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# Human Amnion-derived Stem Cells have Immunosuppressive Properties on NK cells and Monocytes

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Running head: Immunosuppressive activity of Human amniotic Cells

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## ABSTRACT

Human amnion-derived cells are considered to be a promising alternative cell source for their potential clinical use in tissue engineering and regenerative medicine because of their proliferation and differentiation ability. The cells can easily be obtained from human amnion, offering a potential source without medical intervention. It has been proved that human amnion-derived cells express immunosuppressive factors CD59 and HLA-G, implying that they may have an immunosuppressive function. To assess the immunosuppressive activity, we investigated the effect of human amnion-derived cells on NK cell and monocyte function. Amnion-derived cells inhibited the cytotoxicity of NK cells to K562 cells. The inhibition depended on the NK/amnion-derived cells ratio. The inhibition of NK cytotoxicity was recovered by continuous culturing without amnion-derived cells. The inhibition of NK cytotoxicity was related to the down-regulation of the expression of the activated NK receptors and the production of IFN-γ, as well as the up-regulation of the expression of IL-10 and $PGE_2$ in human amnion-derived cells. The addition of antibody to IL-10 or $PGE_2$ inhibitor tended to increase NK cytotoxicity. IL-10 and $PGE_2$ might be involved in the immunosuppressive

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activity of amniotic cells toward NK cells. Amniotic cells also suppressed the activity of cytokine production in monocyte analyzed with TNF-α and IL-6. These data suggested that amniotic cells have immunosuppressive activity.

Key words: Amnion mesenchymal stem cells; Amnion epithelial stem cells; immunosuppressive activity; IL-10; $PGE_2$

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## INTRODUCTION

The amniotic membrane is a tissue of fetal origin and is composed of three major layers: a single epithelial layer, a thick basement membrane, and an avascular mesenchyme. It is adjacent to the trophoblast cells and lines the amniotic cavity in which the fetus can grow without distortion by pressure from surrounding structures. Although the fetus has semiallogeneic antigens recognized by the mother, pregnancy is established normally in most cases. It is thought that a special immunologic mechanism may protect the fetus from maternal aggression. Furthermore, it is reported that a kidney allograft avoided allograft rejection in one case when immunosuppression was discontinued during and after pregnancy (8), which implied that an active immunoregulatory mechanism may be generated. Based on this, there has been increasing interest in the investigation of the immunosuppressive activity of amnion-derived cells in vivo and in vitro (23,24,25,33,50,53). The human amniotic membrane contains two principal types of cells: amniotic epithelial cells (hAECs) isolated from epithelial layer and amniotic mesenchymal cells (hAMCs) isolated from the avascular mesenchymal layer. Emerging evidence suggests that both of them retain proliferation and multipotent/pluripotent

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characteristics (18,47,49,50,57), making the amniotic membrane a promising and very attractive source of cells for regenerative medicine. The amnion membrane can provide a sufficient cell supply without any substantial ethical issues, increasing the attractiveness of amnion-derived cells for cell transplantation.

CD59, a negative regulator of complement activation, was reported to be expressed in amnion-derived cells (12), indicating that they may possess a mechanism to escape from complement attack by inhibiting formation of membrane attack complex. It has also been shown that amnion-derived cells express the nonpolymorphic, nonclassical human leukocyte antigen G (HLA-G), but lack the polymorphic antigens HLA-A, HLA-B, and HLA-C (class IA) and HLA-DR (class II) (48,49). HLA-G was thought to protect the fetus from maternal uterine NK cells and is considered to be an important immunosuppressive factor during pregnancy (39). These findings suggest that amnion-derived cells may be immunologically inert and would have a reduced risk of rejection upon transplantation. There are many potential cell sources for regenerative medicine, including bone marrow-derived mesenchymal stem cells, tissue-specific progenitor cells, embryonic stem (ES) cells, and induced pluripotent stem (iPS) cells. Although their biological potentials have been demonstrated, all of them still have some infeasibility for clinical applications. Bone marrow-derived mesenchymal stem cells

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have been shown to ameliorate tissue damage and to improve function after lung injury (28), myocardial infarction (16,17), renal injury (30) and bone injury (15). However, the decrease of cell number depending on the patients' age is one of the limitation (27,37). Tissue-specific progenitor cells are very hard to isolate and grow in vitro. Also, transplantation needs a large cell supply (42). ES cells and iPS cells are considered to be the most promising stem cells because of their tremendous differentiation ability. However, with the pluripotency comes genetic instability, which leads to concerns of tumorigenicity (14,20) and mutation (11). Furthermore, their expansion and maintenance to obtain a therapeutically sufficient number of cells requires time, effort, and cost. As a promising alternative source, use of the amnion membrane can resolve all of these problems. Therefore, human amnion-derived cells have started to be appreciated as an attractive and promising alternative source in the field of regenerative medicine. If amnion-derived cells have immunosuppressive activity, the problem of immune rejection could be overcome by the use of amnion-derived cells.

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## MATERIALS AND METHODS

### *Cell Culture*

A human amniotic membrane was peeled mechanically from the chorion of a placenta obtained from a patient undergoing cesarean section with informed consent. The study and the use of the amnion were approved by the Research Ethics Committee of University of Toyama. Fresh amniotic epithelial cells (fHAEs) and fresh amniotic mesenchymal cells (fHAMs) were isolated from human fresh amniotic membrane by sequential trypsin (Sigma-Aldrich, St. Louis, MO, USA) and collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) digestion as previously described (52). Immortalized human amniotic mesenchymal cells (iHAMs) and immortalized human amniotic epithelial cells (iHAEs) were established previously (47,57). HAMα is a proliferation subpopulation purified from fHAM (female) (32,51). Human Umbilical Vein Endothelial cells (HUVECs, female) were obtained from Lonza (Walkersville, MD, USA). K562 cells (female) were purchased from the ATCC. fHAM, iHAM, HUVECs and K562 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories

