Age-specific different immune responses due to capabilities of secreting primal immunoglobulins responding to unexperienced antigens

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Abstract

Among numerous studies on COVID-19, we noted that the infection and mortality rates of SARS-CoV-2 increased with age and that fetuses known to be particularly susceptible to infection were better protected despite various mutations. Hence, we established the hypothesis that a new immune system exists that forms before birth and decreases with aging. To prove this, we analyzed the components from early pregnancy fetal stem cells cultivated in various ex-vivo culture conditions simulating the environment during pregnancy. Resultingly, we confirmed that IgM, a natural antibody produced only in early B-1 cells, immunoglobulins including IgG3, which has a wide range of antigen-binding capacity and affinity, complement proteins, and antiviral proteins are induced. Our results suggest that fetal stem cells can form an independent immune system responding to unlearned antigens as a self-defense mechanism before establishing mature immune systems. Moreover, we propose the possibility of new solutions to cope with various infectious diseases based on the factors therein.

Introduction

COVID-19, which has generated worldwide pandemics, has caused previously unexperienced phenomena, such as the rapid expansion of viruses before activating the innate immunity (1), increased infection and mortality rates due to continuous mutations (2, 3), severe side-effects across the whole body including the respiratory tract and nervous system (4), induction of incomplete and delayed adaptive immune response (5, 6), re-infection after cure (7, 8), and post-vaccination breakthrough infection (9). Particularly, we paid attention to the patterns of the asymptomatic infection rate in the young and the relatively high fatality rate of COVID-19 in the elderly. The case fatality rate (CFR) of seasonal influenza was less than 0.1%, whereas the CFR of COVID-19 was 1.38%, but was reported to be much higher at 13.4% in those over 80 years of age (10). In contrast, the CFRs of 0–9 years old children and 10–19 years old adolescents were reported to be very low compared to adults, 0.0026% and 0.0148%, respectively (10). In addition, many studies (11–14) reported that children's relatively less experienced immune system could eliminate SARS-CoV-2 much faster than adults'. In particular, fetuses and newborns considered to be susceptible to viral infection also show a relatively low infection rate (15), but the exact cause is not yet known.

However, from the studies that children infected with seasonal coronavirus already have antibodies that cross-react to unexperienced SARS-CoV-2 (16), that live vaccines vaccinated in newborns activate non-specific immunity that has a protective effect against a variety of antigens within 2–3 days, much faster than target pathogen-specific immunity, which is typically activated over several weeks in adults who need to get the flu vaccine every year (17), and that SARS-CoV-2-specific IgM with a macromolecular structure that cannot be transmitted from the mother infected with SARS-CoV-2 exists in infant blood (18), we have established a hypothesis that another immune system may exist even before birth capable of protecting the fetus before the existing innate and adaptive immune systems can respond to new antigens after birth.
To prove this, we focused on fetal stem cells (FSCs) in early pregnancy before the mature immune systems were formed by various immune cells. For the first time, we confirmed that various immunoglobulins (Igs) such as IgM, IgG, IgA, and IgE could be secreted from FSCs around 10 weeks gestation, before 12–13 weeks when B cells, known as the only cells secreting Igs, appear in fetal blood and spleen (19). In addition, unlike stem cells from other origins, we demonstrated that only FSCs cultured under established ex-vivo culture conditions simulating in vivo environment during pregnancy were induced to secrete IgM, a natural antibody (NAb) that binds to and removes various foreign antigens as soon as infection occurs (20), and IgG3 that has the broadest range of antigen-binding, variability capable of binding to various antigens, the highest activation capability of complement system after antigen binding, and binding affinity with various immune cells among other Igs (21). Furthermore, we confirmed that secretions of complement proteins contributing to the innate immune system with NAbs, including IgM and IgG3, and various anti-viral proteins inhibiting infection on cell membranes and inside cells, not Igs’ activity area, were also induced from them. This study suggests the possibility of the existence of a new immune system, named “Primal Immune System” that can protect FSCs from unexperienced external infectious agents by themselves before forming innate and adaptive immune systems through the interaction of various types of immune cells, and propose the mechanism by which the severity of COVID-19 increases with aging by decreased adaptability to new antigens based on the primal immune system. Furthermore, through the considerations of maternal mechanism to protect the fetus, fetal self-protection mechanism, multilayer defense mechanism depending on the infection route, and the blocking mechanism to immune evasion of the virus, we ultimately present the possibility of new solutions to promptly respond to various infectious diseases and disturbances in the immune system without causing unnecessary immune rejection, based on multiple factors that constitute the primal immune system, especially immune-tolerized extracellular vesicles containing newly proposed “Primal Immunoglobulin” which can promptly respond to unexperienced antigens.

Results

Igs secreted by B lymphocytes through somatic hypermutation (SHM) and class-switch recombination (CSR) are found in fetal tissues and serum after 20 weeks of gestation (22), and the period of formation of antibodies through T cell-dependent B cell responses is known after birth (23). However, it is known that IgG, the only Ig that can pass through the placenta, is delivered through the umbilical cord formed around 15 weeks of pregnancy to protect the fetus from various sources of infection (24). Still, it is not precisely known whether Igs were formed in fetuses before that time.

Therefore, we established the flowchart as shown in [Fig. 1] to confirm the possibility of secretion of Igs from FSCs in early pregnancy and to clarify the concept of a new immune system built up by FSCs by developing an ex-vivo culture system that can induce the secretion of various factors, including Igs, which can protect themselves from foreign infections.
Confirming the possibility that FSCs in early pregnancy can produce immunoglobulins

There have been reports that FSCs expressed the characteristics of hematopoietic stem cells (HSCs) (25–27). However, no study has reported that FSCs could secrete Igs produced only from B cells of HSCs lineage. To confirm the possibility of Igs production in the fetus in the early pregnancy, we obtained FSCs from amniotic fluid samples donated from a healthy pregnant woman at around 10 weeks of gestation for amniocentesis. Through flow cytometry analysis on the obtained FSCs, we confirmed the expressions of various stem cell markers and Igs. Then, we performed Protein Antibody Microarray and ELISA analysis on the conditioned media obtained by culturing FSCs on a typical 2D plate. As a result, we confirmed that IgM, IgG, IgA, and IgE were expressed at very low levels in microarray analysis. Still, no Ig, especially NAbs acting on the innate immunity, was detected in ELISA analysis. From these results, we confirmed the possibility of Ig expression in FSCs. However, it was confirmed that establishing a new culture condition simulating in vivo environment capable of inducing NAbs secretion from FSCs is necessary to verify the possibility of secretion of NAbs.

