Original Article

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IL-10-Producing Potential Treg Precursor in Placenta

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Summary: The maternal immune system needs to be tolerant of allogeneic fetal tissue for reproductive success. The regulatory immune cell network plays an essential role in maintaining maternal tolerance to the fetus. We herein demonstrate in a green fluorescent protein (GFP)/IL-10 reporter mouse system that unique IL-10-expressing cells exist presumably in chorionic villi within the placenta. Flow cytometric analysis revealed that these IL-10-expressing cells exhibit a unique CD19 negative, CD3 negative, and B220 positive phenotype. Interestingly, these cells were enriched during *in vitro* culture, but well-known stimuli for T cells and B cells failed to enhance their growth, suggesting that the $CD19^-$ $CD3^-$ $B220^+$ cells were self renewing. Unexpectedly, in an adoptive cell transfer experiment, IL-10 production was detected in Sca-1^+ $CD4^+$ $CD25^+$ regulatory T cells (Treg). To our knowledge, this is the first report to identify IL-10-producing $CD19^-$ $CD3^-$ $B220^+$ cells in the fetus. These cells may represent a potential progenitor of Sca-1^+ Treg or pluripotent precursor cells for immune tolerance.

Key words placenta, immune tolerance, Breg, Treg, hematopoietic stem cell

INTRODUCTION

The immune system is tightly controlled to eliminate pathogenic antigens as well as to maintain tolerance to non-pathogenic self-antigens. Failure to maintain tolerance leads to exaggerated immune responses, eliciting harmful effects. Immune tolerance is mainly mediated by interleukin-10 (IL-10), which is an immune-suppressive and anti-inflammatory cytokine produced by many kinds of immune cells including both adaptive and innate immune cells [1]. A well-known IL-10-producing cell subset is regulatory T cells (Treg). These are induced by the transcription factor Foxp3 and are characterized by high expression of CD25 [2]. Another IL-10-producing cell subset is regulatory B cells (Breg), which develop depending on micro environmental factors such as inflammation [3].

During pregnancy the mother's immune system should be tolerant of paternal alloantigens derived from her fetus to prevent harmful immune responses. Indeed, the failure of the induction of immunological tolerance against fetal alloantigens results in placental inflammation, recurrent miscarriage, and pregnancy complications such as preeclampsia [4]. Treg has been demonstrated to maintain the maternal immune tolerance to fetus alloantigens [5]. In addition, several studies proposed that Breg, which is enriched in the umbilical cord blood [6], also plays an active role in maternal immune tolerance [7].

Although immunosuppressive drugs are necessary to avoid allograft rejection, very few patients are able to induce spontaneous operational tolerance to the alloantigens in organ transplantation [8]. The tolerant recipients who maintain normal graft function without immunosuppressive drugs are characterized by presence of three genes (IGKV4-1, IGLLA, and IGKV1D-13) in the peripheral blood [9]. Interestingly, these genes are expressed by pre-B cells, suggesting

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Abbreviations: BAFF, B cell activation factor; GFP, green fluorescent protein; LIF, leukemia inhibitory factor; Sca-1, Stem cell antigen-1; TLR, Toll-like receptor; TNF, tumor necrosis factor.

the involvement of pre-B cells in the tolerance to alloantigens.

In this report we utilized IL-10 reporter mice to identify a novel immune cell population in mouse placenta that is capable of producing IL-10 spontaneously (without *in vitro* stimulation). Although these IL-10-producing fetal cells are characterized by the expression of B cell marker B220, they can differentiate into $Sca1^+$ Treg cells in immune compromised recipients. These findings indicate the presence of a unique Treg precursor population in the mouse fetus.

METHODS

Mice: GFP/IL-10 reporter mice (C57BL/6 background) were obtained from Jackson Laboratory. NOD-scid IL2rγ^{null} (NSG) mice were obtained from Charles River. All mice were maintained under specific pathogen-free conditions at Kurume University. All experiments were approved by the Board for Animal Experiments of the Kurume University Animal Centre (Kurume, Japan).

Isolation and stimulation of placental cells: After euthanizing mice by inhalation of isoflurane, we peeled away the endometrial tissue, isolated the embryo conceptus, and separated the yolk sac from the placenta. Single-cell suspensions from placenta were prepared by mechanical dissociation using forceps. The cells were resuspended in 10ml RPMI and collected by centrifugation at 1200 rpm for 5 minutes. Red blood cells were lysed in ammonium-chloride-potassium bicarbonate (ACK) lysing buffer for 5 minutes at room temperature, and the reaction was stopped by the addition of the same volume of RPMI. The cells were collected by centrifugation at 1200 rpm for 5 minutes, and the single cell suspensions were filtered through 50μm filters and subjected to analysis.

