The Effect of IL-22 and IL-28 in Induction of Type 1 Regulatory T (Tr1) Cells

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ABSTRACT

Cytokines have been introduced as critical inducers in the development of Th subpopulations.Cytokines like IL-10 are involved in inducing regulatory T cells such as Type 1 regulatory T (Tr1) cells cells. IL-22 is a member of IL-10 family of cytokines, and IL-28A is a member of IFN-γ family.

In this study, cord blood mononuclear cells (CBMC) from normal healthy individuals were isolated by Ficoll and then naïve T cells were purified by CD4+CD25+ Regulatory T cell Isolation kit. The effect of these two cytokines on production of IL-5, TGF-β, IL-10, IL-4 and IFN-γ cytokines from cord blood T cells was investigated to identify Tr1 cells as well as Th1 and Th2 polarization.

Flow cytometric analysis showed that IL-28A and IL-22 were not effective in expression of IL-5 and TGF-β either alone or in synergy, but in view of IL-10, IL-4 and IFN-γ, the results showed that IL-22 increased IL-10 and IL-4 but had a decreasing effect on IFN-γ. The results showed that IL-28A was not effective in increasing or decreasing the level of IL-10, IL-4 and IFN-γ.

Therefore, according to these results, IL-22 and IL-28A were not effective in inducing Tr1 cells.

Keywords: Cord blood; IL-22; IL-28A; Polarization; Tr1 cells

INTRODUCTION

Among the induced T regulatory cells, type 1

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regulatory T (Tr1) cell subpopulation has been extensively described.

It has been demonstrated that murine CD4+ T cells in mouse and humans in the presence of IL-10 generate T cell clones with a cytokine profile different than T helper (Th)1 and Th2 cells. Th1 cells secrete IL-2, IFN-γ and TNF-β cytokines, and are effective in elimination of intracellular pathogens such as Listeria by activation of

macrophages. Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 cytokines, causing humoral immunity mainly against worms. [1-3]

Tr1 cells are characterized based on their unique pattern of cytokine production that is distinct from Th1 and Th2 cells. These cells produce high levels of IL-10, significant levels of IFN-γ, TGF-β and IL-5 but lack IL-4, and produce no IL-2 or only produce low levels of it. [1-3]

Overall, Th1 cells produce pro-inflammatory cytokines enhancing cellular immunity, while Th2 cells promote anti-inflammatory cytokines such as IL-10, which enhance humoral immunity. Tr1 cells also have regulatory effects. [4]

IL-22 (also known as IL-10-related T cell-derived inducible factor) is a member of IL-10 cytokine family. This cytokine is secreted by T cells (especially Th1 cells) upon their activation by IL-9 as well as thymic and brain mast cells after activation by Concanavalin A (Con A), indicating the pleiotropic property of this cytokine both within and outside the immune system. [5]

The receptor of this cytokine is composed of two parts, which are both a member of type II family of cytokine receptors known as CRF2-9 (IL-22 R1) and CRF2-4 (IL-10 RB or IL-10 R2). The former is specific for IL-22 while the latter is common in human IL-10 and IL-22, and is necessary for signaling. [5,6]

Tissue expression of IL-22 R1 is very limited (it is present in high levels in the pancreas and in low levels in gastrointestinal tract, skin, kidneys and liver), whereas IL-10 R2 is extensively expressed. [7-9]

IL-28A is also a member of INF-λ family, and is known as INF-λ2. This cytokine, along with IL-28B (INF-λ3) and IL-29 (INF-λ1), is a type III interferon (IFN-III). These cytokines are very similar in terms of structure and are functionally antiviral. [10,11] They all signal the target cells by a receptor complex composed of IL-28R1 and IL-10R2. IL-28R1 is a class II receptor family member (IFN-I and IFN-II receptors and IL-10 family cytokines also belong to this category). IL-10R2 is a member of the IL-10, IL-22 and IL-26 family of receptors. [11-13] The overlap and some similarities of IL-22 and IL-28A cytokines with IL-10 in receptor subunits and signal transduction pathways posed the question of the ability of these cytokines in induction of regulatory cells similar to IL-10. For this reason, in this study, the cytokines

TGF-β, IL-10, IL-5, IL-4 and IFN-γ were studied to evaluate the possibility of induction of Tr1 cells in T CD4$^+$ cord blood cells.

MATERIALS AND METHODS

Cord Blood Sample

All cord blood samples were obtained after normal full term delivery from mothers with a history of normal pregnancy at Valiasr Obstetrics and Gynecology Hospital Tehran, Iran. The cord blood samples had each a volume between 20 to 50 ml, and were screened for genetic disorders, hematologic abnormalities or infectious diseases. This study was approved by the Ethics Committee of Tehran University of Medical Sciences and Health Services, and informed consent was obtained before samples were collected.

