The effect of IL-28A on human cord blood CD4+ T cells

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Abstract

Background and aim: The utilization of umbilical cord blood transplantation (UCBT) has been increasing because of the potential advantage of rapid accessibility and the lesser risk of graft-versus-host disease (GVHD), thus allowing less strict HLA matching. IL-28A, also known as IFN-λ2, has been regarded as a member of a new cytokine family that shares some features with type I interferon (IFN) and was shown to have antiviral activity. The aim of this study was to identify biological activity of IL-28 on cord blood CD4+ T cells.

Materials and methods: In this study, we cultured CD4+ T cells with IL-28A (20 ng/ml), IL-2 (20 ng/ml) and 5 μg/ml MACS Anti-Biotin MACSiBead Particles (bead-to-cell ratio 1:2) for 2 weeks.

Results: Flow cytometry analyses showed that IL-28A cannot be effective on CD25 and Foxp3 expression on cord blood CD4+ T cells, and it is not involved in proliferation of these cells. Treg suppression assay also showed that this cytokine cannot induce production of regulatory T cells.

Conclusion: We showed that IL-28A is not involved in expression of CD25 and Foxp3 markers and proliferation of CD4+CD25+ T cells, and that our findings also suggest that induction of Foxp3 in T cells activated by anti-CD3/anti-CD28 does not result in the regulatory activity in these cells.

Keywords: Interleukin 28A; CD4+ T cell; cord blood; regulatory T cells; Foxp3

Introduction

Umbilical cord blood transplantation (CBT) has increasingly been performed as an alternative to human leukocyte antigen (HLA)-matched sibling or unrelated bone marrow transplantation (BMT).[1–3] The advantages of CBT in comparison to BMT include rapid accessibility of cryopreserved cells, a less stringent HLA matching between donors and recipients, and a low risk of inducing severe graft-versus-host disease (GVHD).[4] On the other hand, cord blood contains a large population of immature unprimed highly functional regulatory T lymphocytes,[5, 6] that may actively and specifically suppress unwanted alloimmune immune responses post-UCBT.[7, 8] Therefore, expansion or induction of Treg cells may be the most important reason for exploring therapeutic applications of UCB.

The IFN-λ gene family is comprised of three different genes: IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B).[9, 10] IFN-λ2 is very similar to IFN-λ3 (96%), and IFN-λ1 exhibits 81% similarity with IFN-λ2.[11] They are expressed by human peripheral blood mononuclear cells and dendritic cells upon infection with viruses or stimulation with poly(I:C).[9, 10, 12] Even though nearly any cell type following viral infection can express IFN-λ, dendritic cells (DC) seem to be its most significant producers, specifically plasmacytoid DC express high levels of IFN-λ following viral infection.[12]

IFN-λs act through a cell surface receptor composed of two chains, the first one (IFNLR1) being IFN-λ specific and the second one (IL10R2) was shared among IL-10, IL-22 and IL-26.[9, 10, 13]

Both receptor subunits are constitutively expressed in a wide variety of human cell lines and tissues.[9, 10]
and signal through the Jak/STAT pathway.[9, 10, 13] Both IFNLR1 and IL10R2 are essential to make a functional IFN-λ receptor. If one of these chains is not present on the cell surface or is neutralized by an antibody, cells are not responding to IFN-λ.[9, 10]

The distinctive receptor use of type I IFNs and IFN-λs could cause special signaling pathways in addition to recruitment of Stat1 and Stat2, both of which are activated by type I IFN and IFN-λs through the Tyk2 and Jak1 kinases. Another significant difference between type I IFN and IFN-λs is the regulation of their receptor expression. Since IFN-AR1 and IFN-AR2, the 2 chains of the type I IFN receptor, and IL-10R2, the second chain of the IFN-λ receptor, are ubiquitously expressed, IFNLR1 expression is cell-type dependent. Indeed, a limited variety of tissues are responding to IFN-λ.[14, 15]

IFN-λs display a number of common characteristics with type I IFNs, including capacity of modulating the Th1/Th2 response,[16] antiviral, antiproliferative, and antitumor activities.[17, 18] The antiproliferative effects of IFN-λ have been demonstrated in various tumor cell lines that express endogenous or ectopic IFN-λ receptors.[15, 19, 20] IFN-λ also exhibit antiviral activities against EMCV, VSV, CMV, HSV1, IAV and West Nile virus in ad hoc in vitro cellular systems.[9, 10, 12, 21, 22]

