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Immune tolerance induced by the inhibition of CD18 alone or both signal transducer and activator of transcription 3 (Stat3) and islet/duodenum homeobox-1 (IDX-1) at the time of rat bone marrow (BM) transplantation in which thymus T lymphopoiesis occurred post-transplantation

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Ανοσιακή ανοχή προκαλούμενη από την αναστολή μόνο του CD18 ή και του Stat3 και του IDX-1 κατά τη μεταμόσχευση μυελού οστών όπου εμφανίζεται λεμφοποίηση Τ-λεμφοκυττάρων του θύμου μετά από τη μεταμόσχευση

Περίληψη στο τέλος του άρθου

OBJECTIVE **To elucidate the mechanism of immune tolerance and demonstrate its occurrence clinically and *in vitro*.** METHOD **Rat bone marrow (BM) transplantation was performed using F_1 (DA×LEW) hybrid hosts and DA donors, which were followed for 127–157 days post-transplantation. BM cells were injected together with both signal transducer and activator of transcription 3 (Stat3) and islet/duodenum homeobox-1 (IDX-1) antibodies (Abs) or CD18 Ab alone into the host spleen onto which a piece of donor spleen was transplanted.** RESULTS **When DA BM cells were injected with Stat3 and IDX-1 Abs or CD18 Ab, chronic graft versus host disease (GvHD) symptoms, such as body weight loss, thymus atrophy, intestinal disturbances and alopecia, were not displayed. The F_1 females without clinical GvHD showed increased numbers of megakaryocytes in their BM, no extramedullary hematopoiesis, and flow cytometer (FCM) activation of Stat3 in the BM and thymus, and CD11b, CD18 and IDX-1 in the host and graft spleens. The correlation coefficient between the Stat3$^+$ thymus cells and DIX-1$^+$ spleen cells of the females without GvHD signs was 0.58, while that of the control females was 0.4. Immuno-chemical electron micrographs showed mitochondrial IDX-1 proteins specifically, as well as Stat3 in the females with immune tolerance.** CONCLUSIONS **Immune tolerance was induced by the inhibition of Stat3 and IDX-1, which has a negative interaction with Stat3, at the time of BM transplantation. Post-transplant CD18 rebound stimulated thymus T cell proliferation. Spleen production of insulin-like growth factor 1 (IGF-1) must promote immune tolerance without clinical GvHD symptoms.**

Key words

Bone marrow transplantation
CD18
Chronic graft versus host disease
Immune tolerance
Spleen graft
Thymus

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Animal experimental systems of bone marrow (BM) transplantation have been used to explore possible preventive measures for graft versus host disease (GvHD). Host thymectomy prevented acute and chronic GvHD, but induced graft rejection when donor BM cells were injected into the peripheral veins of rats.[1] Donor BM cell injection into the host spleen onto which a donor spleen graft was also transplanted was found to be a good experimental design for preventing not only GvHD, but also graft rejection.[2] It has been shown that immature rat BM cells have the potential to develop into pancreatic β-cells expressing islet/duodenum homeobox-1 (IDX-1) protein.[3] When IDX-1 protein is inhibited in immature BM cells, they may be able to differentiate into other cell types with immune tolerance. Many aspects of immune tolerance, however, remain unknown.

In humans, BM transplantation therapy is selected not only for BM cell malignancies, but also for congenital myeloid cell anomalies. Leukocyte adhesion deficiency type 1 (LAD-1) is characterized clinically by impaired migration and adhesion of neutrophils. LAD-1 is caused by β_2-integrin CD18 mutations. Patients with this problem lack, or have

markedly reduced expression of CD11c/CD18 (LFA-1), CD11b/CD18 (MAC-1, CR3), and CD11c/CD18 (p150, 95).

In 4 patients with LDA-1, unrelated BM transplantation resulted in a successful recovery,[4,5] but 3 of the 4 transplants were complicated by grade 1 to 2 acute GvHD.

In this study using rats, 5 experimental groups of F_1 (DA×LEW) hybrid hosts were used to investigate chronic GvHD-free systems using DA donor cells, and to elucidate the mechanism behind immune tolerance through clinical and laboratory tests. When either CD18 antibody (Ab) alone or both signal transducer and activator of transcription 3 (Stat3) Ab and IDX-1 Ab were injected together with DA BM cells into the host spleen onto which a segment of donor spleen was grafted at the time of BM transplantation, chronic GvHD was prevented in all the rats. Immune tolerance was induced with high levels of mitochondrial Stat3 and IDX-1, which has a negative interaction with Stat3. High levels of CD18 were detected post-transplantation, which resulted in host thymus T lymphopoiesis. However, IDX-1 Ab combined with CD18 Ab induced chronic GvHD with intestinal perforation, and Stat3 Ab alone also induced chronic GvHD with thymus atrophy. A piece of BM transplantation into the host pankreas induced cutaneous GvHD.

MATERIAL AND METHOD

Animals

DA and Lewis (LEW/SsN) rats were purchased from Japan SLC Co, Ltd (Hamamatsu, Japan). F_1 (DA×LEW) hybrid rats were bred and maintained in the animal center of Hamamatsu University School of Medicine, and were used as hosts in this study. All experiments were started when the F_1 hybrid rats were 8 weeks old. Maternal DA females became the donors in this study.

Experimental designs

The F_1 hybrid rats were classified into 5 experimental (Exp) groups: Exp A comprised 4 females which had a piece of DA bone marrow (BM) transplanted into their pancreas and a piece of donor spleen attached onto their spleens, all which were obtained from a DA female rat. The DA BM section was approximately half as long as the upper (femur) or lower (tibia) leg, and the DA spleen section was equivalent to a quarter of the spleen. Exp B comprised 4 females which received 3.5×10^7 DA BM cells per rat mixed with 2 µL (0.5 µg) of signal transducer and activator of transcription 3 (Stat3) antibody (Ab) (BD Biosciences, Tokyo, Japan) per rat into their spleens, to each of which was attached a quarter of the donor DA spleen. Exp C comprised 4 Exp C males, each of which had a quarter of the donor DA spleen attached to the host spleen and received 3.1×10^7 DA BM cells per rat mixed with 0.75 µL (0.75 µg) anti CD18 Ab, per rat (Serotec, Oxford, DX5 1GE, UK) and 1.5 µL rabbit anti islet/duodenum homeobox-1 (IDX-1), which is PDX-1/SFT1/IPF1, polyclonal Ab per rat (CHEMICON international, Inc, Temecula, CA, USA). Exp D comprised 4 female rats which received 2.6×10^7 DA BM cells per rat mixed with 2 µL anti Stat3 Ab per rat (BD Biosciences) and 1.5 µL anti IDX-1 Ab per rat (CHEMICON International, Inc) into their spleens, to each of which was attached a quarter of the donor DA spleen. Exp E consisted of 5 females which received 2.9×10^7 DA BM cells per rat mixed with 0.76 µL anti CD18 Ab per rat (Serotec) into their spleens, to which 1/5 of the donor DA spleen had been attached. Exp A, Exp B, Exp C, Exp D, and Exp E rats were followed for 159, 164, 107, 127, and 157 days post-transplantation, respectively. Clinical symptoms were monitored until the time of sacrifice in all the F_1 hybrid rats.