Cell stimulation: Cells were incubated for 12 or 24hr at 37˚C and 5% CO2 in F12/DMEM medium containing 25mM HEPES (Nacalai Tesque, Tokyo, Japan), 3.2mM L-glutamine (Gibco, Burlington, Canada), Antibiotic-Antimycotics (Nacalai Tesque), 1% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO), 1X SITE3 (Sigma-Aldrich), 10mmol/l sodium pyruvate (Sigma-Aldrich), and 50μM 2-mercaptoethanol (Gibco). In some experiments, cells were stimulated with 50ng/ml B cell activating factor (BAFF) (R&D systems), IL-4 (10ng/ml) (PEPROTECH, Rocky Hill, NJ), IL-33 (10ng/ml, PEPROTECH), Calcipotriol hydrate (100nM, Sigma-Aldrich), 1μM CpG ODN 2006 (InvivoGen, San Diego, CA), LPS (50ng/ml, InvivoGen), IL-2 (50ng/ml, PEPROTECH), IL-7 (10ng/ml, PEPROTECH), Leptin (20ng/ml, PEPROTECH) or LIF (5ng/ml, PEPROTECH), either singly or in combination.

Cell transfer: For reconstitution of placental-derived cells, cells were extracted from placentas of 14-18 pooled GFP/IL-10 reporter mice, after which 2.3 - 5.5 $\times$ 10^7 cells were intraperitoneally injected into 4-month old NSG mice. The cell injections were repeated twice, at Days 1 and 16. The recipient mice were sacrificed at Day 33.

Immunohistological staining: The placentas of GFP/IL-10 mice were placed in OCT compound, frozen on dry ice, and stored at -80˚C. Frozen sections (4-μm thick) were air-dried for 2hrs, fixed in acetone for 10 min, air dried again, and stained by the avidin-biotin complex method as described previously [10]. Antibodies used were rat anti-mouse CD4 (clone: RM4-5) (BD Biosciences, San Jose, CA), rat anti-mouse CD45R/B220 (clone: RA3-6B2) (BD Biosciences) at 1: 50 followed by biotinylated rabbit anti-rat Ig (Vector) at 1: 200 dilution and a 1: 100 dilution of avidin-biotinylated peroxidase complex (Vector, Burlingame, CA). Each incubation was followed by three washes in phosphate-buffered saline. Endogenous peroxidase activity was blocked by a 30 min incubation in 0.3% hydrogen peroxide in phosphate-buffered saline. The tissue sections were stained by incubation in a solution of 3-amino-9-ethylcarbazole (Aldrich, Milwaukee, Wisconsin), counterstained with hematoxylin, and mounted in Glycerogel (Dako, Wiesentheid, Germany).

Immunofluorescence staining: The primary monoclonal antibodies used were rabbit anti-GFP antibody (Invitrogen, Carlsbad, CA) and rat anti-mouse CD45R/B220. Secondary antibodies used were CF594-rabbit anti-rat IgG (Biotium, Fremont, CA) at 1: 200 dilution. For nuclear staining, specimens were counterstained with DAPI (BioRad, Herbules, CA) at 1: 100 dilution. The stained tissues were analyzed by confocal microscope.

Flow cytometry staining and analysis: After washing, cells were pre-incubated in blocking buffer containing 10% normal rat and hamster serum (Jackson ImmunoResearch Laboratories) and unconjugated CD16/CD32 (clone 93; Biolegend, San Diego, CA). Cells were then subjected to surface staining with a cocktail

mixture of PerCP/Cy5.5-anti-CD19 (ID3), PE/Cy7-anti-CD3 ϵ (145-2C11) and APC-anti-B220 (RA3-6B2). In addition, a combination of PE-anti-CD19 (ID3) and APC-anti-CD335 (29A1.4) were used. After washing, the cells were immediately subjected to flow cytometric analysis. These antibodies were purchased from Invitrogen, Biolegend and eBioscience.

