3. Results

3.1. Frequencies of Foxp3$^+$ cells and Treg (naïve Treg + effector Treg) cells among CD4$^+$ cells in the decidua

The frequency of decidual Foxp3$^+$ cells among CD4$^+$ cells in miscarriage cases with normal fetal chromosomal content was significantly lower ($P=0.039$) than that in normal pregnancies (Fig. 2A). However, this frequency in miscarriage cases with abnormal chromosomal content was similar to that in normal pregnancies. Next, we measured the frequency of Treg cells (naïve Treg cells + effector Treg cells) among CD4$^+$ T cells. The frequency of the true Treg cell population excluding Foxp3$^+$ Teff cells in the decidua was significantly lower in subjects with miscarriage and a normal embryo karyotype than in those with normal pregnancies, and in subjects with miscarriage and an abnormal embryo karyotype ($P=0.0258$ and $P=0.0389$ respectively; Fig. 2B). We have reanalyzed the frequency of decidual Foxp3$^+$ cells and true Treg cells in subjects with a first pregnancy (○), subjects who have had a previous live birth (●), and the subjects with miscarriage who have had previous miscarriage(s) and no live births (△). There were no significant differences in Foxp3$^+$ cells and true Treg cells among the three groups, although the sample size is small.

3.2. Frequencies of decidual naïve Treg cells, effector Treg cells and Foxp3$^+$ Teff cells among CD4$^+$Foxp3$^+$ cells

The frequency of decidual effector Treg cells in subjects with miscarriage and a normal embryo karyotype was significantly lower ($P=0.0105$) than that in subjects with normal pregnancies (Fig. 3B). On the other hand, the frequency of decidual Foxp3$^+$ Teff cells in subjects with miscarriage and a normal embryo karyotype was significantly higher than that in subjects with normal pregnancies and in subjects with miscarriage and an abnormal embryo ($P=0.0258$ and $P=0.0389$ respectively; Fig. 3C). The ratio of effector Treg cells to Foxp3$^+$ Teff cells in subjects with miscarriage and a normal embryo was significantly lower ($P=0.0106$) than that in subjects with normal pregnancies.

The frequencies of naïve Treg cells were similar among the three groups (Fig. 3A).

3.3. Frequency of naïve Treg cells, effector Treg cells and Foxp3$^+$ Teff cells among CD4$^+$Foxp3$^+$ cells in the peripheral blood

The frequencies of naïve Treg cells, effector Treg cells and Foxp3$^+$ Teff cells in the peripheral blood were similar among the three groups (Fig. 4A–C). The frequency of effector Treg cells in the peripheral blood in subjects with miscarriage and a normal karyotype embryo appeared to be higher, but the difference did not reach significance (Fig. 4B). Foxp3$^+$ Teff cell population in peripheral blood did not increase in subjects with miscarriage and a normal karyotype embryo. Furthermore, the ratio of effector Treg cells to Foxp3$^+$ Teff cells in subjects with miscarriage and a normal embryo karyotype appeared to be higher, but the difference did not reach significance (Fig. 4D). The frequency of effector Treg cells and Foxp3$^+$ Teff cells was different between decidua and peripheral blood. We have calculated the ratio of the frequency of decidual naïve Treg cells to that of peripheral Treg cells (Fig. 5A), frequency of

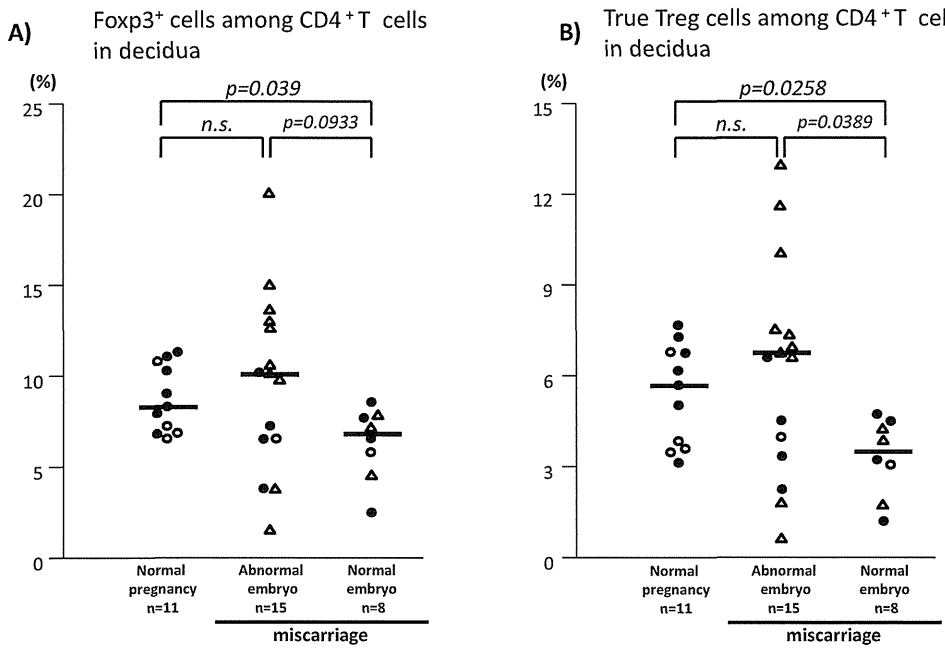

Fig. 2. Frequencies of Foxp3$^+$ cells (A) and true Treg (naïve Treg and effector Treg) cells (B) among CD4$^+$ cells in the decidua of normal pregnancy, miscarriage with abnormal karyotype embryo and miscarriage with normal karyotype embryo. Horizontal bar is the median value. n.s. means not significant. Open circle shows subjects with their first pregnancy. Closed circle shows subjects who have had a previous live birth. Open triangle shows the subjects with miscarriage who have had a previous miscarriage(s) and no live births.

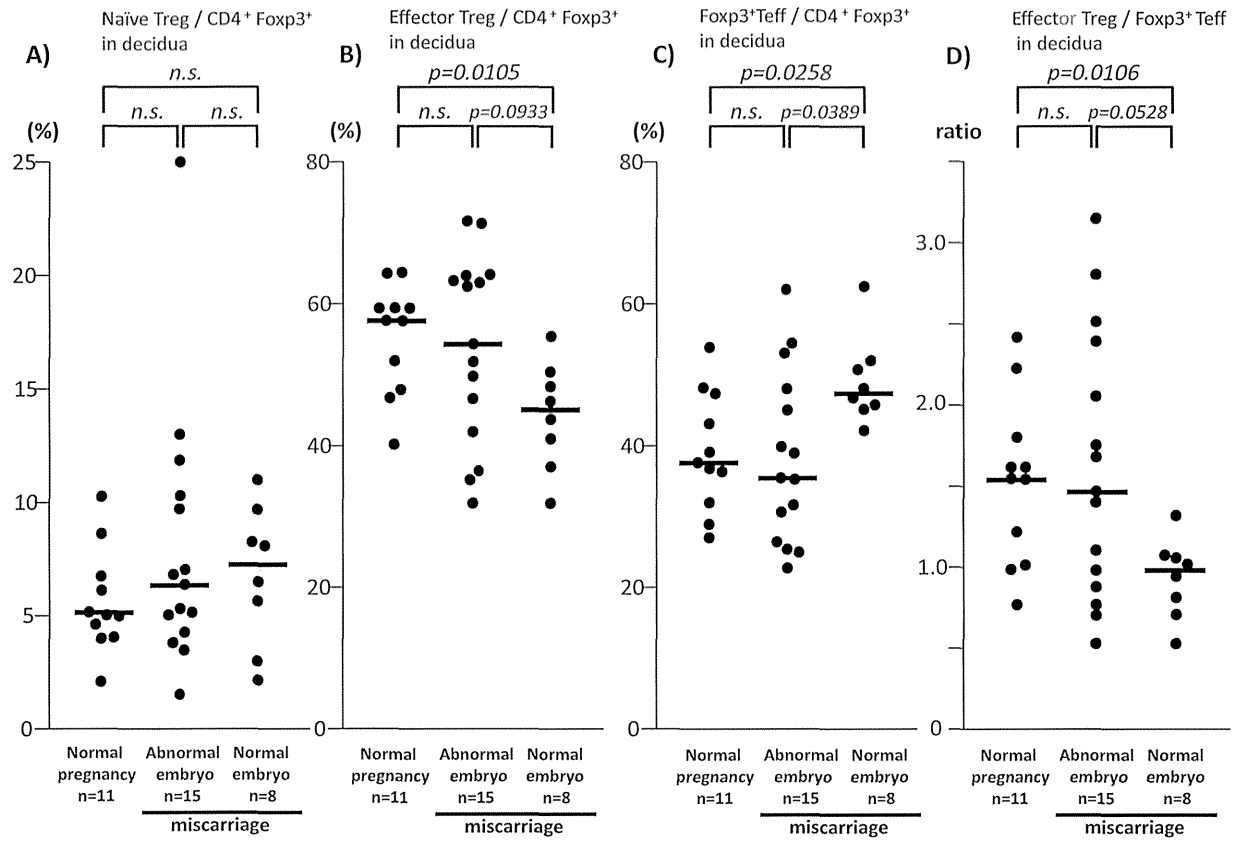

Fig. 3. Frequencies of decidual naïve Treg cells (A), effector Treg cells (B), and Foxp3+ Teff cells (C) among CD4+Foxp3+ cells, and the ratio of effector Treg cells/Foxp3+ Teff cells (D) in normal pregnancy, miscarriage with abnormal karyotype embryo, and miscarriage with normal karyotype embryo. Horizontal bar is the median value. n.s. means not significant.

decidual effector Treg cells to that of the peripheral blood (Fig. 5B), and frequency of decidual Foxp3+ Teff cells to that of the peripheral blood (Fig. 5C). The ratio of effector Treg cells of decidua to peripheral blood in subjects with miscarriage and a normal karyotype embryo was significantly lower than that in subjects with normal pregnancies and in subjects with miscarriage and an abnormal karyotype embryo ($P=0.0128$ and $P=0.0123$ respectively; Fig. 5B). The ratio of Foxp3+ Teff cells of the decidua to those in the peripheral blood was significantly higher ($P=0.0265$) in subjects with miscarriage and a normal karyotype embryo than the ratio in subjects with normal pregnancies, and in subjects with miscarriage and an abnormal karyotype embryo ($P=0.0265$ and $P=0.0066$ respectively; Fig. 5C).

3.4. Frequency of Helios+ effector Treg cells and naïve Treg cells in the decidua and peripheral blood of normal pregnancies, miscarriages with an abnormal karyotype embryo, and miscarriages with a normal karyotype embryo

The frequency of Helios+ Treg cells in the peripheral blood was around 80% of normal pregnancies, miscarriages with an abnormal karyotype embryo, and in miscarriages with a normal karyotype embryo, as previously reported (Thornton et al., 2010; Fig. 6A). The frequencies of Helios+ naïve Treg cells were similar between decidua and peripheral blood in normal pregnancies, miscarriages with abnormal karyotype embryo, and miscarriages with normal karyotype embryo (Fig. 6A). However, the frequency of Helios+ effector Treg cells in the decidua was over 90%, and this frequency in the decidua was significantly higher than in peripheral blood in normal pregnancies ($P=0.02$), miscarriages with abnormal karyotype embryo ($P=0.008$), and miscarriages with a normal karyotype embryo ($P=0.02$) in contrast to the data for naïve Treg cells (Fig. 6B), suggesting the selective migration of effector Treg cells into the decidua from peripheral blood. However, the frequencies of Helios+ cells among effector Treg cells in the decidua and peripheral blood were similar among the three groups. On the other hand, the frequencies of Helios+-naïve Treg cells were similar to those in decidua and peripheral blood (Fig. 6A).

Next, we studied the frequency of Helios-positive or Helios-negative naïve Treg cells and effector Treg cells in the decidua (Table 2). The frequency of decidual Helios+ effector Treg cells among CD4+Foxp3+cells in subjects with miscarriage and a normal embryo was significantly lower compared with that in normal pregnancy subjects ($P=0.0258$). These frequencies in the peripheral blood were similar among the three groups (Table 3).

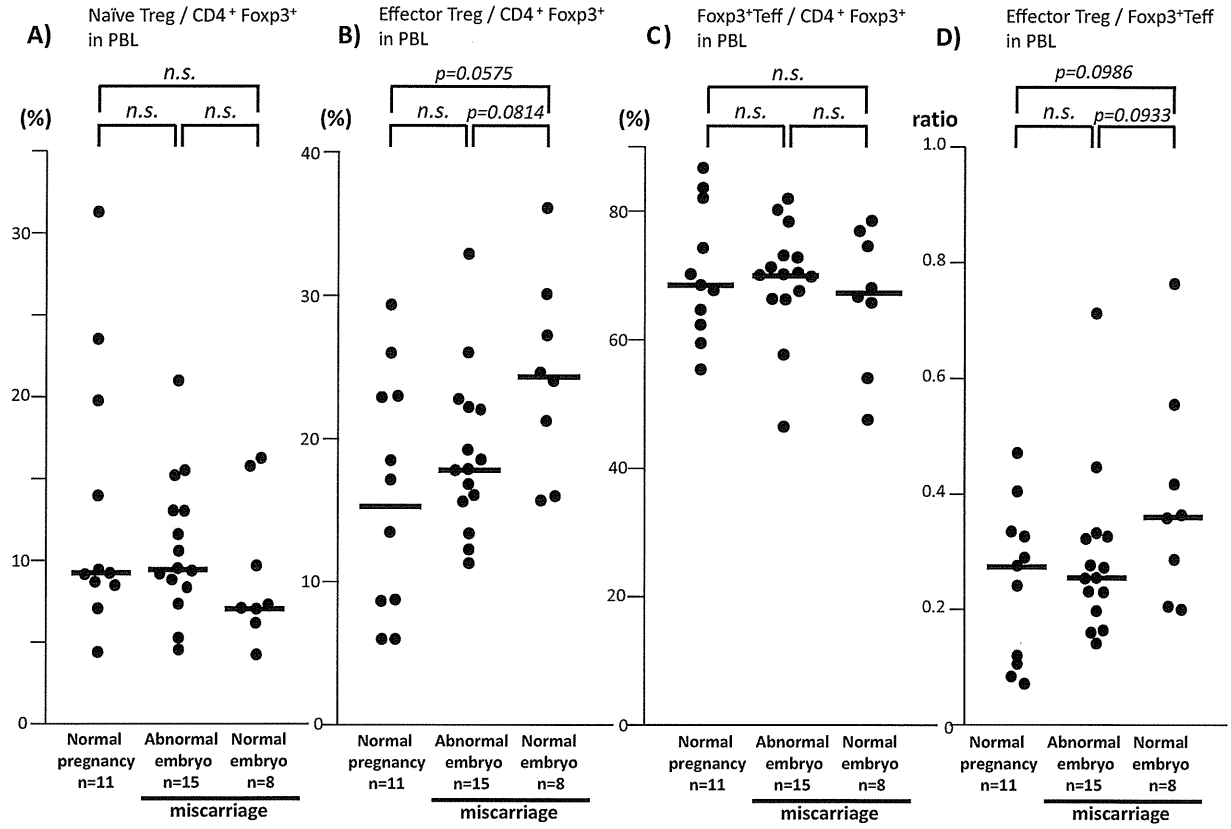

Fig. 4. Frequencies of peripheral blood naïve Treg cells (A), effector Treg cells (B), and Foxp3+ Teff cells (C) among CD4+Foxp3+ cells, and the ratio of effector Treg cells/Foxp3+ Teff cells (D) in normal pregnancy, miscarriage with abnormal karyotype embryo, and miscarriage with normal karyotype embryo. Horizontal bar shows the median value. n.s. means not significant.