Flow cytometer (FCM) analyses

One million cells separated from the BM, host spleen, graft spleen, and thymus were assessed using an EPICSR XL-MCL system III FCM (Beckman Coulter, Fullerton, CA, USA) after being stained with Abs labeled with fluorescein. The BM cells were stained with anti Stat3 Ab (BD Biosciences) for 30 minutes at 4 °C and then with monoclonal mouse IgG_1 fluorescein isothiocyanate (FITC) (Ancell Co, Bayport, MN, USA) for 15 minutes at 4 °C. The BM cells were also stained with R-phycoerythrin (R-PE)-conjugated mouse anti-rat CD90 (Thy-1)/mouse CD90.1 (Thy-1.1) monoclonal antibody (mAb) for 30 minutes at 4 °C (BD Biosciences, Tokyo, Japan). The host and donor spleen cells were stained with CD11b/FITC (AbD serotec, Oxford, OX5 1GE, UK) for 30 minutes at 4 °C, and also with anti Stat3 Ab (BD Biosciences) and anti IDX-1 Ab (CHEMICON International, Inc) for 30 minutes at 4 °C, respectively. They were then doubly stained with monoclonal mouse IgG_1/FITC (Ancell Co) for 15 minutes at 4 °C. The thymus cells were stained doubly with anti Stat3 Ab (BD Biosciences) and IgG_1/FITC (Ancell Co) in the same manner as described above. For the determination of Stat3 and IDX-1 Abs, the threshold between the positive and negative peaks was judged from the data of a peak not stained with fluorescein, because their peaks could not be separated. The positive cell percentage in all rats was decided mainly from fluorescein intensity. Statistical analyses were performed using EXCEL functions.

Histopathological findings

The intestine (ileum), BM, host spleen, and graft spleen were fixed in 20% formalin and stained with hematoxylin-eosin (H-E) for light microscopic analyses. The number of megakaryocytes in the BM sections was counted on a ×40 field of a light microscope. A piece of donor BM from Exp A-2 survived for 159 days in the host pancreas. The donor BM of Exp A-2, the ileum of Exp C-3, which was cut at 1 cm above the ileocecum, and the donor spleens of Exp D-1 and D-4, which survived well for 127 days post-transplantation, were fixed in 2% glutaraldehyde for 2 hours and then post-fixed with 1% osmium tetroxide. Apart from the organs stated above, the cell suspensions stained with Abs before

fixing were also prepared for electron micrograph analyses. The donor BM cells of Exp A-1 were stained with anti Stat3 Ab (BD Biosciences) for 15 minutes at 4 °C and subsequently with sheep and mouse IgG_1 conjugated with 15 nm-gold colloidal particles (EY Laboratories, Inc, San Mateo, CA, USA) for 10 minutes at 4 °C. The host spleen cells of Exp C-3 were stained with anti CD18 Ab (Serotec) for 15 minutes at 4 °C and then with IgG_1 conjugated with gold colloidal particles (EY Laboratories, Inc) for 10 minutes at 4 °C. The graft spleen cells of Exp B-1 and Exp E-1 were stained with anti IDX-1 Ab (CHEMICON International, Inc) for 15 minutes at 4 °C and then with IgG_1 conjugated with gold colloidal particles (EY Laboratories, Inc) for 10 minutes at 4 °C. All the cell suspensions stained with Abs were fixed in 2% glutaraldehyde and post-fixed with 1% osmium tetroxide for the electron micrograph analyses. All fixed and sectioned materials were doubly stained with uranyl acetate-lead citrate and were observed using a JEM 1220 transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS

Table 1 shows the frequency of chronic GvHD symptoms found in the experimental groups of F_1 (DA×Lewis) hybrid rats. All Exp A females, which had DA BM transplanted into their pancreas, had skin alopecia (100%), but none of them showed thymus atrophy (0%) (cutaneous GvHD). The mean thymus weight of the Exp A group was 0.25±0.02 g and their mean body weight (BW) was 223±5 g at the time of sacrifice, while the 4 control females had a mean thymus weight of 0.24±0.03 g and a mean BW of 215±5 g. Eighty-six days post-transplantation, alopecia was confirmed on the upper side of the right foreleg in an Exp A rat, which had partially recovered at the time of sacrifice (73 days after the first alopecia detection). The Exp B females, which had DA BM cells transplanted into their spleen and were injected with Stat3 Ab, had a higher frequency of thymus atrophy (50%) than of skin alopecia (25%). The mean atrophic thymus weight of the 2 affected Exp B females (50%) was 0.15 g, and their mean BW was 208 g. The Exp C males, which had DA BM cells transplanted into their spleen and were injected with CD18 and IDX-1 Abs, showed the severest chronic GvHD in this study. One of the Exp C males died of ileocecal perforation 63 days post-transplantation. This male showed an approximately 80 g loss of BW and thymus atrophy of less than 0.10 g. The other 3 Exp C males had a mean atrophic thymus weight of 0.18±0.05 g and a mean BW of 351±16 g at the time of sacrifice, while the 6 control males had a mean thymus weight of 0.28±0.02 g and a mean BW of 396±33 g. On histopathological examination, the lower ileum of the Exp C rats showed intestinal villous atrophy and epithelial cell destruction. The Exp D females, which had DA BM cells transplanted into their spleens combined with Stat3 and IDX-1 Abs, and the Exp E females, which had DA BM cells transplanted into their spleens combined with CD18 Ab, showed no clear clinical signs of chronic GvHD for 127 and 157 days post-transplantation, respectively. The Exp D females had a mean thymus weight of 0.26±0.02 g and a mean BW of 212±8 g. The Exp E females had a mean thymus weight of 0.26±0.04 g and a mean BW of 233±4 g. The mean thymus weight of the 2 Exp E rats increased to 0.30 g. Figure 1 is an electron micrograph of the ileum of an Exp C-3 rat cut at 1 cm above the ileocecum, showing intestinal destruction. Crypt stem cell hyperplasia was observed together with degenerated intestinal epithelial cells. As control females showed enlarged lymph node (LN) with destroyed lamina propria (LP), LN findings of lower ileum were not used as a sign of chronic GvHD in the female hosts.