RESULTS

Spontaneous IL-10 production by the labyrinth B220$^+$ cells: We used a green fluorescent protein (GFP) / IL-10 reporter mouse system, which can express GFP when IL-10 is produced, to visualize spontaneous IL-10 production in the placenta. Interestingly, cells with strong GFP expression were detected in relatively large ducts in the chorionic plate, which we considered to represent umbilical veins (Figure 1A). To identify the cells present in the placenta, immunohistochemical analysis was performed after staining with anti-B220 (B cell marker) Abs. There were relatively large numbers of B220$^+$ cells, presumably in the chorionic villi, which contain fetal blood (Figure 1B, C). To further confirm whether the B220$^+$ cells were the cellular source of IL-10 in the placenta, immunofluorescence analysis was carried out. Of note, cells co-expressing GFP and B220 were observed in the labyrinth zone (Figure 2). These findings suggest the spontaneous production of IL-10 by B220$^+$ cells, presumably in the fetal vessels within the labyrinth zone.

Unique CD19$^-$ B220$^+$ cells produce IL-10 in the placenta: To further characterize the surface phenotype of the IL-10-producing B220$^+$ cells, cells were isolated from the placenta and subjected to flow cytometric analysis. Consistent with the data obtained from tissue analysis, the cells isolated from placenta spontaneously produced IL-10 without *in vitro* stimulation. Some GFP$^+$ cells expressed very low levels of IgM and high levels of B220 (Figure 3A, B). Since strong autofluorescent signals, which may be caused by adherent cells such as mesenchymal cells, were detected, adherent cells were removed by culturing cells on plastic plates. Although this approach was unable to remove the cells with autofluorescence, we unexpectedly found that the *in vitro* culture enriched the lymphocyte population. As shown in the cytogram of Figure 3C and 3D, the lymphocyte population was hard to recognize in freshly isolated cells, but was clearly detectable after *in vitro* culture. Flow cytometric analysis showed strong autofluorescence, particularly with PerCP/Cy5.5-anti-CD19 staining, and IL-10 production was detected in PerCP/Cy5.5-CD19-negative cell population (Figure 3E). Therefore, to minimize the influence of autofluorescent signals, we pre-gated a negative cell population after staining with PerCP/Cy5.5-anti-CD19. Interestingly, the CD19-negative cell population contained a large number of GFP-expressing cells. Further analysis confirmed that, among the CD19-negative cell population, IL-10 was produced predominantly by B220$^+$ cells, with additional production by some CD3$^+$ cells (Figure 3F, G). These findings suggest that a unique CD19$^-$ B220$^+$ cell population, derived from the fetus, spontaneously produces IL-10 in the placenta.

No effect of B cell-associated stimuli on placental CD19$^-$ B220$^+$ cells: To induce the expansion of CD19$^-$ B220$^+$ cells or enhance their expression of IL-10 *in vitro*, cells isolated from placenta were stimulated with either or a combination of the following cytokines and bacterial products. B cell activation factor (BAFF), which plays a role in B cell maturation and function, has previously been reported to induce IL-10 production from B cells [11]. IL-4 has been shown to induce the pre-B cells maturation process [12]. IL-33 has been reported to induce the expansion of Bregs with IL-10 production [13]. Toll-like receptor (TLR) 4 and TLR9 ligands and calcitriol (used as a vitamin D analog) have been demonstrated to induce IL-10 production [14,15]. However, none of these stimuli were able to expand CD19$^-$ B220$^+$ cells or enhance their expression of IL-10 (data not shown).

Since a strong association of immature B cell gene signatures (IGKV4-1, IGLLA, and IGKV1D-13) with immune tolerance to alloantigens has previously been reported [9], DNA microarray analysis was carried out before versus after in vitro culture of placental cells. However, there was no difference in the expression of these genes.

No effect of T cell-related stimuli on placental CD19$^-$ B220$^+$ cells: We next used single or combinations of cytokines that have an effect on T cells or are produced in the placenta. These cytokines included IL-2, which is well-known to induce the survival of Treg [16], IL-7, which is essential for T- and B cell development [17], leukemia inhibitory factor (LIF), which is produced in the placenta for maintaining healthy pregnancy [18], and leptin, which is secreted from placental trophoblasts to induce the expression of IL-6, IL-10, and TNF-α [19,20]. Although multiple combinations of these cytokines were used, none of them induced the expansion of CD19$^-$ B220$^+$ cells or en-