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GmbH, Pasching, Austria), 1% Penicillin-Streptomycin Mixed Solution (Nacalai Tesque, Kyoto, Japan) and 1% 200 mmol/l-L-Glutamine Stock Solution (Nacalai Tesque). HAMα cells were cultured in DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin Mixed Solution, 1% 200 mmol/l-L-Glutamine Stock Solution and 10 ng/ml basic Fibroblast Growth Factor (Wako Pure Chemical Industries, Ltd.). fHAE and iHAE cells were cultured with Dulbecco's modified Eagle's medium (DMEM) Nutrient Mixture F12 HAM (Sigma-Aldrich) supplemented with 10% FBS and 1% Penicillin-Streptomycin Mixed Solution at 37 °C with 5% $CO_2$ and 95% air humidity to a confluent state. For further expansion passage of confluent cells, fHAM, iHAM, HAMα, HUVEC, fHAE and iHAE cells were detached by treatment with trypsin/di-Sodium Dihydrogen Ethylenediamine tetraacetate Dihydrate (Nacalai Tesque) and replated in a 10 cm dish (Greiner Bio-One GmbH, Frickenhausen, Germany). NK-92MI cells (NK cells, male), with stable expression of interleukin-2 (29,41,43,45,46), were obtained from the ATCC. Fresh human peripheral blood samples were from healthy volunteers of 3 male and 3 female with age range from 24 years to 34 years. Approval for this study was obtained from the Research Ethics Committee of University of Toyama. Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples by Lymphoprep™ (Fresenius Kabi Norge AS, Bad

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Humburg, Germany). NK cells and PBMCs were cultured with Alpha Minimum Essential Medium (Life technologies, Carlsbad, CA, USA) containing 0.2 mM inositol (Wako Pure Chemical Industries, Ltd.), 0.1 mM 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd.), 0.02 mM folic acid (Wako Pure Chemical Industries, Ltd.), 1% Penicillin-Streptomycin Mixed Solution and 12.5% FBS.

***Cytotoxicity Assay***

The cytotoxicity of NK cells was evaluated by the 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution method. Briefly, NK cells were plated ($1\times10^5$ cells) in flat-bottomed 12-well tissue-culture plates (Greiner Bio-One GmbH) with or without amnion-derived cells or HUVECs with numbers $2.5\times10^4$, $5\times10^4$, $1\times10^5$, $2\times10^5$, $5\times10^5$, $1\times10^6$ cells for 1 day, 3 days or 5 days. NK cells were collected as effectors in the cytotoxic assays. K562 cells were labeled with 4.5 µM CFSE (Sigma-Aldrich) in 0.01% FBS/Dulbecco's PBS (-) (Wako Pure Chemical Industries, Ltd.) at room temperature for 8 minutes and continuously incubated at 37 °C for 10 minutes. Labeled cells were washed three times with 2% FBS/PBS and then were coincubated with NK cells (with NK/K562 ratios 1/2) at 37 °C for 4 hours. The cytotoxicity of NK cells against K562 cells was assessed by Fluorescence-Activated Cells Sorter Analysis using FACS

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Calibur (BD Bioscience, Franklin Lakes, New Jersey, USA) by quantifying apoptosis of K562 with 3,8-Diamino-5-[3-(diethylmethyl-ammonio)propyl]-6-phenyl-phenanthridinium diiodide solution (propidium iodide, PI, DOJINDO, Kumamoto, Japan). IL-10 neutralizing antibody (Abcam, Cambridge, UK), indomethacin (Sigma-Aldrich) or neutralizing antibody to thioredoxin (ADF11, a kind gift from Dr. Yoshiyuki Matsuo and Dr. Junji Yodoi) was added to the coculture. Cytotoxicity of NK cells was investigated at 3 days.

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***Reverse Transcription Polymerase Chain Reaction (RT-PCR)***

Amnion-derived cells were cultured with NK cells at a ratio of 10:1 for 1 day. Amnion-derived cells and NK cells were separated for RNA isolation with ISOGEN II (NIPPON GENE CO., LTD., Tokyo, Japan). cDNA was synthesized from 1 μg of total RNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). cDNA was subjected to polymerase chain reaction (PCR) using a Taq PCR Core Kit (QIAGEN, Tokyo, Japan). The primer sequences, annealing temperature and amplification cycle were as follows: NKp30 (forward: CTCATCTTGATCATGGTCCATCC, reverse: TGAGGATGTTCTTTCTCCACCAC; 57 °C; 27 cycles); NKp44 (forward:

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CTTCAGCACTTGTGTGCATCAG, reverse: TCACGGTTTTCCACCATATGTC; 55 °C; 35 cycles); NKp46 (forward: ATCTGAGCGATGTCTTCCACACTC, reverse: AGACCAGGCATGGTTGTTATAGGAG; 59 °C; 40 cycles); NKG2D (forward: TGAGAGTAAAAACTGGTATGAGAGCCA, reverse: GCAT GCAGATGTATGTATTTGGAG; 57 °C; 38 cycles); CD69 (forward: CATAGCTCTCATTGCCTTATCAGT, reverse: CCTCTCTACCTGCGTATCGTTT; 60 °C; 30 cycles); IFN-γ (forward: TGCAGGTCATTCAGATGTAG, reverse: AGCCATCACTTGGATGAGTT; 59 °C; 30 cycles); IL-4 (forward: GCGATATCACCTTACAGGAG, reverse: TTGGCTTCCTTCACAGGACA; 50 °C; 40 cycles); IL-6 (forward: AAATTCGGTACATCCTCGAC, reverse: CAGGAACTGGATCAGGACTT; 50 °C; 34 cycles); IL-8 (forward: CTTGGCAGCCTTCCTGATTT, reverse: CTCAGCCCTCTTCAAAAACT; 51 °C; 34 cycles); IL-10 (forward: AGATCTCCGAGATGCCTTCA, reverse: TTTCGTATC TTCATTGTCATGTA; 50 °C; 40 cycles); HLA-G (forward: TATGCCTACGATGGCAAGG, reverse: CTACAGCTGCAAGGACAACC; 50 °C; 22 cycles); COX-2 (forward: AACAGGAGCATCCTGAATGG, reverse: GGTCAATGGAAGCCTGTGAT; 50 °C; 32 cycles); TGF-β (forward: CAAGTGGACATCAACGGGTT, reverse: GCTCCAAATGTAGGGGCAGG; 62 °C;

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26 cycles); GAPDH (forward: GTCAACGGATTTGGTCGTATTG, reverse: CATGGGTGG AATCATATTGGAA; 57 °C; 30 cycles).