Establishment of an ex-vivo culture condition simulating in vivo environment during pregnancy to induce the secretion of NAbs in FSCs

In a previous study (28), we established the maternal-fetal interface-like ex-vivo culture condition to investigate the immune tolerance mechanism that completely protects the fetus from the maternal immune system during pregnancy. In addition, we demonstrated for the first time that extravillious trophoblasts (EVTs) cultured in this condition promoted the continuous expression and secretion of HLA-G, which indues an immune tolerance environment through the activation of self-secretion of pregnancy-related hormones, including human chorionic gonadotropin (hCG) and progesterone. In particular, we confirmed that the secretion of hCG and progesterone from trophoblast cells could be promoted cultured in maternal-fetal interface-like ex-vivo culture condition by applying temperature profile based on the woman's body temperature change, pH, and vibration conditions to replace the internal nervous system that regulates the secretion of hormones promoting the expression and secretion of HLA-G protein that protects the fetus from the mother's immune cells. As a result, we could establish immune-tolerized trophoblasts (iTBCs) that express and secrete HLA-G consistently. Based on this, we tried to establish new ex-vivo culture conditions to induce the secretion of NAbs from FSCs in early pregnancy.

FSCs in the first trimester of pregnancy are protected by layers of trophoblasts in direct contact with the maternal blood and decidua. Moreover, they coexist with immature immune cells of the fetus (Fig. 2A). To simulate this in vivo environment of FSCs in the early stages of pregnancy, we devised three in vitro culture environment modules as shown in [Fig. 2B]. The first is the maternal-fetal interface-like in vitro culture condition established in a previous study (28), in which we cultured trophoblasts on hyaluronic acid (HA)-based matrix for in vitro culture containing extracellular vesicles (EVs) obtained through co-culture of amniotic fluid and amnion-derived stem cells for pH and physical conditions similar to in vivo environment during pregnancy. The second is an in vitro culture condition for inducing the secretion of
pregnancy-related hormones. From the possibility of Ig production of FSCs that we have identified, we hypothesized that pregnancy-related hormones secreted from trophoblast cells could induce HLA-G secretion protecting the fetus from the maternal immune system, and at the same time, affect the secretion of Igs, especially NAbs, protecting the fetus from foreign antigens. To prove this, we obtained iTBC-derived EVs (iTBC-EVs) containing various pregnancy-related hormones by applying the temperature profile and vibration conditions similar to women's internal environment to maternal-fetal interface-like culture conditions, as shown in [Fig. 2C, 2D]. Lastly, to stimulate the secretion of NAbs from FSCs, we obtained the EVs (HSC/UCB-MSC\(^{CO}\)-EVs) from serum-free co-cultivation of HSCs and umbilical cord blood mesenchymal stem cells (UCB-MSCs) under hypoxic conditions by replacing undeveloped fetal immune cells. And by applying these EVs together with iTBC-EVs, we prepared a new matrix for in vitro culture. Moreover, by applying the above temperature change, pH, and vibration conditions, we finally established the new ex-vivo culture condition simulating the in vivo environment to confirm the induction of NAbs secretion in FSCs.

**Establishment and characterization of FSCs secreting NAbs**

The expression and secretion of NAbs in FSCs (FSCs\(^{Ex-vivo}\)) cultured in new ex-vivo culture conditions established for inducing NAbs secretion were compared with those of FSCs (FSCs\(^{Control}\)) cultured in general 2D-plate and verified. [Fig. 3A] is a schematic diagram proving the expression and secretion of NAbs in FSCs\(^{Ex-vivo}\) through various analysis methods. [Fig. 3B] shows the comparison of the expression patterns of stem cell-specific markers for FSCs\(^{Control}\) and FSCs\(^{Ex-vivo}\) through flow cytometry. As a result of performing flow cytometry using mesenchymal stem cell (MSC) markers (CD73, CD90, CD105) and embryonic stem cell (ESC) markers (SSEA4) to investigate the cellular characteristics of FSCs in early pregnancy, which are known to have both ESC and MSC characteristics (25), it was confirmed that FSCs\(^{Control}\) and FSCs\(^{Ex-vivo}\) showed almost the same expression patterns. These results indicate that FSCs\(^{Ex-vivo}\) cultured in new ex-vivo culture conditions established by us fully maintain the inherent MSC and ESC characteristics. [Fig. 3C] shows the flow cytometry analysis of the expression patterns of IgG and NAbs (IgM and IgG3) for FSCs\(^{Control}\) and FSCs\(^{Ex-vivo}\). As a result of analyzing the cell membrane expression of IgM, the only membrane-bound Ig, interestingly, some membrane-bound IgM was expressed in FSCs\(^{Control}\), but few expressed in FSCs\(^{Ex-vivo}\). However, as a result of analyzing intracellular expressions, the expression pattern of IgG was similar in FSCs\(^{Control}\) and FSCs\(^{Ex-vivo}\), but NAbs were expressed higher in FSCs\(^{Ex-vivo}\) than FSCs\(^{Control}\).

[Fig. 3D] shows the analysis of the Ig secretion patterns of FSCs\(^{Control}\) and FSCs\(^{Ex-vivo}\) through Protein Antibody Microarray. As a result of analyzing the expression pattern of Igs in the culture supernatant obtained through the serum-free culture of each FSC, we confirmed that the expression of all Igs such as IgG, IgM, IgE, and IgA included in the microarray were higher in FSCs\(^{Ex-vivo}\) than in FSCs\(^{Control}\). These results indicate that our ex-vivo culture conditions induced the expression and secretion of Igs in FSCs.
Finally, we verified the secretion of NAbs in FSCs^Ex-vivo through IgM and IgG3 ELISA analysis. To prove whether the expression and secretion characteristics of Igs in stem cells, not immune cells, are inherent to FSCs and whether new ex-vivo culture conditions induce the secretion of NAbs, IgM and IgG3 concentrations were analyzed by ELISA for each conditioned medium obtained by serum-free cultivation of various stem cell lines under normal 2D-plate culture conditions (2D) and new ex-vivo culture conditions (Ex-vivo). As shown in [Table.1], IgG3 and IgM were not detected in the conditioned medium of FSCs cultured under normal culture conditions (2D), but only in the conditioned medium of FSCs cultured under newly established in vitro culture conditions (Ex-vivo). In addition, IgG3 and IgM were not detected in the conditioned medium of cord blood, bone marrow, adipose, and umbilical cord-derived MSCs and HSCs regardless of the culture conditions. Moreover, we confirmed that IgG3 and IgM were not detected in all EVs used in new ex-vivo culture conditions, including iTBC-EVs, AF/AM-MSC\textsuperscript{CO}-EVs, and HSC/UCB-MSC\textsuperscript{CO}-EVs. These results verified that only FSCs in the early stages of pregnancy have the characteristic of secreting NAbs to protect themselves from external infections, and FSCs cultured in established ex-vivo culture conditions are NAb-secreting FSCs (NAb-FSCs).