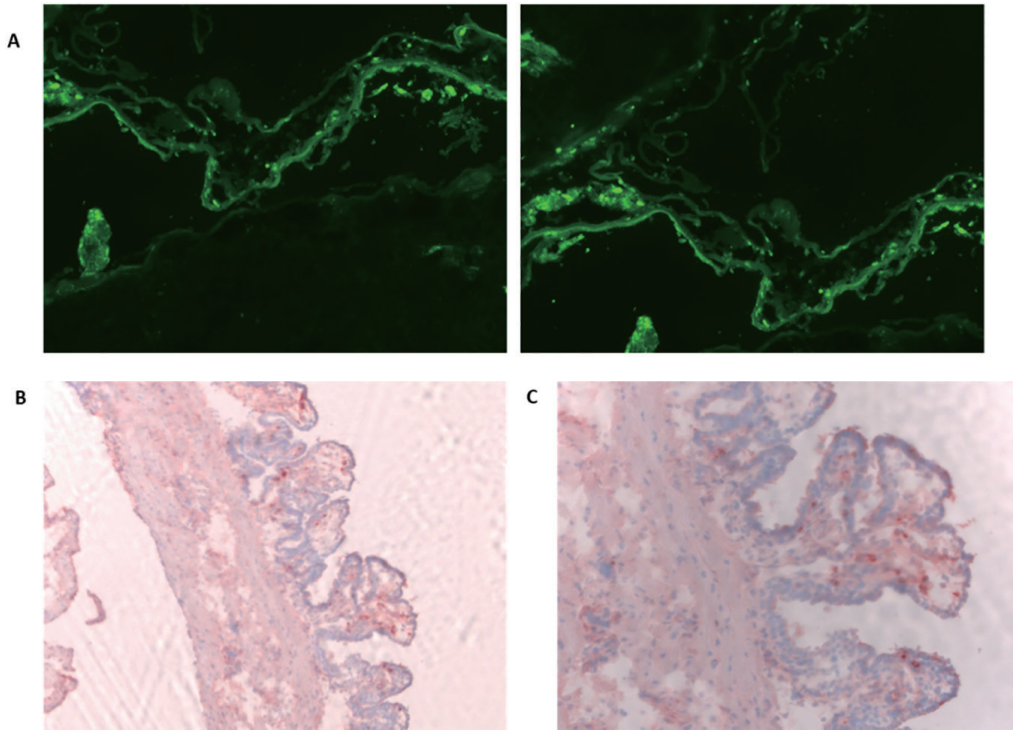

Fig. 1. IL-10-producing cells in the placenta
(A) Confocal fluorescence microscopy of placenta from GFP/IL-10 mouse showed GFP-expressing cells at the fetal-maternal interface, particularly on the surface of the labyrinth zone (objective ×200).
(B) (C) Immunohistological analysis showed B220 (+) cells in the labyrinth region (B: 100×, C: 200×).