IFN-λs also involves induction of tumor apoptosis,[23] and furthermore, they appear to have immunomodulatory functions. This phenomenon may describe why IFN-λs have suppressive activity on CD4+ T cells induced by respiratory syncytial virus.[24, 25]

On the other hand, IL-28 exhibits low homology to IL-10 and it also uses (similar to IL-10, IL-22, and IL-26) the IL-10R2 chain as a component of its receptor complex.[9, 10] As we know IL-10 is a well-known immunoregulatory cytokine and a potent inhibitor of proinflammatory cytokine production by monocytes and has been shown to downregulate the production of IL-1α, IL-1β, IL-6, and TNF-α. IL-10 strongly reduces the antigen specific proliferation of T cells.[26, 27] Treatment of T cells with IL-10 results in a long-lasting state of antigen-specific T-cell unresponsiveness. IL-10 can also induce the in vitro differentiation of a novel class of CD4+ T cells, termed T-regulatory 1 (Tr1) cells.[28] Therefore, with regard to these similarities and differences between IFN-λs and type I IFNs or IL-10, and considering reports on receptor expression on cell populations we intend to shed light on the affect of IL-28A upon the CD4+CD25+ T cells.

**Subjects and methods**

**Cord blood samples**

Cord blood specimens were obtained from the placentas of healthy full-term newborns at Valiasr Obstetrics and Gynecology Hospital Tehran, Iran. These newborns were screened for genetic disorders, hematologic abnormalities or infectious complications.

**Media and reagents**

Complete media composed of RPMI 1640 supplemented with 2mM L-glutamine, 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin was used. All reagents were purchased from Gibco (Life Technologies, Grand Island, NY, USA). Ficol-Paque was purchased from Cedarlane (Hornby, Ontario, Canada). Cell Proliferation ELISA, BrdU kit was bought from Roche (Mannheim, Germany).

CD4CD25 regulatory T cell isolation kit; T Cell Activation Expansion Kit: The kit consists of MACS Anti-Biotin MACSiBead Particles and biotinylated antibodies against human CD2, CD3 and CD28.

All the following were purchased from Miltenyi Biotec GmbH (Gladbach, Germany): FOXP3 Staining Buffer Set; mouse anti-human CD4 FITC-conjugated (cloneVIT4); mouse anti-human CD25 PE-conjugated (clone 4E3); mouse anti-human Foxp3 APC-conjugated (clone 3G3); isotype controls - mouse IgG2a FITC-conjugated; mouse IgG2b PE-conjugated and mouse IgG1 APC-conjugated. IL-2 and IL-28A were purchased from R&D Systems.

**Isolation of cell subsets**

Cord blood mononuclear cell (CBMC) were isolated from heparinized cord blood by density gradient sedimentation over Ficoll-Paque. Freshly isolated CBMC (98% viable by Trypan blue exclusion) were used for isolation of CD4+CD25+ T cells and CD4+CD25- T cells by CD4CD25 regulatory T cell isolation kit. In brief, CD4+ T cells were first isolated through negative selection by removing all other cell types using LD column. Then CD4+ T cells were incubated with 10 μl of magnetic beads conjugated with anti-CD25 antibody to separate CD4+CD25+ and CD4+CD25- T cell populations using MS column. Purity of sorted populations, as determined by flow cytometric analysis, was ≥90%.

**CD4 T cell culture**

Isolated CD4+ CD25- T cells were divided into four groups and cultured in 48-well microtiter plates (Nunc, Copenhagen, Denmark) as follows:

**Group1:** 1 × 10⁶ CD4 T cells + 20ng/ml IL-2

**Group2:** 1 × 10⁶ CD4 T cells + 5μg/ml MACS Anti-Biotin MACSiBead Particles (bead-to-cell ratio 1:2)

**Group3:** 1 × 10⁶ CD4 T cells + 20ng/ml IL-2 + 5μg/ml MACS Anti-Biotin MACSiBead Particles

**Group4:** 1 × 10⁶ CD4 T cells + 20ng/ml IL-2 + 5μg/ml MACS Anti-Biotin MACSiBead Particles + 20ng/ml IL-28A
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Then these cells were cultured for two weeks at 37°C and 5% CO₂ in incubator. Every 3–4 days, IL-2 and IL-28A were added to corresponding groups.