Table 1. Clinical and pathological findings of chronic graft versus host disease (GvHD) expressed using rat number (%).

				Pathological findings in the lower ileum	
Exp group (Sex)	Total rats (n)	Skin alopecia (%)	Thymus atrophy (%)	Enlarged LN with destroyed LP (%)	Crypt hyperplasia with villous atrophy (%)
A (F)	4	100	0	25	0
B (F)	4	25	50	0	0
C (M)*	4	50	100	50	100
D (F)	4	0	0	0	0
E (F)	5	0	0	20	0
Contr (M)	6	0	0	0	0
Contr (F)	4	0	0	50	0

LN: Lymph nodes, LP: Lamina propria, F: Female, M: Male; * One of the Exp C rats died of ileocecal perforation

Table 2 shows the results of the BM studies in the F_1 hybrid hosts. From the BM megakaryocyte number and BM FCM analyses using Thy-1 and Stat3 Abs, chronic GvHD phase was evaluated. The length of the post-transplantation follow-up period was taken into account when considering the phase. The Exp C males, which had the severest chronic GvHD, showed a decrease in megakaryocyte number to 7±1 per a ×40 field on a light microscope, while the count of the control males was 9±1. In the Exp C males, the percentage of Thy-1^+ BM cells increased to 28±7%, and the percentage of Stat3 decreased to 24±2%. The Exp C males were judged to be in the progressive phase. The levels of Thy-1^+ BM cells (more than 19±6% in females and 10±2% in males) and those of Stat3^+ BM cells (less than 59±10% in females and

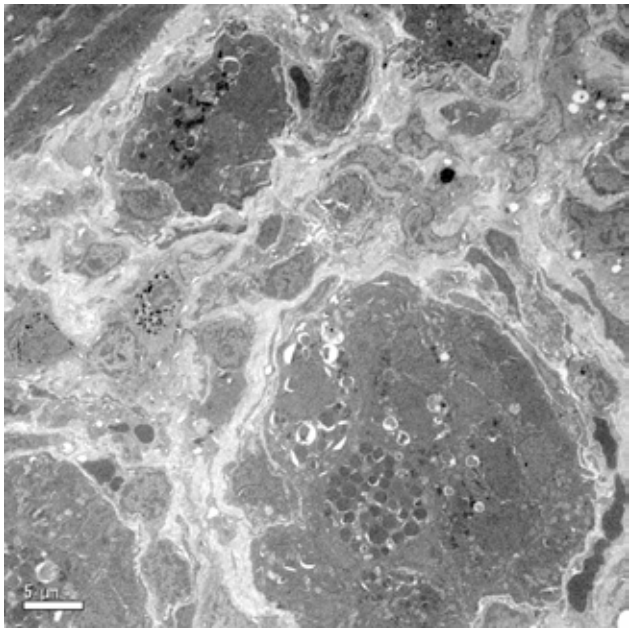

Figure 1. Electron micrograph of the lower ileum of the Exp C-3 male cut 1 cm above the ileocecum, showing chronic GvHD. In the destroyed epithelial cells, Paneth cells and goblet cells are confirmed. In the proliferating crypt stem cells, argentaffin stem cells are present. On the upper left side, the inner muscularis mucosa is recognizable, which is shown as a disorganization of mucosal architecture. This destructive phase of intestinal GvHD was accompanied by donor cytotoxic T lymphocytes.

52±11% in males) were good markers of chronic GvHD. The other Exp A, B, D, and E rats were in the recovery phase of chronic GvHD. Megakaryocyte numbers of the Exp A, B, D, and E females were higher than that (11±1) of the control females. The Exp A females showed high Thy-1$^+$ cell (25±4%) and a low Stat3$^+$ cell (44±11%) percentages, but high numbers (16±2) of BM megakaryocytes. The Exp B females showed high numbers (18±4) of BM megakaryocytes and a near normal percentage of Thy-1$^+$ cells (16±3%), but still had a low Stat3$^+$ cell percentage (49±5%). In the Exp D females, which had clinically undetectable chronic GvHD, a high Thy-1$^+$ cell percentage (28±4) was found, along with a near normal Stat3$^+$ cell percentage (60±15). The high percentage of Thy-1$^+$ cells (greater than 19±6 in females and 10±2 in males) indicated that a recovery from anti-apoptotic degenerative changes was occurring in the BM. The Exp D females showed a persistent recovery from anti-apoptotic changes 127 days post-transplantation. Although both the Exp B and Exp D females were injected with Stat3 Ab, the Exp D females had milder chronic GvHD than the Exp B females because of the combined IDX-1 Ab given to only the Exp D. The Exp E females showed the mildest chronic GvHD, in which they had a Thy-1$^+$ cell % of 17±7 together with a high Stat3$^+$ cell % of 69±4. Figure 2 shows the donor BM of rat Exp A-2, which survived in the donor pancreas, and the piece of the donor spleen grafted onto the host spleen. The donor T cells shown in figure 2a strongly activated in the BM graft. The donor T cell cytoplasm had long artificial legs projecting outside the cell. The T cell must have come into contact with host T cells and then rejected host T cells. Figure 2b shows an activated myeloblast in the BM graft. The GvH reactions that occurred in the donor BM transplanted into their pancreas triggered rejections against the host skin epithelial cells, but not the epithelial cells of the thymus medulla. The hypoplastic BM graft showed remaining mast cells. Figure 3 shows the grafted donor spleen of Exp D-1. The nuclei of the rejected host T cells have become apoptotic. The cytoplasm of the rejected T cells survived for longer after the nuclear apoptosis. The remaining cytoplasm contained surviving mitochondria. The spleen graft of Exp D-1 showed milder GvH reactions. Figure 4 shows the grafted donor spleen of Exp D-4, another rat from the same Exp D group as shown in figure 3. In figure 4a, the donor T cell on the right side rejected the host cell on the left side. The left host T cell had both dense cytoplasm containing developed

Table 2. Bone marrow (BM) studies to evaluate chronic graft versus host disease (GvHD) stage.