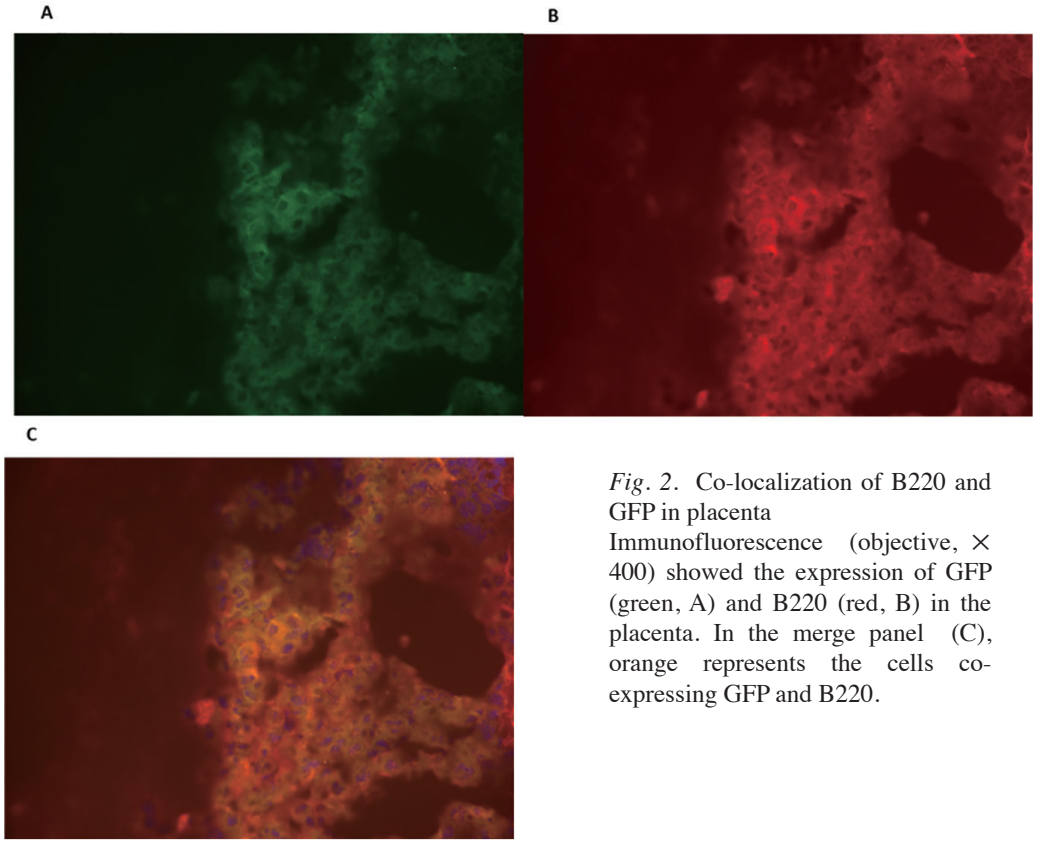

Fig. 2. Co-localization of B220 and GFP in placenta
Immunofluorescence (objective, × 400) showed the expression of GFP (green, A) and B220 (red, B) in the placenta. In the merge panel (C), orange represents the cells co-expressing GFP and B220.

hanced their expression of IL-10 (data not shown). These findings raise the possibility that the fetus-derived IL-10 producing cells in the placenta possess an ability of self-renewal similar to that of hematopoietic stem cells.

Differentiation of placental IL-10-producing cells to Sca1$^+$ Treg: Since the placenta has been identified as a major hematopoietic organ [21], we next employed a cell transfer system to identify the cell type that is differentiated or maturated from the placental IL-10 producing cells. Since the CD19$^-$ B220$^+$ cell population was enriched during *in vitro* culture without stimulation, we initially used the cultured cells from the placenta of GFP/IL-10 reporter mice. The cultured cells were intraperitoneally transferred into NSG mice lacking T cells, B cells, and NK cells. The cell transfers were carried out twice and the recipient mouse was euthanized 5 weeks after the second cell transfer. Interestingly, GFP-expressing cells were clearly detectable in the spleen and peritoneal cavity in the recipient mice. To test whether the *in vitro* culture somehow allows CD19$^-$ B220$^+$ cells to reconstitute in the recipient, we next used freshly isolated cells from placenta for the cell transfer experiment. However, no difference in the reconstitution pattern was seen when freshly isolated versus cultured cells were used for cell transfer. Interestingly, the main producer of IL-10 in the recipient spleen and peritoneal cavity expressed CD3 (freshly isolated cell transfer data were shown in Figure 4). In contrast, reconstitution of CD19$^+$ B cells was seen only in the recipient peritoneal cavity but not

