow cytometry analysis of CD4<sup>+</sup> T cells for Foxp3 expression. For Foxp3 expression, comparison of different groups with control group showed that except for group 1, other groups showed significant increase relative to group control ( $P < 0.05$ ). In comparison of anti CD2/CD3/CD28 coated bead containing groups (groups 2, 3 and 4) with group without anti CD2/CD3/CD28 coated bead (group 1) there was a significant increase in Foxp3 expression ( $P < 0.05$ ).

Comparison of anti CD2/CD3/CD28 coated bead and IL-2 containing groups (groups 3 and 4) with group containing only anti CD2/CD3/CD28 coated bead (group 2) also showed this significant increase.

There was no significant difference in increasing expression of Foxp3 in comparison between anti CD2/CD3/CD28 coated bead, IL-2 and IL-22 containing group (group 4) with group containing only anti CD2/CD3/CD28 coated bead and IL-2 (group 3).

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**Figure 2.** The frequency of Foxp3<sup>+</sup> T cells in different groups. **A)** Histogram shows the percentage of Foxp3 expression in cultured cell groups in comparison with control group (unstimulated CD4+CD25- T cells). **B)** Diagram showing percentage of Foxp3 expression in different cell groups after two weeks of culture. **C)** Evaluation of Foxp3 gene expression using RT-PCR showed that only in groups containing anti CD2/CD3/CD28 coated bead (groups 2, 3 and 4) band 421 bp is visible

Therefore, the results showed that presence of anti CD2/CD3/CD28 coated bead alone or together with IL-2 in culture medium can cause Foxp3 expression, but for IL-22 the results showed that it had no role in increasing Foxp3 expression. Evaluation of Foxp3 gene expression using RT-PCR technique showed that only in anti CD2/CD3/CD28 coated bead containing groups (groups 2, 3 and 4) the 421 bp is visible (Figure 2C).

#### Evaluation of CD4<sup>+</sup> T Cell Proliferation in Presence of IL-22

For T cell proliferation in all groups except group1, our results showed significant increase in comparison with control group (T cells alone) ( $P < 0.05$ ). In comparison of anti CD2/CD3/CD28 coated bead containing groups (groups 2, 3 and 4) with group without anti CD2/CD3/CD28 coated bead (group 1), significant increase was observed in T cell proliferation ( $P < 0.05$ ).

This significant difference was also observed in groups 3 and 4 (groups containing anti CD2/CD3/CD28 coated bead and IL-2) in comparison with group 2 (containing only anti CD2/CD3/CD28 coated bead). Comparison of group 4 (containing anti

CD2/CD3/CD28 coated bead, IL-2 and IL-22) with group 3 (containing anti CD2/CD3/CD28 coated bead and IL-2) indicated no significant increase in proliferation of T cells.

Therefore, the results clearly showed that presence of anti CD2/CD3/CD28 coated bead alone or together with IL-2 in culture medium can increase T cell proliferation but for IL-22 the results showed that it had no effect on proliferation (Figure 3).

#### Suppression Assay of CD4<sup>+</sup> T Cells after Two Weeks of Culture

Figure 4 shows the effect of simultaneous culture of CD4<sup>+</sup> T cells cultured in each group with autologous CD4<sup>+</sup>CD25<sup>-</sup> T cells in 1:1 ratio. The results showed that cultured T cells in four groups showed no suppression effect.

#### DISCUSSION

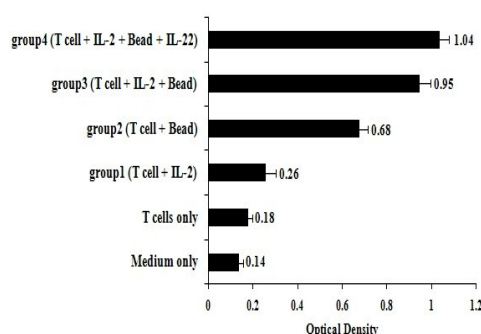
As previously stated, one of the strategies for increasing engraftment is simultaneous injection of cord blood stem cells and T regulatory cells,<sup>4</sup> but due to shortage of T regulatory cells in peripheral and cord