Reagents and Media

RPMI 1640 culture medium containing 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin was used. All reagents were purchased from GIBCO (Life Technologies, Merelbeke), and Ficoll-Paque was purchased from Cedarlane (Hornby, Ontario, Canada). Mouse anti-human TGF-β PE-conjugated, Mouse anti -human IL-5 FITC-conjugated, hIL-2, hIL-22 and hIL-28 were purchased from R&D systems (Minneapolis, MN, USA). CD4CD25 regulatory T cell isolation kit, T Cell Activation Expansion Kit, mouse anti-human IFN-γ FITC-conjugated, mouse anti-human IL-4 PE-conjugated, mouse anti-human IL-10 APC-conjugated, isotype controls, mouse IgG2a FITC-conjugated, mouse IgG2b PE-conjugated and mouse IgG1 APC-conjugated were purchased from Miltenyi Biotec GmbH (Gladbach, Germany), and BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit was purchased from BD.

Isolation of Human Cord Blood CD4+ T Cell

Cord blood mononuclear cells (CBMC) were isolated by density gradient sedimentation using Ficoll-Paque. Then, CMBC were used to isolate CD4+CD25- T cells by CD4+CD25+ Regulatory T cell Isolation kit. In brief, in the first step, CD4+ T cells were isolated through negative selection by removing all other cell types using LD column. In the second step, CD4+CD25+ and CD4+CD25− T cell

populations were isolated using CD25 microbeads using MS columns. Purity of sorted populations was ≥95% as determined by flow cytometric analysis.

CD4 T Cell Culture

Isolated CD4+ CD25− T cells were divided into six groups and cultured in 48-well microtiter plates as follows:

Group1: 1×10^6 CD4+ T cells + 20ng/ml IL-2

Group2: 1×10^6 CD4+ T cells + 5µg/ml MACS Anti-Biotin MACSi Bead Particles (bead-to-cell ratio 1:2)

Group3: 1×10^6 CD4+ T cells + 20ng/ml IL-2 + 5µg/ml MACS Anti-Biotin MACSiBead Particles

Group4: 1×10^6 CD4+ T cells + 20ng/ml IL-2 + 5µg/ml MACS Anti-Biotin MACSiBead Particles + 20ng/ml IL-28A

Group5: 1×10^6 CD4+ T cells + 20ng/ml IL-2 + 5µg/ml MACS Anti-Biotin MACSiBead Particles + 100ng/ml IL-22

Group6: 1×10^6 CD4+ T cells + 20ng/ml IL-2 + 5µg/ml MACS Anti-Biotin MACSiBead Particles + 20ng/ml IL-28A + 100 ng/ml IL-22

The cells were incubated for two weeks at 37°C in 5% CO2. Every three to four days, IL-2, IL-28A and IL-22 (at the above mentioned doses) were added to the corresponding groups.

Intracellular Cytokine Assay Using Flow Cytometry

For evaluation of IL-4, IL-5, INF-γ, TGF-β and IL-10, CD4+ T cells were stained before and after cell culture with the corresponding antibodies as mentioned in the materials and methods section.

In brief, isolated CD4+ T cells were resuspended (1×106 cells/ ml) with Anti-Biotin MACSi Bead particles (5 µg/ml) for 2 hours in 37°C in 5% CO2. Then, monensin (0.6µl/ml) was added to the cells and reincubated for 6 hours in 37^{0}C. The cell suspensions were fixed and permeabilized with the Cytofix/ Cytoperm kit (BD PharMingen) according to the manufacturer's instructions.

Permeabilized cells were stained with different conjugated antibodies as mentioned above for 30 min in 4-8°C. Then, T cells were phenotypically analyzed by three color fluorescence.

In Vitro Suppression Assay

This method was used to assess the suppressive potential of T cells. First, CD4+CD25− T cells were divided into six groups with different treatments as mentioned above. 10^6 cells/mL were cultured in a U-bottomed 96-well plates in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin for 2 weeks. Then, Anti-Biotin MACSiBead particles were removed from culture medium using MACSiMAG separator. To determine the suppressive capacity of cultured cells, 10^5 cultured T cells from each group were cultured together with autologous CD4+CD25- T cells in 1:1 ratio in 96 well plates for three days at 37°C in 5% CO2 incubator. T cells proliferation was induced by stimulation with MACS Anti-Biotin MACSiBead particles (bead-to-cell ratio 1:1) in 96-well round bottomed plates. Thereafter, proliferation assay was performed by Cell Proliferation ELISA, BrdU kit.

