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Single blood transfusion induces the production of donor-specific alloantibodies and regulatory T cells mainly in the spleen

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Abstract

Donor-specific blood transfusion is known to induce alloresponses and lead to immunosuppression. We examined their underlying mechanisms by employing fully allogeneic rat combinations. Transfused recipients efficiently produced alloantibodies of the IgM and IgG subclasses directed against donor class I MHC. The recipients exhibited active expansion of $CD4^+$ T cells and $CD4^+FOXP3^+$ regulatory T cells (T_{reg} cells), followed by $CD45R^+$ B cells and IgM^+ or IgG $subclass^+$ antibody-forming cells mainly in the spleen. From 1.5 days, the resident $MHCII^+CD103^+$ dendritic cells (DCs) in the splenic T-cell area, periarterial lymphocyte sheath, formed clusters with recipient $BrdU^+$ or 5-ethynyl-2′-deoxyuridine$^+$ cells, from which the proliferative response of $CD4^+$ T cells originated peaking at 3–4 days. Transfusion-induced antibodies had donor passenger cell-depleting activity *in vitro* and *in vivo* and could suppress acute GvH disease caused by donor T cells. Furthermore, T_{reg} cells significantly suppressed mixed leukocyte reactions in a donor-specific manner. In conclusion, single blood transfusion efficiently induced a helper T-cell-dependent anti-donor class I MHC antibody-forming cell response with immunoglobulin class switching, and a donor-specific T_{reg} cell response mainly in the spleen, probably by way of the indirect allorecognition via resident DCs. These antibodies and T_{reg} cells may be involved, at least partly, in the donor-specific transfusion-induced suppression of allograft rejection.

Keywords: resident dendritic cells, indirect allorecognition, antibody-forming cell, passenger cell depletion, GvH disease

Introduction

Alloantibodies are produced against MHC antigens of the same species (alloantigens) in organ transplantation. Generation of these alloantibodies in animals usually requires repeated immunization such as donor skin grafts plus intra-peritoneal injection of donor lymphocytes in adjuvants (1). In contrast, donor-specific transfusion (DST), which simply transfuses fresh donor blood intravenously prior to transplantation, is also reported to readily induce alloantibody production against donor antigens, predominantly class I MHC antigens (MHCI), in humans (2). However, few reports are available concerning where and how this antibody (DST-antibody) production occurs so efficiently.

DST is known as one of the tolerance-inducing protocols used not only in experimental (3), but also in clinical (4), solid organ transplantation. Although DST-antibodies are reported to have a deleterious effect on the graft at least partly (2), their role in the DST-induced immunosuppression is still unclear. In addition, production of regulatory T cells (T_{reg} cells) in graft recipients is enhanced when they receive DST in advance (5). However, induction of T_{reg} cells after a single DST only has not yet been reported.

Alloantigens are recognized by T cells mainly via two main distinct pathways, ‘direct’ and ‘indirect’ allorecognition (6). The direct allorecognition involves the ligation of T-cell receptors on alloreactive recipient T cells by foreign intact MHC

molecules on the surface of donor dendritic cells (DCs). Previously, we demonstrated this pathway following rat liver transplantation, in which migrated graft DCs induced diffuse $CD8^+$ T-cell proliferative responses within the cluster between DCs and the recipient's T cells in the secondary lymphoid organs (SLOs) (7). The indirect allorecognition results from the recognition of allogeneic MHC molecules as peptides presented by recipient DCs in the context of self-MHCs. Because the donor blood contains very few donor DCs, DST-antibody may be operated mainly by the indirect allorecognition, which has not been proven *in vivo*. Therefore, how these pathways are involved in the DST responses is still unclear.

The spleen is a major lymphoid organ that produces antibodies against antigens in the blood, suggesting that the DST-antibody response may mainly occur in this organ. In most mammals, including rats, mice, goats and rabbits, the outer margin of the periarterial lymphocyte sheath (outer PALS) is the site of the antibody-forming cell (AFC) response to various types of soluble antigens (8, 9). In the PALS, DCs present antigen peptides on their surface class II MHC (MHCII) molecules to $CD4^+$ T cells, which in turn stimulate and differentiate B cells into AFCs in the outer PALS. T_{reg} cell proliferation also occurs in the PALS (10). However, *in situ* alloresponses including $CD4^+$ T cells, T_{reg} cells and AFCs after DST in the spleen have not been reported thus far.

In this study, we examined the nature of DST-antibodies and T_{reg} cells after single DST, in regards to their production kinetics *in situ*, specificities and functions using rats. The roles of resident DCs, DST-antibodies and T_{reg} cells in the DST-induced immunosuppression are discussed.

Methods

Animals

Inbred ACI rats ($RT1.A^aB^a$) were obtained from the National Bio Resource Project for the Rat in Japan (Kyoto, Japan) and bred at Dokkyo Medical University Animal Research Center (Mibu, Japan). Lewis rats ($RT1.A^lB^l$), BN rats ($RT1.A^nB^n$) and DA rats ($RT1.A^aB^a$) were supplied by Charles River Japan Inc. (Tsukuba, Japan), CLEA Japan Inc. (Tokyo, Japan) and/or SLC Japan Inc. (Shizuoka, Japan), respectively. All rats were reared under specific pathogen-free conditions. The handling and care of animals were approved by the Dokkyo Medical University Animal Experiment Committee and conducted in accordance with the Dokkyo University's Regulation for Animal Experiments and Japanese Governmental Law (No. 451). In most experiments, ACI rats donated blood and thoracic duct lymphocytes (TDLs) and Lewis rats served as recipients. BN rats were used as a third-party strain. In GvH disease (GvHD) experiments, DA rats donated TDLs and Lewis rats served as recipients.

Antibodies and reagents

Monoclonal antibodies (mAbs) and labeled secondary antibodies used for immunohistology or flow cytometry (FCM) analysis are listed in Table 1. Some mAbs were purified from culture supernatants and coupled to Alexa Fluor® 350, 488, 594 or 647 (Life Technologies Japan Ltd, Tokyo, Japan) in house. A rabbit polyclonal antibody to FOXP3 was prepared in house (11).

Experimental design

Freshly heparinized whole blood was aseptically obtained from the donor abdominal aorta and 1 ml was immediately injected intravenously into the tail vein of the recipient rat. At various times from 1 to 14 days after the transfer of blood, recipient rats received an intravenous injection of BrdU (2 mg/100 g body weight, Sigma-Aldrich Japan, Tokyo, Japan) for immunoenzyme staining or 5-ethynyl-2′-deoxyuridine (EdU, 1.67 mg/100 g body weight, Life Technologies) for immunofluorescence staining and FCM 1 h before sacrifice under isoflurane anesthesia. The SLOs, including the spleen, and peripheral lymph nodes (LNs, cervical, axillary and brachial LNs), were excised and fresh cryosections of 4-μm-thickness prepared for the immunohistological examination. Pieces of spleens were also digested by collagenase D (Roche Diagnostics, Indianapolis, IN, USA) and the cells used for FCM analysis (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). T_{reg} cells and DCs were further isolated from spleen cells by FACSAria sorting and autoMACS (Miltenyl Biotech, Gladbach, Germany), respectively. In some experiments, we employed TDLs as a convenient source of donor target cells or donor T cells that could be isolated with ease at high purity and viability. By routine thoracic duct cannulation of a rat (12), up to 10^8 TDLs, containing mature peripheral recirculating T cells (~80%), B cells (~20%) and non-lymphoid cells (<2%) with a viability of >95% could be obtained by overnight collection (13).

In the first set of experiments, the characteristics of DST-antibody were examined in regards to immunoglobulin subclass, antigen epitope and spleen dependency. In the second set of experiments, the immune response to DST in the spleen was studied *in vitro* by FCM and then *in situ* immunohistologically, using multicolor immunoenzyme or immunofluorescence stain. In the third set of experiments, the functions of the DST-antibody itself were examined in regards to cytotoxicity *in vitro* and depleting activity of donor cells *in vivo*, including the suppressive activity of GvHD. In a fourth experiment, T_{reg} cells were examined for their suppressive function and antigen specificity.

Serum antibody detection and antigen epitope

In the first experiment, TDLs from donor ACI or recipient Lewis rats were used as target cells. Aliquots of 50 μl containing 1.25×10^6 cells TDLs in FACS buffer were incubated with 50 μl of DST-treated sera (DST-sera), diluted 10× or 80×, for 1 h at 4°C, and then washed and incubated with 100 μl of mouse mAbs against rat IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, IgA and IgE (each 1.4 μg) for 30 min at 4°C. After washing, cells were labeled for 30 min at 4°C with 100 μl of a 100× dilution of FITC-conjugated goat anti-mouse immunoglobulin. Subsequently, cells in a T-cell gate were analyzed by FCM.