Exp group (Sex)	Rat number (n)	Post-BM-T[i] (Day)	BM megakaryocyte (n/40 time field)	FCM		GvHD Stage
				Thy-1 (%)	Stat3 (%)	
Cont (F)	4	–	11±1	19±6	59±10	–
Cont (M)	6	–	9±1	10±2	52±11	–
A (F)	4	159	16±2	25±4	44±11	Recov[ii]
B (F)	4	164	18±4	16±3	49±5	Recov
C (M)	3	107[iii]	7±1	28±7	24±2	Prog[iv]
D (F)	4	127	15±2	28±4	60±15	Recov
E (F)	5	157	16±3	17±7	69±4	Recov

[i]Post-BM transplantation; [ii]Recovery; [iii]One rat died 64 days post-BM-T; [iv]Progressive, FCM: Flow cytometer, F: Female, M: Male

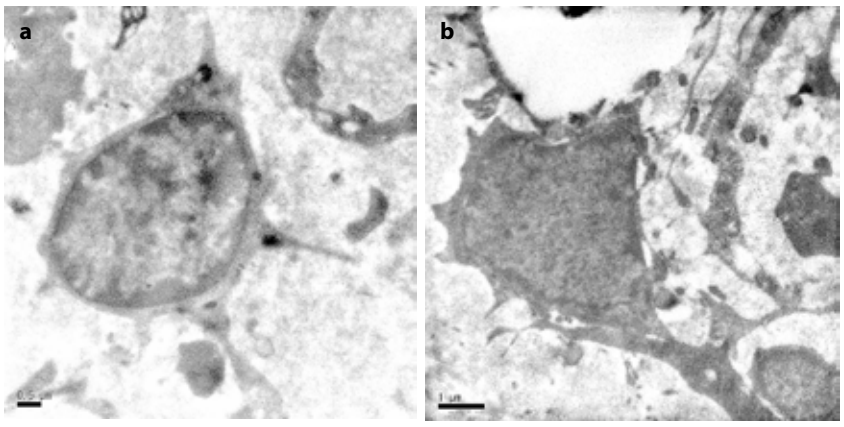

Figure 2. Electron micrographs of the BM graft of Exp A-2. Figures 2a indicate a strongly activated donor T cell in the severely hypoplastic BM transplanted into the host pancreas. The donor T cell shown in figure 2a shows activated cytoplasm with projections into the outside. On both upper sides, 2 apoptotic BM cells are present. The nuclei of the cells show severe apoptotic changes with ribosome-rich cytoplasm. Figure 2b shows an activated myeloblast cytoplasmic projections. The other contracted cells lose their nucleus before showing fatty degeneration.

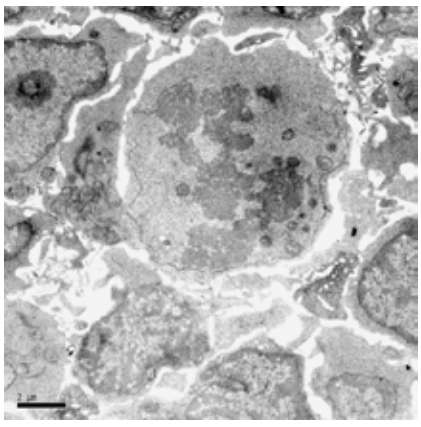

Figure 3. Electron micrograph of spleen graft in Exp D-1 female. Two apoptotic host cells are shown in which the cell nuclei have been lost or become apoptotic, but their cytoplasm still contains many mitochondria. Mild GvH reactions have occurred in the spleen graft.

ribosome and an apoptotic nucleus. The two lymphocytes shown in figure 4b were slightly different from those in figure 4a. In figure 4b, the donor and atrophic host T cells had activated ribosome around their nuclear membranes, which suggested the occurrence of a resistant reaction between them. Newly generated lymphocytes with different antigens were considered to be present. Figures 2, 3, and 4 show chronic GvH reactions, which are continuing in an attempt to reject the F_1 host. The donor BM T cells of Exp A were revealed on histopathology to be stronger than the T cells of Exp D grafted spleen.

Figure 5 shows the FCM findings in the spleen grafts of the Exp C-3 and Exp D-1 rats. The positive threshold of CD11b was decided upon, using the results from the Exp D-1 rat. The spleen graft of the Exp D-1 rat showed a $CD11b^+$ cell level of 92.4%, while the spleen graft of Exp C-3 showed a $CD11b^+$ cell level of 29.6%. The positive threshold of CD18 was decided upon using the result from Exp C-3. The spleen graft of Exp C-3 had a $CD18^+$ cell level of 57.9%, while the spleen graft of Exp D-1 had a $CD18^+$ cell level of 66.6%. Table 3 summarizes the FCM results measured in the F_1 hosts. Figure 6 illustrates the results presented in tables 2 and 3. Panel a of figure 6 shows the relationship between $Stat3^+$ BM cells and $CD11^+$ host spleen cells. The results shown on panel a were classified into 3 groups. The Exp C males, which had the severest chronic GvHD had a low percentage (24±2%) of $Stat3^+$ BM cells and a low percentage (76±4%) of $CD11b^+$ host spleen cells, while the control males showed $Stat3^+$ MB cells of 52±11% and a $CD11b^+$ host spleen cells of 83±3%. The Exp A and Exp B rats, which had cutaneous and thymic GvHD, respectively, showed a slightly low levels of $Stat3^+$ BM cells (44±11% and 49±5%, respectively) and high levels of $CD11b^+$ host spleen cells (92±4% and 87±3%, respectively), while the control females had a $Stat3^+$ BM cell level of 59±10% and a $CD11b^+$ host spleen level of 75±1%. The Exp D and Exp E rats, which had the second mildest and mildest forms of chronic GvHD, respectively, had a near normal level (60±15%) and a high level (69±4%) of $Stat3^+$ BM cells, respectively, and higher levels of $CD11b^+$ host spleen cells (86±2% and 85±5%, respectively). A high percentage of $CD11b^+$ host and graft spleen cells indicated a sign of protective chronic GvH reactions. The spleen graft of Exp D had higher levels of $CD11b^+$ cells than the host spleen at the time of sacrifice, with the reverse situation in Exp A and Exp C. The spleen grafts of Exp C showed severe rejection with low expression of CD11b, one of which became necrotic with pus discharge. Panel b of figure 6 shows the relationship between $Stat3^+$ thymus cells and $IDX\text{-}1^+$ spleen cells. The percentage of $Stat3^+$ thymus cells was positively correlated with the percentage of $IDX\text{-}1^+$ spleen cells in Exp D and Exp E. The correlation coefficient of the Exp D and Exp E rats was 0.58, which was higher than that (0.40) of the control females. Although in the Exp D females the IDX-1 Ab and Stat3 Ab reacted to suppress the Stat3 and IDX-1 proteins at the time of BM transplantation, the Exp D females demonstrated Stat3 and IDX-1 activation at the time of sacrifice.