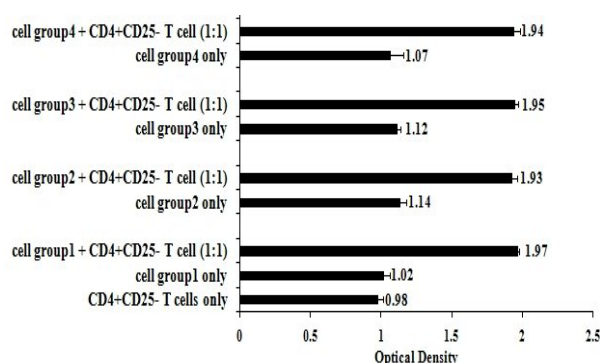
blood, various methods such as proliferation or induction of T regulatory cells have been assigned to expand T regulatory cells without compromising their suppression effect.<sup>12-16</sup> In this study, IL-22 a cytokine with a shared receptor chain with IL-10 was used for probable induction of T regulatory cells.

Several markers such as GITR, CTLA-4, CD25 and Foxp3 have been suggested which identify T regulatory cells,<sup>17</sup> the last two markers were used in our research.

CD25 is a marker of T cell function, therefore T regulatory cells cannot be distinguished from CD4<sup>+</sup>CD25<sup>-</sup> T cells by this molecule, and CD25 can only be used as an indirect marker for CD4 T regulatory cells.<sup>12, 18-20</sup>



**Figure 3. Proliferation assay of T cells in different groups.**  $2 \times 10^4$  CD4<sup>+</sup>CD25<sup>-</sup> T cells with different combinations of anti CD2/CD3/CD28 and cytokines (IL-2 and IL-22) as already mentioned were treated in 96 well plates. The cells were cultured at 37°C and 5% CO<sub>2</sub> for 72 hours. Then, T cell proliferation assay using Cell Proliferation ELISA, was performed.



**Figure 4. Suppression assay of T cells cultured in diverse groups.** In order to evaluate the suppression effect,  $10^5$  cultured T cells in each group were cultured with autologous CD4<sup>+</sup>CD25<sup>-</sup> T cells incubated for three days at 37°C and 5% CO<sub>2</sub> in 1:1 ratio in 96 well plates. Then, to assay proliferation of T cells the Cell Proliferation ELISA, was used.

The results depicted in Figure 1 also showed that T cells activated by anti CD2/CD3/CD28 coated bead (groups 2, 3 and 4) showed a significant increase in CD25 expression comparing with groups without anti CD2/CD3/CD28 coated bead (groups 1 and control).

These results seem logical because for full T cell proliferation both TCR and co-stimulatory signals such as CD28 are required<sup>21-23</sup> (anti CD2/CD3/CD28 coated beads mimic this signal). Therefore, an obvious increase in expression of activity markers such as CD25 and consequently increase in CD4<sup>+</sup>CD25<sup>+</sup> T cell percentages were observed. Addition of IL-2 to culture medium can cause further increase in CD25 expression since studies have shown that anti CD3 and anti CD28 can increase IL-2 expression in T cells.<sup>24-26</sup> Therefore, these data show that significant increase in CD25 marker expression in anti CD2/CD3/CD28 coated bead and IL-2 containing groups compared with group containing anti CD2/CD3/CD28 coated bead alone are rational.