***Enzyme-linked Immunosorbent Assay (ELISA)***

NK cells were cultured with amnion-derived cells at a 1:10 ratio for 3 days. The supernatant was collected to detect the secretion of interleukin-10 (IL-10) or Prostaglandin $E_2$ ($PGE_2$) by enzyme-linked immunosorbent kit (eBioscience, San Diego, CA, USA and Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturers' instructions.

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***Monocyte Assay***

PBMCs were plated ($1\times10^5$ cells) in flat-bottomed 6-well tissue-culture plates (Greiner Bio-One GmbH) in the presence of amnion-derived cells ($1\times10^6$ cells) with Lipopolysaccharides (LPS, 0.01 μg/ml, Sigma-Aldrich) as a stimulator for 4 hours (9). Brefeldin A (5 μg/ml, Sigma-Aldrich) was added to stop the reaction among PBMCs, LPS and amnion-derived cells, and to inhibit the protein secretion. After 4-hour inhibition, PBMCs were collected to be analyzed for the expression of TNF-α (APC anti-human TNF-α, BioLegend, San Diego, CA, USA) and IL-6 (APC anti-human IL-6,

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BioLegend) on monocytes labeled by CD14 (CD14IOM2, BECKMAN COULTER, Brea, CA, USA) by Fluorescence-Activated Cells Sorter Analysis with BD FACS Canto$^{TM}$II (BD Bioscience).

***Statistical Analysis***

The Student's *t*-test was performed using statistical software (SPSS Statistics ver. 20 for Mac; IBM, Tokyo, Japan). All experiments were performed with at least 3 independent assays. Data are presented as means ± SE. Comparisons were two-sided, with a significance level of 5% (*) and 1% (**).

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## RESULTS

### *Amnion-derived cells-mediated inhibition of NK cytotoxic activity*

To assess the immunosuppressive activity of amnion-derived cells, NK cytotoxic activity was analyzed by targeting K562 cells after coculturing for 1, 3 or 5 days with or without HUVECs (Figure 1A) (35), iHAM cells (Figure 1B), iHAE cells (Figure 1C) or HAMα cells (Figure 1D). iHAM, iHAE and HAMα cells inhibited NK cytotoxic activity with an amnion-derived cell/NK cell ratio of 10:1 at day 3, declining with lower amnion-derived cell content, whereas in Figure 1A, HUVECs did not inhibit NK cytotoxic activity. However, the NK cytotoxic activity was little decreased at day 1. The NK cytotoxic activity decreased artificially at day 5. This may have been because of damaged NK activity through coculturing without changing the media for a long time. fHAM cells suppressed NK cytotoxicity significantly, fHAE cells also had the tendency to suppress NK cytotoxicity (data not shown). Furthermore, the suppression of iHAM, fHAM and HAMα to NK cytotoxicity was much more than iHAE and fHAE. In summary, amnion-derived cells suppressed NK cytotoxic activity to K562 cells in a dose-dependent manner.

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***Inhibition by amnion-derived cells to the expression of activated NK receptors and NK cell function***

NK cell activation depends on the balance between activating and inhibitory signals mediated by cell surface receptors (34). To further analyze the inhibition of NK cytotoxic activity by amnion-derived cells, the expression of NK activated receptors NKp46, NKp30, NKp44, NKG2D, and CD69, which are involved in the NK cell activation and target cell killing (7,22,56), was analyzed by RT-PCR. Significant down-modulation of NK receptors NKp30, NKp44, NKp46, CD69 after coculturing with amnion-derived cells was observed (Figure 2). The expression of NKG2D was partly decreased after coculturing with amnion-derived cells. The expression of NK activated receptors also was decreased in NK cells after coculturing with fHAM, fHAE and HAMα (data not shown). These data suggested that the amnion-derived cell-mediated inhibition of NK cytotoxic activity correlated with down-modulation of NK activated receptors. Together with cytotoxic activity, cytokine production is another main NK cell function. To better characterize the inhibitory effect exerted by amnion-derived cells, IFN-γ production by NK cells after coculturing was analyzed by RT-PCR (10). The expression of IFN-γ in NK cells significantly decreased after

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stimulation with iHAM and iHAE cells. Also, the expression of IFN-γ in NK cells decreased after stimulation with fHAM, fHAE and HAMα (data not shown). These data indicated that amnion-derived cells can inhibit not only NK cytotoxic activity but also cytokine production. IFN-γ, as a proinflammatory, was involved in innate immunity and can trigger immunosuppressive activity (2,21,44). The decrease of production of IFN-γ in NK cells further confirmed that amnion-derived cells have immunosuppressive activity.

***Increase of the expression of anti-inflammatory factors in amnion-derived cells by interaction with NK cells***

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To further investigate the factors involved in this immunosuppressive activity, the expression of immunosuppressive factors in amnion-derived cells was analyzed. Amnion-derived cells were harvested and RNA was isolated for RT-PCR after coculturing with NK cells for 1 day. The expression level of IL-4, IL-6, IL-8, HLA-G, TGF-β was not changed, while the expression of IL-10 and COX-2 was remarkably increased after coculturing with NK cells (Figure 3). The expression of IL-10 and PGE2 also was increased in fHAM and fHAE cells after coculturing with NK cells (data not shown).