The Exp E females, which were injected with CD18 Ab, also showed Stat3 and IDX-1 activation post-transplantation. The normal values of $CD18^+$ spleen cells differed between the control males (71±17%) and control females (23±3%)

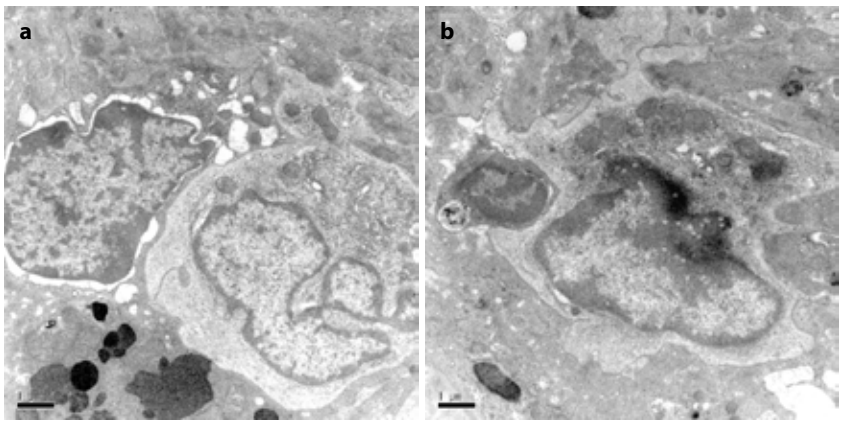

Figure 4. Electron micrographs of the spleen graft of the Exp D-4 female. Figure 4a shows 2 lymphocytes, one of which is an activated donor T cell, and the other is a decreasing host T cell. Both cells have membrane attachments. The cytoplasm of the apoptotic cell contains a well-developed ribosome. The GvH reaction in the spleen graft is mild. Figure 4b also shows 2 lymphocytes. The donor T cell demonstrates strongly activated reactions in its nuclear membrane, and the host cell, which has become atrophic, also displays an activated nuclear membrane reaction, shown by a densely stained ribosome. The cytoplasm of the atrophic host cell has been almost completely enclosed by the donor T cell. In the mild GvH reaction shown here, a kind of resistance is present between the two cells.

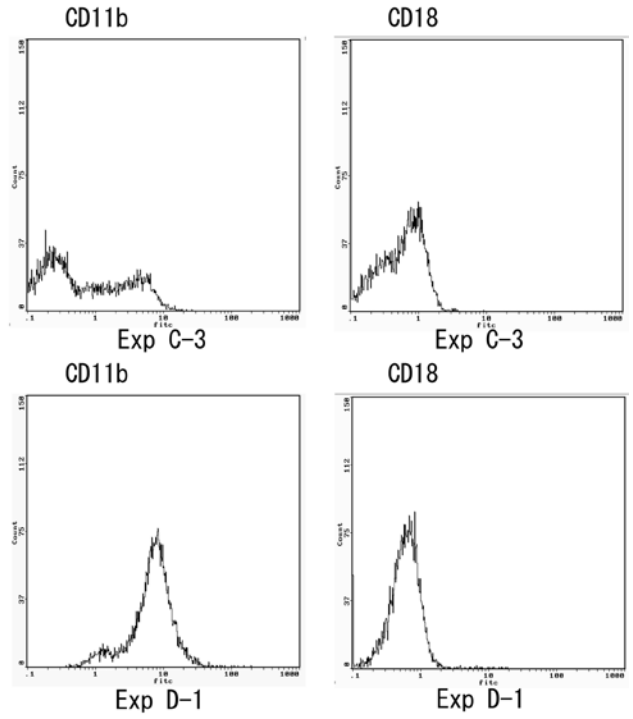

Figure 5. Flow cytometry (FCM) findings from the graft spleens of the Exp C-3 male and Exp D-1 female. Both of the rat spleen grafts have been stained with CD11b-FITC and CD18-FITC. The spleen graft of the Exp C-3 male had a $CD11b^+$ cell level of 29.6%, and the spleen graft of the Exp D-1 female had a $CD11b^+$ cell level of 92.4%, while the spleen graft of the Exp C-3 male had a $CD18^+$ cell level of 57.9%, and the spleen graft of the Exp D-1 female had a $CD18^+$ cell level of 66.6%. The spleen graft of the Exp C-3 male was rejected more strongly than that of the Exp D-1 female.