Statistical Analysis

The experiments were conducted in triplicate. To evaluate the mean variation for data analysis for significance, Student T test was used. A p value of <0.05 was considered significant.

RESULTS

Flow Cytometry Analysis of CD4+ T Cells for Expression of IFN-γ and IL-4

In comparison of the mentioned groups with control for the expression of IL-4, significant difference (p <0.05) was only observed in group 5 (T cells + IL-2 + bead + IL-22) and group 6 (T cells + IL-2 + bead + IL-22 + IL-28A), while for the expression of IFN-γ, group 3 (T-cell + IL-2 + bead) and 4 (T-cell + IL-2 + bead + IL-28A) showed significant difference compared to control (p <0.05).

In comparison to the groups with bead (groups 2, 3, 4, 5 and 6) and the group without it (group1), only groups 5 and 6 showed a significant increase in the expression of IL-4 (p<0.05). For IFN-γ, only groups 3 and 4 showed a significant increase in the expression of this cytokine (p<0.05).

Moreover, in comparison between groups with bead, IL-2 and the IL-22 and IL-28A cytokines (groups 4, 5 and 6) with the group having only bead and IL-2 (Group 3), it was shown that the groups 5 and 6, unlike group 4, showed a significant increase in the expression of IL-4 (p<0.05), while this comparison for IFN-γ showed a significant difference with group 3 (Figure 1).
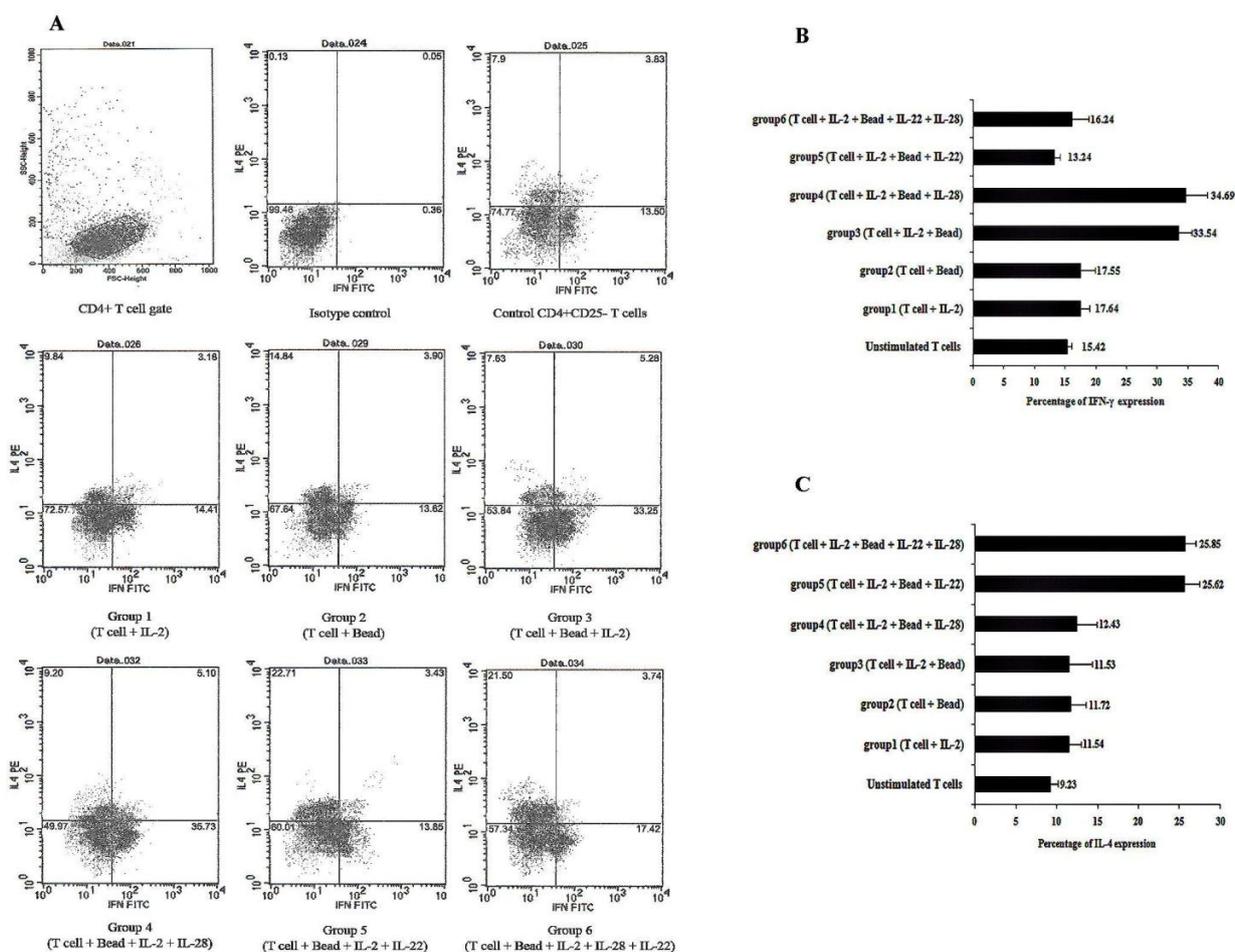
Figure 1. Evaluation of IL-4 and IFN-γ expression in four groups after two weeks of culture. Isolated CD4+ CD25− T cells were divided in four groups as described in section 2 and cultured in 48-well microtiter plates. Then, these cells were cultured for two weeks at 37°C and 5% CO2 in incubator. After this time, T cells were stained for intracellular cytokines (IL-4, IFN-γ). (A) Flow cytometry analysis of cultured cells in four groups. (B, C) Percentage of IL-4 and IFN-γ expression among different groups (*p*<0.05)