Because DST-antibodies have been suggested to recognize donor MHCI (RT1.A) antigen in rats (14), we performed FCM analysis using $RT1.A^a$-transfectant (15). cDNAs encoding the full-length polymorphic rat class I MHC molecule, $RT1.A^a$ in the pCR™3 plasmid, were a kind gift from Dr Simon Powis (Glasgow University). Plasmids were transfected into mouse L cells using Lipofectamine 2000 (Life Technologies). Cell surface expression of $RT1.A^a$ was confirmed by FCM

Table 1. Antibodies and probes used in this study

First antibodies and probe

Antigen	Clone/probe	Source	Conjugate (nil; purified)
CD4, for FCM	W3/25	Biolegend	FITC
CD4, for immunohistochemistry	OX-38	AbD Serotec	
CD8β	341	Biolegend	FITC
CD25	OX-39	Biolegend	R-PE
CD45R (B220)	HIS24	Biolegend	FITC
CD103 (αE2 integrin)	OX-62	ECACC[a]	
CD161 (NK1.1)	3.2.3	Biolegend	
FOXP3, for FCM	FJK-16s	eBioscience	Alexa Fluor 647
FOXP3, for immunohistochemistry	Rabbit polyclonal	Produced in house[b]	
IgA	MARA-2	AbD Serotec	
IgG_1	MARG1	AbD Serotec	
IgG_{2a}	MARG2a	AbD Serotec	
IgG_{2b}	MARG2b	AbD Serotec	
IgG_{2c}	MARG2c	AbD Serotec	
IgE	MARE-1	AbD Serotec	
IgM	MARM-4	AbD Serotec	
RT1.A[a] (polymorphic donor MHCI)	$MN_{4\text{-}91\text{-}6}$	ECACC[a]	
RT1.B[l] (polymorphic recipient MHCII)	OX-3	ECACC[a]	Alexa Fluor 488[c]
T-cell receptor αβ	R73	Biolegend/ECACC[a]	FITC/Alexa Fluor 647[c]
BrdU	BU1/75	AbD Serotec	
EdU	Click-iT® Azide	Life Technologies	Alexa Fluor 488, 594
Type IV collagen	Rabbit polyclonal	LSL	

Second antibodies and probe

Product	Source	Conjugate
Donkey IgG to rabbit IgG	Jackson ImmunoResearch	AMCA[d]
Donkey IgG to rat IgG	Jackson ImmunoResearch	Alkaline phosphatase
Goat IgG to mouse IgG	Sigma/Life Technologies/Biolegend	Alkaline phosphatase/ Alexa Fluor 647/R-PE, PerCP-Cy5.5
Goat $F(ab')_2$ to rabbit IgG	MP Bioscience	Peroxidase
Goat $F(ab')_2$ to rat IgG, Fc	Jackson ImmunoResearch	R-PE
Goat $F(ab')_2$ to rat IgM	Jackson ImmunoResearch	FITC
Sheep $F(ab')_2$ to mouse IgG	Sigma	FITC

[a]The European Collection of Authenticated Cell Cultures.
[b]Reference (11).
[c]Self conjugation.
[d]7-amino-4-methylcoumarin-3-acetic acid.

using a mAb clone $MN_{4\text{-}91\text{-}6}$, which is specific for RT1.A[a], as the probe. Transfectants were incubated with sample sera or antibody, followed by reaction with FITC-conjugated anti-rat IgM or R-PE-conjugated anti-rat IgG, respectively.

Spleen dependency

Three experiments were performed to examine the spleen dependency of the DST response. First, recipient Lewis rats were aseptically splenectomized via left flank incision just before the DST and serum DST-antibody response was examined. Second, short-term culture of the spleen cells after DST was examined. Five days after DST, splenocytes from recipient Lewis rat were prepared, placed in a 24-well plate at a density of 5×10^6 cells per well and cultured overnight at 37°C in a 5% CO_2 atmosphere. The next day, culture supernatants were harvested and used for the DST-antibody detection. Third, recipient splenocytes 4 days after DST were fused with X63-Ag8.653 mouse myeloma cells. Hybridoma cells were selected in hypoxanthin-aminopterin-thymidine medium and their culture supernatants were screened for the secretion of anti-donor MHCI IgM antibody by hemagglutination assays using donor erythrocytes as a target because rat erythrocytes constitutively express MHCI on their surface (14). The hybridomas were cloned by a routine limiting dilution method.

FCM analysis of the splenic immune response

In the second set of experiments, the proliferative response of lymphocytes was examined in recipient spleens 0, 3, 5 and 7 days after DST. Briefly, after *in vivo* labeling with EdU, cell suspensions were prepared from spleens by collagenase D digestion (Roche Diagnostics) and lymphocyte fractions purified by a density gradient using OptiPrep. The isolated cells were stained for mAbs to $CD4^+$ T cells, $CD8\beta^+$ T cells, or $CD45R\ (B220)^+$ B cells, followed by PerCP-Cy5.5-conjugated anti-mouse IgG secondary antibody. Cells were then fixed and permeabilized. For $FOXP3^+$ T_{reg} cells, lymphocyte fractions were incubated with Alexa Fluor 647-conjugated

anti-FOXP3 mAb after fixation and permeabilization. Next, EdU was stained with Click-iT® 488 kit (Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit; Life Technologies) according to the manufacturer's instruction. Cells were analyzed by FCM using Cell Quest software (BD Biosciences). The proliferating responses were quantified as follows: % proliferation = [EdU^+mAb^+ cells/total lymphocytes] × 100.

Immunohistological analysis of the splenic immune response

For the *in situ* analysis of immune responses, spleen cryosections were triple immunostained for TCRαβ, CD4, FOXP3, CD45R, CD161a, IgM, IgG subclasses, or donor MHCI (alkaline phosphatase, blue), and type IV collagen (peroxidase, brown), and BrdU (alkaline phosphatase, red). For the AFC response in the outer PALS, the number of either $BrdU^+IgM^+$ or $BrdU^+IgG_{2b}^+$ cells in the outer PALS (mm^2) was counted. The outer PALS area was defined as a continuous belt with a width of 45 μm in the peripheral margin of the PALS just inside of the marginal zone. The number of the germinal centers was also counted as the number of $BrdU^+IgM^+$ cell aggregates in the lymph follicle/surface area of the spleen sections (mm^2).

A mutual relationship between proliferating cells and resident DCs in the PALS

To examine the involvement of recipient DCs in the induction of immune responses against donor alloantigens in the PALS, we tried to depict the cluster formation of DCs with proliferating cells by the triple immunostaining for recipient MHCII (blue), BrdU (red) and type IV collagen (brown) (16). The number of total $BrdU^+$ cells and of recipient $MHCII^+$ cells clustering with either one $BrdU^+$ cell or two or more $BrdU^+$ cells in the PALS area (mm^2) was counted. The phenotype of the cluster-forming recipient $MHCII^+$ cells and proliferating cells was examined by four-color immunofluorescence staining for the recipient MHCII, DC or T-cell markers, EdU and type IV collagen using fluorescent dye-conjugated antibodies as described previously (10). Multichannel color fluorescence images were captured using an Axioskop 2 Plus fluorescence microscope equipped with an AxioCam MRm camera (Zeiss, Oberkochen, Germany). We assigned pseudocolors to each channel to create more comprehensible merged images by maximizing contrast using AxioVision software (Zeiss).

Complement-dependent cytotoxicity in vitro and donor cell clearance in vivo

The cytotoxicity of DST-sera was determined by a calcein-acetylmethylester (Calcein-AM; Dojin) retention assay. TDLs from donor ACI or recipient Lewis rats were used as target cells and labeled with 7 μM Calcein-AM in PBS containing 0.1% BSA for 20 min at 37°C in the dark. The labeled cells were washed and a 50 μl aliquot containing 2×10^5 cells was incubated with 50 μl of 20× diluted heat-inactivated DST-sera in FACS buffer for 1 h at 37°C. The cells were washed twice and incubated with 100 μl of 20× diluted guinea pig complements (Cedarlane Inc.) for 3 h at 37°C. After incubation, the target cells were washed and the fluorescence in the remaining cells was measured on a FACSCalibur. In all experiments, negative control incubations were performed using medium without sera, and the maximum attainable cytolysis of target cells was determined by measuring the residual fluorescence of 1% saponin-treated cells. Cytotoxicity was expressed as the relative viability calculated from the mean value as follows: % relative viability = [(residual fluorescence of the remaining cells – residual fluorescence of the maximally lysed cells)/(residual fluorescence of the negative control cells – residual fluorescence of the maximally lysed cells)] × 100.