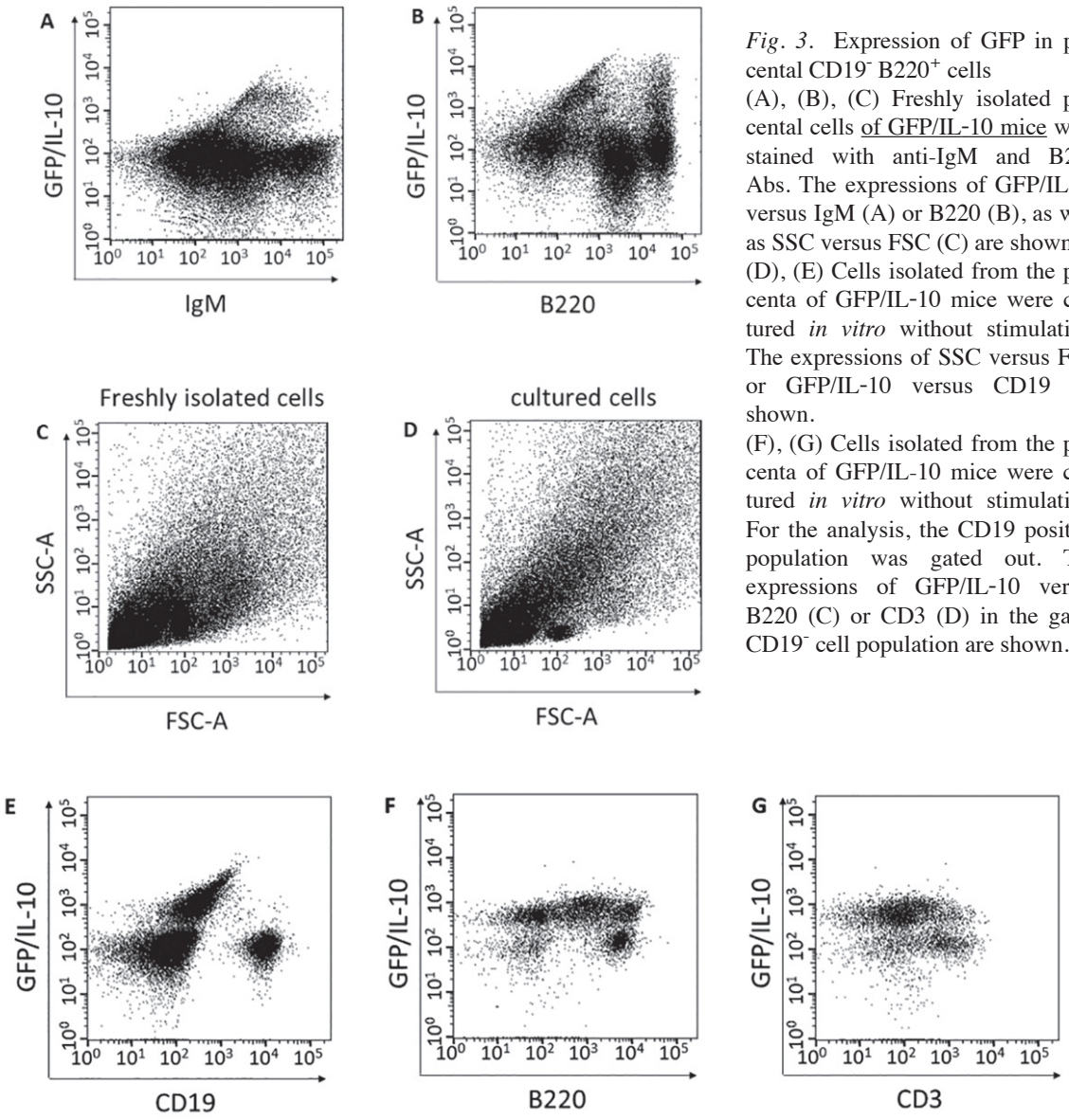

Fig. 3. Expression of GFP in placental CD19$^-$ B220$^+$ cells
(A), (B), (C) Freshly isolated placental cells of GFP/IL-10 mice were stained with anti-IgM and B220 Abs. The expressions of GFP/IL-10 versus IgM (A) or B220 (B), as well as SSC versus FSC (C) are shown.
(D), (E) Cells isolated from the placenta of GFP/IL-10 mice were cultured *in vitro* without stimulation. The expressions of SSC versus FSC or GFP/IL-10 versus CD19 are shown.
(F), (G) Cells isolated from the placenta of GFP/IL-10 mice were cultured *in vitro* without stimulation. For the analysis, the CD19 positive population was gated out. The expressions of GFP/IL-10 versus B220 (C) or CD3 (D) in the gated CD19$^-$ cell population are shown.