Table 2
Frequencies of decidual Helios-positive or Helios-negative naïve Treg cells and effector Treg cells in normal pregnancy, miscarriage with an abnormal embryo and miscarriage with a normal embryo.

	Normal pregnancy $n=11$	Miscarriage with an abnormal embryo $n=15$	Miscarriage with a normal embryo $n=8$
Helios+ naïve Treg cells/CD4+Foxp3+ cells (%)*	4.03 ± 0.20 (0.81–9.08)	6.77 ± 0.37 (1.22–24.07)	5.48 ± 0.35 (1.12–8.53)
Helios− naïve Treg cells/CD4+Foxp3+ cells (%)*	1.53 ± 0.18 (0.49–7.33)	1.13 ± 0.054 (0.23–3.15)	1.14 ± 0.088 (0.54–2.44)
Helios+ effector Treg cells/CD4+Foxp3+ cells (%)*	52.99 ± 0.78 (37.84–64.03)	51.69 ± 0.90 (30.16–71.29)	42.83 ± 0.98 (30.11–54.54)†
Helios− effector Treg cells/CD4+Foxp3+ cells (%)*	2.30 ± 0.17 (0.58–6.81)	2.12 ± 0.18 (0.26–8.74)	1.42 ± 0.12 (0.31–3.47)

* Mean ± SEM (range).
† $P<0.05$ vs. normal pregnancy.

Table 3
Frequencies of peripheral blood Helios-positive or Helios-negative naïve Treg cells and effector Treg cells in normal pregnancy, miscarriage with an abnormal embryo and miscarriage with a normal embryo.

	Normal pregnancy $n=11$	Miscarriage with an abnormal embryo $n=15$	Miscarriage with a normal embryo $n=8$
Helios+ naïve Treg cells/CD4+Foxp3+ cells (%)*	10.41 ± 0.96 (4.77–23.10)	8.09 ± 0.21 (3.09–14.60)	6.74 ± 0.39 (4.10–12.01)
Helios− naïve Treg cells/CD4+Foxp3+ cells (%)*	4.21 ± 0.40 (1.55–8.25)	2.53 ± 0.15 (0–6.8)	2.49 ± 0.19 (0–5.27)
Helios+ effector Treg cells/CD4+Foxp3+ cells (%)*	7.25 ± 0.37 (4.14–11.11)	7.41 ± 0.34 (2.40–20.8)	5.78 ± 0.41 (1.91–10.30)
Helios− effector Treg cells/CD4+Foxp3+ cells (%)*	1.28 ± 0.13 (0.53–2.80)	1.76 ± 0.093 (0–5.08)	0.83 ± 0.099 (0–2.11)

* Mean ± SEM (range).

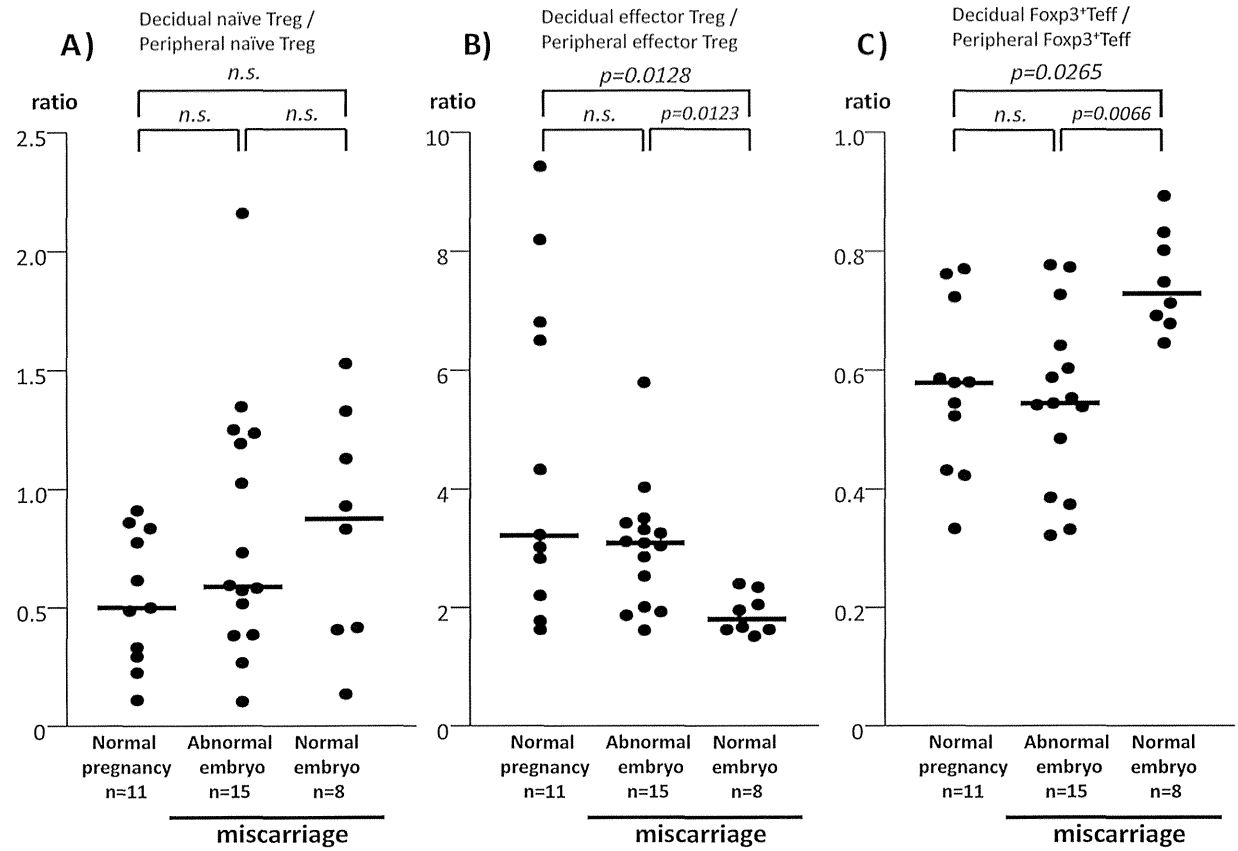

Fig. 5. The ratio of naïve Treg cells in decidua to those in peripheral blood (A), the ratio of effector Treg cells in decidua to those in peripheral blood (B), and the ratio of Foxp3$^+$ Teff cells in decidua to those in peripheral blood (C). Horizontal bar shows the medium value. n.s. means not significant.

4. Discussion

Foxp3 is believed to be a specific marker for Treg cells (Hori et al., 2003), but recent data show that Foxp3 expression transiently occurs in IL-2- and IFN-γ-producing Teff cells in humans (Walker et al., 2003; Gavin et al., 2006; Allan et al., 2007). It is possible that these activated Teff cells might react to fetal antigens presented by antigen-presenting cells (APCs) in the decidua and create harmful inflammation causing demise of the embryo. Many papers have reported that Foxp3$^+$ cells increase in normal pregnancy, but decrease in miscarriage (Sasaki et al., 2004; Yang et al., 2008; Jin et al., 2009; Mei et al., 2010; Wang et al., 2010, 2011; Lee et al., 2011) and preeclampsia (Sasaki et al., 2007; Santner-Nanan et al., 2009). However, there are no reports describing which types of Foxp3$^+$ cell increase or decrease in miscarriage. Foxp3$^+$ cells are classified into effector Treg cells that contribute to immune tolerance, naïve Treg cells that show a weak capacity for immunoregulation, and Teff cells that break immune tolerance. This is the first report that shows that decidual effector Treg cells decreased in miscarriage with a normal karyotype embryo, but not in miscarriage with an abnormal karyotype embryo. Interestingly, Foxp3$^+$ Teff cells in the decidua were increased in miscarriage with a normal karyotype embryo. The immunological environment is controlled by the balance between immunoactivation and immunoregulation. In the miscarriage cases with a normal karyotype embryo, the balance seemed to shift to an immunoactivation-dominant state, and dysregulation of tolerance to the fetus might occur. We compared the percentage of effector Treg cells and Foxp3$^+$ Teff in CD4$^+$Foxp3 cells in decidua and peripheral blood. Our findings suggest that immunological abnormality might be limited in the pregnant uterus in miscarriage with a normal karyotype embryo. Unfortunately, we did not measure the weight of decidual samples; therefore, we could not calculate the numbers of these cells in milligrams of decidua. This is a limitation of our study.

The etiology of recurrent pregnancy loss (RPL) is unknown in 40–60% of cases (Clifford et al., 1994). In a murine model, decreased Treg cells could induce implantation failure (Darrasse-Jèze et al., 2006; Shima et al., 2010) and early pregnancy loss by activation of T cells and NK cells (Aluvihare et al., 2004; Zenclussen et al., 2005; Thaxton et al., 2013), suggesting that immune dysregulation might be one of the etiologies of fetal resorption. These findings suggest that a proportion of sporadic miscarriage cases or RPL cases with a normal fetal karyotype might be associated with the immune etiology of miscarriage in humans. However, further studies are needed to prove this.

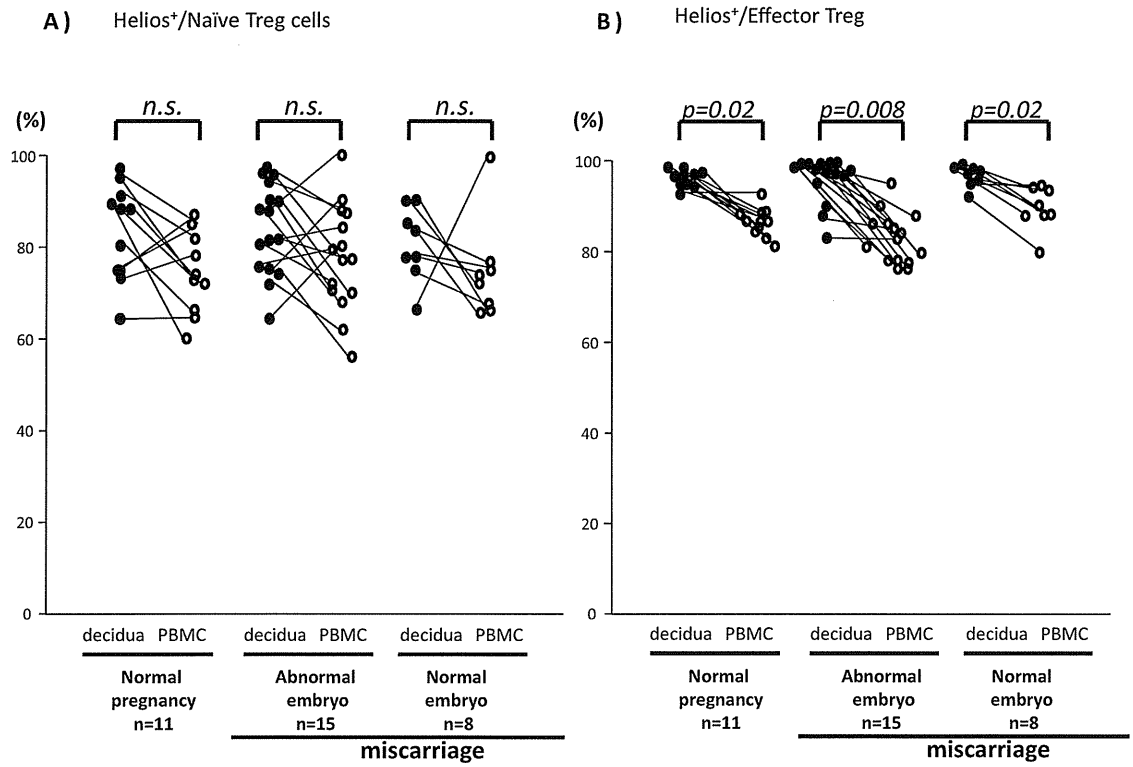

Fig. 6. Helios-positive naïve Treg cells (A) and effector Treg cells (B) in the decidua and peripheral blood of subjects with normal pregnancy, subjects with miscarriage and an abnormal karyotype embryo, and subjects with miscarriage and a normal karyotype embryo. PBMC, peripheral blood mononuclear cells.

Samstein et al. (2012) recently reported that a Foxp3 enhancer, CNS1, is essential for the induction of iTreg cells and that iTreg cells play an essential role in the maintenance of allogeneic pregnancy in placental animals. Thornton et al. (2010) reported that Helios, an Ikaros transcription factor family member, is a specific marker of nTreg cells. Therefore, we analyzed Helios$^+$ nTreg cells and Helios$^-$ iTreg cells among effector Treg cells in the decidua and peripheral blood. Our data first showed that there were very few Helios$^-$ iTreg cells in the decidua of subjects with normal pregnancy and also in that of human subjects with miscarriage, and that the frequency of Helios$^-$ iTreg did not change in miscarriage. Furthermore, the frequency of Helios$^+$ functional nTreg in the decidua was significantly higher than that in peripheral blood in normal pregnancies, miscarriages with an abnormal karyotype embryo and miscarriage with a normal karyotype embryo, but the frequency of Helios$^+$ naïve nTreg cells in the decidua was similar to that in peripheral blood. This finding may be explained by the fact that Helios$^+$ functional nTreg cells selectively accumulated in the pregnant uterus from the periphery. Indeed, selective migration of fetus-specific Treg cells from the peripheral blood to the decidua was reported in human pregnancy (Tilburgs et al., 2008) and murine pregnancy (Kallikourdis et al., 2007). We have already reported that there were decreased numbers of Foxp3$^+$ cells at the decidua basalis were decreased, but not at the decidua parietalis, in subjects with miscarriage and a normal embryo karyotype, showing the failure of the migration of Treg cells at the fetomaternal interface (Inada et al., 2013). The level of effector Treg cells in peripheral blood was slightly elevated in subjects with miscarriages and a normal karyotype embryo (Fig. 4B), which may indirectly support this idea. Indeed, Chen et al. (2013) recently showed that CD44^{high} CD62L^{low} activated memory Treg cells specific for self-antigen were rapidly recruited to the uterus-draining lymph nodes and activated in the first days after embryo implantation. Further studies are necessary to discover whether self-specific memory Treg cells are nTreg or iTreg cells. In summary, Helios$^+$ effector Treg cells were decreased in the decidua of subjects with miscarriage and a normal karyotype embryo. Effector nTreg may play an important role in the maintenance of pregnancy in humans, although Helios is not a strict marker for nTreg (Zabransky et al., 2012).