in this study. However, it could be said that CD18 expression was exaggerated in the recovery phases of chronic GvHD as for CD11b. The progressive phase of chronic GvHD showed lower expression of CD18 with thymus atrophy as found as in Exp C. The Exp D and Exp E females displayed elevated levels of CD18 (66±13% and 73±12%). Figure 7 shows an electron micrograph of the Exp C-3 host spleen. Two clumps of CD18 Abs labeled with gold particles can be seen binding to two T lymphocytes to form a bridge-like structure. Many clumps of CD18 Abs labeled with gold particles were bound to host T cells, in which CD18 expression on T cells induced cell to cell interactions and rejection of the host cells by donor T cells. CD18 was therefore found to be related to graft rejection in chronic GvHD with thymus atrophy. As shown in table 3, the graft spleen T cells of Exp C-3 expressed CD18 more strongly with weaker expression of CD11b than the host spleen T cells. The Exp D and Exp E females, which demonstrated high levels of CD18 did not show clinical chronic GvHD with thymus T lymphopoiesis. Figures 8a and 8b show electron micrographs of Exp B-1 and Exp E-1, respectively, both of which were stained with IDX-1 Abs labeled with gold particles. In figure 8a, many spleen cells in the graft have lost their nuclei, but the cytoplasm of the ghost cells contain mitochondria. The clumps of IDX-1 Abs labeled with gold particles were bound to the surface membrane and were then transported into the cytoplasm. Nuclear transport of IDX-1 Ab caused severe damage to spleen cell nuclei in the Exp B-1 rat with the signs of chronic GvHD. As shown in figure 8b, the Exp E-1 rat, which had the mildest chronic GvHD, IDX-1 Abs masses were detected in mitochondria specifically, and also in the nuclear membrane and nucleolus. During immune tolerance, mitochondrial IDX-1 was a characteristic finding and explained why the spleen cells of the Exp D and Exp E females showed a high percentage of $IDX\text{-}1^+$ cells. IDX-1 Abs was subjected to a similar, but negative interaction pathway to that of Stat3 Abs (data not shown in figure). Masses of Stat3 Abs labeled with gold particles could be more rarely observed in the cytoplasm and mitochondria of the T lymphocytes in the markedly hypoplastic BM graft of the Exp A-1 rat.

Table 3. Flow cytometer (FCM) findings in the thymus, host spleen, and graft spleen.

Exp group (Sex)	Rat number (n)	Thymus Stat3 (%)	Host spleen			Spleen graft		
			CD11b (%)	CD18 (%)	IDX-1 (%)	CD11b (%)	CD18 (%)	IDX-1 (%)
Cont (M)	6	NT[a]	83±3	71±17	NT	–	–	–
Cont (F)	4	31±7	75±1	23±3	31±7	–	–	–
A (F)	4	NT	92±4	60±13	NT	86±7	62±22	NT
B (F)	4	NT	87±3	41±12	NT	87±1	57±2	47[b]
C (M)	3[c]	NT	76±4	48±10	NT	30[d]	58[d]	NT
D (F)	4	66±10	86±2	66±13	NT	92±3	75±8	76[e]
E (F)	5	46±9	85±5	73±12	59±15[f]	NT	69±14	59±19[g]

[a]Not tested; [b]Measured only in the Exp B-1 rat; [c]Among 4 rats, 1 rat died; [d]Measured only in the Exp C-3 rat; [e]Measured in the Exp D-1 and 4 rats; [f]Measured in the Exp E-2, 3, and 5 rats; [g]Measured in the Exp-1, 2, 3, and 5 rats, F: Female, M: Male

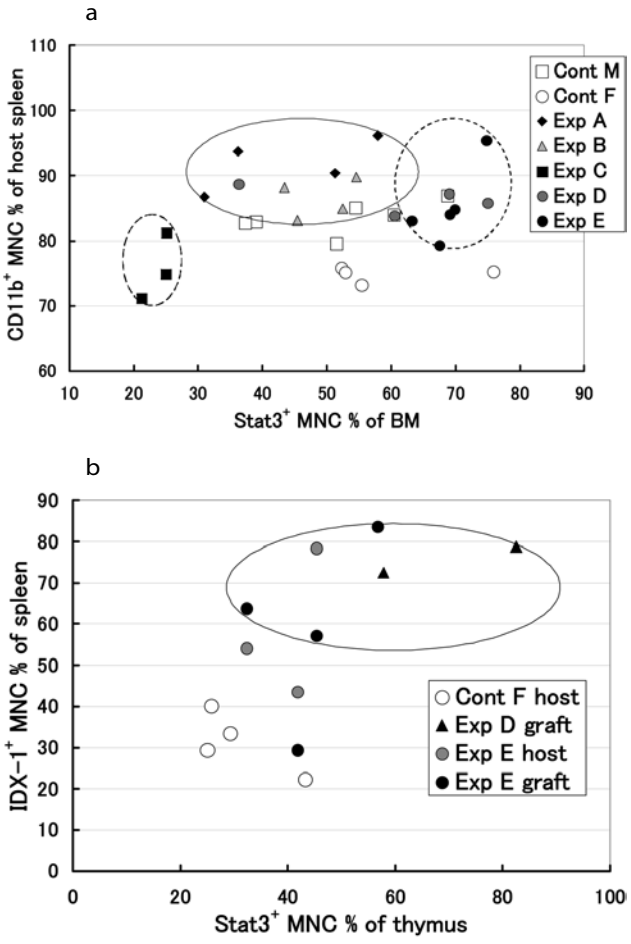

Figure 6. Flow cytometry evidence of immune tolerance: Comparison bone marrow (BM), spleen and thymus cells. Panel a shows the relationship between Stat3+ BM cells and CD11b+ spleen cells. The Exp D and Exp E rats demonstrated immune tolerance, which resulted in the activation of Stat3+ BM cells. Panel b shows the relationship between Stat3+ thymus cells and IDX-1+ spleen cells. The Exp D and Exp E rats displayed immune tolerance, which resulted in the activation of Stat3+ thymus cells and IDX-1+ spleen cells with a correlation coefficient of 0.58.

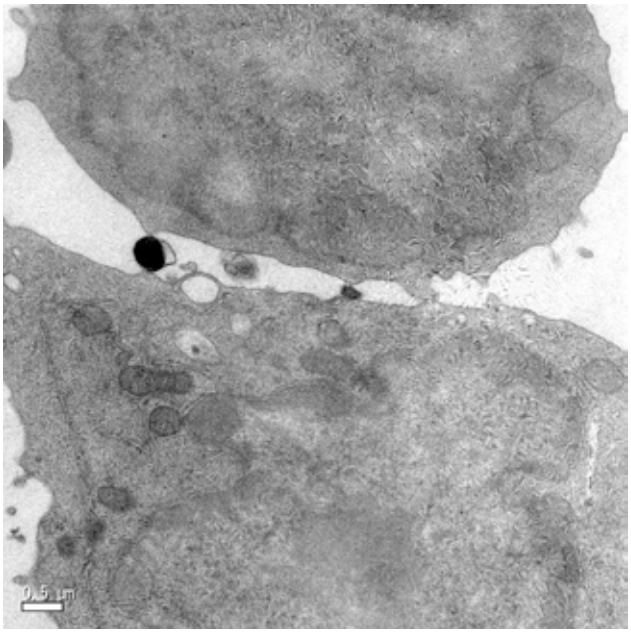

Figure 7. Immuno-chemical stain of electron micrograph depicting the host spleen of the Exp C-3 male with chronic GvHD. Two clumps of CD18 Abs coupled with gold particles, which look very dense are observed between the 2 lymphocytes. CD18 antigens are shown to be involved in GvHD through their attachment to each other. The upper host cell with the apoptotic nucleus has been rejected by the donor T cell below.