Flow Cytometry Analysis of CD4+ T Cells for Expression of TGF-β and IL-5

After two weeks of culture of CD4+ CD25- T cells in different groups, the percentage of TGF-β and IL-5 expression in these groups was compared with controls (unstimulated CD4+ CD25- T cells).

For the expression of TGF-β and IL-5 in cultured cells, comparison of the different groups with control revealed that except for group 1 (T-cell + IL-2), the other groups showed significant differences (*p*<0.05). In comparison to the groups with bead (groups 2, 3, 4, 5 and 6) and without bead (group 1), a significant increase in the expression of these cytokines was

observed (*p*<0.05). In comparison to the groups with bead and IL-2 (Group 3, 4, 5 and 6) and the group with only bead (group 2), no significant difference was seen. The comparison between groups with bead, IL-2 and IL-22 and IL-28A cytokines (groups 4, 5 and 6) with the group having only bead and IL-2 (Group 3) showed no significant difference (Figure 2).

Results of Flow Cytometry Analysis of CD4+ T Cells for IL-10 Expression

Figure 3 shows flow cytometry analysis of CD4+ T cells for expression of IL-10. In this regard, comparison of different groups with control showed no
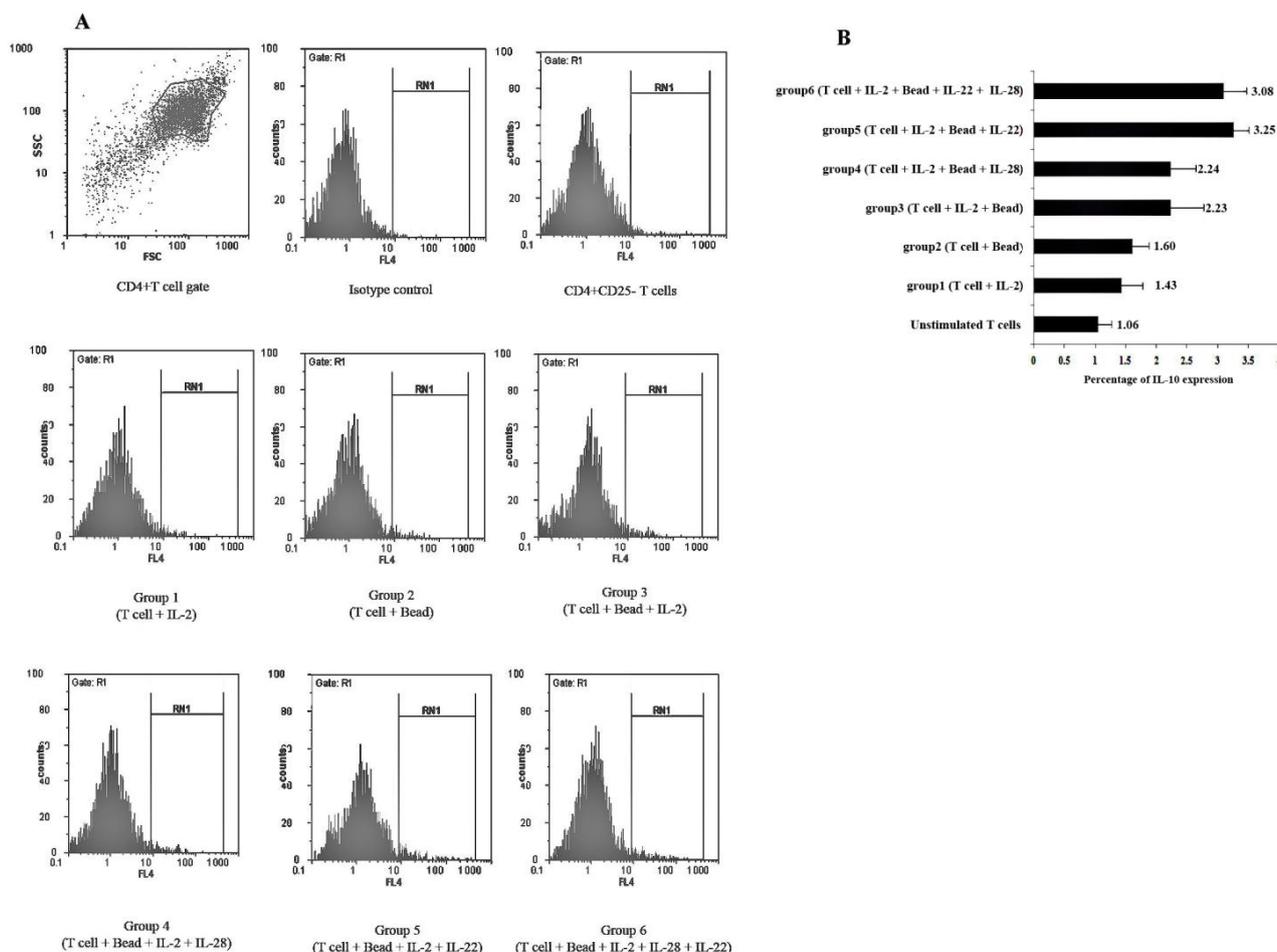
Figure 2. The percentage of IL-5 and TGF-β in different groups. A) Histogram shows the percentage of Foxp3 expression in cultured cell groups in comparison with control group (unstimulated CD4+CD25- T cells). B,C) Diagram showing percentage of IL-5 and TGF-β expression in different cell groups after two weeks of culture (p<0.05).