It is well known that the Treg cell pool is decreased in preeclampsia (Sasaki et al., 2007; Santner-Nanan et al., 2009) and recent data showed that Helios$^-$ iTreg cells, but not Helios$^+$ nTreg cells, were decreased in peripheral blood in preeclampsia (Hsu et al., 2012). iTreg cells may be important for the maintenance of pregnancy at a late stage. Indeed, Rowe et al. (2012) reported that the frequency of Helios$^+$ fetal antigen-specific nTreg cells in mid-gestation (11.5 days post-coitus) was 70%, and this frequency in late gestation (18.5 days post-coitus) was reduced to 40%, suggesting that Helios$^-$ fetal antigen specific iTreg cells expanded in late pregnancy. Neuropilin 1 is expressed on nTreg cells (Weiss et al., 2012; Yadav et al., 2012). We have

tried to study the expression of neuropilin 1 on Treg cells, but the immunostaining of neuropilin 1 was weak; thus, we could not classify Treg cells into nTreg and iTreg cells using neuropilin 1 staining.

Rowe et al. (2012) reported that memory Treg cells that sustain anergy to fetal antigen play a role in the rapid induction of fetomaternal tolerance. Importantly, when Treg cells from female mice were transferred into pregnant mice soon after delivery, they quickly expanded after pregnancy, but when Foxp3$^-$CD4$^+$ T cells were transferred into pregnant mice, donor-derived fetal antigen-specific Treg cells did not appear (Rowe et al., 2012). If iTreg cells play an essential role in the maintenance of allogeneic pregnancy, donor-derived fetal antigen-specific iTreg cells should be increased, but Helios$^+$-fetal antigen-specific nTreg cells were the major population in mid-gestation (Rowe et al., 2012). Furthermore, when CNS1-deficient female mice were mated with allogeneic male mice, the resorption rate was only 10% (Samstein et al., 2012). More than 50% resorption was observed when total Treg cells were depleted in mice (Shima et al., 2010; Rowe et al., 2012). This finding suggests that not only iTreg cells, but also nTreg cells, might play a role in a successful pregnancy. Further studies are needed on the types of Treg cell that play an important role in successful implantation and pregnancy in mice and humans.

In conclusion, we are, to our knowledge, the first to show that Helios-positive functional Treg cells decreased and Foxp3$^+$ Teff cells increased in the decidua of miscarriage cases with normal fetal chromosomal content. These findings suggest that dysregulation of fetomaternal tolerance might be one of the etiologies of miscarriage in humans.

Disclosure

None of the authors has any conflict of interest related to this manuscript.

Acknowledgements

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan [a Grant-in-Aid for Scientific Research (B) 23390386, a Grant-in-Aid for Young Scientists (B) 23791818, and a Grant-in-Aid for Young Scientists (B) 24791107].

References

Allan, S.E., Crome, S.Q., Crellin, N.K., Passerini, L., Steiner, T.S., Bacchetta, R., Roncarolo, M.G., Levings, M.K., 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int. Immunol. 19, 345–354.

Aluvihare, V.R., Kallikourdis, M., Betz, A.G., 2004. Regulatory T cells mediate maternal tolerance to the fetus. Nat. Immunol. 5, 266–271.

Beacher-Allan, C., Brown, J.A., Freeman, G.J., Hafler, D.A., 2001. CD4+CD25high regulatory cells in human peripheral blood. J. Immunol. 167, 1245–1253.

Chen, T., Darrasse-Jèze, G., Bergot, A.S., Courau, T., Churlaud, G., Valdivia, K., Strominger, J.L., Ruocco, M.G., Chaouat, G., Klatzmann, D., 2013. Self-specific memory regulatory T cells protect embryos at implantation in mice. J. Immunol. 191, 2273–2281.

Clifford, K., Rai, R., Watson, H., Regan, L., 1994. An informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. Hum. Reprod. 9, 1328–1332.

Darrasse-Jèze, G., Klatzmann, D., Charlotte, F., Salomon, B.L., Cohen, J.L., 2006. CD4+CD25+ regulatory/suppressor T cells prevent allogeneic fetus rejection in mice. Immunol. Lett. 102, 106–109.

Gavin, M.A., Torgerson, T.R., Houston, E., DeRoos, P., Ho, W.Y., Stray-Pedersen, A., Ocheltree, E.L., Greenberg, P.D., Ochs, H.D., Rudensky, A.Y., 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc. Natl. Acad. Sci. U.S.A. 103, 6659–6664.

Guerin, L.R., Moldenhauer, L.M., Prins, J.R., Bromfield, J.J., Hayball, J.D., Robertson, S.A., 2011. Seminal fluid regulates accumulation of FOXP3+ regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. Biol. Reprod. 85, 397–408.

Hori, S., Nomura, T., Sakaguchi, S., 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science 299, 1057–1061.

Hsu, P., Santner-Nanan, B., Dahlstrom, J.E., Fadia, M., Chandra, A., Peek, M., Nanan, R., 2012. Altered decidual DC-SIGN+ antigen-presenting cells and impaired regulatory T-cell induction in preeclampsia. Am. J. Pathol. 181, 2149–2160.

Inada, K., Shima, T., Nakashima, A., Aoki, K., Ito, M., Saito, S., 2013. Characterization of regulatory T cells in decidua of miscarriage cases with abnormal or normal fetal chromosomal content. J. Reprod. Immunol. 97, 104–111.

Jin, L.P., Chen, Q.Y., Zhang, T., Guo, P.F., Li, D.J., 2009. The CD4+CD25 bright regulatory T cells and CTLA-4 expression in peripheral and decidual lymphocytes are down-regulated in human miscarriage. Clin. Immunol. 133, 402–410.

Kahn, D.A., Baltimore, D., 2010. Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. Proc. Natl. Acad. Sci. U.S.A. 107, 9299–9304.

Kallikourdis, M., Andersen, K.G., Welch, K.A., Betz, A.G., 2007. Alloantigen-enhanced accumulation of CCR5+ 'effector' regulatory T cells in the gravid uterus. Proc. Natl. Acad. Sci. U.S.A. 104, 594–599.

Kleinewietfeld, M., Starke, M., Di Mitri, D., Borsellino, G., Battistini, L., Rötzschke, O., Falk, K., 2009. CD49d provides access to untouched human Foxp3+ Treg free of contaminating effector cells. Blood 113, 827–836.

Lee, S.K., Kim, J.Y., Hur, S.E., Kim, C.J., Na, B.J., Lee, M., Gilman-Sachs, A., Kwak-Kim, J., 2011. An imbalance in interleukin-17-producing T and Foxp3$^+$ regulatory T cells in women with idiopathic recurrent pregnancy loss. Hum. Reprod. 26, 2964–2971.

Mei, S., Tan, J., Chen, H., Chen, Y., Zhang, J., 2010. Changes of CD4+CD25high regulatory T cells and FOXP3 expression in unexplained recurrent spontaneous abortion patients. Fertil. Steril. 94, 2244–2247.

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., Mathian, A., Nakahata, T., Yamaguchi, T., Nomura, T., Ono, M., Amoura, Z., Gorochov, G., Sakaguchi, S., 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 30, 899–911.

Robertson, S.A., Guerin, L.R., Bromfield, J.J., Branson, K.M., Ahlström, A.C., Care, A.S., 2009. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol. Reprod. 80, 1036–1045.

Rowe, J.H., Ertelt, J.M., Aguilera, M.N., Farrar, M.A., Way, S.S., 2011. Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens. Cell Host Microbe 10, 54–64.

Rowe, J.H., Ertelt, J.M., Xin, L., Way, S.S., 2012. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature 490, 102–106.

Saito, S., Nishikawa, K., Morii, T., Narita, N., Enomoto, M., Ichijo, M., 1992. Expression of activation antigens CD69, HLA-DR, interleukin-2 receptor-alpha (IL-2R alpha) and IL-2R beta on T cells of human decidua at an early stage of pregnancy. Immunol. 75, 710–712.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., Toda, M., 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. 155, 1151–1164.

Sakaguchi, S., 2005. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat. Immunol. 6, 345–352.

Samstein, R.M., Josefowicz, S.Z., Arvey, A., Treuting, P.M., Rudensky, A.Y., 2012. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal–fetal conflict. Cell 150, 29–38.

Santner-Nanan, B., Peek, M.J., Khanam, R., Richarts, L., Zhu, E., Fazekas de St Groth, B., Nanan, R., 2009. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. J. Immunol. 183, 7023–7030.

Sasaki, Y., Sakai, M., Miyazaki, S., Higuma, S., Shiozaki, A., Saito, S., 2004. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Mol. Hum. Reprod. 10, 347–353.

Sasaki, Y., Darmochwal-Kolarz, D., Suzuki, D., Sakai, M., Ito, M., Shima, T., Shiozaki, A., Rolinski, J., Saito, S., 2007. Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia. Clin. Exp. Immunol. 149, 139–145.

Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S.I., Nanan, R., Kelleher, A., Fazekas de St Groth, B., 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J. Exp. Med. 203, 1693–1700.

Shima, T., Sasaki, Y., Itoh, M., Nakashima, A., Ishii, N., Sugamura, K., Saito, S., 2010. Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. J. Reprod. Immunol. 85, 121–129.

Steinborn, A., Schmitt, E., Kisielewicz, A., Rechenberg, S., Seissler, N., Mahnke, K., Schaier, M., Zeier, M., Sohn, C., 2012. Pregnancy-associated diseases are characterized by the composition of the systemic regulatory T cell (Treg) pool with distinct subsets of Tregs. Clin. Exp. Immunol. 167, 84–98.

Thaxton, J.E., Nevers, T., Lippe, E.O., Blois, S.M., Saito, S., Sharma, S., 2013. NKG2D blockade inhibits poly(I:C)-triggered fetal loss in wild type but not in IL-1 0-/- mice. J. Immunol. 190, 3639–3647.

Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., Shevach, E.M., 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J. Immunol. 184, 3433–3441.

Tilburgs, T., Roelen, D.L., van der Mast, B.J., de Groot-Swings, G.M., Kleijburg, C., Scherjon, S.A., Claas, F.H., 2008. Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. J. Immunol. 180, 5737–5745.

Tilburgs, T., Scherjon, S.A., van der Mast, B.J., Haasnoot, G.W., Versteeg, V.D., Voort-Maarschalk, M., Roelen, D.L., van Rood, J.J., Claas, F.H., 2009. Fetal–maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. J. Reprod. Immunol. 82, 148–157.

Walker, M.R., Kasprowicz, D.J., Gersuk, V.H., Benard, A., Van Landeghen, M., Buckner, J.H., Ziegler, S.F., 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25– T cells. J. Clin. Invest. 112, 1437–1443.

Wang, W.J., Hao, C.F., Q. u., Q.L., Wang, X., Qiu, L.H., Lin, Q.D., 2010. The deregulation of regulatory T cells on interleukin-17-producing T helper cells in patients with unexplained early recurrent miscarriage. Hum. Reprod. 25, 2591–2596.

Wang, W.J., Hao, C.F., Lin, Q.D., 2011. Dysregulation of macrophage activation by decidual regulatory T cells in unexplained recurrent miscarriage patients. J. Reprod. Immunol. 92, 97–102.

Weiss, J.M., Bilate, A.M., Gobert, M., Ding, Y., Curotto de Lafaille, M.A., Parkhurst, C.N., Xiong, H., Dolpady, J., Frey, A.B., Ruocco, M.G., Yang, Y., Floess, S., Huehn, J., Oh, S., Li, M.O., Niec, R.E., Rudensky, A.Y., Dustin, M.L., Littman, D.R., Lafaille, J.J., 2012. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. J. Exp. Med. 209, 1723–1742.

Winger, E.E., Reed, J.L., 2011. Low circulating CD4(+) CD25(+) Foxp3(+) T regulatory cell levels predict miscarriage risk in newly pregnant women with a history of failure. Am. J. Reprod. Immunol. 66, 320–328.

Yadav, M., Louvet, C., Davini, D., Gardner, J.M., Martinez-Llordella, M., Bailey-Bucktrout, S., Anthony, B.A., Sverdrup, F.M., Head, R., Kuster, D.J., Ruminski, P., Weiss, D., Von Schack, D., Bluestone, J.A., 2012. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. J. Exp. Med. 209, 1713–1722.

Yang, H., Qiu, L., Chen, G., Ye, Z., Lü, C., Lin, Q., 2008. Proportional change of CD4+CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients. Fertil. Steril. 89, 656–661.

Yin, Y., Han, X., Shi, Q., Zhao, Y., He, Y., 2012. Adoptive transfer of CD4+CD25+ regulatory T cells for prevention and treatment of spontaneous abortion. Eur. J. Obstet. Gynecol. Reprod. Biol. 161, 177–181.

Zabransky, D.J., Nirschl, C.J., Durham, N.M., Park, B.V., Ceccato, C.M., Bruno, T.C., Tam, A.J., Getnet, D., Drake, C.G., 2012. Phenotypic and functional properties of Helios+ regulatory T cells. PLoS ONE 7, e34547.

Zenclussen, A.C., Gerlof, K., Zenclussen, M.L., Sollwedel, A., Bertoja, A.Z., Ritter, T., Kotsch, K., Leber, J., Volk, H.D., 2005. Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. Am. J. Pathol. 166, 811–822.

Zenclussen, A.C., Gerlof, K., Zenclussen, M.L., Ritschel, S., Zambon Bertoja, A., Fest, S., Hontsu, S., Ueha, S., Matsushima, K., Leber, J., Volk, H.D., 2006. Regulatory T cells induce a privileged tolerant microenvironment at the fetal–maternal interface. Eur. J. Immunol. 36, 82–94.