DISCUSSION

The mechanism of immune tolerance in BM transplantation was investigated using rat experimental systems involving donor BM cell injection into the host spleen together with the transplantation of a piece of spleen graft, as described previously.[2]

Insulin-like growth factor 1 (IGF-1), which is produced mainly by hepatocytes, but also in the pancreas and spleen, promotes improvement of islet β-cell survival with insulin-

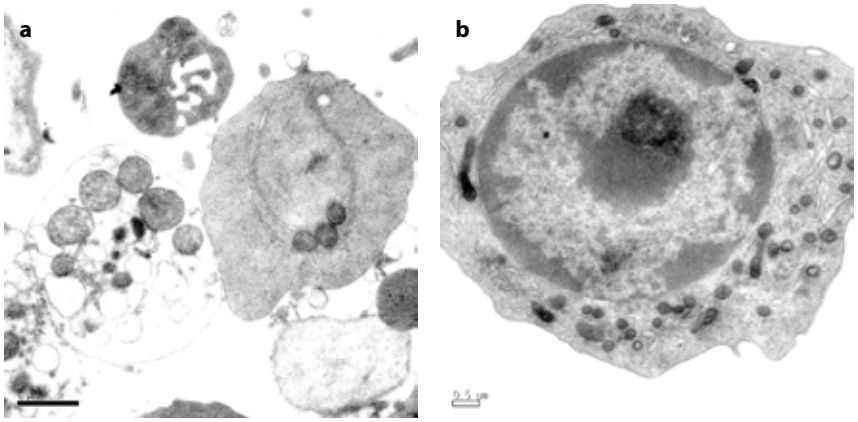

Figure 8. Immuno-chemical stain of electron micrograph of spleen grafts. Figure 8a shows the Exp B-1 spleen graft, and figure 8b shows the Exp E-1 spleen graft. Both have been stained with IDX-1 Abs coupled with gold particles to compare chronic graft versus host disease (GvHD) features observed. Figure 8a (Exp B-1 female with clinical chronic GvHD) shows stronger rejection; several ghost cells have lost their nucleus, but have retained cytoplasmic functions. Figure 8b shows a lymphocyte with widespread mitochondrial IDX-1. A part of section of nuclear membrane and nucleolus also contain the IDX-1 Abs-gold particles. IDX-1 proteins in the immune-tolerate T cell were shown to have mitochondrial metabolic functions.

like effects.[6] IGF-1 also exerts a predominantly anti-apoptotic effect on primitive multi-lineage $CD34^+CD38^-$ hematopoietic progenitor cells and $CD34^+CD38^+CD10^+$ lymphoid progenitor cells.[7] IGF thus acts to reverse thymus involution and enhance T lymphopoiesis in rodents. Immune systems can be reconstructed by IGF stimulation.[8] The donor T cells in a piece of BM transplanted into the host pancreas together with a spleen graft attached onto the host spleen caused cutaneous GvHD. Cutaneous GvHD was associated with the proliferation of megakaryocytes in the host BM and did not cause thymus atrophy. In this study, a different feature of chronic GvHD was considered, based on IGF-1 production in the spleen and pancreas. IGF-1 was produced actively to reestablish lymphopoiesis in the spleen as well as β-cells in the pancreas. The cutaneous GvHD rats had high percentages of $CD11b^+$ spleen cells and Thy-1^+ BM cells. As $CD11b^+$ spleen cells are associated with allogenic immune suppression *in vivo*, donor T cell function must be impaired by proliferated $CD11b^+$ spleen cells. However, $CD4^+CD25^+$ donor cells showing reduced CD3 expression induced alopecia.[9] It was concluded that the cutaneous GvHD might be regulated by myeloid suppressor cells (MSC) of $CD11b^+Gr\text{-}1^+$cells showing strong expression of Thy-1 antigen, which showed a relationship with IGF1 production. Activated myelobrasts observed in the donor BM of cutaneous chronic GvHD (Exp A) might be concerned with cutaneous chronic GvHD.

The male rats with the severest chronic GvHD had a lower percentage of $CD11b^+$ spleen cells than the normal males. The male rats (Exp C) that were injected with both CD18 Ab and IDX-1 Ab into their spleens, and which had a donor spleen graft and donor BM cells, suffered from intestinal perforation. The homeodomain transcription factor IDX-1 (PDX-1/STF1/IPF1) is required for pancreas development. However, IDX-1 also plays an important role in determining the development of the distal stomach, Brunner's glands, the intestinal epithelium of the duodenum, and the spleen.[10] It was hypothesized that the IDX-1 proteins expressed on ileum or ileocecum epithelial cells had become activated in the males with the severest GvHD and that the epithelial cells of the ileum or ileocecum, which demonstrated a marked development of lymph nodes (LN), were destroyed by donor T cell infiltration through the CD18 interactions. In the immune systems that did not induce immune tolerance after the BM transplantation, IDX-1 Ab caused activated IDX-1 nuclear transcription in intestinal epithelial cells as a form of chronic GvHD. As found in the spleen graft with chronic GvHD, when IDX-1 was activated by GvHD, many of the intestinal cells lost their nucleus, which resulted in intestinal perforation. The females (Exp E) that received donor BM cell transplantation in the same way as the males with the severest GvHD, but were injected with CD18 Ab alone, showed clinically unrecognized chronic GvHD. Furthermore, the females (Exp D) that were transplanted with donor BM cells in the same way as above, but were injected with IDX-1 Ab combined with Stat3 Ab, had clinically unrecognizable chronic GvHD with immune tolerance. A recent report suggested that intestinal epithelial Stat3 activation regulated immune homeostasis in the gut by promoting interleukin (IL)-22-dependent mucosal wound healing.[11] Stat3-induced IL-22 was expressed by activated T cells, especially by Th17 cells, as well as by dendroid cells (DC), and natural killer (NK) cells expressed CD11c. IL-22 reacts with IL-22 receptors on the small and large intestines to promote intestinal epithelial cell (IEC) proliferation and IEC protection against apoptosis. IEC perforation has been protected in the immune tolerant rats with activated Stat3 at post-transplantation.