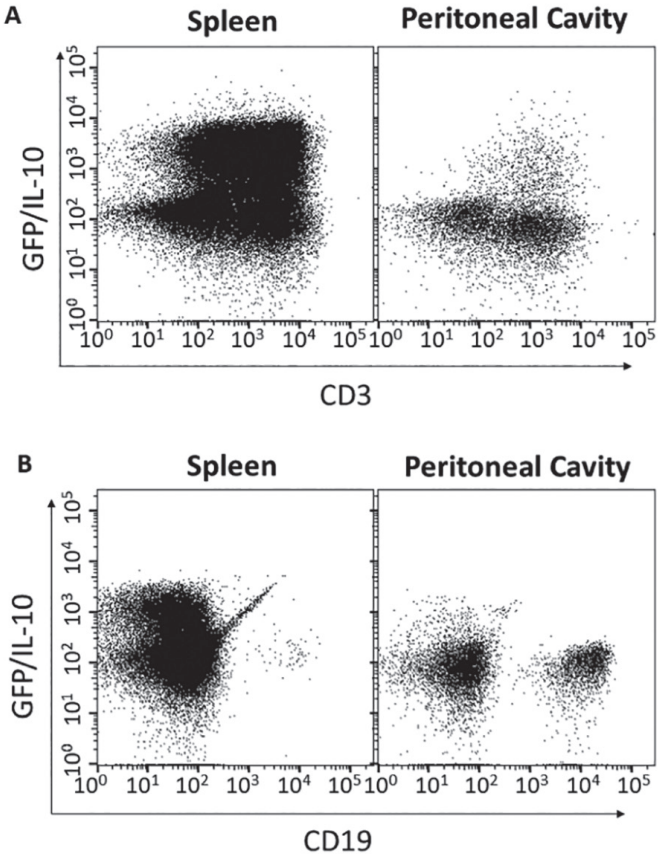

Fig. 4. IL-10-producing T cells in recipient mice after transfer of placental cells
Freshly isolated placental cells from GFP/IL-10 reporter mice were transferred twice into NSG mice. The expression patterns of GFP versus CD3 (A) and GFP versus CD19 (B) in the reconstituted cells from recipient spleen and peritoneal cavity are shown.

spleen, and IL-10 production as judged by GFP expression was undetectable in the reconstituted $CD19^+$ B cells (data not shown).

Another interesting finding was that IL-10-producing T cells in the recipient expressed CD4 and CD25, suggesting that Treg is derived from placental cells (Figure 5). In addition, the reconstituted $IL\text{-}10^+$ $CD4^+$ $CD25^+$ $CD3^+$ T cells also expressed Sca-1 (Stem cell antigen-1), a marker of hematopoietic cells (Figure 5). Since Sca-1 expression on Treg has not been reported previously, we next examined the Treg in native mice to see whether $Sca\text{-}1^+$ Treg is unique to the placenta-derived Treg or not. Sca-1 expression was detectable in some Treg in the spleen of GFP/IL-10 mice (data not shown). These findings suggest that some Treg is derived from the IL-10-producing cells originating from fetal blood in the placenta.

DISCUSSION

In this report, we demonstrate the presence of a novel $CD19^-$ $B220^+$ cell population capable of spontaneously producing IL-10, presumably in the chorionic villi of the placental labyrinth zone. Interestingly, the $CD19^-$ $B220^+$ cell population did not respond to BAFF, IL-4, IL-33, IL-2, IL-7, LIF, leptin, calcitriol, TLR4L or TLR9L, all of which are well-known stimuli for B cells and T cells. In contrast, the $CD19^-B220^+$ cell population was enriched during *in vitro* culture without stimulation. These findings suggest that the $CD19^-$ $B220^+$ cell population possess an ability of