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Intestinal Microbiota is Different in Women with Preterm Birth: Results from Terminal Restriction Fragment Length Polymorphism Analysis

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Abstract

Preterm birth is a leading cause of perinatal morbidity and mortality. Studies using a cultivation method or molecular identification have shown that bacterial vaginosis is one of the risk factors for preterm birth. However, an association between preterm birth and intestinal microbiota has not been reported using molecular techniques, although the vaginal microbiota changes during pregnancy. Our aim here was to clarify the difference in intestinal and vaginal microbiota between women with preterm birth and women without preterm labor. 16S ribosomal ribonucleic acid genes were amplified from fecal and vaginal DNA by polymerase chain reaction. Using terminal restriction fragment length polymorphism (T-RFLP), we compared the levels of operational taxonomic units of both intestinal and vaginal flora among three groups: pregnant women who delivered term babies without preterm labor (non-PTL group) (n = 20), those who had preterm labor but delivered term babies (PTL group) (n = 11), and those who had preterm birth (PTB group) (n = 10). Significantly low levels of *Clostridium* subcluster XVIII, *Clostridium* cluster IV, *Clostridium* subcluster XIVa, and *Bacteroides*, and a significantly high level of *Lactobacillales* were observed in the intestinal microbiota in the PTB group compared with those in the non-PTL group. The levels of *Clostridium* subcluster XVIII and *Clostridium* subcluster XIVa in the PTB group were significantly lower than those in the PTL group, and these levels in the PTL group were significantly lower than those in non-PTL group. However, there were no significant differences in vaginal microbiota among the three groups. Intestinal microbiota in the PTB group was found to differ from that in the non-PTL group using the T-RFLP method.

Citation: Shiozaki A, Yoneda S, Yoneda N, Yonezawa R, Matsubayashi T, et al. (2014) Intestinal Microbiota is Different in Women with Preterm Birth: Results from Terminal Restriction Fragment Length Polymorphism Analysis. PLoS ONE 9(11): e111374. doi:10.1371/journal.pone.0111374

Editor: Ana C. Zenclussen, Medical Faculty, Otto-von-Guericke University Magdeburg, Medical Faculty, Germany

Received June 20, 2014; **Accepted** September 25, 2014; **Published** November 5, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: The author(s) received no specific funding for this work from Toa Pharmaceutical Co., Ltd. Toa Pharmaceutical Co., Ltd., provided support in the form of salaries for authors TM and GS, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan [a Grant-in-Aid for Exploratory Research 26670717, a Grant-in-Aid for Scientific Research (B) 23390386, a Grant-in-Aid for Scientific Research (C) 24592462]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: TM and GS are employees of Toa Pharmaceutical Co., Ltd., whose company offered materials for sample collection and collected the samples. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

We harbor more than 100 trillion microbes in and on our body and these microbes constitute our microbiota [1,2]. Although only a small part of this microbiota can be cultured in a medium, culture-independent analyses, like 16S ribosomal ribonucleic acid (rRNA)-based genomic analysis [3,4] and metagenomic analysis [5], have recently revolutionized our understanding of the microbiota in our body. The microbiota in our intestine plays a major role in health and disease [1] in our body. The gut microbiota interacts with the immune system, providing signals to promote the maturation of immune cells and the normal development of immune functions [6,7,8].

Preterm birth (PTB) is the leading cause of perinatal morbidity and mortality in developing and developed countries [9]. The frequency of preterm births is about 12 to 13% in the USA and 5 to 9% in many other developed countries including Japan. Spontaneous PTB is regarded as a syndrome resulting from multiple causes, including intrauterine infection or inflammation, stress, socioeconomic environment, and uterine over-distension. Risk factors for PTB in Western countries include a previous preterm birth, black ethnicity, periodontal disease, low maternal body mass index, short cervical length, and an elevated cervical-vaginal fetal fibronectin concentration, while multiple pregnancy, short cervical length, part-time worker, steroid use for asthma or collagen disease, low educational level, and male fetus were shown to be risk factors for PTB in Japan [10]. Although antibiotic treatment can eradicate bacterial vaginosis (BV) in pregnancy, the overall risk of PTB in pregnant women with BV was found not to be significantly reduced by antibiotic treatment [11].

An association between preterm birth and intestinal microbiota has not been reported using molecular techniques, although the vaginal microbiota changes during pregnancy. Aagaard et al. [12] showed that the vaginal microbiome signature in pregnancy was distinct from that in non-pregnant women with variation of taxa across vaginal subsites and depending on gestational age. Romero et al. reported that, in a longitudinal study, *Lactobacillus* spp. were the predominant members of the microbial community in normal pregnancy [13], and they did not detect a difference in human vaginal microbiota between women who subsequently had a spontaneous preterm delivery and those who delivered at term [14]. Koren et al. described dramatic remodeling of the gut microbiota over the course of pregnancy [15]. These changes of intestinal microbiota induced insulin resistance during pregnancy. Although abnormal vaginal microbiota such as BV was studied using 16S rDNA-based genomic analysis [16], there are no reports on a link between PTB and gut microbiota as determined by genomic analysis.

Terminal restriction fragment length polymorphism (T-RFLP) analysis has used non-targeted approaches to identify differences and similarities in microbial communities, but it does not provide direct sequence information. Because of its relative simplicity, T-RFLP analysis has been applied to the analysis of bacterial 16S rRNA genes and provides a facile means to assess changes in microbial communities [17]. In this prospective and cross-sectional study using T-RFLP analysis, we examined bacterium-derived 16S rRNA genes in feces and vaginal discharge to determine whether the microbiota differs among three groups as follows: pregnant women who delivered term babies without preterm labor (non-PTL group), those who had preterm labor but delivered term babies (PTL group), and those who had preterm birth (PTB group).

Materials and Methods

This study is a prospective and cross-sectional study. The study protocol and informed consent documents were reviewed and approved by the University of Toyama Institutional Review Board. Written informed consent was obtained from all subjects prior to participation in the study. From 2011 to 2013, the participants were categorized into three groups as follows: (1) pregnant women who delivered term babies without preterm labor during pregnancy (non-PTL group) (n = 20), (2) those who had regular uterine contraction and received tocolytic agents but delivered term babies (PTL group) (n = 11), and (3) those who had regular uterine contractions, received tocolytic agents, and finally delivered preterm babies (PTB group) (n = 10). Women who were at earlier than 22 weeks of gestation and those who took any antibiotics, tocolytics and steroids during pregnancy were excluded. Clinical characteristics of all participants are shown in Table 1. Vaginal samples were obtained from the posterior vaginal fornix using a swab. Swabs were immersed in vaginal discharge for 10 seconds, then immediately placed into 3 mL of a buffer medium (100 mM Tris-HCl, pH 9.0, 40 mM Tris-EDTA, pH 8.0, and 4 M guanidine thiocyanate), and stored in a freezer at −20°C. Fecal samples were collected at home or our hospital by the participants. Briefly, the toilet was covered with a sterile sheet of paper, which was temporarily waterproof but would dissolve in the water of the toilet within a few minutes and could be flushed away with the remaining stool. The defecated fecal samples were quickly collected using a sterile spoon or a swab, immediately placed in 3 mL of buffer medium, and stored in a freezer at −20°C.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

In order to investigate the microbiota of the fecal and vaginal samples obtained from all the subjects, terminal restriction fragment length polymorphism (T-RFLP) analysis was performed, as previously reported [18].

Fecal and vaginal samples were suspended in a solution containing 100 mM Tris-HCl, pH 9.0, 40 mM Tris-EDTA, pH 8.0, and 4 M guanidine thiocyanate, and kept at −20°C until deoxyribonucleic acid (DNA) extraction. An aliquot of 0.8 mL of the suspension was homogenized with zirconia beads in a 2.0 mL screw cap tube using a FastPrep FP120A Instrument (MP Biomedicals, Irvine, CA) and placed on ice. After centrifugation (at 5000×g, for 1 min), the supernatant was transferred to the automated nucleic acid isolation system 12GC (Precision System Science, Chiba, Japan). Thereafter, DNA was extracted from the bead-treated suspension using the Magtration-MagaZorb DNA Common Kit 200 N (Precision System Science, Chiba, Japan).

The 16S rDNA was amplified from human fecal DNA using fluorescent-labeled 516f primer (5′-(6-FAM)-TGCCAGCAGCCGCGGTA-3′) and 1492r primer (5′-GGTTACCTTGTTACGACTT-3′) and from human vaginal DNA using fluorescent-labeled 27f (5′-(6-FAM)-AGAGTTTGATCCTGGCTCAG-3′) and 1492r primer (5′-GGTTACCTTGTTACGACTT-3′), with Hot-starTaq DNA polymerase using Gene Amp PCR system 9600 (Applied Biosystems, CA, USA). The amplification program used was as follows: preheating at 95°C for 15 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute; and finally terminal extension at 72°C for 10 minutes. The amplified DNA was purified using a MultiScreen^{R} PCRμ96 Filter Plate (Millipore, MA, USA) and verified by electrophoresis.

The restriction enzymes were selected according to Nagashima *et al.* [19]. In brief, the PCR product purified from fecal samples was digested with 10 U of *Bsl* I (New England BioLabs, Inc., Ipswich, USA) at 37°C for 3 hours or that from vaginal samples with 10 U of *Msp* I (TAKARA, Shiga, Japan) at 37°C for 3 hours. The resultant DNA fragments, namely, fluorescent-labeled terminal restriction fragments (T-RFs), were analyzed using an ABI PRISM 3130xl genetic analyzer, and their length and peak area were determined using the genotype software GeneMapper (Applied Biosystems).

According to the methods described by Nagashima *et al.* [19], the T-RFs were divided into 29 operational taxonomic units (OTUs) for fecal samples or 22 OTUs for vaginal samples. The OTUs were quantified as the percentage values of individual OTU per total OTU areas, which were expressed as the percent of the area under the curve (%AUC). The bacteria predicted for each classification unit OTU were identified with reference to Human Fecal Microbiota T-RFLP profiling (http://www.tecsrg.co.jp/t-rflp/). To minimize inter- and intra-observer coefficient of variations of the OTU, 1 author (TM) conducted all T-RFLP analyses.

For objective interpretation of the difference in T-RF patterns, cluster analyses were performed using the software SPSS (IBM Statistics, ver. 20.0, NY, USA). T-RF patterns produced by digestion with restriction enzymes (*Bsl* I or *Msp* I) were quantified as the proportion of the total peak area of all T-RFs. The levels of similarity among fecal and vaginal samples were calculated as correlation coefficients, and represented graphically by a scatter plot using principal component analysis.

Table 1. Clinical characteristics of all participants.

Characteristics	Non-PTL (N = 20)	PTL (N = 11)	PTB (N = 10)	Analysis of variance Bonferroni's multiple comparison
Maternal age (year), median (range)	34.0 (27–41)	30.5 (22–37)	33.4 (22–41)	$P = 0.2134$
Gestational weeks at sample collection, average (range)	28.6 (23–34)	28.5 (22–33)	28.0 (22–34)	$P = 0.9351$
Gestational weeks at birth, average (range)	39.2 (37–41)	37.9 (37–40)	33.4 (27–36)	$P = 0.0000$
Previous PTB, percent (n/N)	10.0 (2/20)	9.1 (1/11)	10.0 (1/10)	$P = 0.7897$
Smoking during pregnancy, n/N	0/20	0/11	0/10	-
Parity number, average (range)	0.9 (0–2)	1.1 (0–2)	0.4 (0–2)	$P = 0.1651$
Nulliparous, percent (n/N)	40.0 (8/20)	27.3 (3/11)	70.0 (7/10)	$P = 0.3169$
BMI at sample collection, average (range)	23.3 (17.6–29.1)	23.2 (18.9–29.4)	21.7 (17.0–27.2)	$P = 0.3263$

BMI: Body mass index (kg/m^2).
non-PTL: non-preterm labor.
PTL: preterm labor.
PTB: preterm birth.
doi:10.1371/journal.pone.0111374.t001

Statistical analysis

All values of OTU are expressed as the mean ± standard error (SE). Comparisons of quantitative data among the three groups were carried out by a multiple comparison technique (Bonferroni). False discovery rates were calculated using Benjamini-Hochberg method. Differences were considered significant at P values of less than 0.05. For multivariate analysis of the data, principal component analysis and cluster analysis were used. The principal component analysis was performed unsupervised.

Results

Of 21 women with preterm labor (the PTL group plus the PTB group), 10 women (47.6%) finally delivered their babies before 37 weeks of gestation.

Principal component analysis (PCA)

Fecal samples. PCA was performed based on the relative abundance of OTUs in fecal samples. The first two principal component scores, which accounted for 19.7% and 10.4% of the total variation, were calculated. Hierarchical clustering of fecal samples on the basis of their first two principal component scores separated the fecal samples into two primary clusters (Figure 1A). One cluster (labeled as 'cluster 1', thick dotted line, left in Figure 1A) included all 10 cases of the PTB group, as well as 2/20 of the non-PTL group and 7/11 of the PTL group. The other cluster (labeled as 'cluster 2', thick solid line, right in Figure 1A) was dominated by 90% of the non-PTL group (18/20) and 36.4% of the PTL group (4/11). No PTB cases were included in cluster 2 (0/10). The components of the PTL group occupied an intermediate position between the non-PTL group and the PTB group.

Vaginal samples. In vaginal samples, the first two principal component scores were calculated (the first and second principal components accounted for 18.5% and 15.8% of the total variation, respectively) (Figure 1B). The PCA showed that there was similarity among these three groups.

Terminal restriction fragment length polymorphism profile

The percentages of OTU (percentage values of individual OTU area per total OTU) of representative OTUs for fecal samples after *Bsl* I digestion are shown in Table 2 and those for vaginal samples after *Msp* I digestion in Table 3.

Fecal samples. When comparing respective OTUs of fecal samples between the non-PTL group and the PTB group, the peak areas of the OTU 650, OTU 657, OTU 749, OTU 853, and OTU 955 (digested with *Bsl* I) were significantly different (Table 2). The amounts of *Clostridium* cluster XVIII (OTU 650), *Clostridium* cluster IV (OTU 749), *Bacteroides* (OTU 853), and *Clostridium* subcluster XIVa (OTU 955) were significantly lower in the PTB group than those in the non-PTL group ($P = 0.0125$, $P = 0.0289$, $P = 0.0348$, and $P = 0.0005$, respectively). The amounts of *Clostridium* cluster XVIII and *Clostridium* subcluster XIVa in the PTL group were significantly higher than those in the non-PTL group ($P = 0.0449$ and $P = 0.0125$, respectively).