Meanwhile, trophoblast cells secrete estrogen, another critical sex hormone, together with progesterone and hCG during pregnancy (29). We found that hCG, progesterone, and estrogen were induced in iTBC-EVs used in new ex-vivo culture conditions. A study (30) showed that estrogen promotes Ig production in human PBMCs, but no study has been reported in other types of cells. Therefore, our results that Igs were induced in FSCs^Ex-vivo cultured in ex-vivo culture conditions applying iTBC-EVs containing estrogen at a higher level than in FSCs\textsuperscript{Control} derived from cultivation without iTBC-EVs verify that estrogen also contributes to the promotion of Ig transcription in FSCs before B cell differentiation. Besides, our results demonstrate our hypothesis that hormones secreted during pregnancy can induce the secretion of HLA-G and Igs, thereby simultaneously protecting the fetus from the maternal immune system and foreign antigens.

**Characterization of NAb-secreting FSCs-derived EVs (NAb-FSC-EVs)**

To verify whether EVs (NAb-FSC-EVs) secreted from FSCs (NAb-FSCs) cultured under new ex-vivo culture conditions contained NAbs, we performed various characterization and compared the results with FSCs-derived EVs (FSCs\textsuperscript{Control}-EVs) cultured in a typical 2D plate. [Fig. 4A] shows a schematic diagram demonstrating whether NAb-FSC-EVs contain various factors, including NAbs, that can protect FSCs from external infection. [Fig. 4B] shows the results of nanoparticle tracking analysis (NTA) to determine the size distribution and concentration of FSC\textsuperscript{Control}EVs and NAb-FSC-EVs isolated by multi-stage filtration from the serum-free culture supernatant of 1.0 x 10^5 FSCs, respectively (n = 3). As a result of NTA analysis, we confirmed that the concentration, average size, and distribution of FSC\textsuperscript{Control}EVs and NAb-FSC-EVs showed almost similar patterns. [Fig. 4C] shows scanning microscope (SEM, left) and transmission microscope (TEM, right) images of FSC\textsuperscript{Control}EVs and NAb-FSC-EVs, respectively, and we confirmed that they show very similar size distributions and shapes.
[Fig. 4D] represents the results of flow cytometry analysis of the expression patterns of exosome (small EVs) markers, such as CD9, CD63, and CD81, in FSC\textsuperscript{Control}-EVs and NAb-FSC-EVs. Compared to CD63 and CD81, which show similar expression levels in both EVs, we confirmed that the patterns showing a somewhat low expression level of CD9 were similar in both EVs. This result seems to be due to the unique characteristics of FSCs. In addition, [Fig. 4E] shows the results of re-verifying the co-expression patterns of exosome markers such as CD9, CD63, CD81, and syntenin in each EV using ExoView\textregistered, which is very similar to the flow cytometry results shown in [Fig. 4D]. These results show no difference in the expression characteristics of the exosome markers of EVs secreted from each FSCs regardless of the culture conditions and also show that EVs used in the analysis are all normal exosomes.

[Fig. 4F] show the comparison of IgG and NAb (IgG3 and IgM) content in FSC\textsuperscript{Control}-EVs and NAb-FSC-EVs through flow cytometry. Similar to the results shown in [Fig. 3C], we confirmed that the levels of IgG content were similar in FSC\textsuperscript{Control}-EVs and NAb-FSC-EVs, but IgM and IgG3 were contained at slightly higher levels in NAb-FSC-EVs than in FSC\textsuperscript{Control}-EVs. In addition, [Fig. 4G] and [Fig. 4H] show the results of exosomal cargo analysis using Exoview\textregistered to determine whether each EV contains NAb. We again verified the flow cytometry analysis results (Fig. 4F) that IgM and IgG3 were contained at a relatively higher level in NAb-FSC-EVs than FSC\textsuperscript{Control}-EVs. These results confirmed that the newly established ex-vivo culture conditions could induce the secretion of EVs containing NAb from FSCs in early pregnancy.

Meanwhile, to study the mechanism by which NAb is induced at higher levels in NAb-FSC-EVs, we performed protein antibody array analysis on the components included in FSC\textsuperscript{Control}-EVs and NAb-FSC-EVs and compared the expression patterns of B cell specific proteins related to the transcription of Ig light chain (Fig. 4I).

B cell-specific Ig gene expression and secretion are due to tissue-specific expression of octamer transcription factors (OCT), nuclear proteins that bind to the octamer sequence ATGCAAAT motif in the promoter and enhancer of the Ig heavy chain gene (31), and another transcription factor, NF-kB, is known as an enhancer of the Ig light chain gene (32). In addition, genes specially expressed in early B cells, immunoglobulin lambda-like polypeptide 1 (Igl1, also λ5) and paired box protein 5 (Pax-5), are also known to be expressed during B cell receptor (BCR) development which is converted into a membrane-bound form of IgM by antigen stimulation (33, 34). Furthermore, the cleavage stimulating factor (Cstf), which is known to be involved in the growth and differentiation of B cells, has been reported to convert the membrane-bound form of IgM mRNA to secreted form (35).

Our results shown in [Fig. 4I] confirmed that various B cell-specific and Ig transcription-related proteins were expressed in FSCs at the stage before B cell differentiation and showed that these proteins are induced at higher levels in NAb-FSC-EVs than FSC\textsuperscript{Control}-EVs. In particular, our results that NAb-FSC-EVs contained higher levels of soluble IgM than FSC\textsuperscript{Control}-EVs while the expression of membrane-bound IgM was decreased in FSCs\textsuperscript{Ex-\textit{vivo}} (NAb-FSCs) than in FSCs\textsuperscript{Control} were due to the effect of Cstf induced at higher levels in NAb-FSC-EVs. Our results represented that NAb-FSCs established by environmental
factors simulated to induce secretion of NAbs may have the ability to secrete higher levels of IgG3 and IgM.

**Self-defense mechanism by NAbs and complement proteins induced in NAbs-FSC-EVs**

Innate immunity is a primary defense system that removes the foreign antigens by non-specifically immediately reacting to them and activates antigen-specific adaptive immunity. Various immune cells, including macrophages, dendritic cells (DCs), and natural killer (NK) cells, are involved in innate immunity, but their cytotoxicities are mainly induced by NAbs and complement proteins together that can bind to various antigens. Therefore, we investigated whether NAbs-FSCs secrete complement proteins capable of eliminating foreign antigens by binding with NAbs.

The complement system contributes to local inflammation, pathogen elimination and death, and the formation of the subsequent adaptive immune response through three main functions, including opsonization, chemotaxis, and lysis (36). Most of the complement proteins in the serum are produced and secreted by liver cells, and a small amount of them is secreted from endothelial cells, epithelial cells, and immune cells such as monocytes, macrophages, and DCs in a local area where the serum is restricted (37). However, there has been no report on whether stem cells secrete complement proteins.

From the results of protein antibody microarray and protein absolute quantification analysis on the components in FSC\textsubscript{Control}-EVs and NAbs-FSC-EVs, we confirmed that various complement proteins related to three activation pathways were induced in NAbs-FSC-EVs at a higher level than FSC\textsubscript{Control}-EVs (Fig. 5A, B, C). Our results show that FSCs in early pregnancy, before the innate immune system is fully established, can form the self-defense system to protect themselves from unexperienced antigens by secreting NAbs, including IgG3 and IgM, and various complement proteins.