To examine the effect of DST-antibodies *in vivo*, donor lymphocytes were transferred to Lewis rats 7 days after DST and the fate of donor cells was examined. TDLs were labeled with 10 μM CFSE (Life Technologies) for 20 min at 37°C, resulting in stable intracellular fluorescence (17). CFSE-labeled TDLs from ACI or BN rats, 1×10^8 cells in 1 ml of PBS(−), were intravenously injected into Lewis rats that had undergone DST with either ACI or BN blood. A small amount of recipient blood was collected 15 min, 90 min and 6 h after injection and the proportion of $CFSE^+$ cells among blood mononuclear cells was determined. Recipient rats were sacrificed 24 h after TDL transfer and the spleen cryosections were stained for type IV collagen (blue) to outline the white pulp and count the number of $CFSE^+$ cells per mm^2 white pulp under fluorescence microscopy.

Suppression of GvHD in vivo

The depleting effect of DST-antibodies, as described later, suggests that they may suppress the GvHD caused by donor T cells. Therefore, an acute GvHD model was prepared as described previously (13). Briefly, recipient Lewis rats received a total body sub-lethal split X-irradiation (Hitachi MBR-150R, Japan), twice at a dose of 3 Gy at a 4-h interval, and then received 5×10^7 TDLs of fully allogeneic DA rats, which possess identical MHCI (RT1.A^a) as ACI rats. Recipient rats died by days 10–11.

To examine a preventive effect of DST on GvHD, recipient rats underwent DST with donor DA blood 7 days prior to irradiation and cell transfer. Alternatively, 7 ml of DST-sera at 7 days, as an equivalent amount of sera obtainable from one rat with a body weight of ~200 g, was injected 1 day prior to irradiation and cell transfer. To examine the therapeutic effect of DST on GvHD, recipient rats received a purified IgM mAb against donor RT1.A^a, which was newly prepared in this study (DST IgM mAb), 4 or 7 days after the induction of GvHD. We defined rat survival time as the time from the start of the experiment to the time that we observed body weight loss of >10% in 3 days, plus an additional 5 days, which we assumed they would have survived (18). Rats were euthanized 1 day before this defined survival time.

Kinetics and antigen specificity of T_{reg} cells after DST

To determine their regulatory function, $CD4^+CD25^{hi}$ cells were isolated as T_{reg} cells from the recipient spleen 3 days after DST by FACSAria and $CD25^-CD4^+$ T cells were isolated as responder T cells from untreated recipient spleens by FACSAria. Most isolated $CD4^+CD25^{hi}$ cells were confirmed to be $FOXP3^+$. $CD103^+$ cells were isolated from normal donor spleen as stimulator DCs by autoMACS. Different numbers of T_{reg} cells were added to the allogeneic mixed leukocyte cultures of donor DCs and recipient T cells. The proliferation of responder T cells was measured as relative light units (rlu)

per second after 5 days using a cell proliferation ELISA kit for BrdU (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions.

Image analysis and statistics

Each parameter was measured in a blinded fashion and expressed as the mean ± SD (n = 3–4 rats). For estimation of the surface areas, image analysis was performed with digital image analysis software (cellSens; Olympus) or on a personal computer using the public domain NIH Image program (Image J1.36b). For FACS analysis, each assay was repeated three times. Statistical analysis was performed using the Student's t-test.

Results

Characterization of DST-antibodies

As expected, by FCM analysis using donor TDLs as target cells (Fig. 1), we found DST-antibodies specific for donor cells in the sera of recipient rats that underwent DST. DST-antibodies were mainly IgM class and became detectable in the blood from 5 days after DST and reached peak concentration on day 7 (Fig. 1A). The IgM switched to IgG_1, IgG_{2a}, IgG_{2b} and IgG_{2c} subclasses (Fig. 1B) from days 7 to 14, but not to IgA or IgE (data not shown).

DST-sera collected 7 or 14 days after DST bound to donor MHCI plasmid-transfected L cells but not to control untreated L cells (Fig. 1E). These results demonstrate that DST-antibodies are composed of IgM and class-switched IgGs and confirmed that DST-antibodies can specifically react with donor MHCI.

DST-antibodies were produced mainly in the spleen

The spleen dependency of DST-antibody production was examined. First, splenectomy at the time of DST reduced serum IgM antibody production in the recipient. The reactivity of the 20× diluted sera of splenectomized rats was almost the same as that of 1620× diluted sera of control rats (DST only), indicating that the IgM titer was reduced to ~1/80th of that of controls (Fig. 1C). Instead, these rats produced enhanced level of IgG_1 and comparable levels of other IgG subclasses (Fig. 1C). Second, when spleen cells were isolated from the recipients 5 days after DST and cultured overnight, the supernatant yielded a significant amount of DST-antibody of mainly IgM class (Fig. 1D). Third, DST-antibody-producing IgM hybridomas could be generated with ease from the same spleen cells (Fig. 1D and E). These results indicate that the spleen is the major site for IgM-class DST-antibody production. Other SLOs mainly produce IgG-class DST-antibodies.

FCM analysis of the splenic immune response

FCM analysis of the recipient spleen cells after DST revealed the proliferative responses of $CD4^+$ T cells at days 3–5 and $CD8\beta^+$ T cells and $CD45R^+$ B cells on day 5 after DST (Fig. 2A and B). In addition, a significant increase in the number of EdU^+FOXP3^+ cells, representing proliferating rat T_{reg} cells (10), was observed on day 3 (Fig. 2), as described later.

Immunohistological analysis of the splenic immune response

Immunohistology of the spleens (Fig. 3) revealed that the proliferative response of T-cell lineages began in the PALS at 1.5 days with a peak 3–4 days (Fig. 3A–E) after DST. On day 3, the proliferating cells were mostly $TCR\alpha\beta^+CD4^+$ T cells (Fig. 3E–G), with fewer $CD8^+$ T cells (Fig. 3H), $CD45R^+$ B cells (Fig. 3I) and $CD162a^+$ NK/NKT cells (data not shown). These results confirmed the FCM data.

On the other hand, $CD45R^+$ B cells showed active proliferation in the outer PALS on day 5 (Fig. 4C and D) and in the germinal centers on days 5 (Fig. 4C) and 7. The proliferative response of AFCs observed in the outer PALS, peaked at day 5 (Fig. 4A and B). $BrdU^+IgM^+$ AFCs (Fig. 4E–G) gradually increased in number, peaking at day 5. Notably, $BrdU^+IgG_{2b}^+$ (Fig. 4I and J) and $BrdU^+IgG_{2c}^+$ (data not shown) AFCs appeared and increased in number (Fig. 4H) on day 5. Most AFCs were strongly Ig-positive probably due to an abundance of cytoplasmic immunoglobulin. These AFCs further migrated to the red pulp thereafter. These results indicate a proliferative response of B cells and of AFCs with sequential class switching of IgM to IgG subclasses in the outer PALS after the $CD4^+$ T-cell response in the PALS.

On days 5 and 7, the number of germinal centers increased in the follicular area (Fig. 4K and L) and expanded in size with many $BrdU^+CD45R^+$ B cells (data not shown). Notably, granular staining of both donor MHCI (Fig. 4M) and IgG (data not shown) was observed, suggesting the immune complex formation of donor MHCI antigen and DST-antibodies. The number of the germinal centers/surface area of the spleen (mm^2) significantly increased by days 5 and 7 (Fig. 4K).

Recipient proliferative response occurs in the DC–T-cell cluster

We examined a mutual relationship between proliferating cells and resident DCs in the PALS (Fig. 5A–D). Notably, the majority of proliferating ($BrdU^+$) cells clustered with recipient $MHCII^+$ cells (Fig. 5B), which were mostly $CD103^+CD11b^+CD4^-$ DCs (Fig. 5F–H). The number of $BrdU^+$ cells (Fig. 3A) and the clusters in the PALS significantly and simultaneously increased on day 1.5 with a peak 3–4 days (Fig. 5E). Because the proliferating cells in the PALS at the early stage were mostly $CD4^+$ T cells (Figs 2B, 3E and G) and most EdU^+ cells in the cluster were $CD4^+$ (Fig. 5I), these results indicate that recipient $CD4^+$ T cells become activated and start proliferation within the clusters with recipient DCs.