On the other hand, it was shown in the two experiments involving the injection of CD18 Ab or IDX-1 Ab combined with Stat3 Ab that medullary thymus epithelial cells were induced to initiate T-cell lymphopoiesis. It is known that

the expression of self-antigen occurs in medullary thymus epithelial cells expressing the Aire and insulin (Ins2) genes. Interferon γ (IFN-γ) causes a decrease in the Ins2 mRNA, while lymphotoxin beta receptors (tumor necrosis factor receptor-3) (LTβR) upregulate both Aire and Ins2 genes.[13] As immune tolerance is not observed in human LAD-1 cases, activated β_2-integrin CD18 must be involved in immune tolerance by upregulating both Aire and Ins2 genes.[4,5] β_2-integrin CD18 levels rebounded strongly post-transplantation and the high CD18 stimulated thymus T lymphopoiesis without clinical GvHD. It was also shown clinically that when both Stat3 and IDX-1 antigens were suppressed by their Abs in the host spleen, donor BM cells had acquired immune tolerance in the host spleen. As single Stat3 Ab injection in the same way as above caused chronic GvHD, IDX-1 must have an important negative interaction with Stat3 to induce immune tolerance. The findings of BM stem cell development into pancreatic β-cells in the host pancreas suggested the importance of stem cell IDX-1.[3] The suppressed activation of IDX-1 must inhibit the activation of nuclear protein IκB-ζ, which is a negative regulator of Stat3 as previously reported.[12] The transcriptional activity of Stat3 is inhibited by overexpressed IκB-ζ, but suppression of IκB-ζ leads to greater resistance to apoptosis. Activated mitochondrial Stat3 may indicate the inhibition of IκB-ζ, and the inhibited IκB-ζ may be indicated by mitochondrial IDX-1 activation. On electron micrographs, Stat3 and IDX-1 were also detected in many mitochondria during immune tolerance, which was indicated by FCM and their expression was shown to be correlated. The two proteins regulated cell metabolic functions in the cytoplasm in addition to nuclear transcription. As mitochondrial Stat3 suppresses the small molecule inhibitors of oxidative phosphorylation, mitochondrial Stat3 may play an important role in adenine triphosphate (ATP)-dependent glucose metabolism.[14] Large amounts of mitochondrial Stat3 and IDX-1 indicated the occurrence of immune tolerance.

ΠΕΡΙΛΗΨΗ

Ανοσιακή ανοχή προκαλούμενη από την αναστολή μόνο του CD18 ή και του Stat3 και του IDX-1 κατά τη μεταμόσχευση μυελού ποντικών όπου εμφανίζεται λεμφοποίηση Τ-λεμφοκυττάρων του θύμου μετά από τη μεταμόσχευση

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ΣΚΟΠΟΣ Διευκρίνιση του μηχανισμού ανοσιακής ανοχής και ανάδειξη της εμφάνισής της τόσο κλινικά όσο και *in vitro*. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Έγινε μεταμόσχευση μυελού με δέκτες F_1 (DA×LEW) και δότες υβριδικά ποντίκια, με παρακολούθηση για 127–157 μέρες μετά από τη μεταμόσχευση. Τα μυελικά κύτταρα χορηγήθηκαν μαζί με αντισώματα Stat3 και IDX-1 ή μόνο με αντίσωμα CD18 στο σπλήνα του δέκτη, στον οποίο είχε επίσης μεταμοσχευθεί τμήμα σπλήνα του δότη. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Όταν χορηγήθηκαν μυελικά κύτταρα από DA ποντίκια μαζί με αντισώματα Stat3 και IDX-1 ή αντίσωμα CD18, δεν εμφανίστηκαν συμπτώματα χρονίας αντίδρασης του μοσχεύματος κατά του ξενιστή (GvHD), όπως απώλεια βάρους, ατροφία θύμου, γαστρεντερικές διαταραχές και αλωπεκία. Τα F_1 θηλυκά ποντίκια χωρίς κλινική GvHD παρουσίασαν αυξημένο αριθμό μεγακαρυοκυττάρων στο μυελό, μη παρουσία εξωμυελικής αιμοποίησης και κυτταρομετρικά ενεργοποίηση του Stat3 στο μυελό και στο θύμο και των CD11b, CD18 και IDX-1 στο σπλήνα του δέκτη και του μοσχεύματος. Ο συντελεστής συσχέτισης μεταξύ των $Stat3^+$ κυττάρων του θύμου και των $DIX\text{-}1^+$ κυττάρων του σπλήνα σε αυτά τα θηλυκά ποντίκια χωρίς σημεία GvHD ήταν 0,58, ενώ των αντίστοιχων μαρτύρων ήταν 0,4. Οι ανοσοχημικές μικροφωτογραφίες με ηλεκτρονικό μικροσκόπιο έδειξαν ειδικά μιτοχονδριακές IDX-1 πρωτεΐνες καθώς και Stat3 στα θηλυκά ποντίκια με ανοσιακή ανοχή. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Η ανοσιακή ανοχή προάγεται με την αναστολή του Stat3 και του IDX-1, ενώ εμφανίζει μια αρνητική αλληλεπίδραση με το Stat3, κατά τη στιγμή της μεταμόσχευσης μυελού. Το CD18 αντανακλά τον πολλαπλασιασμό των διεγερμένων Τ-κυττάρων του θύμου. Η σπληνική παραγωγή του IGF-1 θα πρέπει να προάγει την ανοσιακή ανοχή χωρίς την εμφάνιση συμπτωμάτων GvHD.

Λέξεις ευρετηρίου: Ανοσιακή ανοχή, CD18, Θύμος, IDX-1, Μεταμόσχευση μυελού, Μιτοχόνδρια, Σπληνικά μοσχεύματα, Stat3, Χρόνια αντίδραση μοσχεύματος κατά ξενιστή

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