**Identification of the characteristics as an immune system of the self-defense mechanism formed by NAbs-FSCs**

To identify the characteristics as an immune system of the self-defense mechanism formed by NAbs-FSCs and to investigate the effect of HSC/UCB-MSC\textsuperscript{CO}-EVs applied in ex-vivo culture conditions as the replacement of immature fetal immune cells to establish NAbs-FSCs, we performed cytokine array analysis on FSC\textsubscript{Control}-EVs, immune-tolerized FSCs-derived EVs (itFSC-EVs), and NAbs-FSC-EVs secreted from three FSCs established under different culture conditions as shown in Fig. 6A. As a result, we confirmed that the secretion patterns of pro-inflammatory cytokines such as interleukin (IL)-1\(\beta\), IL-6, and tumor necrosis factor (TNF)-\(\alpha\) were similar in EVs from three FSCs, but Th1 cytokine, IL-2 and interferon (IFN)-\(\gamma\), were secreted in NAbs-FSC-EVs at a higher level than itFSC-EVs and FSC\textsubscript{Control}-EVs (Fig. 6B). In these results, we noted that IFN-\(\gamma\) was secreted at a higher level in NAbs-FSC-EVs, obtained from FSCs cultured in new ex-vivo culture conditions including HSC/UCB-MSC\textsuperscript{CO}-EVs, than in itFSC-EVs, because IFN-\(\gamma\) promotes the transcription of the HLA-G gene together with progesterone (38), and at the same time,
acts as a transcriptional factor for various anti-viral proteins that affect mainly on the cell membrane and inside the cell, unlike Igs, NAbs, and complement proteins which function outside the cell.

To verify the effect of IFN-γ on HLA-G expression and secretion in FSCs, we compared the expression aspects of HLA-G proteins in each FSC through flow cytometry analysis. As a result, we confirmed that the expressions of membrane-bound HLA-G1 and intracellular soluble HLA-G5/G6 were more increased in NAbs-FSCs than itFSCs (Fig. 6C). Moreover, to compare the secretion aspects of HLA-G proteins in each FSC, we performed ELISA analysis for the content of HLA-G proteins on each EVs. The results showed that sHLA-G (shedding HLA-G1 and soluble HLA-G5) were secreted more in in ifFSC-EVs than in FSCControl-EVs, and in NAbs-FSC-EVs than in itFSC-EVs, but HLA-G5/G6 were secreted at similarly higher levels in NAbs-FSC-EVs and ifFSC-EVs than FSCControl-EVs (Fig. 6D), which can be interpreted as a difference due to the membrane-bound HLA-G1. Since iTBC-EVs, including progesterone which promotes HLA-G gene transcription, were equally applied in both ex-vivo culture conditions to establish ifFSCs and NAbs-FSCs, we hypothesized that the difference in shedding HLA-G1 in NAbs-FSC-EVs and ifFSC-EVs was due to the effect of different amounts of IFN-γ secreted from each FSC.

To verify this, we performed protein antibody microarray and protein absolute quantification analysis and compared the contents of interferon-inducing proteins in NAbs-EVs and ifFSC-EVs. As a result, we confirmed that the contents of interferon-inducing proteins, including interferon-inducible transmembrane protein 3 (IFITM3) and lymphocyte antigen 6 family member E (LY6E), known to play an anti-viral role in the cellular and endosomal membranes (39, 40), were induced at a higher level in NAbs-FSC-EVs than ifFSC-EVs (Fig. 6E). In addition, we demonstrated that transient receptor potential mucolipin subfamily member 2 (TRPML2), which is expressed only in recycled endosomes similarly to interferon-inducing proteins and is known to inhibit virus replication through activation of anti-viral autophagy (41), was induced at a higher level in NAbs-FSC-EVs than ifFSC-EVs (Fig. 6F).

Our results suggest that a more complex and sophisticated self-defense mechanism in the cell membrane and inside the cell by various anti-viral proteins induced by increased IFN-γ stimulation in addition to the extracellular defense mechanism by secretion of NAbs and complement proteins from NAbs-FSCs exists as an immune system. In addition, our findings that NAbs, complement proteins, and various anti-viral proteins are all contained in immune-tolerized EVs expressing various HLA-G isoforms suggest that they may propose a safe response strategy not causing unnecessary immune rejection to patients in the prevention and treatment of various infectious diseases, as identified through previous studies (28).

**Discussion**

Although many studies have been made through long-term COVID-19 pandemic, conflicting reports have been published on newborns and prenatal fetuses born from mothers infected with SARS-CoV-2 due to many limitations, such as difficulties in designing large-scale studies and ethical issues. However, data accumulated to date on vertical infections (42, 43) were obtained in the last trimester of pregnancy and
after birth. Little study has been done on early pregnancy before the fetal immune system was fully established, especially on Igs that protect the fetus against external pathogens.

As a result of culturing FSCs in the first trimester of pregnancy in ex-vivo culture conditions simulating in vivo environment of FSCs co-existing with the trophoblast layer as a protective barrier against external pathogens from mother and undeveloped fetal immune cells, we confirmed that various Igs, including IgG3, IgG, IgM, and IgA, known to be produced only in mature B cells, are induced from FSCs. We also demonstrated that the B cell-specific proteins such as OCT-1/2, NF-kb, CstF, and Igll1, which promote the transcription and secretion of Igs, complement proteins playing an essential role in innate immunity along with NAbs, including IgM and IgG3, and various anti-viral proteins are induced. From these findings, we verified that FSCs, before the immune system was thoroughly developed, can form a perfect primal immune system to protect themselves from foreign pathogens.

As below, we propose the following three mechanisms for the new immune system, which we demonstrated, never mentioned in the existing immunity.

First, we suggest the new perspective of fetal protection mechanism by hormones secreted for maintaining normal pregnancy. In other words, just as the expression and secretion of HLA-G proteins that protect the fetus from the maternal immune system are induced by hCG and progesterone secreted from trophoblasts (28), we demonstrated the transcription and secretion of Igs that protect the fetus from foreign antigens could be induced by estrogen, another pregnancy-related hormone. In addition, our results proved for the first time that the mechanism that estrogen increases the secretion of IL-10, which promotes Igs production in B cells (44, 45), was already applied to FSCs before B cell differentiation (Supplemental Fig. 1).