When comparing respective OTUs between the PTL group and the PTB group, the amount of *Clostridium* cluster XI and *Clostridium* subcluster XIVa (OTU 919) was significantly lower in the PTB group than that in the PTL group ($P = 0.0285$).

Vaginal samples. In contrast, the % OTU for vaginal samples after *Msp* I digestion from the non-PTL group was quite similar to that from the PTL or the PTB group (Table 3).

Discussion

In this study, we first showed that the fecal microbiota in the PTL group was substantially different from that in the non-PTL group using T-RFLP analysis. Average scores of OTU 650, OTU 749, OTU 853, and OTU 955 were significantly lower in the fecal microbiota from the PTB group than those from the non-PTL group. Nagashima *et al.* [19] reported that OTU 749 and OTU 955 most likely represent *Clostridium* cluster IV species and *Clostridium* subcluster XIVa species, respectively, as determined by the 16S rDNA clone library method. Taking these findings together, it was revealed that the levels of *Clostridium* cluster XVIII, *Clostridium* cluster IV, *Clostridium* subcluster XIVa, and *Bacteroides* were significantly reduced in the fecal microbiota from the PTB group. Our result first showed that there was a significant difference in the average OTU scores of *Clostridium* cluster XVIII, *Clostridium* cluster IV, and *Clostridium* subcluster XIVa between mothers with non-PTL and those with PTB, while mothers with PTL had intermediate scores between them. This finding does not mean that the intestinal microbiota accidentally

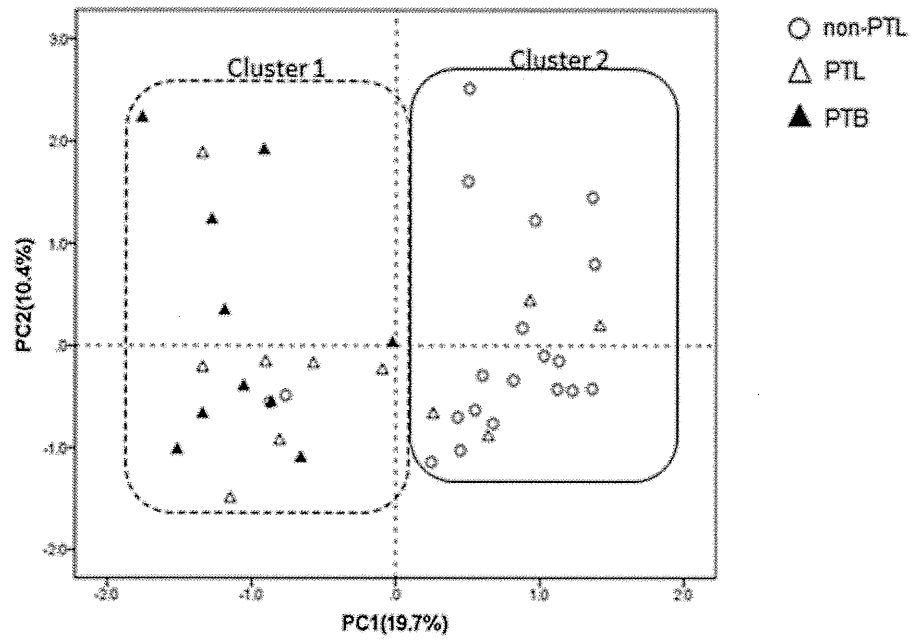

Figure 1B. Principal component analysis for vaginal microbiota

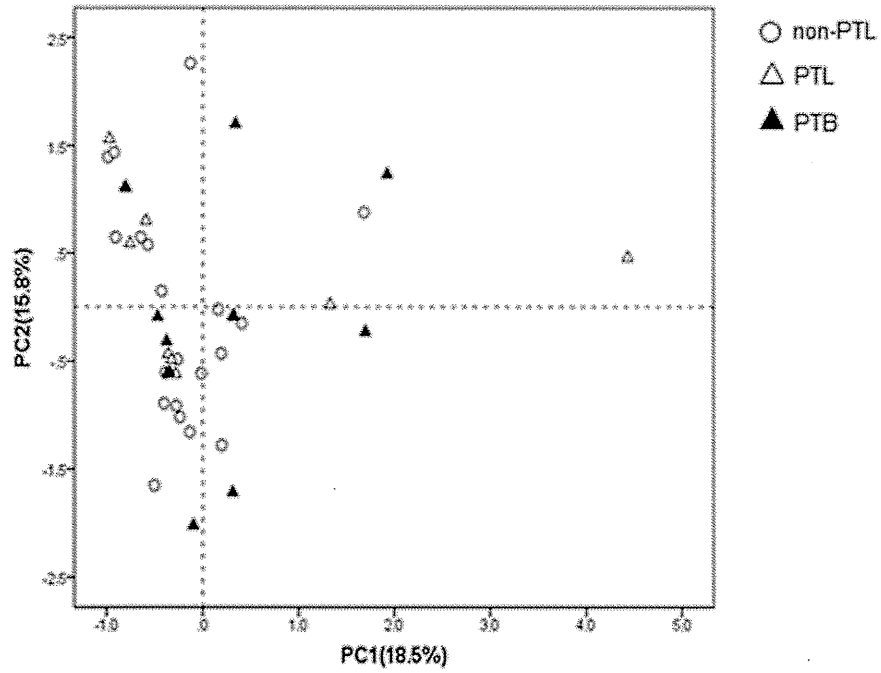

Figure 1. A. Principal component analysis of fecal microbiota. Principal component analysis scores are plotted based on the relative abundance of OTUs of vaginal microbiota. The percentage of variation explained by the principal coordinates is indicated on the axis. Open circles (○) represent the non-PTL group, open triangles (△) the PTL group, and closed triangles (▲) the PTB group. A dotted line, on the left in Figure 1A, shows 'cluster 1', which contains all 10 cases of the PTB group, as well as 2/20 of the non-PTL group and 7/11 of the PTL group. A solid circle, on the right in Figure 1A, shows 90% of the non-PTL group (18/20) and 36.4% of the PTL group (4/11). The PTL group occupied an intermediate position between the non-PTL group and the PTB group. B. Principal component analysis of vaginal microbiota. Principal component analysis scores are plotted based on the relative abundance of OTUs of vaginal microbiota. The percentage of variation explained by the principal coordinates is indicated on the axis. Open circles (○) represent the non-PTL group, open triangles (△) the PTL group, and closed triangles (▲) the PTB group.
doi:10.1371/journal.pone.0111374.g001

changed in the case of the PTB group, but rather it suggests that some alterations in intestinal microbiota are associated with the clinical findings of mothers with PTB.

Our result is compatible with the report by Romero, who found no differences in human vaginal microbiota between women who subsequently had a spontaneous preterm delivery and those who

Table 2. Average OTU sores in fecal samples.

OTU	Bacteria predicted by T-RF length	Non-PTL (N = 20)	PTL (N = 11)	PTB (N = 10)	Bonferroni's adjustment *P* value	False Discovery Rate *P* value
106	*Clostridium* subcluster XIVa	1.85±0.75	0.33±0.27	0.19±0.13	NS	NS
110	*Clostridium* cluster IV	1.25±0.42	3.89±2.99	3.03±1.30	NS	NS
124	*Bifidobacterium*	11.19±1.98	14.10±4.08	5.91±2.61	NS	NS
168	*Clostridium* cluster IV	0.52±0.15	0.18±0.18	0.08±0.08	NS	NS
317	*Prevotella*	4.58±2.79	0.31±0.16	0.99±0.60	NS	NS
332	*Lactobacillares*	1.29±0.34	1.91±0.60	0.87±0.42	NS	NS
338	*Clostridium* cluster IV	0.57±0.16	0.37±0.19	0.00±0.00	NS	NS
366	*Bacteroides*	3.56±1.13	4.62±1.96	5.71±2.38	NS	NS
369	*Clostridium* cluster IV	0.27±0.18	0.89±0.86	2.76±1.32	NS	NS
469	*Bacteroides*	37.10±3.33	30.44±5.66	22.76±4.44	NS	NS
494	*Clostridium* subcluster XIVa	5.90±1.51	10.49±4.46	10.51±5.04	NS	NS
520	*Lactobacillares*	0.79±0.33	0.18±0.12	0.00±0.00	NS	NS
650	*Clostridium* cluster XVIII	2.12±0.34	0.70±0.35	0.26±0.21	Non-PTL vs. PTB, $P=0.0016$; non-PTL vs. PTL, $P=0.0150$	Non-PTL vs. PTB, $P=0.0125$; non-PTL vs. PTL, $P=0.0449$
657	*Lactobacillares*	5.18±2.54	17.63±8.29	24.24±7.17	Non-PTL vs. PTB, $P=0.0497$	NS ($P=0.1089$)
749	*Clostridium* cluster IV	6.41±1.15	2.90±1.22	0.80±0.80	Non-PTL vs. PTB, $P=0.0060$	Non-PTL vs. PTB, $P=0.0289$
754	*Clostridium* subcluster XIVa	1.70±0.28	1.63±1.03	0.00±0.00	NS	NS
853	*Bacteroides*	0.84±0.13	0.60±0.27	0.07±0.07	Non-PTL vs. PTB, $P=0.0087$	Non-PTL vs. PTB, $P=0.0348$
919	*Clostridium* cluster XI, *Clostridium* subcluster XIVa	1.30±0.23	0.36±0.16	2.06±0.55	PTL vs. PTB, $P=0.0047$	PTL vs. PTB, $P=0.0285$
940	*Clostridium* subcluster XIVa, *Enterobacteriales*	3.79±0.68	3.62±1.10	11.43±3.72	NS	NS
955	*Clostridium* subcluster XIVa	5.37±0.64	1.72±0.80	0.34±0.34	non-PTL vs. PTB, $P=0.0000$; non-PTL vs. PTL, $P=0.0012$	non-PTL vs. PTB, $P=0.0005$; non-PTL vs. PTL, $P=0.0125$
990	*Clostridium* subcluster XIVa	3.61±0.69	2.87±0.86	7.98±6.66	NS	NS
Others		0.83±0.42	0.26±0.20	0.00±0.00	NS	NS

OTU: operational taxonomic unit,
T-RF: terminal restriction fragment.
non-PTL: non-preterm labor.
PTL: preterm labor.
PTB: preterm birth.
doi:10.1371/journal.pone.0111374.t002

delivered at term. In addition, more recent studies have shown that bacteria from the oral cavity are most often found in the amniotic fluid of patients with preterm labor, demonstrating that periodontal pathogens/byproducts may reach the placenta and spread to the fetal circulation and amniotic fluid [20]. Taken together, these findings may suggest that oral and/or intestinal, not vaginal, microbiota could induce pathogens and the secretion of elevated levels of inflammatory mediators, which in turn may cause premature birth or suggest that dysbiosis caused by oral and/or intestinal, not vaginal, microbiota may render the uterus and/or the placenta susceptible to infection.

Recently, bacterial vaginosis-associated bacteria (BVAB) in the order *Clostridiales:* BVAB1, BVAB2, and BVAB3, were identified in association with bacterial vaginosis [21]. Subsequently, Foxman et al. reported that, in vaginal fluids, one of the Clostridia-like bacteria, BVAB-3, was consistently associated with a reduction in the risk of preterm birth for all ethnic groups (risk ratio, 0.55; 95% confidence interval, 0.39–0.78) [22]. Their result is compatible with our findings in that the level of *Clostridium* was significantly lower in preterm birth, although its level in our studies was lower in the samples not from vaginal fluid but from feces.

Among the microbiota indigenous to the murine and human colon, the genus *Clostridium* belonging to clusters XIVa and IV is reported to be an outstanding inducer of colonic $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells [7,8]. *Bacteroides fragilis* has also been shown to induce Treg cells in mouse [23]. It was suggested that polysaccharide A (PSA) produced by *Bacteroides fragilis* potentiates the suppressor activity of Treg cells. The balance between beneficial and potentially harmful species in the commensal microbial community, known as dysbiosis, has often been linked to the development of inflammatory bowel disease (IBD) in humans and analogous intestinal inflammation in mice [18]. Importantly, intestinal Treg cells play a key role in regulating inflammation by the production of IL-10 [8,23,24,25] and decreased intestinal Treg cells were observed in IBD patients. Preterm labor may be viewed as inflammation caused by

Table 3. Average OTU scores in vaginal samples.

OTU	Bacteria predicted by T-RF length	non-PTL (N = 20)	PTL (N = 11)	PTB (N = 10)	*P* value
57	Unknown	8.78±1.77	12.58±4.12	10.12±2.61	NS
74	*Enterococcus*	0.47±0.27	0.36±0.31	0.72±0.49	NS
93	*Chitinophagaceae*	1.20±0.53	4.25±2.74	4.16±2.41	NS
98	*Prevotella*	0.54±0.24	2.39±2.19	5.45±2.30	NS
134	*Bifidobacterium*	0.89±0.86	0.05±0.05	2.23±1.78	NS
147	*Firmicutes*	2.37±1.56	0.28±0.13	3.94±3.55	NS
163	*Actinobacteria*	3.17±1.67	0.01±0.01	5.78±4.93	NS
170	*Firmicutes*	0.33±0.17	0.74±0.35	0.53±0.28	NS
179	*Lactobacillus*	27.42±8.09	34.34±12.45	25.40±8.72	NS
189	*Lactobacillus*	43.41±8.38	39.68±13.11	29.01±10.90	NS
230	*Firmicutes*	0.41±0.15	0.59±0.38	0.37±0.25	NS
281	*Actinobacteria*	6.34±4.12	1.16±1.04	2.95±2.95	NS
300	*Clostridiales*	1.03±0.59	0.08±0.08	5.73±4.40	NS
563	*Streptococcus*	0.18±0.15	0.78±0.78	0.92±0.41	NS
573	*Lactobacillus*	3.24±1.29	2.39±1.35	2.62±0.91	NS
Others		0.20±0.15	0.33±0.33	0.05±0.05	NS

OTU: operational taxonomic unit.
T-RF: terminal restriction fragment.
non-PTL: non-preterm labor.
PTL: preterm labor.
PTB: preterm birth.
doi:10.1371/journal.pone.0111374.t003

inappropriately expanded components of the normal bacterial community. In IL-10 knockout mice, a very small amount of lipopolysaccharide (LPS) induces preterm delivery [26]. These findings suggest that decreased intestinal *Clostridium* cannot induce an adequate volume of Treg cells, resulting in susceptibility to inflammation. Indeed, decreased Treg cell volume and function in the peripheral blood have been reported in preterm labor [27,28,29,30,31], although the level of intestinal Treg cells is unclear in these papers and this study. Further studies are needed to clarify the relationship between intestinal microbiota and peripheral Treg cells or intestinal Treg cells.