significant difference between group 1 (T-cell + IL-2), group 2 (cells T + bead), group 3 (T + cell IL-2 + bead) and 4 (T cells + IL-2 + bead + IL-28A) with the control group, while in group 5 (T cells + IL-2 + bead + IL-22)and 6 (T cells + IL-2 + bead + IL-22 + IL-28A), the difference was statistically significant ($p<0.05$).

In comparison of groups with bead (groups 2, 3, 4, 5 and 6) with those without bead (group1), only groups 5 and 6 showed a significant increase in the expression of IL-10 (P <0.05). In comparison of groups with bead and IL-2 (group 3, 4, 5 and 6) with the group with only bead (group 2), only groups 5 and 6 showed a significant increase in the expression of IL-10. Comparison between groups with bead, IL-2, IL-22 and

IL-28A (groups 4, 5 and 6) with the group only having bead and IL-2 (Group 3) showed that unlike group 4, the groups 5 and 6 showed a significant increase in the expression of IL-10 ($p<0.05$).

The Effect of IL-22 and IL-28A on Suppressive Effect of Cultured CD4+ CD25- Cells

Figure 4 shows the effect of coculture of CD4+ T cells with autologous CD4+ CD25- T cells in each group with a ratio of 1:1. The results showed that T cells cultured in the six groups showed no suppressive effect.
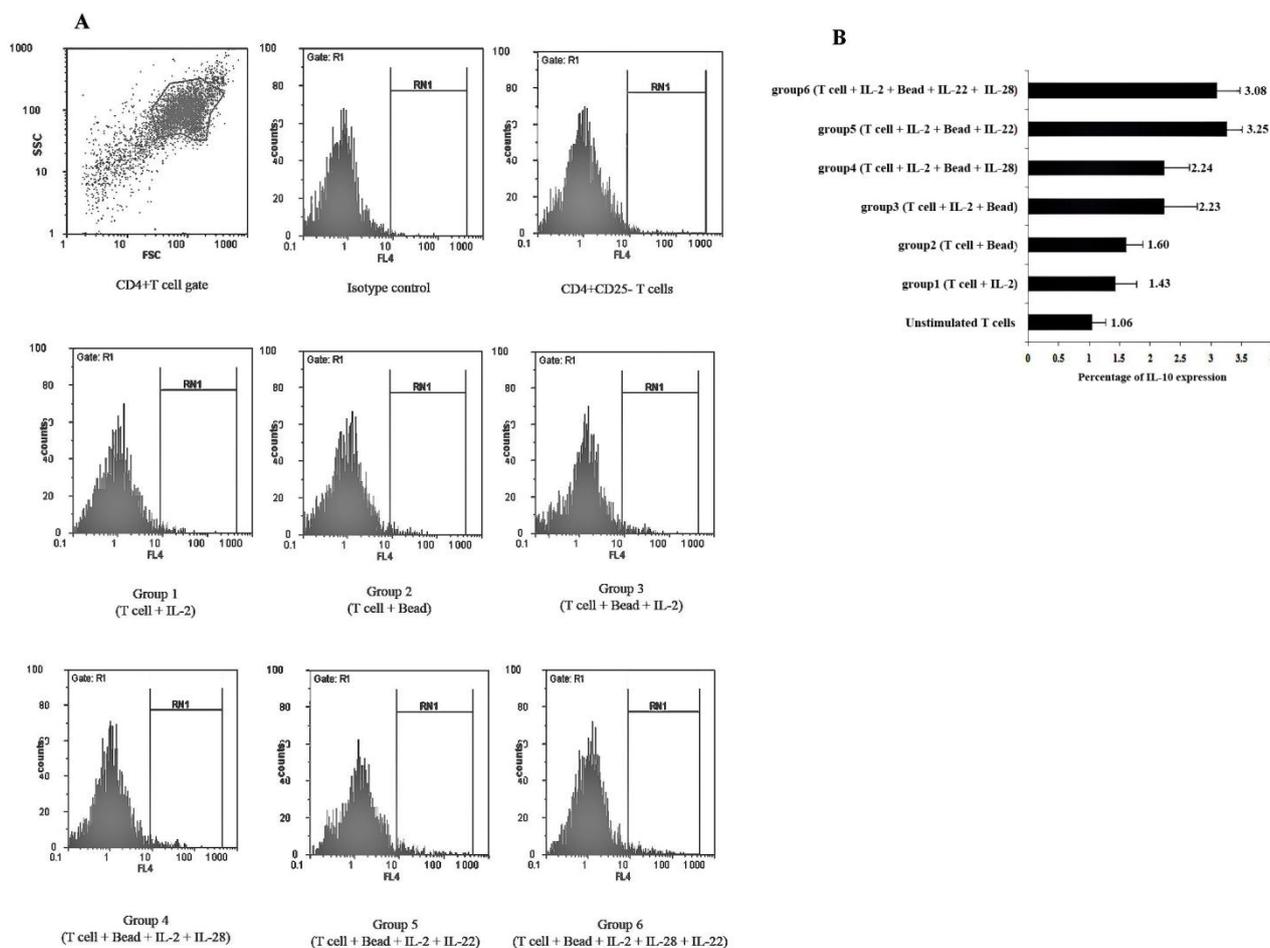
Figure 3. CD4+CD25− T cells upregulated IL-10 following activation. Activated T cells in four groups were fixed and stained intracellularly for IL-10 expression with anti IL-10 APC, then analyzed on a Becton-Dickinson FACSCalibur. (A) Representative histogram showing IL-10 expression on CD4+ T cells before and after activation. (B) Graphs display the percentage of IL-10+ T cells among four groups ($p<0.05$).
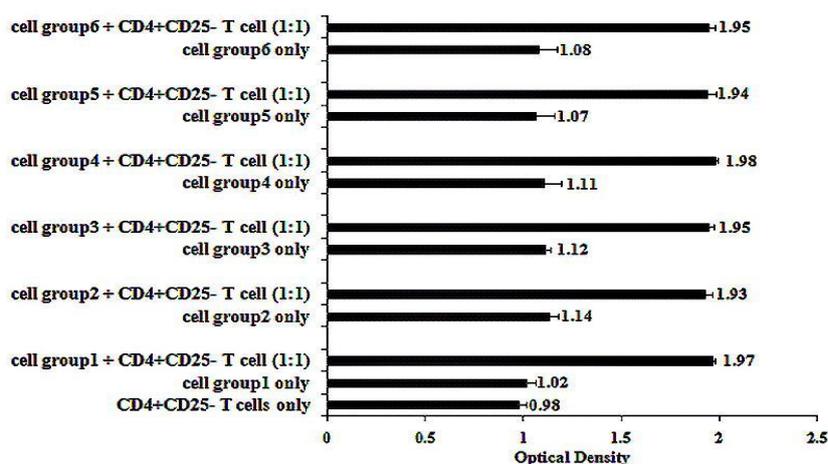

Figure 4. Suppression assay of T cells cultured in diverse groups ($p<0.05$). In order to evaluate the suppression effect, 10^6 cultured T cells in each group were cultured with autologous CD4+CD25- T cells incubated for three days at 37°C and 5% CO2 in 1:1 ratio in 96 well plates. Then, to assay proliferation of T cells the Cell Proliferation ELISA, BrdU kit was used.