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***The detection of IL-10 and $PGE_2$ by ELISA***

The secretion of IL-10 and $PGE_2$ protein from amnion-derived cells was analyzed by ELISA. The secretion of IL-10 in the supernatant from amnion-derived cells cultured without NK cells was very low, but significantly increased after coculturing with NK cells (Figure 4A). The secretion of $PGE_2$ in fHAE cells and HAMα cells cocultured with NK cells was significantly increased compared to that without NK cells (Figure 4B). The production of $PGE_2$ from iHAM, iHAE and fHAM cells after coculturing with NK cells had the tendency to increase. Moreover, the amount of $PGE_2$ in iHAM, fHAM and HAMα cells is much higher than that in iHAE or fHAE cells. This result is consistent with the result of the NK cytotoxicity assay. IL-10 and $PGE_2$ may be involved in the immunosuppressive activity of amnion-derived cells.

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***Blocking of IL-10, $PGE_2$ and thioredoxin with specific inhibitors***

The addition of anti-IL-10 antibody to the amnion-derived cells and NK cells coculture had the tendency to increase the NK cytotoxicity that had been decreased by coculturing with amnion-derived cells (Figure 5A). The addition of indomethacin, single inhibitor specific to $PGE_2$, blocked the suppression to NK cells from interaction with

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amnion-derived cells (Figure 5B). The neutralizing antibody to thioredoxin also had a tendency to restore the decreased NK cytotoxicity by amnion-derived cells (data not shown).

***Recovery of NK cytotoxic activity***

To confirm the immunosuppressive activity of amnion-derived cells, a recovery assay was performed. After interaction with amnion-derived cells for 3 days, NK cells were cultured continuously in the absence of amnion-derived cells for 2 days prior to exposure to K562 cells (35). Compared to inhibition of 3-day coculture of amnion-derived cells and NK cells, extending the incubation without amnion-derived cells significantly restored NK cytotoxic activity (Figure 6). Therefore, the decreased NK cytotoxic activity after interaction with amnion-derived cells was not because NK cells were damaged by coculturing. Amnion-derived cells suppressed NK cytotoxic activity.

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***Amnion-derived cells-mediated inhibition of cytokine production in monocyte***

As immune system cells, monocytes play an important role in immunomodulation. To further confirm the immunosuppressive activity of amnion-derived cells, the inhibition

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of monocyte cytokine production mediated by amnion-derived cells was also investigated. The expression level of TNF-α in monocytes after coculturing with iHAM, iHAE and HAMα cells was significantly decreased (Figure 7A). The expression level of IL-6 after coculturing with iHAM and HAMα cells was significantly decreased (Figure 7B). The expression of IL-6 after coculturing with iHAE cells also had tendency to decrease. Interaction with amnion-derived cells decreased the production of cytokine TNF-α and IL-6 in LPS-stimulated monocytes. The data showed that amnion-derived cells suppressed the cytokine production of monocytes by coculturing. This result suggested that amnion-derived cells have immunosuppressive activity.

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## DISCUSSION

Amnion-derived cells have been demonstrated as a promising alternative source, for their immunosuppressive activity (23,24,25,33,50,53). Although the mechanism underlying the immune-modulation of amnion-derived cells remains to be elucidated, it is now clear that these cells suppress allogeneic immune responses. Several studies have recently shown that amniotic membrane-derived cells display immunomodulatory properties similar to those described for mesenchymal cells obtained from other sources such as bone marrow and adipose tissue (5,23,50,53). Similar to other MSCs, the mechanism of immune-modulation by amnion-derived cells was yet to be clarified. Here, we reported a more accurate analysis of amnion-derived cells-induced inhibition of NK cell function and defined the molecular basis of their inhibitory effect (4). First, we showed that amnion-derived cells sharply inhibited the NK-mediated cytotoxic activity. Inhibition of expression of NKp30, NKp44, NKp46, NKG2D and CD69, which are involved in the NK cytotoxicity to K562 cells, occurred in NK cells cultured with amnion-derived cells. The expression of IL-10 and COX-2 in human amnion-derived stem cells was

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increased after culturing with NK cells. In addition, the secretion of IL-10 and $PGE_2$ in the supernatant from NK and amnion-derived cell coculture was increased. This observation is particularly evident in the inhibition of NK cytotoxic activity by using neutralizing IL-10 antibody or single inhibitor specific to $PGE_2$ or neutralizing thioredoxin antibody. The decreased NK cytotoxicity by interaction with amnion-derived cells was recovered by continuous culturing in the absence of human amnion-derived cells; this means that the decreased NK cytotoxicity is recoverable, implying that the decreased NK cytotoxicity is not because the NK cells were damaged by coculturing. Moreover, amnion-derived cells also suppressed the activity of cytokine production in monocytes analyzed by TNF-α and IL-6.

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In our study, amnion-derived cells exerted an inhibitory effect on NK cell and monocyte activation and function. These data showed that amnion-derived cells have immunosuppressive activity and that IL-10 and $PGE_2$ may be involved in the immunosuppressive activity of amnion-derived cells.

IL-10 is a well-known cytokine involved in cell regulation and promotion of proliferation and activation of regulatory cells or anti-inflammatory cells. Previous studies have demonstrated that IL-10 production was increased in a mixed lymphocyte population cocultured with human bone marrow-derived MSCs (hBM-MSCs)

(1,3,19,21,38) and in plasmacytoid dendritic cells (DCs) cocultured with BM-MSCs and human adipose tissue-derived MSCs (hAD-MSCs) (55). However, other groups found no change in IL-10 production when hBM-MSCs and PBMCs were cocultured, or IL-10 was not detected in the anti-inflammatory of AD-MSCs (26,36). Some previous studies also investigated induction of IL-10 production by umbilical cord lining-derived cells (6). Our data showed increased levels of IL-10 in the amnion-derived cells and supernatant from coculture of amnion-derived cells and NK cells. Our results showed that the addition of IL-10 antibody in the NK cells and amnion-derived cells coculture has the tendency to recover the decreased NK cytotoxicity. So, it is considered that IL-10 was involved in the function of amnion-derived cells to NK cells.