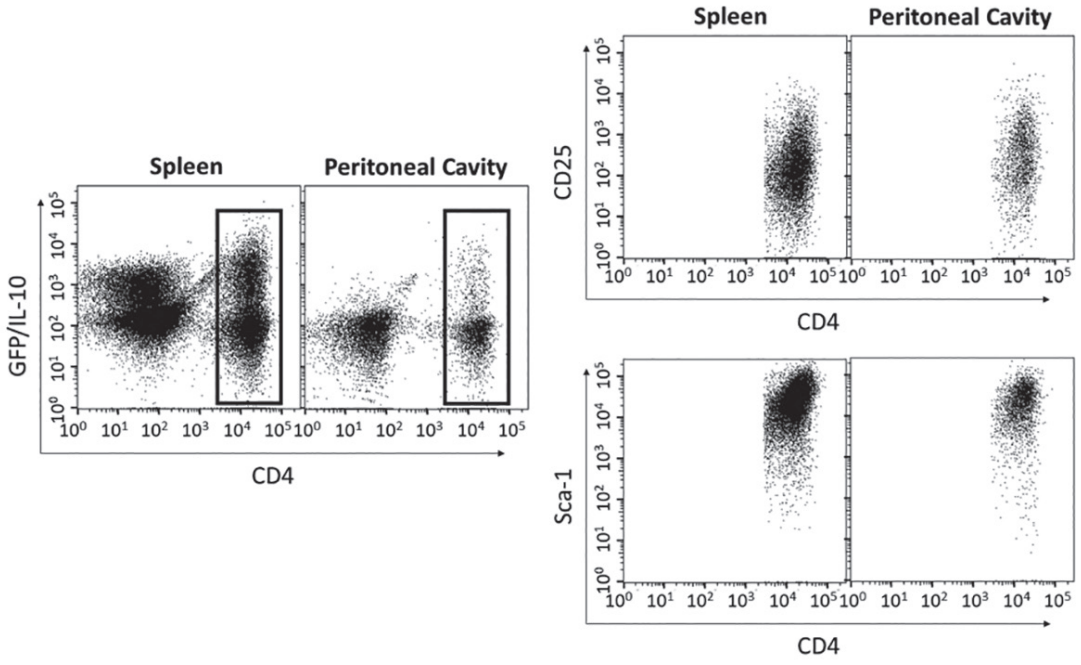

Fig. 5. Reconstituted CD4+ CD25+ Sca-1+ cells expressed IL-10 in the recipient
Among the reconstituted cells in the recipient spleen and peritoneal cavity after transfer of placental cells, $CD4^+$ cells expressed GFP. After gating the CD4 positive population, the majority of cells expressed CD25 (upper panels) and Sca-1 (lower panels)

self-renewal, which is a well-known characteristic of hematopoietic stem cells. Indeed, the placenta has been reported to contain hematopoietic stem cells in the labyrinth region for the development of the hematopoietic system [22]. Alternatively, placenta contains multiple soluble factors, and cells isolated from the entire placenta were used for our culture system. Therefore, it is also possible that certain placenta-derived soluble factors enhanced the growth of $CD19^-$ $B220^+$ cells during the culture. Future studies to neutralize potential placenta-derived factors would be necessary to address this issue.

The existence of Breg in the placenta has been reported [6,7], and the IL-10-producing cells we identified in the labyrinth region of placenta, although CD19 was negative, expressed B cell marker B220. Therefore, we initially hypothesized that the $CD19^-$ $B220^+$ cells represent a neonatal type of Breg. However, cell transfer experiments revealed that placental cells containing fetal blood can differentiate into Sca-1^+ $CD3^+$ $CD4^+$ $CD25^+$ IL-10^+ Treg rather than Breg in the recipient. Therefore, it is possible that fetal-derived IL-10-producing $CD19^-$ $B220^+$ cells represent a precursor of Treg, particularly Sca-1^+ Treg. Since cells isolated from the entire placenta were used for the cell reconstitution experiments, both maternal and fetal components may be potential sources of reconstituted cells. However, differentiation of $CD19^-$ $B220^+$ cells into Sca-1^+ $CD3^+$ $CD4^+$ $CD25^+$ IL-10^+ Tregs does not occur in a physiological setting. Therefore, it is unlikely that the reconstituted cells originate from maternal-derived placental cells, suggesting that fetal components in the placenta contain a precursor with the potential to differentiate into Tregs. Indeed, previous reports suggested $CD19^-$ $B220^+$ cells as pluripotent precursor cells in fetal liver [23]. Future studies designed to transfer purified GFP^+ $CD19^-$ $B220^+$ cells from the fetus versus mother into different genetically engineered recipients would be helpful to clarify these possibilities.

There are at least two types of Treg, one is thymic derived Treg (tTreg) and the other is extrathymically differentiated Treg, which is known as peripherally induced Treg (pTreg). Our data suggest that Sca-1^+ Treg can be differentiated in the recipient from $CD19^-$ $B220^+$ cells. Since B220 has been considered to represent a marker of extrathymically differentiated T cells [24], Sca-1 expression may reflect such extrathymically differentiated Treg. Indeed, Samstein et al. previously demonstrated that extrathymically differentiated Treg plays an important role in maternal-fetal immune tolerance [25]. In addition, Alijotas-Reig et al. reported that only pTreg, which previously exposed paternal alloantigens, are essential to create maternal-fetal tolerance [26].

In summary, this study identified a unique $CD19^-$ $B220^+$ cell population, located presumably in chorionic villi within the placenta, which is capable of spontaneously producing IL-10. The $CD19^-$ $B220^+$ population may have the ability to self-renew, and may differentiate into Sca-1^+ extrathymic derived Treg. Future studies of the fetal placental immune system may help lead to the development of new therapeutic and preventive strategies capable of inducing immune tolerance.

COI: The authors declare no conflicts of interest.

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