DST-antibodies can deplete donor lymphocytes in vitro and in vivo

IgM-dominant sera at day 7 and the IgG fraction at day 14 had a complement-dependent cytolytic activity (Fig. 6A). Among rat IgG subclasses, IgG_{2b} and IgG_{2c} are complement-fixing types (19). Thus, DST-antibodies may have depleting effects on donor cells *in vivo*. To examine this, recipient rats received an intravenous injection of donor TDLs as target cells 7 days after DST. Donor TDLs quickly disappeared from the blood by 15 min and did not migrate to the lymphoid organs of DST recipients (Fig. 6B). When TDLs were transferred to naive

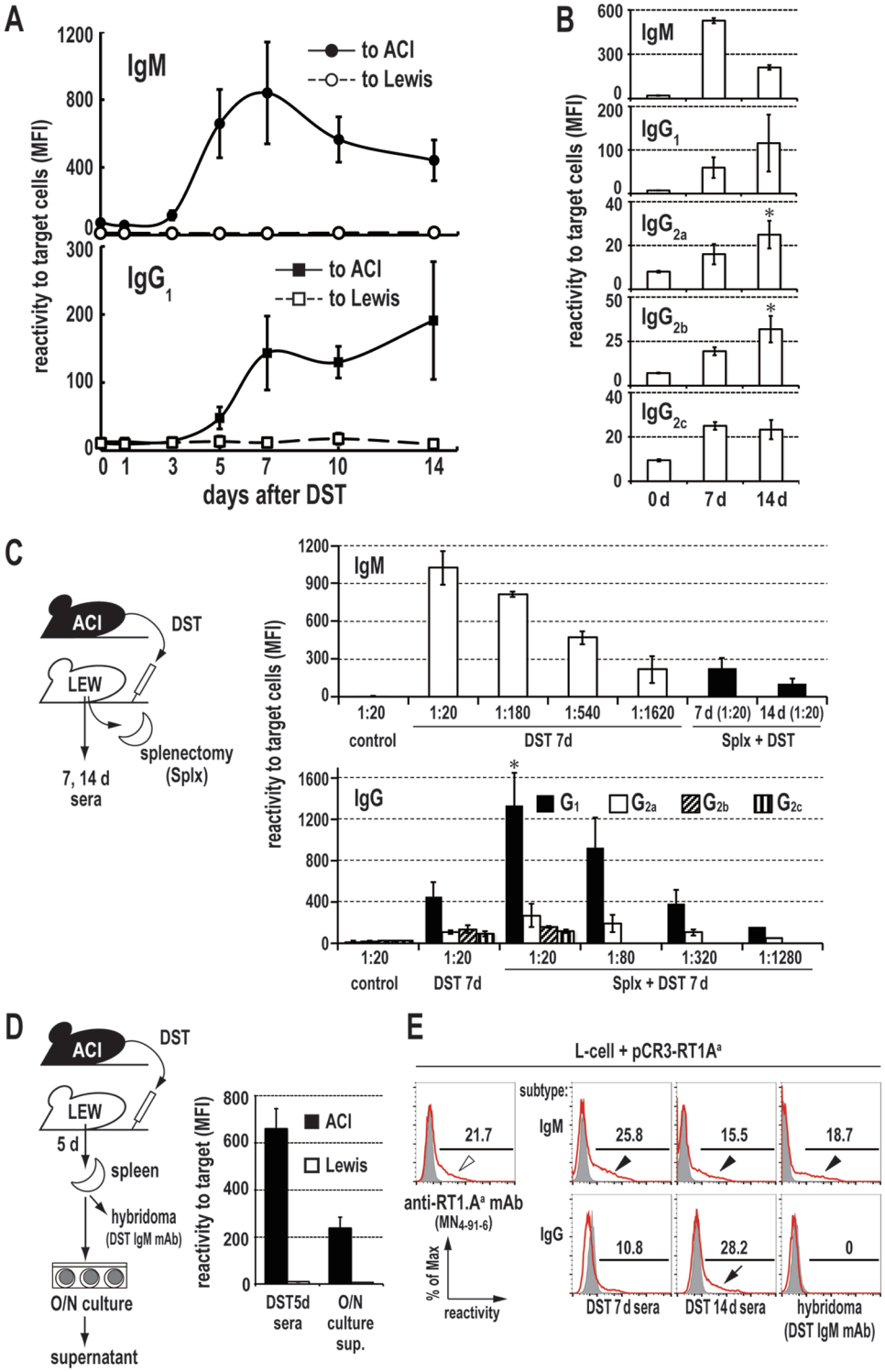


Fig. 1. Characterization of DST-induced alloantibodies. (A) Presence of donor-specific alloantibodies in the recipient blood after DST. IgM or IgG_1 antibodies reacted with TDLs from ACI rats but not those of Lewis rats. (B) Immunoglobulin subclass of DST-induced antibodies. The IgM was switched to IgG_1, IgG_{2a}, IgG_{2b} and IgG_{2c} subclasses but not to IgA or IgE (data not shown), from days 7 to 14. (C) Effect of splenectomy (Splx) in the recipient at the time of DST. The IgM reactivity of the 20× diluted sera in splenectomized rats was almost the same as that of 1620× diluted sera in recipients without Splx. In contrast, the splenectomized rats produced enhanced levels of IgG_1 and comparable levels of other IgG subclasses. (D) Overnight (O/N) culture or hybridoma production (DST IgM mAb) of spleen cells from recipients 5 days after DST. The culture supernatant yielded a significant amount of DST-antibody, mainly IgM class. (E) Binding of antibodies to donor MHCI ($RT1.A^a$) plasmid-transfected L cells (L cell + pCR3-$RT1.A^a$). Not only anti-$RT1.A^a$ mAb but also DST-sera and DST hybridoma culture supernatant (DST IgM mAb) bound to the transfected L cells (red line) but not to control untreated L cells (filled histogram). The values in A–D represent the mean ± SD, $n = 3$ rats each. *$P < 0.05$ (B, 7 days versus 14 days; C, DST 7 days versus Splx + DST 7 days).