The peak area of the 919-bp OTU (*Clostridium* cluster XI, *Clostridium* subcluster XIVa) in the PTB group was significantly larger than that in the PTL group ($P = 0.0285$). This is a limitation of the T-RFLP method. We should thus reevaluate the bacterial species using metagenomic analysis.

Several limitations were identified in this study. Because of the small number of cases (fewer than 30) in each group, information that can only come from patient participation in well-designed clinical trials is needed to improve the management of PTB. Second, we did not check the level of Treg cells in peripheral blood and therefore could not compare the relationship between the fecal microbiota of the mother and the level of Treg cells. Further investigation is needed to address whether the difference in gut microbiota is related to the expression of immunoregulatory Treg cells or inflammation-induced Th17 cells that are induced by gut *Clostridium* strains and segmented filamentous bacteria, respectively. Third, it is possible that the unidentified confounders may have an effect on changes in the microbial communities. An alternative interpretation is that, as Aagaard et al. [12] reported, the vaginal microbial 16S rRNA gene catalogue may uniquely differ in pregnancy, with variation of taxa across vaginal subsites and depending on gestational age. Further study is required to determine the relationship between the difference in average OTU for some phylotypes and PTB. Fourth, owing to the cross-sectional nature of the study, our finding about the causal relationships between fecal microbiota and PTB should be interpreted with caution. Fifth, we didn't measure the concentrations of organic acids, indole, and ammonia. And we also didn't measure pH and moisture and didn't compare these values with those from normal pregnancy. Sixth, the selection of optimal T-RFLP probes is a subject of considerable ongoing discussion in the field of microbiome research. T-RFLP analysis only identify differences and similarities in microbial community and the resulting OTUs aren't assigned to a specific species. Unfortunately, there is no perfect set of T-RFLP probes for fecal and vaginal microbiome. Yet, the T-RFLP data can readily be analyzed using various statistical algorithms to quantitatively ascertain similarities and differences among communities and to infer plausible community of intestine and vagina. Seventh, we are unable to grasp the importance of the results that patients had preterm babies had a higher average OTU for Lactobacillares phylotypes than those who delivered term babies. Yet, the identification of significant differences in average OTUs provides evidence that the study of the intestinal microbiota during pregnancy can yield important insights into the relationship between the fluctuation of microbial communities and adverse pregnancy outcome like PTB. Further studies are required to confirm this finding and elucidate the role of intestinal microbiota in PTB. Eighth, the principal component analysis is not a prefect technique to analyze the T-RFLP data. Therefore, the performance of principal component analysis should be interpreted with caution since there were no validation samples to test.

Many studies have been performed to identify the differences in microbial diversity between healthy individuals and patients with rheumatoid arthritis (RA) [32], inflammatory bowel disease (IBD) [33], and type 1 diabetes [34]. However, the results about the fecal level of *Clostridium* in these patients are conflicting (decrease [32],

no change [33], and increase [34]). When abnormality in fecal microbiota in the PTL group is found, the question arises of whether microbiotic alteration, dysbiosis, is a cause or a result of preterm delivery. We speculate that the changes in the immune system through fecal dysbiosis may change the uterine activity. In addition, the underlying mechanisms resulting in alteration of the microbiota remain to be clarified. Recently, intake of probiotic food was found to be associated with a reduced risk of spontaneous preterm delivery and preeclampsia [35,36]. Prospective studies are needed to clarify whether intestinal dysbiosis before pregnancy might cause uterine inflammation and induce uterine contraction or cervical ripening during pregnancy.

Conclusion

Disturbance of the intestinal flora, dysbiosis, during pregnancy was first observed in the PTB group in this study. This may cause inflammatory reactions in the uterus leading to PTB. Further study is needed to clarify the relationship between PTB and dysbiosis of intestinal bacterial flora.

References

1. Clemente JC, Ursell LK, Parfrey LW, Knight R (2012) The impact of the gut microbiota on human health: an integrative view. Cell 148: 1258–1270.
2. Human Microbiome Project Consortium (2012) Structure, function and diversity of the healthy human microbiome. Nature 486: 207–214.
3. Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, et al. (1995) Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol 61: 3069–3075.
4. Suau A1, Bonnet R, Sutren M, Godon JJ, Gibson GR, et al. (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 65: 4799–4807.
5. Shendure J, Ji H (2008) Next-generation DNA sequencing. Nat Biotechnol 26: 1135–1145.
6. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, et al. (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139: 485–498.
7. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, et al. (2011) Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. Science 331: 337–341.
8. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, et al. (2013) Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 500: 232–236.
9. Goldenberg RL, Culhane JF, Iams JD, Romero R (2008) Epidemiology and causes of preterm birth. Lancet 371: 75–84.
10. Shiozaki A1, Yoneda S, Nakabayashi M, Takeda Y, Takeda S, et al. (2014) Multiple pregnancy, short cervix, part-time worker, steroid use, low educational level and male fetus are risk factors for preterm birth in Japan: A multicenter, prospective study. J Obstet Gynaecol Res 40: 53–61.
11. Brocklehurst P, Gordon A, Heatley E, Milan SJ (2013) Antibiotics for treating bacterial vaginosis in pregnancy. Cochrane Database Syst Rev 1: CD000262.
12. Aagaard K, Riehle K, Ma J, Segata N, Mistretta TA, et al. (2012) A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. PLoS One 7: e36466.
13. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, et al. (2014) The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. Microbiome 2: 4.
14. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, et al. (2014) The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. Microbiome. 2: 18.
15. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, et al. (2012) Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell 150: 470–480.
16. Fredricks DN, Fiedler TL, Marrazzo JM (2005) Molecular identification of bacteria associated with bacterial vaginosis. N Engl J Med 353: 1899–1911.
17. Schütte UM, Abdo Z, Bent SJ, Shyu C, Williams CJ, et al. (2008) Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. Appl Microbiol Biotechnol 80: 365–380.
18. Hayashi H, Sakamoto M, Kitahara M, Benno Y (2003) Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP. Microbiol Immunol 47: 557–570.
19. Nagashima K, Hisada T, Sato M, Mochizuki J (2003) Application of new primer-enzyme combinations to terminal restriction in human feces. Appl Environ Microbiol 69: 1251–1262.
20. Madianos PN, Bobetsis YA, Offenbacher S (2013) Adverse pregnancy outcomes (APOs) and periodontal disease: pathogenic mechanisms. J Periodontol. 84(4 Suppl): S170–80.
21. Foxman B, Wen A, Srinivasan U, Goldberg D, Marrs CF, et al. (2014) Mycoplasma, bacterial vaginosis-associated bacteria BVAB3, race, and risk of preterm birth in a high-risk cohort. Am J Obstet Gynecol. 210: 226. e1–7.
22. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM (2007) Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. J Clin Microbiol 45: 3270–3276.
23. Round JL, Mazmanian SK (2010) Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci USA 107: 12204–12209.
24. Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, et al. (2011) Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity 34: 794–806.
25. Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, et al. (2012) Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Rep 13: 440–447.
26. Murphy SP, Fast LD, Hanna NN, Sharma S (2005) Uterine NK cells mediate inflammation-induced fetal demise in IL-10-null mice. J Immunol 175: 4084–4090.
27. Bizargity P, Del Rio R, Phillippe M, Teuscher C, Bonney EA (2009) Resistance to lipopolysaccharide-induced preterm delivery mediated by regulatory T cell function in mice. Biol Reprod 80: 874–881.
28. Kisielewicz A, Schaier M, Schmitt E, Hug F, Haensch GM, et al. (2010) A distinct subset of HLA-DR+-regulatory T cells is involved in the induction of preterm labor during pregnancy and in the induction of organ rejection after transplantation. Clin Immunol 137: 209–220.
29. Steinborn A, Schmitt E, Kisielewicz A, Rechenberg S, Seissler N, et al. (2012) Pregnancy-associated diseases are characterized by the composition of the systemic regulatory T cell (Treg) pool with distinct subsets of Tregs. Clin Exp Immunol 167: 84–98.
30. Gomez-Lopez N, Laresgoiti-Servitje E (2012) T regulatory cells: regulating both term and preterm labor? Immunol Cell Biol 90: 919–920.
31. Schober L, Radnai D, Schmitt E, Mahnke K, Sohn C, et al. (2012) Term and preterm labor: decreased suppressive activity and changes in composition of the regulatory T-cell pool. Immunol Cell Biol 90: 935–944.
32. Vaahtovuo J, Munukka E, Korkeamäki M, Luukkainen R, Toivanen P (2008) Fecal microbiota in early rheumatoid arthritis. J Rheumatol 35: 1500–1505.
33. Reiff C, Kelly D (2010) Inflammatory bowel disease, gut bacteria and probiotic therapy. Int J Med Microbiol 30: 25–33.
34. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, et al. (2013) Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC Med 11: 46.
35. Myhre R, Brantsæter AL, Myking S, Gjessing HK, Sengpiel V, et al. (2011) Intake of probiotic food and risk of spontaneous preterm delivery. Am J Clin Nutr 93: 151–157.
36. Myhre R, Brantsæter AL, Myking S, Gjessing HK, Sengpiel V, et al. (2011) Intake of probiotic food and risk of preeclampsia in primiparous women: the Norwegian Mother and Child Cohort Study. Am J Epidemiol 174: 807–815.

Acknowledgments

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan [a Grant-in-Aid for Exploratory Research 26670717, a Grant-in-Aid for Scientific Research (B) 23390386, a Grant-in-Aid for Scientific Research (C) 24592462]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The author(s) received no specific funding for this work. Toa Pharmaceutical Co., Ltd., provided support in the form of salaries for authors TM and GS, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Author Contributions

Conceived and designed the experiments: AS SY NY RY TM GS SS. Performed the experiments: AS SY NY RY TM GS SS. Analyzed the data: AS TM GS SS. Contributed reagents/materials/analysis tools: AS SY NY RY TM GS SS. Contributed to the writing of the manuscript: AS TM GS SS.

Cytokine 66 (2014) 1–6

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Extensive serum biomarker analysis in patients with enterohemorrhagic *Escherichia coli* O111-induced hemolytic-uremic syndrome

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ARTICLE INFO

Article history:
Received 13 September 2013
Received in revised form 14 November 2013
Accepted 9 December 2013
Available online 31 December 2013

Keywords:
Biomarker
Enterohemorrhagic
Escherichia coli
Cytokine
Hemolytic-uremic syndrome

ABSTRACT

Proinflammatory cytokines are related to the pathogenesis of enterohemorrhagic *Escherichia coli* infection and hemolytic-uremic syndrome (HUS). We employed an antibody array that simultaneously detects 174 serum cytokines. We identified five serum biomarkers, namely insulin growth factor-binding protein-2, angiopoietin-2, soluble interleukin-6 receptor, soluble tumor necrosis factor receptor type II, and matrix metalloprotease protein-3 whose levels increased with the development of HUS. Furthermore, the levels of these cytokines were significantly increased in severe HUS compared with mild HUS. These cytokines might play an important role in the pathogenesis of HUS and may also be used to predict the severity of HUS.

1. Introduction

Hemolytic-uremic syndrome (HUS) is a multisystem disease characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia, and renal failure. HUS occurs after a prodrome of hemorrhagic colitis (HC) caused by Shiga toxin-producing *Escherichia coli*, also known as enterohemorrhagic *E. coli* (EHEC) [1,2]. The most prevalent serotype is EHEC O157 however EHEC O111 can also cause these complications [3,4]. An outbreak of EHEC O111 occurred in Toyama and other prefectures in Japan between late April and early May 2011. The courses in some patients were extremely aggressive and some cases were fatal [3].

Some laboratory parameters including increased number of white blood cells, decreased serum sodium and total protein levels, and increased serum alanine aminotransferase levels have been used for predicting the severity of HUS [5,6]. Recently, however, roles of several cytokines and chemokines in EHEC-induced HUS have been identified [7–15].

Enzyme-linked immunosorbent assay (ELISA)-based methods are considered to be the most robust platforms for biomarker discovery and are known for their high degree of sensitivity [16]. Recent advancement in protein array technology has created a high-throughput platform for biomarker screening by ELISA. In this study, we employed the Raybiotech C-Series 2000 antibody array system, an antibody array that simultaneously detects 174 cytokines, to identify potential predictive markers for EHEC-induced HUS.

We report the identification of five serum biomarkers, namely insulin growth factor-binding protein (IGFBP)-2, angiopoietin-2 (Ang-2), soluble interleukin-6 receptor (sIL-6R), soluble tumor necrosis factor receptor type II (sTNFRII), and matrix metalloprotease protein-3 (MMP-3), that were significantly elevated in patients with developing and severe HUS.

2. Materials and methods

2.1. Patients and samples

Serum samples were obtained from nine patients during the outbreak of EHEC O111 HUS that occurred in Toyama, Japan

Abbreviations: EHEC, enterohemorrhagic *Escherichia coli*; HUS, hemolytic-uremic syndrome; HC, hemorrhagic colitis; ELISA, enzyme-linked immunosorbent assay; IGFBP, insulin growth factor-binding protein; IL, interleukin; TNF, tumor necrosis factor; sTNF-RII, soluble tumor necrosis factor receptor type II; MMP, matrix metalloprotease protein.

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http://dx.doi.org/10.1016/j.cyto.2013.12.005

Table 1
Clinical characteristics of patients with EHEC O111/HUS.

Case	Age (years)	Sex	STx1	STx2	ARF	Encephalopathy	Severity	Outcome
1	7	M	–	+	+	+	Severe	Died
2	14	M	–	–	+	+	Severe	Died
3	7	F	+	+	+	+	Severe	Alive
4	7	F	–	+	+	+	Severe	Alive
5	26	F	+	+	+	+	Severe	Alive
6	13	M	+	+	–	+	Severe	Alive
7	6	F	–	+	–	–	Mild	Alive
8	8	F	–	+	–	–	Mild	Alive
9	16	M	+	+	–	–	Mild	Alive

between late April and early May 2011. Samples from six patients with EHEC O111 infection were serially obtained from the HC phase to the HUS phase. The clinical characteristics of patients with HUS are shown in Table 1. EHEC O111 infection was diagnosed when one or both of the following criteria were fulfilled: bloody diarrhea, vomiting, or bowel cramps with microbiological identification of EHEC O111 infection. The presence of HUS was defined by thrombocytopenia (platelet count of <150,000/mm^3), hemolytic anemia, and acute renal dysfunction. Acute renal dysfunction was defined as renal injury evidenced by hematuria, proteinuria, or elevated creatinine levels ($\geqslant$1.0 mg/dl in children aged <13 years, $\geqslant$1.5 mg/dl in patients $\geqslant$13 years old, $\geqslant$50% increase over baseline) [17]. The severity of HUS was classified according to Gianantonio's criteria as follows: (i) mild, no anuria; (ii) moderate, <7 days of anuria; or (iii) severe, $\geqslant$7 days of anuria [18]. One patient (case 2) did not have anuria but died of acute encephalopathy with diffuse brain edema 2 days after the diagnosis of HUS. This patient was classified as having severe HUS. Another patient (case 6) also did not have anuria but suffered from acute encephalopathy, acute lung injury, and acute pancreatitis. This patient was also classified as having severe HUS.