The results of this research indicated that IL-22 has no role in increasing CD25 expression. In our previous study we have reported that IL-28 with shared receptor with IL-22 has no role in increasing CD25 expression.<sup>27</sup> Oral et al also showed in a study that stimulation of CD45RA<sup>+</sup> T cells in peripheral blood using anti CD2, anti CD28 and anti CD3 in presence of IL-19, IL-20, IL-22 and IL-24 had no effect on CD25, CD69, HLA-DR, GITR or PD-1 expression.<sup>28</sup>

Our results on Foxp3 expression also showed that significant difference was observed in anti CD2/CD3/CD28 coated bead treated groups compared to control group. Different results have been reported about Foxp3 molecule. Some researchers showed that in humans, Foxp3 expression is seen in active CD4 cells with no suppressive effect.<sup>29-31</sup> Pillai et al showed that all CD4<sup>+</sup>CD25<sup>-</sup> T cells stimulated by anti CD3 and anti CD28 transiently express Foxp3.<sup>32</sup> Thus, significant increase in Foxp3 in groups stimulated by anti CD2/CD3/CD28 coated bead seems to be logical. There are of course reports indicating lack of Foxp3 expression by stimulated T cells. Yagi et al have reported that stimulation of naïve human CD4<sup>+</sup>CD25<sup>-</sup> T cells cannot cause Foxp3 expression.<sup>33</sup>

In vitro study of naïve T cells showed that these cells were converted to Foxp3<sup>+</sup> cells in presence of TGF-β and IL-2,<sup>34, 35</sup> but the results of this study on the effect of IL-22 on Foxp3 expression showed that it had no effect on expression of this molecule in activated CD4 T cells. In our previous study on IL-28A also

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similar results were observed.<sup>27</sup> Other studies indicated that IL-22 may be used in indirect induction of Foxp3. In a study, analysis of Foxp3 expression by Real time PCR in CD4 T cells of peripheral blood showed that when these cells were cultured with dendritic cells treated with IFN- $\lambda$  and IFN- $\beta$ , Foxp3 was expressed in these CD4 T cells.<sup>36</sup>

Regarding CD4 T cell proliferation, the results showed that although groups treated with anti CD2/CD3/CD28 coated bead compared with groups lacking it exhibited significant increase, the results of this research showed that IL-22 had no role in proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells of cord blood activated by anti CD2, anti CD3 and anti CD28 in presence of IL-2. Oral et al also treated peripheral blood mononuclear cells with various stimuli such as PLA, bee venom, anti CD3 and anti CD28 to evaluate the effect of IL-10 and IL-10 family cytokines (IL-19, IL-20, IL-22 and IL-24) on T cell proliferation. They showed that IL-10 suppressed proliferation of T cells activated by PLA and anti CD28 in PBMC. However, no cytokine of this family showed suppression effect. They also observed that neither IL-10 nor IL-10 family cytokines had any suppression effect on proliferation of T cells induced by anti CD3.<sup>28</sup> Therefore, it seems that stimulus type and cytokine amount can be effective on proliferation of T cells. However, in some cultures their results showed that IL-19, IL-20 and IL-24 increased T cell proliferation.<sup>28</sup>

Therefore, according to data and results of this research, although IL-22 is produced through stimulation of T cells by some stimuli such as Con A,<sup>37</sup> it did not induce proliferative effect on cord blood T cells stimulated by anti CD2/CD3/CD28 coated bead. Considering the fact the molecules such as CD25, Foxp3, CTLA-4, GITR and CD127 alone or in combination cannot be used in definitive T regulatory cell identification<sup>17</sup> therefore, in this research, for elucidating the suppressive effect of cultured T cells, the suppression assay was also used. The results indicated that these cells had no suppressive effect. In our previous research the results showed that these cells have no suppressive effect. In our previous study also the results showed that stimulation of cord blood T cells with anti CD2, anti CD3 and anti CD28 in presence of IL-2 and IL-28A had no effect in inducing suppression effect of these cells.<sup>27</sup> Thus, it seems that IL-22, unlike many cytokines, instead of being effective on cord blood CD4<sup>+</sup> T cells, is secreted by immune system cells

and is effective upon other tissues and cells such as hepatocytes, pancreatic cells, etc.<sup>38</sup> On the other hand, it may be possible to get better results through studying the effect of this cytokine on other immune cells such as dendritic cells for inducing T regulatory cells. Thus, it may be due to the lack of IL-22R1 on the surface of umbilical cord blood T cells.

Further studies on the signaling mechanisms and mediators involved could elucidate the similarities between members of this family of cytokines and the effects that they exert on various cell populations.

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