Flow cytometric analysis
CD4+ T cells were stained for cell surface molecules (CD4, CD25) and intracellular marker (Foxp3) before and after cell culture. T cells were phenotypically analyzed by three color fluorescence.

Surface staining was performed by adding 10 μl anti-CD4 FITC and anti-CD25 PE antibodies to cells and incubated for 10 min at 4°C. Then cells were washed with buffer with 0.5% BSA and 2mM EDTA. For intracellular staining of Foxp3, 1 ml freshly prepared Fixation/Permeabilization (1:4) working solution (Miltenyi Biotec, Gladbach, Germany) was added to each sample, followed by twice washing with 1–2 ml 1× Permeabilization Buffer (Miltenyi Biotec, 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 1× Permeabilization Buffer, and then resuspended in suitable amount of buffer for analysis by flow cytometry.

Semi quantitative reverse transcription PCR
RNA from T cells in different groups was extracted using RNA extraction kit (Isogene Laboratory, Ltd., Moscow, Russia) and reverse transcribed into cDNA (Isogene Laboratory Ltd., Moscow, Russia). cDNA samples were amplified using primers specific for Foxp3 (Sense: 5′-AGTGCTTTTGTGCGGGTGAGAG-3′; anti-sense: 5′-TCGTTGCGTGGTTGTAAGGC-3′). PCR conditions were as follows: 95°C for 10 min, 40 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min and then 72°C for 10 min.

PCR products were separated by electrophoresis through a 2% agarose gel and detected by ethidium bromide staining and photographed with gel documentation (Vilber Lourmat, Marne-la-Vallée, France).

Proliferation assays
CD4+CD25− T cells were divided into four groups with different treatment, as mentioned under “CD4 T Cell Culture” section, at a final concentration of 10⁶ cells/ml in a U-bottomed 96-well plate and cultured in RPMI 1640 media, supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin for 72 hours. After this time, cultured cells were reincubated with Brdu for 8 hour. Then anti-Brdu-POD was added to the cells and, finally, the reaction product was quantified by measuring the absorbance using an ELISA reader. All tests were conducted in triplicate.

In vitro suppression assay
First, CD4+CD25− T cells were divided into four groups with different treatment, as mentioned in “CD4 T Cell Culture” section, at a final concentration of 10⁶ cells/ml in a U-bottomed 96-well plate and cultured in RPMI 1640 media, supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin for 2 weeks. After that, MACSBead Particles were removed from culture using MACSIMAG Separator. Then, to determine the suppressive capacity of cultured cells, 10⁶ cells per well of the cells of each group were cocultured with autologous CD4+CD25− T cells (ratio 1:1) for 3 days at 37 °C and 5% CO₂. T cells proliferation was induced by stimulation with MACS Anti-Biotin MACSBead particles (bead-to-cell ratio 1:1) in 96-well round bottomed plates. Thereafter, proliferation assay was performed by Cell Proliferation ELISA, BrdU kit as mentioned in “Proliferation Assays” section.

Statistical analysis
Experiments were conducted at least three times. Differences between samples were analyzed by using the Student’s t-test. A p-value of ≤0.05 was considered significant.

Results

The effect of IL-28 on the frequency of CD4+CD25+ T cells
After a two-week culture of CD4+ CD25− T cells in the previously mentioned groups, we investigated the number of CD4+CD25+ T cells in these cell culture groups compared to control CD4+ CD25− T cells (Figure 1).

Flow cytometry analysis of CD4 and CD25 in all of the groups except group 1 showed that the number of CD4+ CD25+ T was significantly increased as compared to control CD4+ CD25− T cells. Amongst these four groups, increase in CD4+ CD25+ T cells in groups 2, 3 and 4 was greater than group 1 (p<0.05), and this significant increase was found in groups 3 and 4, in comparison to group 2, and also in group 2 versus group 1. There was also no significant difference in the number of CD4+ CD25+ T cells between groups 3 and 4.