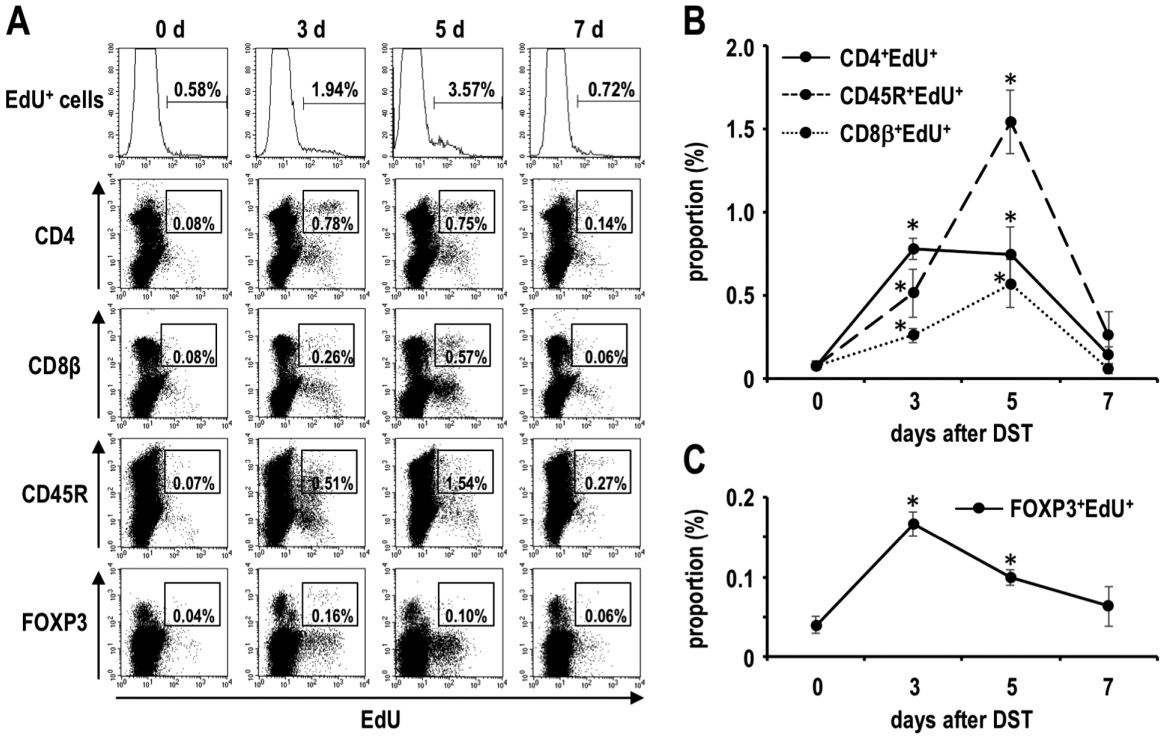


Fig. 2. Two-color FCM analysis of the recipient splenic lymphocytes after DST, for $CD4^+$ T cells, $CD8\beta^+$ T cells, $CD45R^+$ B cells or T_{reg} cells ($FOXP3^+$) and for proliferating cells (EdU^+). (A) Whole view of FCM data 0, 3, 5 and 7 days after DST. Representative data from three individual experiments are shown. (B, C) The time kinetics for proportions of each proliferating cell type/total splenocytes. The proliferation of $CD4^+$ T cells and T_{reg} cells peaked at day 3, whereas B cells and $CD8\beta^+$ T cells peaked at day 5 (mean ± SD, $n = 3$ rats each, $^*P < 0.05$).

recipients, donor cells remained in the blood for longer than 6 h (Fig. 6B) and migrated systemically to recipient lymphoid organs (Fig. 6D). This inhibition was at least partly donor-specific because third-party donor cell migration was also partly affected (Fig. 6C). Some cross-reactivity among different MHCI epitopes is often observed in rodents (20).

DST-antibodies can suppress acute GvHD caused by donor passenger T cells

In GvHD after bone marrow transplantation, the major pathology is caused by graft-derived donor passenger T cells that have migrated to recipient lymphoid organs and become activated by recipient DCs via direct allorecognition (13). Because DST pre-treatment could eliminate donor TDLs, we investigated an effect of DST in an acute GvHD model (13) in which donor T cells are transferred to immunocompromised recipients as passenger leukocytes. Control recipients without pre-treatment suffered from GvHD with a mean survival time of 11 ± 1 days (Fig. 7A). When recipient rats received DST or DST-treated sera 7 days or 1 day, respectively, prior to irradiation and cell transfer (Fig. 7A), the donor cell migration was completely suppressed (Fig. 7C and D) and the recipients did not exhibit any sign of GvHD, surviving >100 days (Fig. 7B).

When a newly generated DST IgM mAb specific for donor MHCI (Fig. 1E) was intravenously injected (5 mg/100 g body weight) once on day 4 at the beginning of the effector stage, it suppressed the GvHD, whereas the same treatment on day 7, after effector cells entered the target organs, only partly suppressed GvHD (Fig. 7F and G). Thus, the results show that DST-antibodies have a depleting effect on donor cells in the blood, demonstrating a preventive effect at the time of cell transfer and a therapeutic effect at an early stage after the onset of the disease.

T_{reg} cells suppress allogeneic mixed leukocyte cultures in a donor-specific manner

FCM analysis showed a significant increase in splenic EdU^+FOXP3^+ proliferating T_{reg} cells 3 days after DST (Fig. 2A). This response was confined to the PALS, being immunohistologically revealed as a significant increase in the number of $BrdU^+FOXP3^+$ cells on day 3 (Fig. 8A–C). A preliminary study showed a presence of proliferative responses of recipient T cells and T_{reg} cells in the T-cell area of the peripheral LNs (Y. Kitazawa, unpublished data).

Isolated $CD4^+CD25^{hi}$ cells from the recipient spleen 3 days after DST, which were mostly $FOXP3^+$, demonstrated a significant donor-specific inhibition of recipient T-cell proliferation, even with a very low number of cells (~1/128 × 10^5), in the allogeneic mixed leukocyte cultures (Fig. 8D and E). In contrast, naturally occurring T_{reg} cells, sorted as $CD4^+CD25^{hi}$ cells from the untreated recipient spleen, exhibited some inhibition only at a high number of cells (1–1/4 × 10^5) (Fig. 8D and E). The results indicate that proliferation of donor-specific functional T_{reg} cells was induced mainly in the PALS solely by DST.

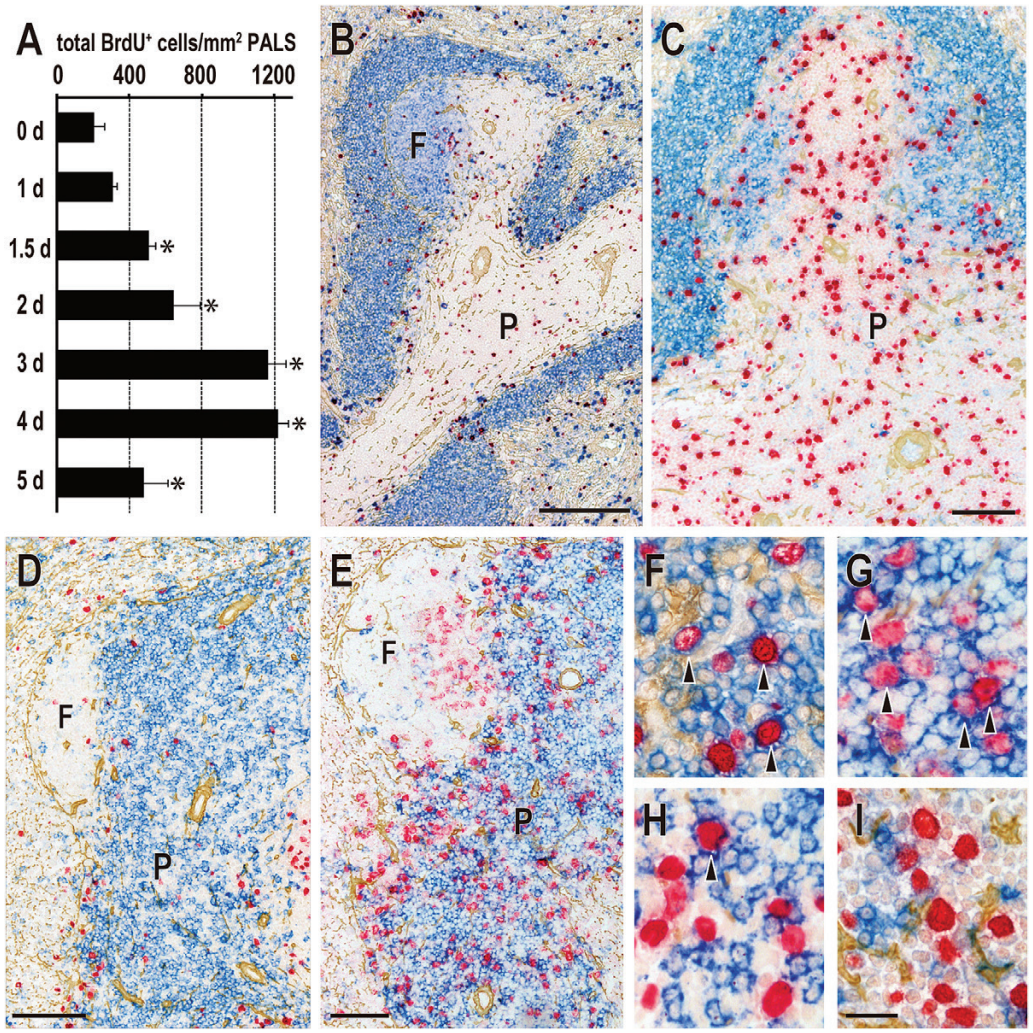


Fig. 3. Proliferative response of CD4⁺ T cells in the splenic PALS after DST. (A) Kinetics of BrdU⁺ cells in the PALS, indicating a significant increase in proliferating cells from 1.5 days with a peak 3–4 days (mean ± SD, $n = 3$ rats each, $^*P < 0.05$). (B–I) Triple immunostaining for IgM (B, C), CD4 (D, E, G), TCRαβ (F), CD8β (H), or CD45R (I) (blue), BrdU (red) and type IV collagen (brown) of spleen, 0 (B, D) and 3 days (C, E–I) after DST. Arrowheads indicate proliferating (BrdU⁺) TCRαβ⁺ (F), CD4⁺ (G) and CD8β⁺ cells (H) in the PALS. F, lymph follicle; P, splenic PALS. Scale bars = 250 μm (B), 100 μm (C–E) or 20 μm (F–I).