Second, we propose that the self-defense mechanism of FSCs is acting as an “immune system”. FSCs in early pregnancy before developing the adaptive immune system by T and B cells should immediately remove the foreign antigens when the infection occurs while minimizing the damage caused by NK cells, which account for most fetal immune cells to maintain normal development. In this regard, we suggest that the following protection mechanisms by various anti-viral factors contained in NAbs-FSC-EVs exist as a sophisticated self-defense system that works organically as below (Supplemental Fig. 2);

1. Protective functions outside cells by NAbs and complement proteins
2. Protective functions in the cell membrane by regulating the expression of receptors as the co-expressions of angiotensin-converting enzyme 2 (ACE 2) and transmembrane protease serin subtype 2 (TMPRSS2), which are essential for intracellular entry of SARS-CoV-2, are downregulated in placental trophoblasts, resulting in a low infection rate (15, 46)
3. Protective functions inside cells that inhibit the replication of infected viruses and induce a delayed immune response by NK cells during the degradation process of activated anti-viral autophagy (47)

Although we couldn't obtain the analysis results for more interferon-inducing proteins in NAbs-FSC-EVs due to the limitation of the protein library in the protein antibody array we used, we expect a much more
elaborate defense system will protect FSCs from foreign infectious agents than our results. Therefore, we propose to define the defense system of FSCs as a new term, "Primal Immune System", to distinguish from the existing innate and adaptive immune systems.

Third, the most notable result is that IgG3, one of NAbs, was detected in NAbs-FSC-EVs only obtained under newly established ex-vivo culture conditions. NAbs are preimmune antibodies generated without antigen stimulation and act as a primary line of defense against infection because they can immediately nonspecifically cross-react with various exogenous antigens (48). IgM is known as a representative NAb, but our study shows that early pregnancy FSCs can secrete IgM, IgA, IgG, and especially IgG3, which is recently attracting attention as an ideal alternative to existing Ab-based immunotherapies (18). IgG3 has many differences that distinguish it from other IgG subclasses.

1. IgG3 has a long hinge region consisting of 11 disulfide bonds to provide extended flexibilities and reaches to bind various antigens of wide ranges.
2. IgG3 has various functions, including significant improvement of Fc effector activity, broader neutralizing efficacy, and intensively inducing adaptive immune responses, by high binding affinities to Fcγ receptors of immune cells and complement protein C1q, which induce antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (49–53).
3. IgG3 has a more remarkable neutralization ability despite structural modification of endocytic virus into cells due to the property to form aggregates more easily than other IgG subclasses at low pH and maintain binding to receptors (54–56).

However, a more critical point than these structural features of IgG3 is that FSCs in early pregnancy possess the most naive, inexperienced Ig repertoire that can immediately recognize and respond to all foreign antigens. The primary Ig repertoire, which is formed in the early stage of B cells development at about 26 weeks of pregnancy (57, 58), gradually has antigen-specific diversity and becomes increasingly difficult to expand the repertoire for unexperienced new antigens because the number of naive B cells contributing to the formation of the primary Ig repertoire decreases, while antigen-experienced B cells accumulate due to various acquired factors with aging (59). These are supported by many studies showing that children's immune systems respond better to COVID-19 than the old and adults (11–14) and that Ig repertoires responding to COVID-19 exist even in some adults not infected with COVID-19 (60).

We have demonstrated that NAbs-FSCs, established in this study by culturing FSCs obtained at around 10 weeks of gestation, that is, the stem cells in the early stage of HSCs that can differentiate into B cells, could secrete a variety of NAbs, including IgG3. Since these NAbs are not almost exposed to any antigens, they may have the "Primal Ig Repertoire" that can form a full range of primary Ig repertoire that expands later in the B cell development stage against all antigens. Therefore, to differentiate IgG3, a NAb secreted from FSCs in the early stages of pregnancy and having a primal repertoire, from the existing IgG3, which is known to be secreted from B-1 cells, we suggest that it should be redefined with a new classification and terminology as "Primal Immunoglobulin (IgP)". In addition, we would like to define IgP-secreting FSCs established under new ex-vivo culture conditions in this study as "immune-tolerized primal
immunoglobulin secreting FSCs (itPG-FSCs)”. Also, considering the characteristics of the primal immune system by the newly defined IgP, we propose a human immune response system against external infections, including SARS-CoV-2, as shown in [Supplemental Fig. 3].

Lastly, through additional studies and clinical trials of “itPG-FSC-EVs” not causing immune rejection, as demonstrated in the previous study (28), and containing all of the various anti-viral factors constituting the newly proposed "Primal Immune System", we expect that our results become new immunological strategies that can be applied to fundamentally preventive protection and treatment against foreign antigens of various infection pathways, including SARS-CoV-2.

| Cell Lines                  | Concentration of IgG3 (ng / ml) | Concentration of IgM (ng / ml) |
|-----------------------------|---------------------------------|-------------------------------|
| FSCs (2D)                   | < Min                           | < Min                         |
| FSCs (Ex-vivo)              | 0.5512 ± 0.0290                 | 1.497 ± 0.417                 |
| UCB-MSCs (2D)               | < Min                           | < Min                         |
| UCB-MSCs (Ex-vivo)          | < Min                           | < Min                         |
| 1)ADSCs (2D)                | < Min                           | < Min                         |
| ADSCs (Ex-vivo)             | < Min                           | < Min                         |
| 2)BM-MSCs (2D)              | < Min                           | < Min                         |
| BM-MSCs (Ex-vivo)           | < Min                           | < Min                         |
| 3)UC-MSCs (2D)              | < Min                           | < Min                         |
| UC-MSCs (Ex-vivo)           | < Min                           | < Min                         |
| HSCs (2D)                   | < Min                           | < Min                         |
| HSC/UCB-MSC\(^{CO}\)-EVs (Co culture) | < Min                   | < Min                         |
| 4)AF/AM-MSC\(^{CO}\)-EVs (Co culture) | < Min                   | < Min                         |
| itTBC-EVs                   | < Min                           | < Min                         |

1) ADSC, Adipose-derived stem cells; 2) BM-MSC, Bone marrow derived mesenchymal stem cells; 3) UC-MSC, umbilical cord-derived mesenchymal stem cells; 4) AF/AM-MSC\(^{CO}\)-EVs, EVs from serum-free co-cultivation of Amniotic fluid (AF) and Amniotic membrane (AM) derived mesenchymal stem cells.
Declarations

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AUTHOR CONTRIBUTIONS

J.L. conceived the idea, built the theory, and supported the funding; J.L. and K.C. conceived and designed the study and all experiments and wrote the manuscript; H.K. and S.K. contributed to EV preparation and characterization and performed in vitro experiments; S.K., Y.L., and J.L. contributed in vitro experiments and analysis; K.C. and S.K. processed and analyzed the data; Y.L. and J.L. contributed to the drawings of the manuscript. All authors reviewed the manuscript and have given approval to the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interest.