Assessment of Foxp3 expression in cultured cells
Given the important role of the Foxp3 transcription factor in identification of Treg cells, in this study Foxp3 expression was evaluated using staining protocols for Foxp3 detection.
In Figure 2(a and b), representative flow cytometry results for Foxp3 expression in cultured and control CD4+ CD25− T cells are shown.

Our results indicated that significant variation was observed in the level of Foxp3 in the aforementioned cell groups of 2, 3 and 4 but not group 1 compared to control CD4+ CD25− T cells. Although up-regulation of Foxp3 in group 1 was also shown, this increase was not statistically significant. The comparison of changes in Foxp3 expression among other groups indicated that there was significant increase of this marker in groups 3 and 4 in contrast to group 2, but we observed no significant difference between group 3 and group 4.

Furthermore, to confirm these data, we tested Foxp3 expression by semiquantitative RT-PCR (reverse transcriptase-PCR), and 421 bp Foxp3 bands were detected in all of the groups (Figure 2c).

Figure 1. Evaluation of the frequency of CD4+CD25+ T cells 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 cell surface molecules (CD4, CD25). (a) Flow cytometry analysis of cultured cells in four groups. (b) Percentage of CD4+CD25+ T cells among different groups.
**IL-28 effect on CD4+ T cell proliferation**

We next examined whether IL-28 can affect on CD4+ CD25- T cell proliferation. As already mentioned, in order to assess the influence of this cytokine, CD4+ CD25- T cells were cultured in four groups with different treatments for 72 hours at 37 °C and 5% CO₂. As indicated in Figure 3, significant differences between bead-treated cells (groups 2, 3 and 4) versus other groups (group 1 and CD4+ CD25- T cells alone) have been indicated (p < 0.05). No difference in proliferation was seen between group 1 and control CD4+ CD25- T cells and also between groups 3 and 4, but significant proliferation was observed in groups 3 and 4 compared to group 2.

![Graphs showing cell proliferation](image)

**Figure 2.** CD4+CD25- T cells upregulate Foxp3 following activation. Activated T cells in four groups were fixed and stained intracellularly for Foxp3 expression with anti Foxp3 APC, then analyzed on a Becton-Dickinson FACS Calibur. (a) Representative histogram showing Foxp3 expression on CD4+ T cells before and after activation. (b) Graphs display the percentage of Foxp3+ T cells among four groups. (c) RT-PCR analysis of CD4+ T-cells for Foxp3 expression is shown. The 421 bp Foxp3 band was detected in all groups in agarose gels.
Cultured cells in different groups did not show suppressive effect

In order to evaluate the *in vitro* suppressive capacity of cultured CD4+ CD25- T cells, we used the same number of these cells and autologous responder T cells in 96-well plates for 3 days at 37°C and 5% CO₂. In suppression assays, cultured cells in the four groups showed no suppression activity (Figure 4).

**Discussion**

As mentioned earlier, IL-28A, as a member of a IFN-λ family,, acts through a cell surface receptor composed of two chains, IFNLR1 and IL10R2.[9, 10, 13] Both receptor subunits are constitutively expressed in a wide variety of human cell lines and tissues. [9, 10, 17]

To identify definitive markers of natural and inducible Treg cells, a number of markers have been described to

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**Figure 3.** *In vitro* proliferation assays. As mentioned in section 2, CD4+CD25- T cells were cultured in triplicate in U-bottomed 96-well plates in four groups with different treatments for 72 hours at 37 °C and 5% CO₂. Then, proliferation of cultured cells was assessed by Cell Proliferation ELISA, BrdU kit.

**Figure 4.** Cultured T cells in four groups can not suppress autologous responder T cells. With regard to expression of Foxp3 on activated T cells, suppressive activity of these cells was evaluated. After a 2-week culture of different cell groups, the same number of these cells and autologous responder T cells were used in 96-well plates for 3 days at 37°C and 5% CO₂. Thereafter, proliferation assay was performed by Cell Proliferation ELISA, BrdU kit.
delineate this subpopulation of T cells. The transcription factor Foxp3 and CD25 have generally been used to define natural Treg cells.