Discussion

The present study has demonstrated several novel findings: first, DST-antibodies of mainly IgM class specific for donor MHCI were produced in the recipient spleen. In splenectomized recipients, DST-antibodies of mainly IgG subclasses were produced. Second, in the rat PALS, a proliferative response of CD4⁺ T cells and FOXP3⁺ T_{reg} cells in the PALS, that of B cells and IgM⁺ or IgG⁺ AFCs in the outer PALS, and the germinal center reaction in the lymph follicle were observed sequentially. Third, the recipient T-cell response originated in the recipient DC–T-cell cluster. Fourth, DST-antibodies had depleting effects on donor lymphocytes in the blood, by which they had preventive and partial therapeutic effects on GvHD induced by donor T-cell injection of the recipients. Finally, DST also induced FOXP3⁺ donor-specific T_{reg} cell responses in the PALS.

As demonstrated here, the recipient spleen is a major site for DST-antibody production. Proliferating AFCs in the outer PALS are considered to be mostly cytoplasmic Ig⁺ plasmablasts because the outer PALS is known as a site of the plasma-cellular reaction in many species (8, 9). These AFCs may mostly produce DST-antibodies, because their kinetics was almost the same as that of the serum DST-antibody response and DST-antibody production is, for the most part, spleen-dependent. Sequential observations of the CD4⁺ T-cell response, the appearance of AFCs with class switching and the germinal center reaction together with the FCM study (Fig. 2) indicate that the CD4⁺ helper T-cell-dependent antibody response occurs in the splenic white pulp after DST. The T-cell dependency of this response in rats was also reported previously (14). Although the CD8β⁺ T-cell response was also observed at day 5, it occurred more slowly and less intensely than that via the direct allorecognition reported previously (data not shown) (7).

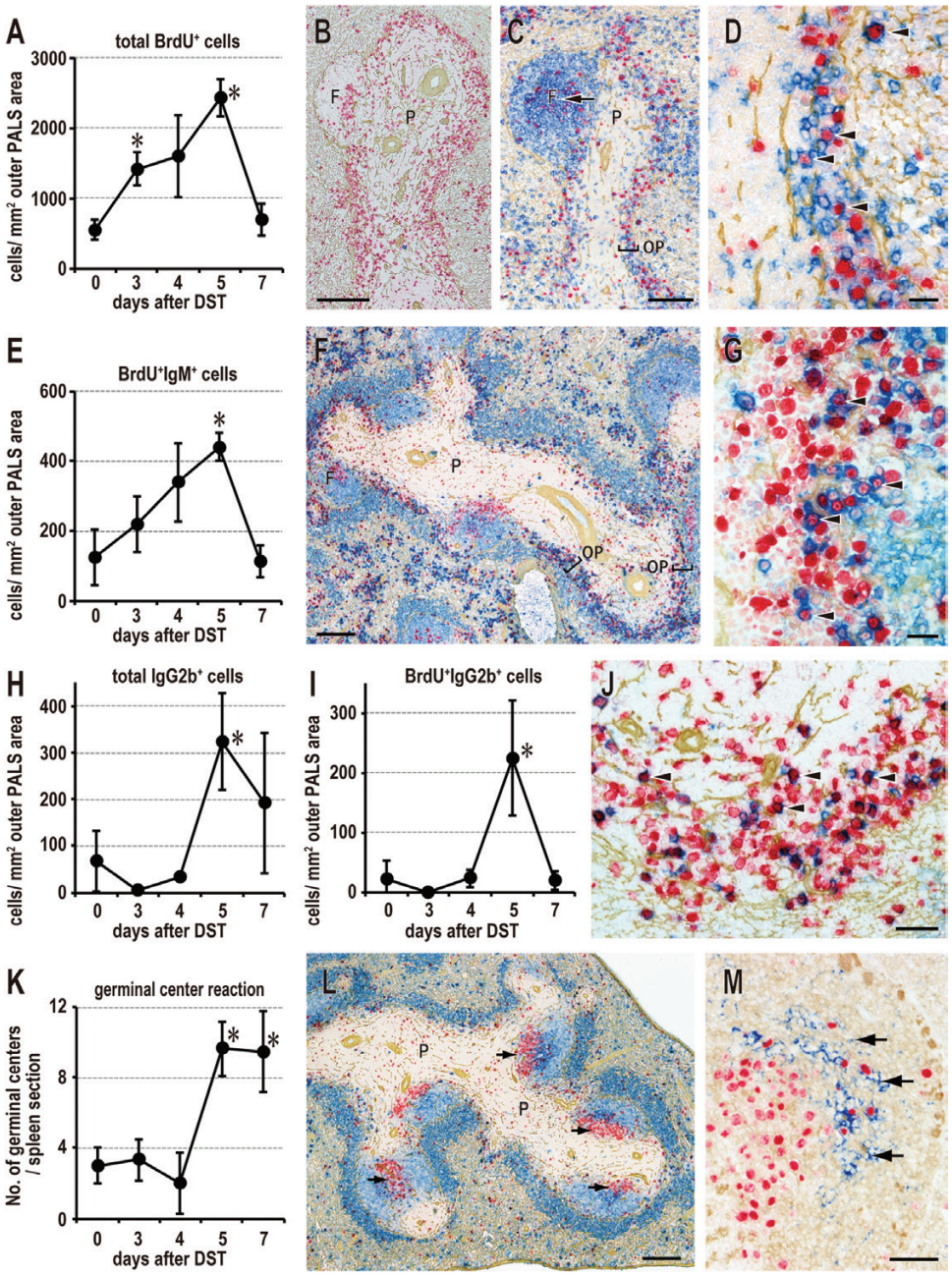
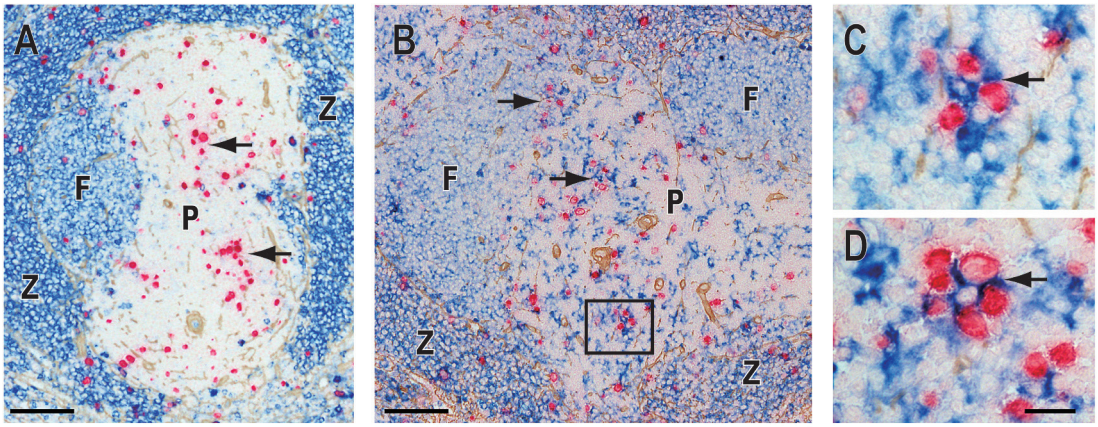
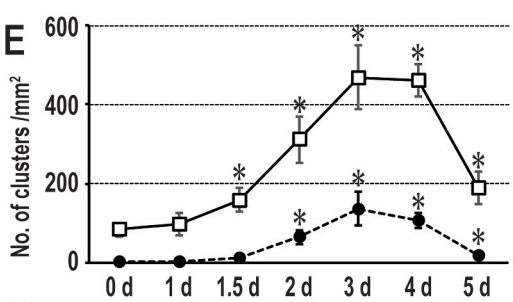


Fig. 4. Proliferative response of B-cell lineages in the spleen after DST. (A, E, I, H) Kinetics of the number of proliferating total cells (A, $BrdU^+$), IgM^+ cells (E), and $IgG_{2b}{}^+$ cells (I) and total $IgG_{2b}{}^+$ cells (H) in the outer PALS (mm^2). (K) Kinetics of germinal center number/spleen section. The values represent the mean ± SD, $n = 3$ rats each, $^*P < 0.05$ compared with day 0. (B–D, F, G, J, L, M) Triple immunostaining for CD45R (C, D), IgM (F, G, L), or donor MHCI (M) (blue), BrdU (red) and type IV collagen (brown) of spleen, at days 5 (B–D, F, G, J, M) and 7 (L) after DST. The number of proliferating total cells (A, B), IgM^+ cells (E–G), and $IgG_{2b}{}^+$ cells (I, J) and of total $IgG_{2b}{}^+$ cells (H) in the outer PALS increased significantly at day 5. $CD45R^+$ B cells exhibited active proliferation in the outer PALS and in the germinal center (arrow) on day 5 (C, D). Arrowheads indicate $BrdU^+CD45R^+$ B cells (D), $BrdU^+IgM^+$ cells (G) or $BrdU^+IgG_{2b}{}^+$ cells (J). The number of germinal centers increased in the follicular area by days 5 and 7 (K) and expanded in size with accumulation of $BrdU^+$ cells (arrow) (L). (M) Blue granular staining of donor MHCI antigen (arrow) suggests immune complex formation with DST-antibodies. F, lymph follicle; P, PALS; OP, outer PALS. Scale bars = 250 μm (B), 200 μm (F, L), 100 μm (C), 50 μm (J, M) or 20 μm (D, G).