Three patients were classified as having mild HUS and six were classified as having severe HUS. No patient was classified as having moderate HUS. Serum samples from the patients were separated, divided into aliquots, frozen, and stored at −80 °C until analysis. This study was approved by the Institutional Review Board of Kanazawa University, and all specimens were used after the receipt of informed patient consent.

2.2. Quantification of serum cytokines

RayBio human cytokine antibody array (C-Series 2000 antibody array, RayBiotech, Norcross, GA) was used for detection of 174 cytokines in frozen stock sera of the patients with EHEC O111 induced HUS, according to the manufacturer's protocol. Relative levels were determined by the ratio of intensity of each sample to that of the internal positive control. The intensity of each sample and control was measured using the software image processing and analysis in Java (Image J).

Serum levels of IGFBP2 and sTNFRII were evaluated by ELISA according to the manufacturer's instructions (IGFBP2: RayBiotech, Inc., Norcross, GA, sTNF-RII: R&D Systems, Minneapolis, MN, USA).

2.3. Statistical analysis

Within-group comparisons were analyzed using the Mann–Whitney *U*-test or paired *t* test. A *p*-value of <0.05 was considered statistically significant.

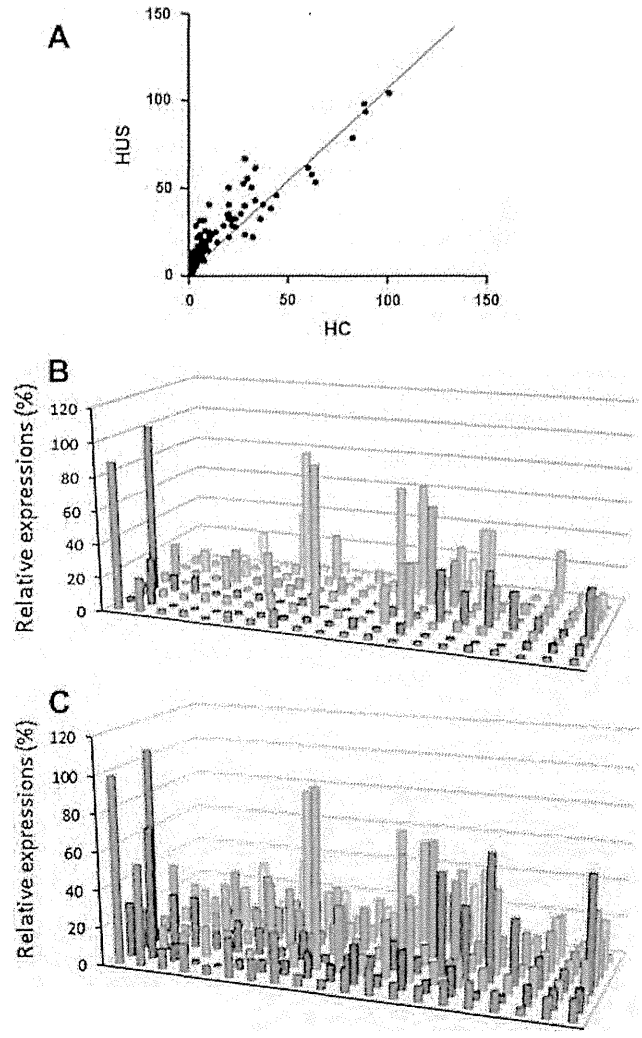

Fig. 1. Changes in the relative levels of 174 cytokines with the development of hemolytic-uremic syndrome (HUS). The relative levels were determined by the ratio of intensity of each sample to that of internal positive control. (A) Plot of the mean relative density values of the 174 cytokines during the hemorrhagic colitis (HC) phase and the HUS phase. (B) Mean relative density values of 174 cytokines of six patients during the HC phase. (C) Mean relative density values of 174 cytokines of nine patients during the HUS phase. The mapping of each bar in Fig. 1B and C are shown in Supplementary Table 1.

3. Results

3.1. The levels of 126 out of 174 cytokines increased with the development of HUS

The levels of 174 cytokines were analyzed during the HC phase (n = 6) and the HUS phase (n = 9) and the mean relative density

values for each group were shown as plot files (Fig. 1A). The levels of 126 out of the 174 cytokines significantly increased during the HUS phase (Fig. 1A–C) compared with the HC phase (Fig. 1A and B). These 126 cytokines were as follows: BDNF, BLC, BMP-4, CKβ8-1, eotaxin-3, FGF-6, FGF-7, Flt-3 ligand, fractalkine, GCP-2, GDNF, I-309, IFN-γ, IGFBP-1, IGFBP-2, IGFBP-4, IL-15, IL-16, IL-1α, IL-1β, IL-1ra, IL-3, IL-4, IL-5, IL-6, IL-7, Leptin, MCP-2, MCP-3, MCP-4, M-CSF, MDC, MIG, MIP-1δ, MIP-3α, NT-3, PARC, SCF, SDF-1, TGF-β1, TGF-β3, TNF-α, TNF-β, AgRP, Ang-2, amphiregulin, Axi, bFGF, bNGF, BTC, CTACK, Dtk, EGF-R, ENA78, Fas/TNFRSF6, FGF-4, FGF-9, GITR, GRO, HCC-4, ICAM3, IGFBP-6, IGF-I SR, IL-1R4/ST2, IL-1R1, IL-11, IL-12p40, IL-12p70, IL-17, IL-2Rα, IL-6R, IL-8, MIF, MIP-1α, MIP-1β, MIP-3β, MSP-α, NT-4, osteoprogerin, oncostatin M, PIGF, sgp130, sTNFRII, sTNFRI, thrombopoietin, TRAIL R3, TRAIL R4, uPAR, VEGF, VEGF D, ActivinA, CD80, BMP-5, BMP-7, cardiotrophin-1, CXCL-16, endoglin, ErbB3, Fas ligand, IL-1RII, IL-10Rβ, IL-13Rα2, IL-18BPα, IL-18Rβ, MMP-3, IL-2Rβ, IL-2Rγ, IL-21R, IL-5Rα, IP-10, LeptinR, LIF, MMP-1, MPIF-1, PDGFAA, PDGFRα, PECAM-1, Prolactin, Siglec-5, TGF-α, TGF-β2, Tie-1, Tie-2, TIMP-4, VE-cadherin, VEGF R2, and VEGF R3.

3.2. Five cytokines were associated with the severity of EHEC O111-induced HUS

To identify potential predictive markers for the severity of EHEC-induced HUS, we evaluated the differences of the levels of these 126 cytokines between the three mild and the six severe cases during the HUS phase. The levels of 11 cytokines including IGFBP-1, IGFBP2, IL-5, IL-6, NT-3, Ang-2, IGFBP-6, sIL-6R, sTNFRII, and MMP-3 significantly increased during the HUS phase in patients with severe HUS compared with those with mild HUS (Fig. 2). We then evaluated how the levels of these 11 cytokines changed from the HC phase to the HUS phase in four patients with severe HUS. The levels of IGFBP2, Ang-2, sIL-6R, sTNFRII, and MMP-3 increased significantly with the development of severe HUS (Fig. 3). To verify these results, we measured serum levels of IGFBP2 and sTNFRII by ELISA. Serum levels of IGFBP2 and sTNFRII significantly increased during the HUS phase in patients with severe HUS compared with those with mild HUS (Supplemental Fig. 1).

4. Discussion

Previous reports have demonstrated that proinflammatory cytokines play an important role in the pathogenesis of EHEC infection and development of severe complications such as HUS [7–15]. In this study, we employed the Raybiotech C-Series 2000 antibody array system and identified five serum cytokines (IGFBP-2, Ang-2, sIL-6R, sTNFRII, and MMP-3) that were significantly elevated in patients with severe HUS. The levels of these five cytokines also increased with the development of HUS. Our findings indicate that these particular cytokines play an important role in the pathogenesis of EHEC infection and the development of HUS. Furthermore, these cytokines might be useful biomarkers to predict the severity of EHEC-induced HUS.

The IGF system plays a key role in the regulation of cellular proliferation, differentiation, and apoptosis [19]. The IGF system comprises the ligands IGF-I and IGFII, their receptors, and a family of six high-affinity IGF-binding proteins (IGFBPs) [19,20]. Most of

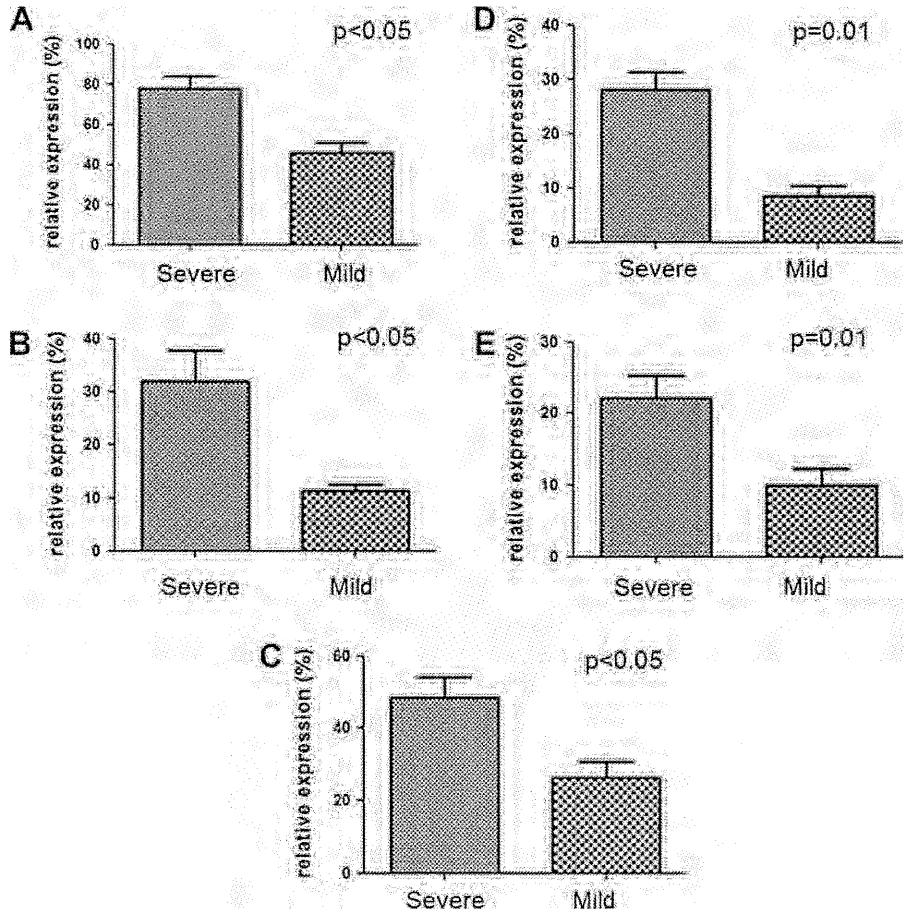

Fig. 2. The levels of five cytokines identified during severe and mild hemolytic-uremic syndrome ((A) IGFBP-2, (B) Angiopoietin-2, (C) sIL-6R, (D) sTNFRII, and (E) MMP-3).

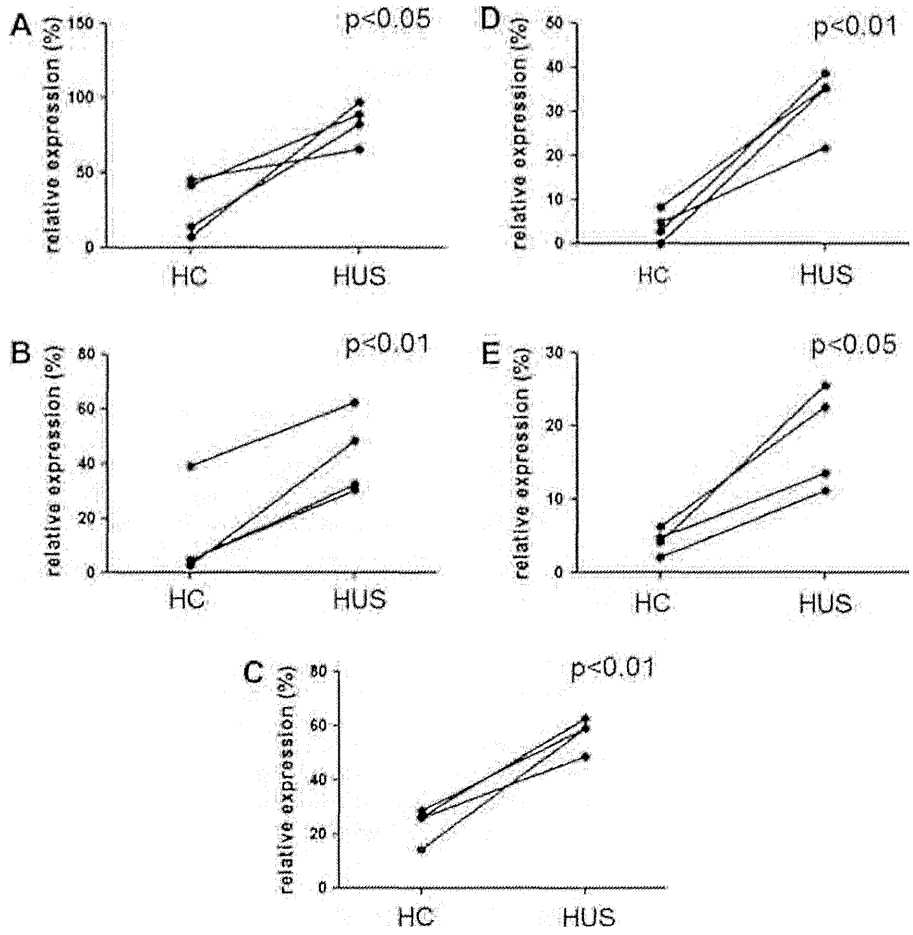

Fig. 3. The changes in levels of five cytokines between hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in four patients with severe HUS ((A) IGFBP-2, (B) Angiopoietin-2, (C) sIL-6R, (D) sTNFRII, and (E) MMP-3).

the biological actions of the IGF are modulated by the IGFBPs. IGFBP-2 is the second most abundant binding protein in the circulation [20–23]. Although the regulation and physiological roles of IGFBP-2 are less well defined, an association with metabolic homeostasis and insulin sensitivity has been reported [24]. In addition to these roles in metabolism, IGFBP-2 has an important role in cell proliferation/survival, migration, and adhesion, through the interaction with critical genes in tumorigenesis [23]. Interestingly, IGFBP-2 is also associated with angiogenesis and enhances VEGF gene promoter activity [25]. Serum IGFBP-2 levels are elevated under conditions where the total IGF-I plus IGF-II level exceeds the capacity of IGFBP-3 including growth hormone deficiency, with an IGF-II-secreting tumor or following IGF-I administration. A number of disease states in critically ill patients are also characterized by an elevated serum IGFBP-2 level [26]. Although there are certainly nutritional influences on IGFBP-2, it is not clear whether the elevation seen in critical illness is primarily related to nutritional disturbance or to a direct cytokine effect. Mice injected with IL-1α or TNF-α show no change in serum IGFBP-2. However, intravenous injection of *E. coli* endotoxin into healthy humans elicits an increase in IGFBP-2 over several hours [27,28]. Furthermore, IGFBP-2 mRNA shows a dramatic increase by 24–48 h following either CNTF or IL-1 beta injection in a rat model of central nervous system injury [29]. The role of IGFBP-2 in the pathogenesis of HUS is still unknown, but IGFBP-2 might have pleiotropic effects through the interactions with other cytokines.