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Methods

Cell Lines and Culture

Human trophoblast BeWo cells and their culture medium were purchased from ATCC (Manassas, VA, USA). BeWo cells were cultured in F-12k medium (ATCC) supplemented with 100 U/mL of penicillin (Sigma-Aldrich, St. Louis, MO, USA), 100 μg/mL of streptomycin (Sigma-Aldrich), and 10% fetal bovine serum (FBS) (Gibco by Thermo Fisher Scientific, Waltham, MA, USA). Human fetal stem cells (FSCs) were established using a known method from amniotic fluid obtained from the healthy pregnant woman at 10 weeks of gestation who performed amniocentesis with written informed consent for utilization of tissues and all experimental procedures approved by the Institutional Bioethics Committee of Stemmedicare Ltd. (SIRB-2020-AUG-01). Human hematopoietic stem cells (HSCs) were purchased from ATCC and were maintained in Hank’s Balanced Salt Solution (HSBS) medium (ATCC) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% heat inactivated FBS. Human amniotic mesenchymal stem cells (hAM-MSCs) and human umbilical cord blood mesenchymal stem cells (UCB-MSCs) were purchased from ScienCell (Carlsbad, CA, USA). Human amniotic fluid-derived mesenchymal stem cells (hAF-MSCs) were established as described in the previous study (28). These MSCs were maintained in DMEM medium (Welgene, Republic of Korea) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% heat-inactivated FBS.

In vivo-like temperature change, pH, and circulation conditions

An in vivo-like culture condition for inducing immune-tolerized cell lines was applied using temperature change, pH, and circulation conditions established in the previous study (28). In summary, temperature change between 36.0 °C and 37.0 °C with a five or six-day cycle and an acidic pH of 6.2 to 6.8 by HA-based matrix were applied to induce the secretion of various pregnancy-related hormones as shown in Fig.2C. Simultaneously, a circulation condition with a 24-hr cycle, as shown in Fig.2D, was applied to facilitate the signal transduction between the culture matrix and immune-tolerized cells to promote autocrine of various soluble factors.

Establishment of co-culture system of HSCs and UCB-MSCs
An indirect co-culture system of HSCs and UCB-MSCs was established using a multi-dish with polycarbonate membrane insert (Thermo Fisher Scientific). HSCs were seeded at a density of $2 \times 10^5 / \text{cm}^2$ in a 6-well plate, and UCB-MSCs were seeded at a density of $2 \times 10^4 / \text{cm}^2$ in a 0.4 μm pore insert (Nunc). They were cultured in DMEM serum-free medium in a 1% O$_2$ and 5% CO$_2$ humidified atmosphere at 37°C for 120 hrs to obtain the conditioned medium to isolate EVs later.

**EVs Preparation**

*Multi-stage Filtration.* To increase the efficiency of the subsequent stage filtration and recovery a high yield of EVs, multi-stage filtration method was applied, established in the previous study (28). In summary, the cell culture supernatant, centrifuged at 1,500 RPM for 5 min, was filtered with a 0.8-μm filter to remove cell debris and apoptotic bodies completely, and the supernatant was filtered with a 0.45-μm filter twice. To obtain small EVs, the supernatant was filtered with a 0.22-μm filter in the same manner as above.

*AF/AM-MSC$^{CO}$-EVs.* The conditioned medium obtained from serum-free co-cultivation of hAM-MSCs and hAF-MSCs in multi-dish plate, as described in the previous study (28), was centrifuged at 1,500 RPM for 5 min at the room temperature, and the supernatant was filtered with a 0.45-mm filter after multi-stage filtration to obtain AF/AM-MSC$^{CO}$-EVs.

*HSC/UCB-MSC$^{CO}$-EVs.* The conditioned medium obtained from serum-free co-cultivation of HSCs and UCB-MSCs in multi-dish plate was centrifuged at 1,500 rpm for 5 minutes at the room temperature, and the supernatant was filtered with a 0.22-μm filter after multi-stage filtration to obtain HSC/UCB-MSC$^{CO}$-EVs.

*itTBC-EVs.* BeWo cells with both syncytiotrophoblast (STB) and EVT phenotypes were sub-cultured in HA-Matrix 3-5 times to establish immune-tolerized trophoblast cells (iTBCs). The conditioned medium, obtained from serum-free cultivation of iTBCs with a seeding density of $2.0 \times 10^4 / \text{cm}^2$ in HA-Matrix containing AF/AM-MSC$^{CO}$-EVs with in vivo-like temperature change, pH, and circulation conditions, was centrifuged at 1,500 RPM for 5 minutes at the room temperature, and the supernatant was filtered with a 0.45-μm filter after multi-stage filtration to obtain iTBC-EVs.

*NAbs-FSC-EVs.* The conditioned medium, obtained from serum-free cultivation of NAb-secreting FSCs (NAbs-FSCs) with a seeding density of $2.0 \times 10^4 / \text{cm}^2$ alone without any matrix after washing with PBS several times to remove any contaminant during the cultivation on HA-matrix containing iTBC-EVs and HSC/UCB-MSC$^{CO}$-EVs with in vivo-like temperature change, pH, and circulation conditions, was centrifuged at 1,500 RPM for 5 minutes at the room temperature, and the supernatant was filtered with a 0.22-μm filter after multi-stage filtration to obtain NAbs-FSC-EVs.

**NTA measurement of EVs with Nanosight NS300**
The concentration and size of EVs were analyzed by NTA using a NanoSight NS300 with a Blue 488 nm laser (Malvern Panalytical, Malvern, UK). All samples were diluted in PBS to reach concentrations inside the precision range of the NTA machine \((2 \times 10^8 \text{ to } 10 \times 10^8 \text{ particles/ml})\). EVs were measured at camera level 14 (camera shutter speed: 21.48 shutters / ms, slider gain: 366). After capture, the videos have been analyzed using the in-build NanoSight Software NTA 3.4 Build 3.4.003 with a detection threshold 5.

**Flow Cytometry**

The expressions of IgS (IgG), NAbs (IgM and IgG3), stem cell and EV-specific markers, and HLA-G proteins in FSCs and FSCs-derived EVs were quantified by flow cytometry. FSCs were harvested and stained with anti-Human CD73 APC (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA), anti-Human CD90 APC (Invitrogen), anti-Human CD105 APC (Invitrogen), anti-Human SSEA-4 AF488 (Invitrogen), anti-HLA-G 87G APC (BioLegend, San Diego, CA, USA), anti-HLA-G 2A12 FITC (Invitrogen), anti-Human IgG Fc AF488 (BioLegend), anti-Human IgG3 Hinge AF555 (SouthernBiotech, Birmingham, AL, USA), anti-Human IgG3 AF488 (Novus Biologicals, Centennial, CO, USA), and anti-Human IgM AF647 (BioLegend) antibodies for 30 min at room temperature. Intracellular staining of FSCs were performed using Intracellular Fixation & Permeabilization Buffer set (Invitrogen) according to the manufacturer's instruction.