DISCUSSION

Combined injection of cord blood stem cells and T regulatory cells in umbilical cord blood transplantation especially in adults is an important strategy to increase graft survival and graft versus leukemia (GVL) and reduce graft versus host diseas (GVHD) as well as transplant-related mortality (TRM). This strategy is adopted to overcome the problem of low number of regulatory T cells in cord blood. [14] A method for the expansion of T regulatory cells is induction of these cells from non-regulatory precursor cells by cytokines in vitro. [15,16] IL-10 has an important role in the induction of Tr1 cells. In this study, the cytokines IL-22 and IL-28A, which share the same receptor with IL-10, have been used to induce Tr1 cells.

As previously mentioned, no specific surface marker has been described for Tr1 cells up to now. These cells are characterized by their unique pattern of cytokine production that is distinct from Th1 and Th2 cells. Tr1 cells produce high levels of IL-10, significant levels of IFN-γ, TGF-β and IL-5 but are short of IL-4, and generate very low levels of IL-2 or do not generate it at all. [1-3]

Tissue expression of IL-22 R1 is very limited (copiously expressed in the pancreas and low levels of expression in gastrointestinal tract, skin, kidneys and liver) while CRF2-4 is extensively expressed. The important function of CRF2-4 in this family of cytokine receptors can justify its high level of expression. IL-10R1 is also extensively present on white blood cells and lymphoid tissues. [7-9]

Resting or activated monocytes, macrophages, DCs, B, T and NK cells do not express IL-22 R1, but IL-10 R2 is expressed on these cells. It has been demonstrated that skin keratinocytes have receptors for this cytokine, and the expression of these receptors (especially IL-10 R2) is increased under IFN-γ influence. [17]

With respect to amino acids, IL-28 and IL-29 are very similar and affiliated with IFN alpha and beta, but from the viewpoint of genomic structure, they have more homology with IL-10 family. [18] Although type I IFN receptor subunits (IFN-α / β) and IFN-λ family do not show any detectable homology, they activate similar signaling pathways. [11] IL-28A also shows low homology with IL-10, and uses the IL-10R2 chain as part of its receptor complex similar to IL-10, IL- 22 and IL-26. [13,19] Under the conditions of our study, IL-28A was not effective in the induction of Tr1 cells and in T

cell polarization. There are other studies, however, which indicate that the type I IFN (IFN-α/β) can be effective in this regard. [11]

In the study of MacRay et al and Roncarlo et al, it was found that in some cases adding IFN-α alone to cord blood T cells was sufficient to induce a population of T cells with Tr1 cells cytokine profile and immunosuppressive properties. They showed that cord blood T cell have the innate ability to produce high amounts of IL-10, which is increased in the presence of IFN-α. [20,21] Therefore, the autocrine IL-10 production by T cells in cord blood reduces the need for exogenous IL-10. In contrast, T cells in the peripheral blood produce 7 to 13 times less IL-10 than umbilical cord blood, and require IL-10 and IFN-α to induce differentiation of functional Tr1 cells. [20,21] In another study, Martin Savdra et al observed a significant increase in mRNA expression upon culture of splenic T cells with IFN-β. They also showed that treatment with IFN-β was effective in promoting inflammatory responses by increasing the expression of IL-4 and STAT6 activity, enhancing Th2 phenotype polarization. [22] Therefore, according to these observations, it is unlikely that IFN-λ family cytokines (which are known to induce a different signaling pathway) have such capacity similar to type I interferons (IFN-α/β).

Lack of effect of this cytokine in polarization of T cells compared with IL-22 may be attributed to the difference in dose used in this study. For IL-28A, 40 ng/ml and for IL-22, 100 ng/ml doses were used. On the other hand, it seems that IL-22 can be produced by T cells in culture, and this can be helpful in enhancing the effects of these cytokines. There are many indications that after T lymphocyte activation, the IL-22 mRNA level is increased in these cells but usually not in other cells. Extensive studies have shown that activation of T cells by IL-12, anti-CD3 plus anti-CD28 or anti-CD3 plus ICAM causes production of IL-22 in these cells. [8] Another reason may be difference in binding affinity of IL-22 and IL-28A to IL-10R2 (CRF2-4), as researchers believe that this receptor (CRF2-4) plays an important role in immunological effects of IL-10 and IL-22. In vivo studies have shown that injection of IL-22 causes a rapid induction of PAP1 (secretory protein associated with Reg family increased in inflammation of the pancreas) in the pancreas, but this phenomenon will not occur in mice lacking CRF2-4. Binding affinity of IL-22 and IL-10 to