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$PGE_2$, one of the immune-modulation candidates synthesized from arachidonic acid by COX-1 and COX-2 enzymes, regulates the maturation and antigen presentation of DC, and inhibits T cell proliferation and cytokine production (54). According to Aggarwal et al. (1), the $PGE_2$ production pattern is bell-shaped and occurs in a time-dependent manner, indicating that this factor might be related to early immune suppression by MSCs. Our results also showed that COX-2 mRNA and $PGE_2$ production increased in amnion-derived cells when they were cocultured with NK cells. Some previous studies showed that $PGE_2$ is involved in the immunosuppression of MSC to NK cell

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cytotoxicity (10,45). Also, it is reported that culture with mixed lymphocyte reaction (MLR) increases the release of $PGE_2$ in umbilical cord tissue-derived cells (13). Moreover, blocking $PGE_2$ production in the NK cells/MSCs coculture counteracted the decreased NK cytotoxicity (10) and blocking $PGE_2$ production fully blocked it in the umbilical cord tissue-derived mesenchymal stem cells (UCMSC)/MLR coculture (13). Our results showed that blocking $PGE_2$ partly recovered the decreased NK cytotoxicity. It suggested that $PGE_2$ may play important role in the immunosuppressive activity of amnion-derived cells to NK cells. Therefore, the immunomodulation of amnion-derived cells is a complicated procedure which is involved by many factors (31), among which IL-10 and $PGE_2$ play important roles (40). Further studies will be necessary to determine the detailed mechanism of the immunomodulation.

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iHAM, fHAM and HAMα cells exhibited more inhibition to NK cytotoxicity than iHAE and fHAE, consistent with more decreased expression in activated NK receptors and IFN-γ, more significant increase in production of IL-10 and COX-2, suggesting that amnion-derived mesenchymal cells exerted more effective immunosuppressive properties than amnion-derived epithelial cells. Monocyte assay indicated that amnion-derived mesenchymal cells exerted more impairment to monocytes response than amnion-derived epithelial cells. The results suggested that

the capacity of immunosuppressive activity may depend on the origin of the cells. HAMα indicated the most immunosuppression on NK and on monocyte and the most secretion of IL-10 and PGE2 after coculuring with NK cells in amnion-derived mesenchymal cells. HAMα is a proliferative subpopulation from fHAM and has shown stem cell characteristics and differentiation ability. HAMα can be considered as the most promising potential cell source for cell-base therapy. Although there is small difference of immunosuppressive activity among mesenchymal, epithelial and subpopulation of mesenchymal cells, the amnion-derived cells overall have immunosuppressive activity and would be good cell sources for cell therapy.

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In conclusion, immunosuppressive activity of amnion-derived cells on NK cells and monocytes was demonstrated. Soluble factors IL-10 and $PGE_2$ produced by amnion-derived cells may suppress allogeneic immune responses. These findings support the hypothesis that these cells have potential therapeutic use. Further study is needed to identify the detailed mechanisms responsible for the immunomodulatory effects of amnion-derived cells. Amnion-derived cells will be transplanted into mice model for further analysis of immunosuppressive activity or anti-inflammatory effects.

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## FIGURE LEGENDS

**Figure 1. Amnion-derived cells-mediated inhibition of NK cytotoxic activity.**

NK cytotoxic activity was analyzed after coculturing for 1, 3 or 5 days with or without HUVECs (A) or iHAM (B), iHAE (C), HAMα (D) at an effector/target ratio of 1:10. The cytotoxic activity of NK cells cultured with HUVECs or amnion-derived cells was expressed as a percentage of K562 dead cells with respect to NK cells alone (100%). The results are representative of three independent experiments and are shown as the means ± SE.

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**Figure 2. Expression of activated NK cell receptors and cytokine.**

The expression of activated receptors (NKp30, NKp44, NKp46, NKG2D and CD69) and cytokine (IFN-γ) in NK cells cultured with or without HUVECs (as control) or amnion-derived cells for 1 day was investigated by RT-PCR. GAPDH was used as a loading control.

**Figure 3. Expression of immunosuppressive factors.**

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The expressions of immunosuppressive factors IL-4, IL-6, IL-8, IL-10, HLA-G, COX-2 and TGF-β in amnion-derived cells cultured with or without NK cells for 1 day were investigated. GAPDH was used as a loading control.

**Figure 4. Detection of IL-10 and $PGE_2$ by ELISA.**

The production of IL-10 (A) or $PGE_2$ (B) by amnion-derived cells cultured with or without NK cells for 3 days was investigated by ELISA. Statistically significant difference between amnion-derived cells cultured alone and cocultured with NK cells is indicated by asterisks. *$P<0.05$, **$P<0.01$.

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**Figure 5. The block of IL-10 and $PGE_2$ with specific inhibitors.**

IL-10 neutralizing antibody (A) was added at concentrations of 1 μg/ml, 5 μg/ml or 10 μg/ml, and indomethacin (B) was added at concentrations of 10 μM or 100 μM to the NK and iHAM coculture. Cytotoxicity of NK cells was investigated at day 3.

**Figure 6. Recovery of NK cytotoxic activity.**

FACS analysis of NK cytotoxic activity was performed after culturing with iHAM (A), iHAE (B) or HAMα (C) for 3 days and without amnion-derived cells for 2 days. The

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cytotoxic activity of NK cells was expressed with respect to NK cells alone (100%). Statistically significant difference between 3 day-inhibition assay and 3+2 day-recovery assay is indicated by asterisks. *$P$<0.05, **$P$<0.01.

**Figure 7. Amnion-derived cells-mediated inhibition of cytokine production in monocytes.**

PBMCs from different donors were plated in the presence of amnion-derived cells with LPS. Four hours later, Brefeldin A was added to stop the reaction. The secretion of TNF-α (A) and IL-6 (B) on monocytes cultured with or without iHAM, iHAE, or HAMα cells was analyzed by FACS. The secretion of TNF-α and IL-6 was expressed with respect to monocytes alone (100%). Statistically significant difference of TNF-α and IL-6 on monocytes with or without amnion-derived cells was indicated by asterisks. *$P$<0.05, **$P$<0.01.