The EVs were stained with anti-Human CD9 FITC (Invitrogen), anti-Human CD63 PE (Invitrogen), and CD81 APC (Invitrogen) antibodies for 30 min at room temperature. Intracellular staining of EVs were performed after EV isolation using Exosome Isolation and RNA Purification Kit (System Biosciences, Palo Alto, CA, USA) according to the manufacturer’s instruction. Isolated EVs were treated with permeabilizing solution for 1 hour at room temperature and stained with anti-HLA-G 2A12 FITC, anti-Human IgG Fc AF488, anti-Human IgG3 AF488, and anti-Human IgM AF647 antibodies for 30 min at room temperature. Then, stained EVs were isolated again using Exosome Isolation and RNA Purification Kit and analyzed through flow cytometry using various sizes of reference beads (Thermo Fisher Scientific).

The flow cytometry analysis of FSCs and their EVs were performed using Novocyte Quanteon (Agilent Technologies, Santa Clara, CA, USA), and the data were analyzed by Novoexpress software ver. 1.43.

**Enzyme-linked immunosorbent assay (ELISA)**

The concentration of sHLA-G (shedding HLA-G1 and HLA-G5) was detected by Human HLA-G ELISA Kit (LSBio, Seattle, WA, USA) using MEM-G/9 antibody according to the manufacturer's instruction. The concentration of soluble HLA-G isoforms (HLA-G5 and HLA-G6) was detected by soluble HLA-G ELISA Kit (MyBioSource, San Diego, CA, USA) using 5A6G7 antibody according to the manufacture's instruction.

The concentrations of IgG3 and IgM were analyzed using Human IgG3 ELISA Kit (MyBioSource) and Human IgM ELISA Kit (Abnova, Taipei, Taiwan) according to the manufacturer’s instructions, respectively.

**Protein Antibody Microarray**
The immunoglobulin and protein profiles in EVs secreted from FSCs were analyzed using SET100 Signaling Explorer Antibody Array (Full Moon Biosystems, Sunnyvale, CA, USA) and Human L493 Array (RayBiotech, Peachtree Corners, GA, USA) according to the manufacturer's instructions.

**Cytokine Array Analysis of EVs**

The concentrations of human growths and cytokines in EVs were analyzed by Quantibody Human Cytokine Array 1000 (RayBiotech) according to the manufacturer's instruction.

**Protein absolute quantification of EVs**

ESI-Q-TOF MS/MS analysis is performed on pre-treated EV sample according to the manufacturer's instructions using Synapt G2-Si HDMS (Waters, Milford, MA, USA) equipment. Protein identification was performed using the human database (version 3.87) of the IPI (International Protein Index). Protein absolute quantification was carried out based on the standard BSA mass value information (SwissProt) according to the manufacturer's instructions and the method suggested by Silva J. et al. (60).

**Characterization of EVs with ExoView™ R100**

Co-expressions of CD9, CD61, CD81, syntenin, IgG3, and IgM in EVs were analyzed using Tetraspanin Custom Kit with ExoView™ R100 (NanoView Biosciences, Boston, MA, USA) according to the manufacture's instruction of Cargo and Surface Membrane Immuno-Fluorescence Staining. Labelling Abs that consist of anti-syntenin Alexa-555, anti-human IgG3 Alexa Fluor 488 (Novus Biologicals), and anti-human IgM Alexa Fluor 647 Abs (BioLegend), were used. The data were then analyzed using ExoViewer Analyzer 3.0 with sizing thresholds set to 50 to 200 nm diameter.

**Statistical analysis**

Statistical analyses were performed using the Student's t-test for the comparison of means in more than two groups. Data presented are mean±S.D. and $P<0.05$ was considered statistically significant.

**Figures**
Figure 1

A schematic diagram of the process of verifying hypotheses to identify a new immune system built up by FSCs in early pregnancy (A) The developing progress of the fetal immune system. Igs-secreting B cell progenitors and pre pro-B cells were detected only in the fetal liver around 9 weeks of gestation, and mature B cells expressing B cell receptors were found in various fetal tissues after 18 weeks of gestation. In addition, maternal IgG can be transferred to the fetus through the umbilical cord after 15 weeks of
gestation. However, it is not known whether the fetus produces Igs before the innate and adaptive immune system are formed. Modified from Fig. 1 in Ref. 23. (B) To investigate the mechanism of self-protection by FSCs before establishing the innate and adaptive immune system by immune cells in early pregnancy, we established hypotheses and prepared a flow chart to verify them. (1) We confirmed whether Igs, particularly NAbs, including IgG3, which act on the innate immune system, were secreted from FSCs under a typical 2D culture condition. (2) We established an ex-vivo culture condition that simulates in vivo environment of FSCs to induce the secretion of NAbs from them. We confirmed the secretions of Igs and NAbs from FSCs cultured in this culture condition and compared them with FSCs cultured in a typical 2D culture condition. (3) We confirmed NAbs, proteins related to the promotion of Ig secretion, and complement proteins in extracellular vesicles (NAbFSC-EVs) secreted from FSCs (NABs-FSCs) cultured in a newly established culture condition to induce the secretion of NAbs and compared them with FSCs-derived EVs (FSC-EVs) cultured in a typical 2D culture condition. In addition, we confirmed the difference in induction aspects according to the culture conditions by comparing the content of HLA-G proteins inducing immune tolerance and various anti-viral proteins induced by interferon in NAbFSC-EVs with FSC-EVs. Moreover, we compared and analyzed the induction aspects of NAbs such as IgG3 and IgM in NAbFSC-EVs and FSC-EVs through Exoview®. This verification process confirmed that FSCs cultivated in a newly established ex-vivo culture condition could secrete NAbs, complement proteins, and various anti-viral proteins, which could construct the new immune system to protect themselves from external infectious agents at the cellular level.
Figure 2

Establishment of new ex-vivo culture conditions to induce the secretion of NAbs in FSCs in early pregnancy. (A) In vivo environment of FSCs in the first trimester of pregnancy and factors inducing the secretion of NAbs in FSCs. (B) A schematic diagram of novel in vivo environment-like ex-vivo culture conditions inducing NAbs secretion in FSCs. (C) An ex-vivo cell culture temperature profile with a 5-6 day-cycle simulating the 28-day cycle of female hormones and body temperature changes applied to new ex-vivo culture conditions to induce hormone secretion in trophoblasts and FSCs replacing the internal nervous system (28). (D) A graph of 24-hour-cycle vibration condition applied to new ex-vivo culture conditions for inducing pregnancy-related hormone secretion in trophoblasts and FSCs (28).
Figure 3

Analysis of NAb secretion characteristics of FSCs cultured in the novel ex-vivo culture conditions for inducing NAb secretion potential in FSCs (FSCsEx-vivo) cultured under new ex-vivo culture conditions. (A) A schematic diagram to verify NAb secretion potential in FSCs cultured under new ex-vivo culture conditions. (B) Flow cytometry results comparing the expression patterns of stem cell-specific markers in FSCs cultured in a typical 2D plate (FSCsControl) and FSCsEx-vivo. The results showed that the expression aspects of MSC
markers (CD73, CD90, and CD105) and ESC marker (SSEA4) were very similar in FSCsControl and FSCsEx-vivo. (C) Flow cytometry results comparing the cell membrane and intracellular expression patterns of Igs and NAbs in FSCsControl and FSCsEx-vivo. The expression of membrane-bound IgM is rather higher in FSCsControl than FSCsEx-vivo, but the expressions of intracellular IgM and IgG3 (NAb) are much higher in FSCsEx-vivo than FSCsControl. The expression pattern of IgG is similar in FSCsControl and FSCsEx-vivo. (D) Results of protein antibody microarray analysis comparing the relative expression levels of Igs in the culture supernatants of FSCsControl and FSCsEx-vivo. Compared to FSCsControl obtained through a typical 2D plate culture, FSCsEx-vivo obtained from the newly established NAb-inducing ex-vivo culture system secreted higher levels of Igs. Error bars represent standard deviation. *, P <0.05; **, P<0.01; ***, P<0.001; Student’s t-test.