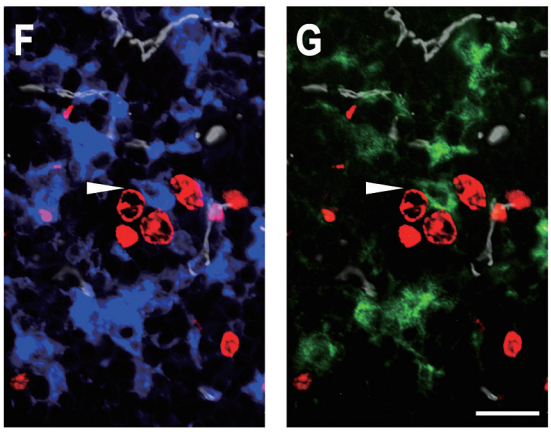
Concerning a site for the differentiation of B cells into AFCs, we speculate two possibilities. First, B cells activated by T cells and antigens may directly migrate to the outer PALS where they differentiate to AFCs. In this context, the early migration of antigen-activated B cells into the B–T boundary including the outer PALS via a new chemokine axis in mice (21) as well as the appearance of $BrdU^+$ B cells in this study (Fig. 4C and D) may support this. Second, a migration pathway to the outer PALS via the germinal center may also exist. Further studies are awaited.





H

	CD103	CD11b	CD4
No. of mAb⁺ cells / recipient MHCII⁺cells forming cluster	35/42, 29/42, 28/44	14/15, 18/19, 24/24	6/21, 3/24, 5/31
% positive	72.0 ± 10.2	96.0 ± 3.5	19.1 ± 8.4



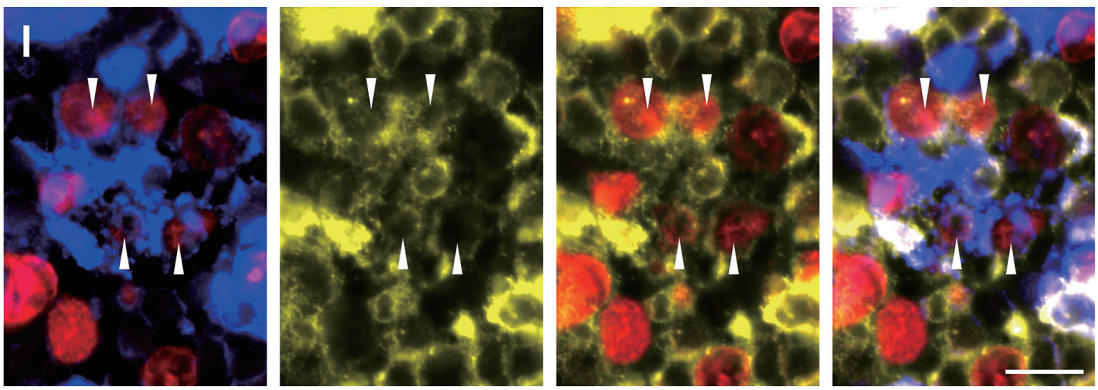


Fig. 5. A mutual relationship between proliferating cells and resident DCs in the PALS. (A–D) Triple immunostaining of the spleen 2 days after DST for IgM (A) or recipient MHCII (blue) (B–D), BrdU (red) and type IV collagen (brown). (A) Focal accumulations of BrdU⁺ cells (arrow) in the PALS (P) suggest cluster formation. (B–D) Most of the proliferating (BrdU⁺, red) cells in the PALS clustered with recipient MHCII⁺ cells (blue, arrow). (C) A closer view of the framed area in B. (E) Kinetics of cluster-forming recipient MHCII⁺ cells per mm² PALS. Open square and closed circle represent the number of recipient MHCII⁺ cells clustering with 1 and ≥2 BrdU⁺ cells, respectively. The kinetics of the clusters (E) was in parallel with that of the BrdU⁺ cell response in the PALS (Fig. 3A). Mean ± SD, $n = 3$ rats each, $^*P < 0.05$ compared with day 0. (F, G) Four-color immunofluorescence staining of the recipient MHCII (blue) or DC marker (CD103, green), EdU (red) and type IV collagen (gray) in the PALS 2 days after DST. The arrowhead indicates an MHCII⁺ cell forming a cluster with EdU⁺ cells is CD103⁺. (H) The phenotype of the cluster-forming MHCII⁺ cells was mostly CD103⁺CD11b⁺CD4⁻. Mean ± SD, $n = 3$ rats each. (I) Four-color immunofluorescence staining of the recipient MHCII (blue), EdU (red), CD4 (yellow), and type IV collagen (gray) in the PALS 2 days after DST showing that EdU⁺ cells (arrow) clustering with a recipient MHCII⁺ cell (blue) were mostly CD4⁺ (yellow). Scale bars = 100 μm (A, B), 20 μm (C, D, F, G) or 10 μm (I).

The beginning of CD4⁺ T-cell response in the recipient DC–T-cell cluster strongly suggests that this cluster represents the site of the indirect allorecognition (7, 22) in which recipient DCs, having captured the donor MHCI antigens, present them on their MHC molecules to recipient T cells. The direct allorecognition may be not involved, because of