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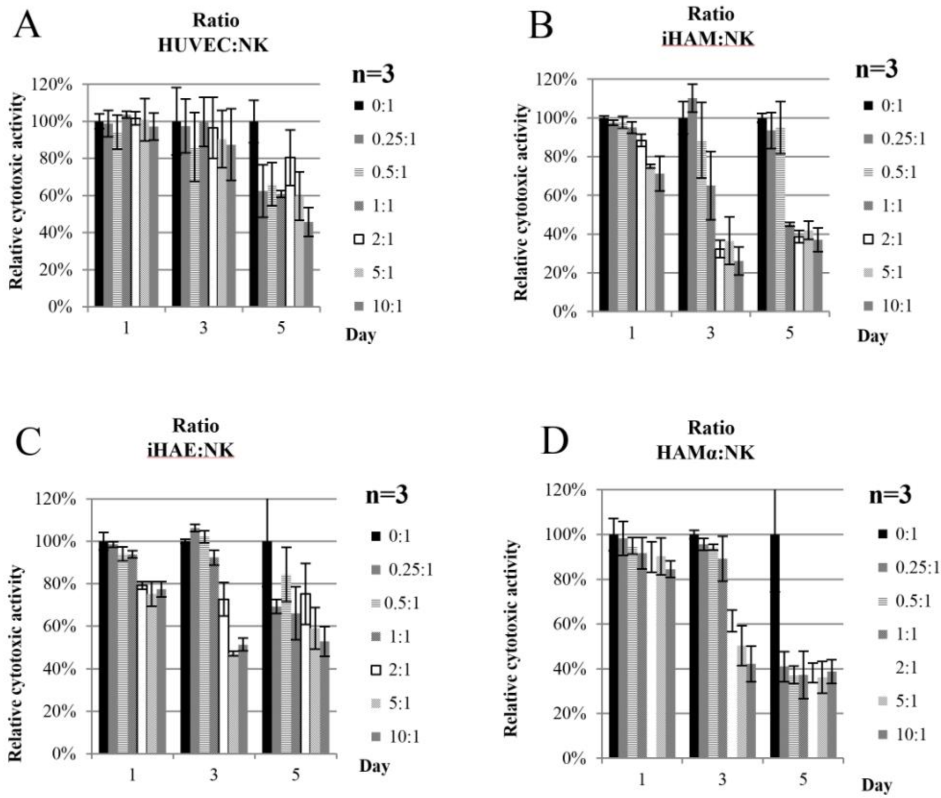


Figure 1.

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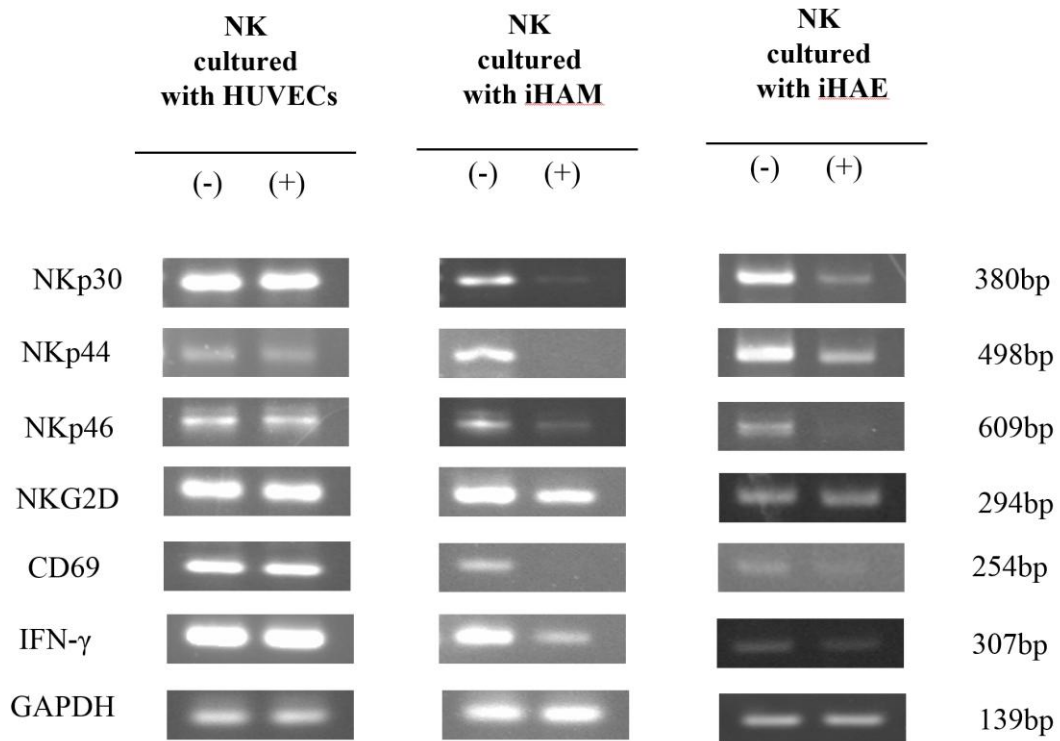


Figure 2.