CRF2-4 is different. CRF2-4 alone is sufficient for binding to IL-22, but the second receptor is required for efficient binding to IL-10. [8,9]

Essentially, the binding affinity of IL-22 to CRF2-4 is low, but it is high for CRF2-9. Intracellular tail of CRF2-9 is long and contains 323 amino acids, while cytoplasmic tail of CRF2-4 is short and has only 24 amino acids. Therefore, it seems that CRF2-4 has the role of recruiting the JAK kinase family, but CRF2-9 is the main signaling agent. [8,9]

Levings et al showed that IL-10 and IFN-α have synergistic effects in differentiation of Tr1 cells from CD4+ T cells.[23,24] Groux et al, using neutralizing antibodies against IL-10R and TGF-β, showed that the immunoregulatory effect of Tr1 cells is mediated by IL-10 and TGF-β.[25] Pot et al as well as Wang et al showed that IL-27 can induce Tr1 cells. [26,27]

Co-engagement of the CD3 and CD46 in the presence of IL-2 also leads to production of Tr1 like CD4+ T cells, which produce high levels of IL-10 and TGF-β.[24,28]

In our work, it was also shown that stimulation of cord blood T cells by anti-CD2, anti-CD3 and anti-CD28 in the presence of IL-2 and IL-22 could enhance IL-4 and decrease IFN-γ levels relative to groups lacking this cytokine. Our results also showed a significant increase in the expression of IL-10 in the presence of this cytokine. So, it seems that IL-22 can enhance the polarization of T cells towards the Th2 profile. Oral et al showed that repeated stimulation of naive peripheral blood T cells by IL-19, IL-20 or IL-22 induces the polarization of these cells to a profile similar to Th2. They also showed that the activity of naive T cells by anti-CD2, anti-CD3 and anti-CD28 in the presence of IL-10 causes polarization of cells toward T cells secreting IL-10 and IFN- γ. [29]

Cytokine production by activated T cells appears to be a function of time, because RT-PCR analysis by Pillai et al showed that in stimulated CD4+ T cells, IFN-γ is detectable in the early stages of activity, while Foxp3 and IL-10 are expressed simultaneously in later times. When expression of Foxp3 and IL-10 is in its highest level, the expression of IFN-γ is at minimum. IL-2 and IL-4 levels are low or undetectable when Foxp3 level is in its peak. [30]

In order to identify regulatory T cells, various indicators and cytokines have been reviewed. For regulatory CD4 + CD25 + T cells, a series of molecules such as CD25, Foxp3, CTLA-4, GITR and CD127 are used for detection[31], most of which are also increased in activated T cells. Therefore, these markers cannot be used for definitive identification of regulatory T cells, either alone or in combination.[32,33] Some types of regulatory T cells are classified based on their cytokine production profile. Th3 cells are identified only by production of TGF-β, and Tr1 cells are identified with high levels of IL-10.[1,32-36] The point is that the suppressive effect of induced cells should be determined. Therefore, in our work, suppression assay was used to clarify the suppressive effect of cultured T cells, and the results showed that these cells have no suppressive effect.

In our previous study, the effect of IL-22 and IL-28A on CD4+ T cells from cord blood was evaluated in order to induce CD4+ CD25+ regulatory T cells, and Foxp3 molecule was used to identify these cells. The two studies also showed that these two cytokines have no effect in the induction of Foxp3 + regulatory T cells.[8,37,38]

Therefore, the results of this study and other studies showed that several factors can be effective in expression of surface molecules, cytokines and T cell activity, including the type of stimulus, antibody and cytokine dose, duration of culture and expression of cytokine receptors on the cells. The change in each of these can create different results.

The survey results showed that in the dose and conditions of this study, none of the cytokines IL-22 and IL-28A can induce Tr1 cells, which are identified based on their cytokine profile.

Considering the importance of Tr1 induction in cases such as cord blood transplantation, although our candidate molecules seem to lack that capacity, further studies including mRNA and signaling mechanisms may be useful for elucidating the role of IL-22 and IL-28a. Cord blood transplantation is an important and preferred strategy, which still faces serious limitations in its clinical applications, and it seems necessary to continue research on cytokine and signaling pathways that could create more favorable conditions for this vital therapeutic intervention.

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