Ang-1 and Ang-2 are important regulators of endothelial cell function and competitive ligands of the endothelial Tie-2 receptor [30]. Ang-1 is produced constitutively and promotes endothelial cell quiescence. Ang-2 is stored in endothelial cells and leads to endothelial activation upon release by noxious or inflammatory stimuli. In healthy states, circulating Ang-1 levels exceed those of Ang-2, and the Ang-2/Ang-1 ratio is low. Recently, dysregulation of Ang-1 and Ang-2 has been reported in EHEC O157:H7 infection and HUS [31]. In patients with EHEC O157-induced HUS, the Ang-2/Ang-1 ratio increases as a result of decreased Ang-1 levels and increased Ang-2 levels. These findings indicate that endothelial dysfunction, manifested as dysregulation of Ang-1 and Ang-2, could underlie HUS pathophysiology and the Ang-2/Ang-1 ratio might be a useful indicator for the severity of HUS. Although we did not evaluate Ang-1 levels, the increased Ang-2 levels in this study support this finding.

IL-6 performs a prominent role during disease and has been described as both a pro-inflammatory and anti-inflammatory cytokine. IL-6 responses are regulated by sIL-6R which forms a ligand–receptor complex with IL-6 that is capable of stimulating a variety of cellular responses including proliferation, differentiation, and activation of inflammatory processes. Increased sIL-6R levels have been documented in several clinical conditions indicating that its production is coordinated as part of a disease response [32–34]. Thus, sIL-6R has the potential to regulate both local and systemic IL-6-mediated events. Previous reports including have shown that serum IL-6 levels are significantly elevated in patients with severe HUS and increase with the development of HUS, consistent with the findings of the present study [8,14,15]. In this study, IL-6 levels were significantly elevated during the HUS phase

in patients with severe HUS compared with those in patients with mild HUS. IL-6 levels tended to increase with the development of HUS in severe cases, although this was not statistically significant because the number of patients in our study is small. On the other hand, sIL-6R levels were significantly increased during the HUS phase in patients with severe HUS compared with those in patients with mild HUS and significantly increased with the development of HUS in severe cases. These findings indicate that IL-6 might play an important role in the pathogenesis of HUS.

The pro-inflammatory cytokine TNF-α also has a prominent role during HUS. For example, TNF-α increases vascular permeability, injures vascular endothelial cells, and induces necrosis of myelin and oligodendrocytes. sTNFR is a shedding form of the extramembranous domain of the TNF receptor that can interfere with the function of TNF-α. It has been reported that sTNFR is the natural homeostatic regulator of the action of TNF-α and that its level, rather than TNF-α levels, reflects the true biological activity of the cytokine. We previously reported that serum sTNFRII levels are significantly elevated in patients with severe HUS and increase with the development of HUS [15]. We also demonstrated that patients with acute encephalopathy showed elevated TNF-α levels during HUS [15]. In the present study, sTNFRII levels significantly increased during the HUS phase in patients with severe HUS compared with those in patients with mild HUS, and they were significantly increased with the development of HUS in severe cases. These findings indicate that TNF-α might play an important role in the pathogenesis of HUS.

MMPs are a group of proteolytic enzymes that can degrade the principal components of the extracellular matrix. On the basis of these degradation activities, MMPs are widely believed to play a central role in tissue degradation. MMPs influence many basic processes, such as cell proliferation, differentiation, angiogenesis, and apoptosis [35]. Notably, MMP family proteins elicit dual roles in the pathogenesis of inflammation, stimulating protective innate and/or adaptive immune functions as well as tissue destruction [36]. MMP-3 overexpression is associated with tissue destruction in the context of chronic inflammation. For example, MMP-3 is involved in extracellular matrix breakdown and tissue remodeling in affected joints of patients with rheumatoid arthritis (RA) and serum MMP-3 levels are widely used in clinics for RA patients as a disease activity marker [37]. In addition to the role of tissue degradation, MMP-3 has a role as a regulator of inflammation through the interaction with inflammatory cytokines [38,39]. The role of MMP-3 in the pathogenesis of HUS is still obscure, but MMP-3 might have pleiotropic effects on the pathogenesis of HUS.

A limitation of the present study was the small number of patients with EHEC O111/HUS. A study with a larger sample including other serotypes of EHEC infection with the correction for multiple comparison, may help define the true diagnostic value of these markers. Despite this limitation, our results indicate that IGFBP-2, Ang-2, sIL-6R, sTNFRII and MMP-3 might play important roles in the pathogenesis of HUS. These cytokines may also be useful to predict the severity of HUS.

Acknowledgements

This work was supported, in part, by the Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics and the Japan Foundation for Pediatric Research. We thank Harumi Matsukawa for technical assistance.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.12.005.

References

[1] Zoja C, Buelli S, Morigi M. Shiga toxin-associated hemolytic uremic syndrome: pathophysiology of endothelial dysfunction. Pediatr Nephrol 2010;25:2231–40.
[2] Scheiring J, Andreoli SP, Zimmerhackl LB. Treatment and outcome of Shigatoxin-associated hemolytic uremic syndrome (HUS). Pediatr Nephrol 2008;23:1749–60.
[3] Matano S, Inamura K, Konishi M, Okumura T, Kawai H, Okamura T, et al. Encephalopathy, disseminated intravascular coagulation, and hemolytic-uremic syndrome after infection with enterohemorrhagic *Escherichia coli* O111. J Infect Chemother 2012;18:558–64.
[4] Piercefield EW, Bradley KK, Coffman RL, Mallonee SM. Hemolytic uremic syndrome after an *Escherichia coli* O111 outbreak. Arch Intern Med 2010;170:1656–63.
[5] Kamioka I, Yoshiya K, Satomura K, Kaito H, Fujita T, Iijima K, et al. Japanese Society for Pediatric Nephrology, Risk factors for developing severe clinical course in HUS patients: a national survey in Japan. Pediatr Int 2008;50:441–6.
[6] Buteau C, Proulx F, Chaibou M, Raymond D, Clermont MJ, Mariscalco MM, et al. Leukocytosis in children with *Escherichia coli* O157:H7 enteritis developing the hemolytic-uremic syndrome. Pediatr Infect J Dis 2000;19:642–7.
[7] Lopez EL, Contrini MM, Devoto S, de Rosa MF, Grana MG, Genero MH, et al. Tumor necrosis factor concentrations in hemolytic uremic syndrome patients and children with bloody diarrhea in Argentina. Pediatr Infect J Dis 1995;14:594–8.
[8] Karpman D, Andreasson A, Thysell H, Kaplan BS, Svanborg C. Cytokines in childhood hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Pediatr Nephrol 1995;9:694–9.
[9] Inward CD, Varagunam M, Adu D, Milford DV, Taylor CM. Cytokines in haemolytic uraemic syndrome associated with verocytotoxin producing *Escherichia coli* infection. Arch Dis Child 1997;77:145–7.
[10] Murata A, Shimazu T, Yamamoto T, Taenaka N, Nagayama K, Honda T, et al. Profiles of circulating inflammatory- and anti-inflammatory cytokines in patients with hemolytic uremic syndrome due *E. coli* O157 infection. Cytokine 1998;10:544–8.
[11] van de Kar NC, Sauerwein RW, Demacker PN, Grau GE, van Hinsbergh VW, Monnens LA. Plasma cytokine levels in hemolytic uremic syndrome. Nephron 1995;71:309–13.
[12] Litalien C, Proulx F, Mariscalco MM, Robitaille P, Turgeon JP, Orrbine E, et al. Circulating inflammatory cytokine levels in hemolytic uremic syndrome. Pediatr Nephrol 1999;13:840–5.
[13] Proulx F, Turgeon JP, Litalien C, Mariscalco MM, Robitaille P, Seidman E. Inflammatory mediators in *Escherichia coli* O157:H7 hemorrhagic colitis and hemolytic-uremic syndrome. Pediatr Infect J Dis 1998;17:899–904.
[14] Shiraishi M, Ichiyama T, Matsushige T, Iwaki T, Iyoda K, Fukuda K, et al. Soluble tumor necrosis factor receptor 1 and tissue inhibitor of metalloproteinase-1 in hemolytic uremic syndrome with encephalopathy. J Neuroimmunol 2008;196:147–52.
[15] Shimizu M, Kuroda M, Sakashita N, Konishi M, Kaneda H, Igarashi N, et al. Cytokine profiles of patients with enterohemorrhagic *Escherichia coli* O111-induced hemolytic-uremic syndrome. Cytokine 2012;60:694–700.
[16] Wulfkuhle JD, Liotta LA, Petricoin EF. Proteomic applications for the early detection of cancer. Nat Rev Cancer 2003;3:267–75.
[17] Elliott EJ, Robins-Browne RM. Hemolytic uremic syndrome. Curr Probl Pediatr Adolesc Health Care 2005;35:305–44.
[18] Gianantonio CA, Vitacco M, Mendilaharzu F, Gallo GE, Sojo ET. The hemolyticuremic syndrome. Nephron 1973;11:174–92.
[19] Russo VC, Gluckman PD, Feldman EL, Werther GA. The insulin-like growth factor system and its pleiotropic functions in brain. Endocr Rev 2005;26:916–43.
[20] Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 2002;23:824–54.
[21] Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 1995;16:3–34.
[22] Binoux M, Lalou C, Zadeh M-S. Biological action of proteolytic fragments of IGF binding proteins. In: Rosenfeld RG, Roberts CTJ, editors. Contemporary endocrinology: the IGF system. Totowa, N.J: Humana Press; 1999. p. 281–313.
[23] Russo VC, Rekaris G, Baker NL, Bach LA, Werther GA. Basic fibroblast growth factor induces proteolysis of secreted and cell membrane-associated insulin-like growth factor binding protein-2 in human neuroblastoma cells. Endocrinology 1999;140:3082–90.
[24] Wheatcroft SB, Kearney MT. IGF-dependent and IGF-independent actions of IGF-binding protein-1 and -2: implications for metabolic homeostasis. Trends Endocrinol Metab 2009;20:153–62.
[25] Azar WJ, Azar SH, Higgins S, Hu JF, Hoffman AR, Newgreen DF, et al. IGFBP-2 enhances VEGF gene promoter activity and consequent promotion of angiogenesis by neuroblastoma cells. Endocrinology 2011;152:3332–42.
[26] Baxter RC. Changes in the IGF±IGFBP axis in critical illness. Best Pract Res Clin Endocrinol Metab 2001;15:421–34.
[27] Benbassat CA, Lazarus DD, Cichy SB, Evans TM, Moldawer LL, Lowry SF, et al. Interleukin-1 alpha (IL-1 alpha) and tumor necrosis factor alpha (TNF alpha) regulate insulin-like growth factor binding protein-1 (IGFBP-1) levels

and mRNA abundance in vivo and in vitro. Horm Metab Res 1999;31: 209–15.
[28] Lang CH, Pollard V, Fan J, Traber LD, Traber DL, Frost RA. Acute alterations in growth hormone–insulin-like growth factor axis in humans injected with endotoxin. Am J Physiol 1997;273:R371–8.
[29] Wood TL, O'Donnell SL, Levison SW. Cytokines regulate IGF binding proteins in the CNS. Prog Growth Factor Res 1995;6:181–7.
[30] van Meurs M, Kumpers P, Ligtenberg JJM, Meertens JHJM, Molema G, Zijlstra JG. Bench-to-bedside review: angiopoietin signaling in critical illness – a future target? Crit Care 2009;13:207.
[31] Page AV, Tarr PI, Watkins SL, Rajwans N, Petruzziello-Pellegrini TN, Marsden PA, et al. Dysregulation of Angiopoietin 1 and 2 in *Escherichia coli* O157:H7 infection and the hemolytic-uremic syndrome. J Infect Dis 2013;208:929–33.
[32] Ivashkiv B. Cytokine expression and cell activation in inflammatory arthritis. Adv Immunol 1996;63:337–76.
[33] Heaney ML, Galde DW. Soluble cytokine receptors. Blood 1996;87:847–57.
[34] Kotake S, Sato K, Kim KJ, Takahashi N, Udagawa N, Nakamura I, et al. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. J Bone Miner Res 1996;11:88–95.
[35] Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2:161–74.
[36] Le NT, Xue M, Castelnoble LA, Jackson CJ. The dual personalities of matrix metalloproteinases in inflammation. Front Biosci 2007;12:1475–87.
[37] Kobayashi A, Naito S, Enomoto H, Shiomoi T, Kimura T, Obata K. Serum levels of matrix metalloproteinase 3 (stromelysin 1) for monitoring synovitis in rheumatoid arthritis. Arch Pathol Lab Med 2007;131:563–70.
[38] Jones GC, Riley GP, Buttle DJ. The role of proteases in pathologies of the synovial joint. Int J Biochem Cell Biol 2008;40:1199–218.
[39] Eba H, Murasawa Y, Iohara K, Isogai Z, Nakamura H, Nakamura H, et al. The anti-inflammatory effects of matrix metalloproteinase-3 on irreversible pulpitis of mature erupted teeth. PLoS One 2012;7:e52523.