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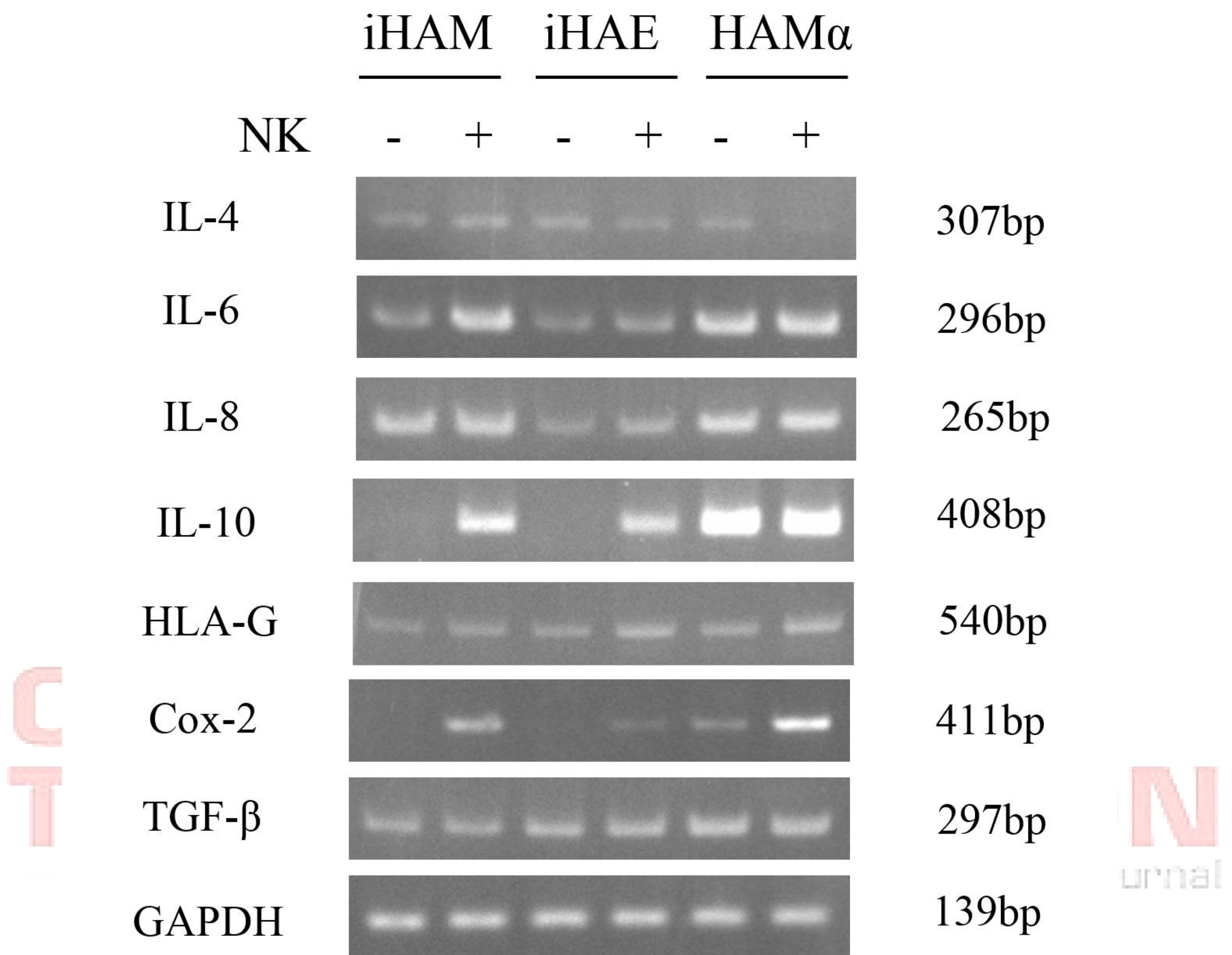


Figure 3.

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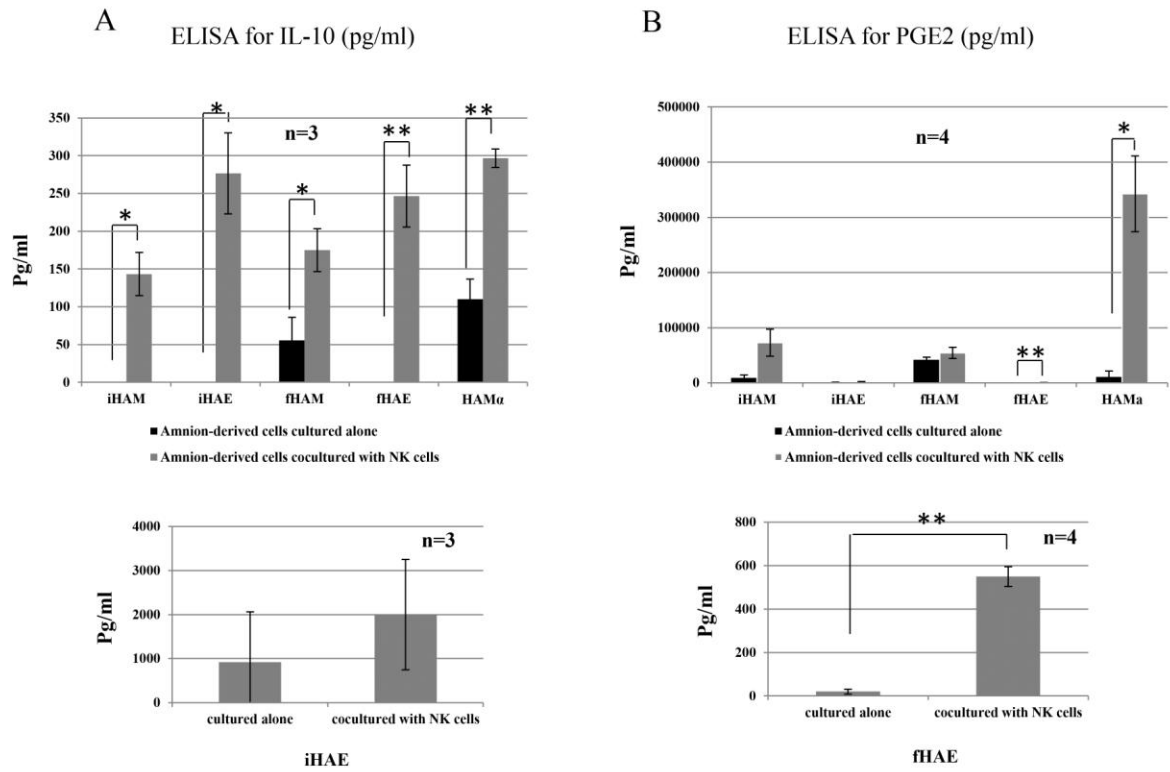


Figure 4.