Figure 4

Characterization of NAb-secreting FSCs-derived EVs (NAb-FSC-EVs) (A) A schematic diagram verifying whether NAbs are contained in EVs (NAb-FSC-EVs) secreted from FSCs (NAb-FSCs) cultured under new ex-vivo culture conditions. (B) The results of NTA analysis on NAb-FSC-EVs and EVs (FSCControl-EVs) derived from FSCs (FSCsControl) cultured on a typical 2D plate. The concentrations, average sizes, and distribution of FSCControl-EVs and NAb-FSC-EVs are almost similar. There is no significant difference in EV secretion characteristics according to the culture conditions. (C) SEM (left) and TEM (right) images of FSCControl-EVs and NAb-FSC-EVs, respectively. There are no significant differences in shapes and size.
distributions in both EVs according to the culture conditions. (D) The results of flow cytometry analysis to compare the expression patterns of exosome markers (CD9, CD63, CD81) in FSCControl-EVs and NAbs-FSC-EVs. The expression aspects that CD63 and CD81 are expressed at higher levels than CD9 are similar in both EVs. (E) The results of co-expression analysis of exosome markers (CD9, CD63, CD81, and syntenin) in FSCControl-EVs and NAbs-FSC-EVs using ExoView equipment. They show expression patterns very similar to the flow cytometry results in [Fig.4D], and these common expression patterns appear to be a unique characteristic of FSCs-derived EVs. (F) The results of flow cytometry analysis comparing the presence of IgG and NAbs (IgG3 and IgM) in FSCControl-EVs and NAbs-FSC-EVs. (G) The results of exosomal cargo analysis to compare the content of NAbs in FSCControl-EVs and NAbs-FSC-EVs using ExoView®. Similar to the flow cytometry results in [Fig.4F], they show that NAbs-FSC-EVs contain higher levels of NAbs than FSCControl-EVs. (H) The results of analyzing the patterns of NAbs content in both EVs captured by each exosomal marker using Exoview®. (I) The results of protein antibody microarray analysis comparing relative expression levels of B cell-specific proteins related to the promotion of Ig transcription induced in FSCControl-EVs and NAbs-FSC-EVs. They show that B cell-specific proteins related to the promotion of Ig transcription are induced in NAbs-FSC-EVs at higher levels than FSCControl-EVs. Error bars represent standard deviation. *, P <0.05; **, P<0.01; ***, P<0.001; Student’s t-test.
Figure 5

Analysis results of various complement proteins contained in NAbs-FSC-EVs. The complement system is activated by the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP). C1 subcomponents such as C1q, C1r, and C1s form the C1 complex in the CP, and the C5, C6, C7, C8, and C9 complement proteins form the membrane attack complex. C3a, C4a, and C5a proteins, generated in this process, recruit various immune cells to spread the inflammatory response (36). Comparisons of relative expression levels of complement proteins contained in NAbs-FSC-EVs and FSCControl-EVs using SET100 protein antibody microarray (A) and human L493 array (B). (C) Comparisons of absolute quantification of
complement proteins contained in NAbs-FSC-EVs and FSCControl-EVs using ESI-Q-TOF MS/MS. We confirm that NAbs-FSC-EVs contain higher levels of various complement proteins compared to FSCControl-EVs in all analysis results. Error bars represent standard deviation. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; Student’s t-test.

Figure 6
Comparison of secretion characteristics of cytokines, HLA-G, and anti-viral proteins in FSCs established in various culture conditions (A) Various FSCs established in different culture conditions to characterize the self-defense mechanism formed by NAbs-FSCs. (B) Cytokine analysis results of EVs from FSCs obtained under each culture condition using human cytokine array. The secretion levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) in FSCControl-EVs, itFSC-EVs, and NAbs-FSC-EVs were similar, but the secretion levels of Th1 cytokines (IFN-γ and IL-2), showing increased expression in asymptomatic infected person to COVID-19, were the highest in NAbs-FSC-EVs. (C) The results of flow cytometry analysis to compare the expression aspects of membrane-bound HLA-G1 and intracellular soluble HLA-G5/G6 in each FSC using 87G (detecting β2m of HLA-G1 and HLA-G5 isoforms) and 2A12 (detecting intron 4 of soluble HLA-G5 and HLA-G6 isoforms) antibodies. Similar to the previous study results (28), all HLA-G expressions were increased in NAbs-FSCs and itFSCs compared to FSCsControl. In addition, the expression of HLA-G in NAbs-FSCs was also higher than in itFSCs due to the effect of increased IFN-γ stimulation. (D) sHLA-G (shedding HLA-G1 and HLA-G5) and HLA-G5/6 concentration analysis results of EVs from FSCs obtained under each culture condition through ELISA analysis using MEM-G/9 (detecting β2m of HLA-G1 and HLA-G5 isoforms) and 5A6G7 (detecting intron 4 of soluble HLA-G5 and HLA-G6 isoforms) antibodies, respectively. Similar to the previous study results (28), all HLA-G concentrations were higher in itFSC-EVs and NAbs-FSC-EVs than in FSCControl-EVs. However, unlike HLA-G5/6 concentrations, sHLA-G (shedding HLA-G1 and soluble HLA-G5) concentration was higher in NAbs-FSC-EVs than itFSC-EVs. (E) A comparison of the concentrations of interferon-induced proteins with anti-viral functions in NAbs-FSC-EVs and itFSC-EVs. IFITM3 protein was expressed at a higher level in NAbs-FSC-EVs than itFSC-EVs using SET100 protein antibody microarray, and LY6E protein was contained at a higher level in NAbs-FSC-EVs than itFSC-EVs according to ESI-Q-TOF MS/MS analysis. (F) A comparison of absolute quantification of another anti-viral protein (TRPML2) in NAbs-FSC-EVs and itFSC-EVs using ESI-Q-TOF MS/MS. Error bars represent standard deviation. *, P <0.05; **, P <0.01;***, P<0.001; ****, P<0.0001; Student’s t-test.

**Supplementary Files**

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- [NaturePrimalImmunoglobulinSupplementalInformationENGFinal2021.08.24.pdf](#)