As shown in Figure 1b, our results demonstrated that CD25 expression was significantly increased in bead activated T cells (groups 2, 3, and 4) compared to group 1 and control CD4+CD25− T cells. Moreover, in comparing groups 3 (CD4+CD25− T cells + bead + IL-2) and 4 (CD4+CD25− T cells + bead + IL-2 + IL-28), our results showed no significant difference in the number of CD4+CD25− T cells. However, IL-28 does not appear to be able to induce CD25 expression on cultured T cells.

Although it has been reported that the suppressive property of Tregs is due to Foxp3, there is still controversy about expression of this marker on T cells. The results of some studies have shown that activated human CD4+ CD25− effector T cells also express Foxp3 while failure of Foxp3 induction in TCR-stimulated naïve CD4+ T cells has also been reported.

Our data showed that T cells cultured with IL-2 plus bead (groups 3 and 4), in comparison to cultured T cells with bead alone (group 2) or IL-2 alone (group 1), had a higher number of Foxp3+ T cells (Figure 2b). It appears, therefore, that IL-2 plus bead is more effective in the increase of the number and viability of Foxp3+ T cells than the effect of bead or IL-2 alone. Moreover, comparing group 3 versus group 4, we found that IL-28A cannot be effective in the induction of Foxp3 in the cultured cells. This is probably due to lack of IL-2R1 on leukocytes.

We then asked whether IL-28 might be involved in the proliferation of CD4+CD25− T cells. In comparing groups 3 and 4, our data showed that IL-28 has no effect on proliferation of CD4+CD25− T cells (Figure 3). Two possible reasons are as follows: First, although receptors for IFN-λs are broadly expressed on non-hematopoietic tissues, they do not seem to be present on leukocytes; and second, IL-28 exerts an antiproliferative activity. For example, some studies have been reported that IFN-λs can induce antiproliferative responses in some cell lines, and Chi et al. also showed that both IFN-α and IFN-λ are critical mediators of the suppression of CD4 T cells associated with RSV infection.

On the other hand, Li et al. have demonstrated that, signaling induced through the type III IFN (IFN-λ) receptor can induce apoptosis in cells similar to type I and type II IFNs. However, apoptotic capability of IFN-λ appears to depend on the strength of IFN-λ-induced signaling in cells. Thus, up-regulation of expression levels of IFN receptor and critical signaling molecules and/or inhibition of negative regulators of IFN signaling can render cells sensitive to IFN-λ-mediated apoptosis.

To ensure regulatory function of Foxp3+ T cells obtained from different groups, cultured cells were evaluated using Treg suppression assay and results demonstrated that none of the activated cell groups had suppressive capacity.

In the present study, we showed that although activated T cells express Foxp3 and CD25 markers, these cells do not have a regulatory role. In agreement with our data some studies indicate that Foxp3 expression does not invariably confer a regulatory phenotype, whereas other studies report consistent correlation of suppressive activity with Foxp3 expression in induced Treg cells in functional assays.

**Conclusion**

We first present here evidence that IL-28A is not involved in expression of CD25 and Foxp3 markers and proliferation of CD4+CD25− T cells, and that our findings also suggest that induction of Foxp3 in T cells activated by anti-CD3/anti-CD28 does not result in the regulatory activity in these cells.

We also concluded that, when CD4+ T cells are treated with an equivalent dose of IL-2 and IL-28, it appears that IL-2 can be more effective with regard to high expression of CD25(II-2Rα) on activated T cells. Therefore, increasing the dose of IL-28 compared to IL-2 may lead to different results.

The use of media containing various cytokines or different agents is on of the new approaches to induce or increase Treg cells. Thus, the previous data summarized above, along with future investigations, will form the foundation for production of Treg cells as a therapeutic strategy in transplantation, autoimmunity and other diseases. The use of these cells will require a further understanding of the factors that influence expansion and induction of Treg cells.

Moreover, the therapeutic potential of IFN-λ is promising, either for its type I IFN-like activities or for its theoretical specific bioactivity. Particularly, IFN-λ could be an interesting replacement to IFN-α. Such an approach may decrease the severe side effects associated with type I IFN therapies.

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References