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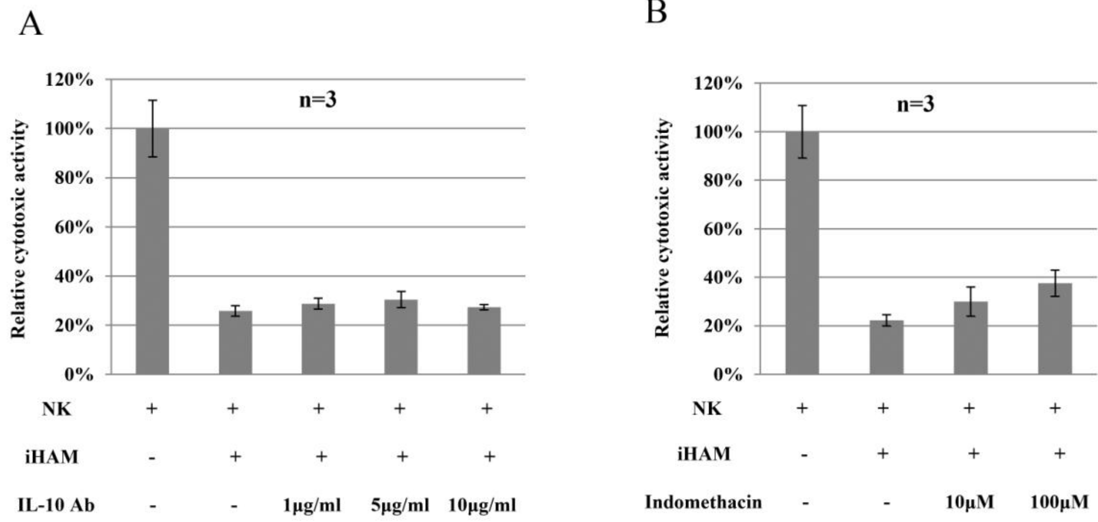


Figure 5.

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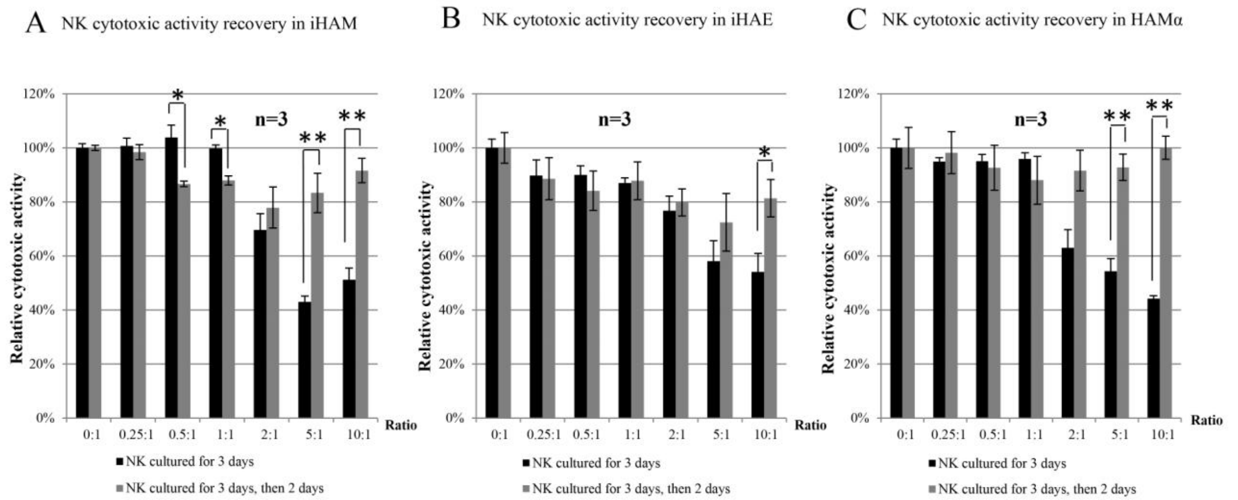


Figure 6.

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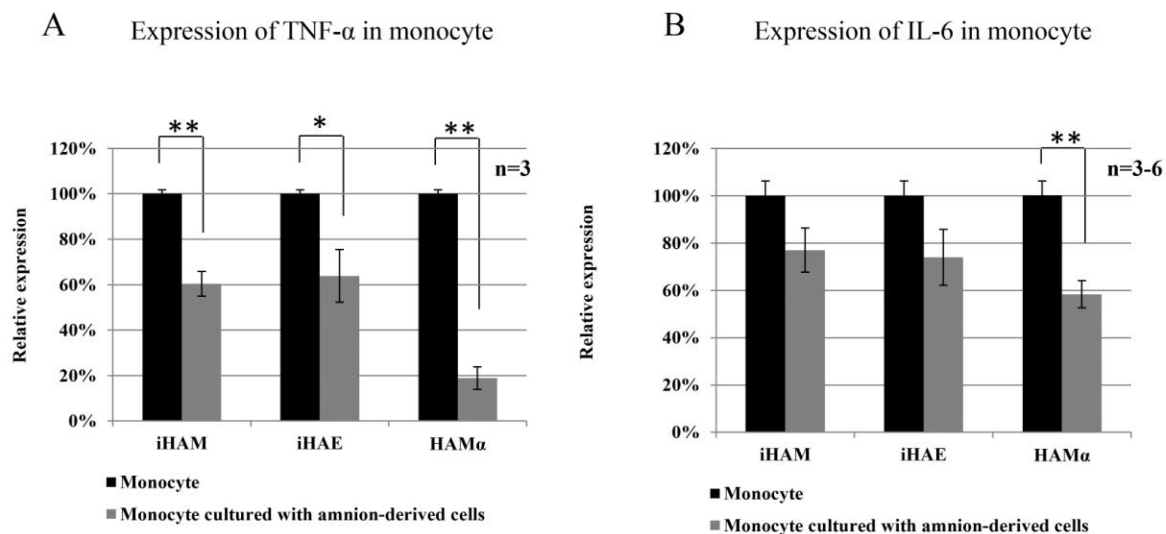


Figure 7.

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