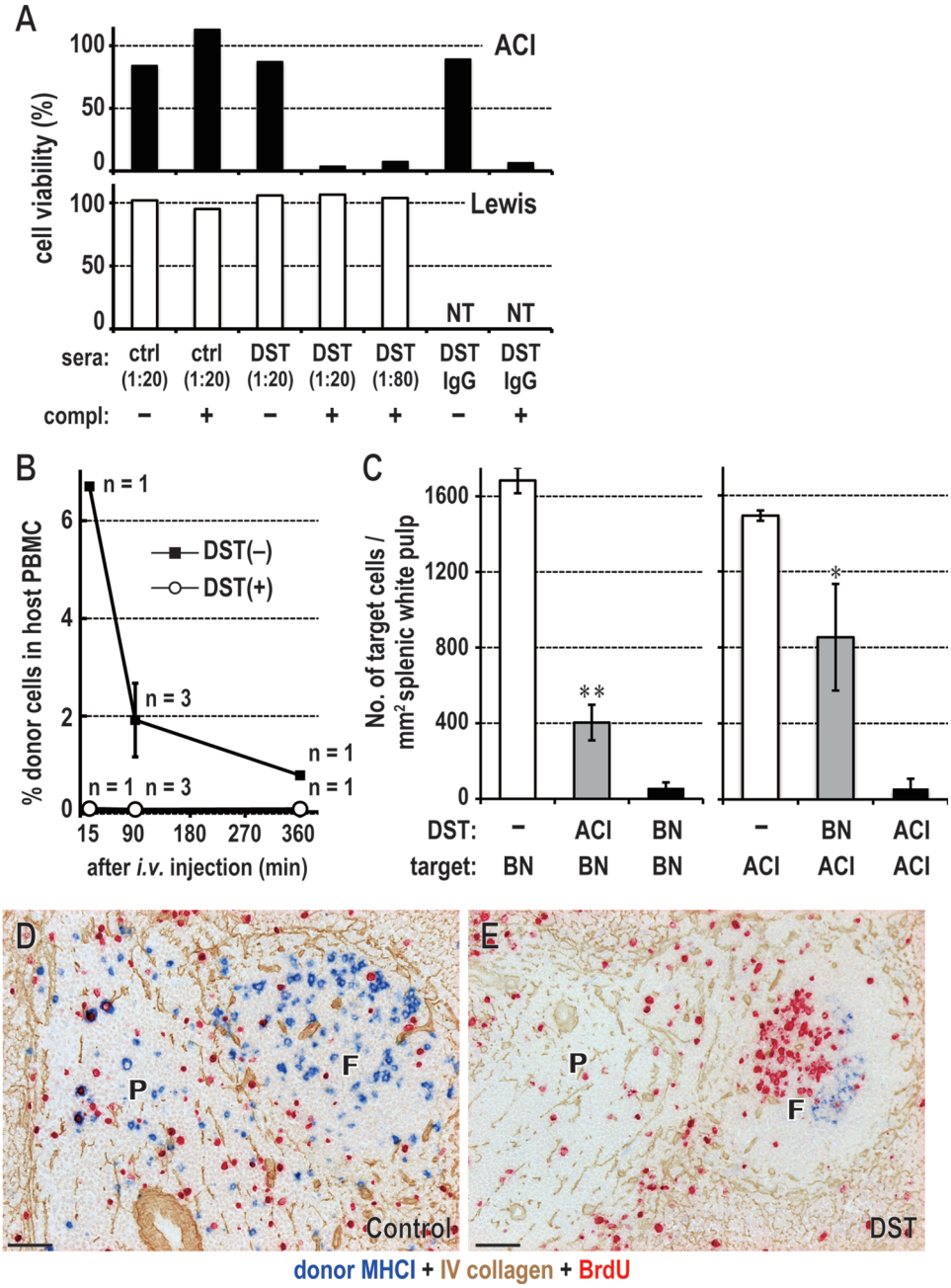


Fig. 6. Specific *in vitro* killing or *in vivo* depletion of donor lymphocytes by DST-sera collected at day 7 after DST. (A) Donor TDLs but not recipient TDLs were killed in a complement-dependent manner *in vitro*. The IgG fraction of day 14 sera (DST-IgG) also exhibited the complement-dependent killing. Representative data from three individual experiments are shown. (B) Donor TDLs quickly disappeared from recipient blood at 7 days after DST compared with their gradual decrease without DST. (C) Rat strain specificity of the DST effect. Lewis rats that underwent DST with ACI blood exhibited inhibition of lymphocyte migration of the ACI strain, and partly that for the BN strain. In contrast, Lewis rats that underwent DST with BN blood exhibited inhibition of lymphocyte migration of the BN strain, and only partly that for the ACI strain (mean ± SD, $n = 3$ rats each, $^*P < 0.05$, $^{**}P < 0.01$). (D, E) Triple immunostaining for donor MHCI (blue), BrdU (red) and type IV collagen (brown). Donor MHCI$^+$ cells accumulated in the splenic PALS (P) and lymph follicle (F) 1 day after TDL transfer in control rats without DST (D). In recipient rats 7 days after DST (E), donor MHCI$^+$ cells were not found in the spleen. Notably, granular MHCI$^+$ fragments were observed suggesting immune complex formation (E). Scale bars = 50 μm.

a paucity of DCs in the DST blood and the weak CD8$^+$ T-cell response.

Concerning T_{reg} cells, it is unclear but they are presumably also induced by the indirect allorecognition, because the donor-specific T_{reg} cell response occurred simultaneously with the CD4$^+$ response in the PALS on day 3 after DST. The numbers of total FOXP3$^+$ cells per mm^2 were not significantly different among the experimental period (Fig. 8C). This is because the proportion of proliferating BrdU$^+$FOXP3$^+$ cells was ~10% of total FOXP3$^+$ cells on day 3 (Fig. 8C), which

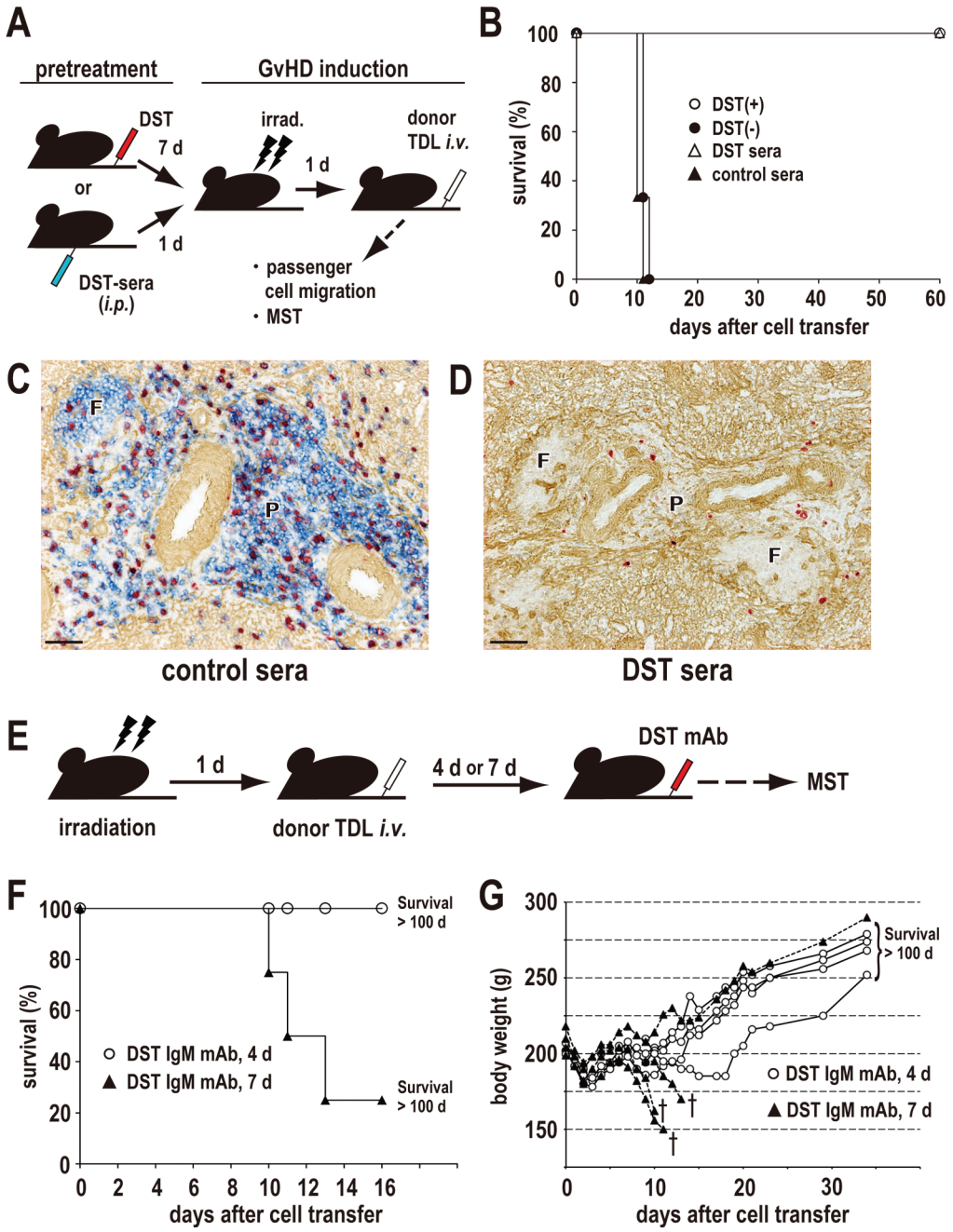


Fig. 7. Suppression of GvHD by DST or DST-antibodies. (A) Scheme showing the experimental protocol for preventive effects. (B–D) Donor cell migration and proliferation in the splenic PALS and death by GvHD in control rats (B, C) were completely blocked by either DST or DST-sera transfer (B, D). *n* = 3 rats each. F, lymph follicle; P, PALS. Scale bars = 50 μm. (E) Scheme showing the experimental protocol for the therapeutic effects of the IgM DST mAb. (F, G) Purified IgM DST mAb (5 mg/100 g body weight) injected at the early stage of GvHD 4 days after induction rescued the rats from the disease completely. When the mAb was injected on day 7 in the late stage of GvHD, only one of four recipients survived. *n* = 4 rats each.

would have little influence on the total number. Although these T_{reg} cells could suppress the allogeneic mixed leukocyte culture, they could not suppress the $CD4^+$ helper T-cell-dependent antibody response *in vivo* in the spleen. This is because the responder cells in the mixed leukocyte culture were naive $CD4^+$ T cells, while $CD4^+$ T cells *in vivo* had been already activated on day 3. Accordingly, we consider that T_{reg} cells on day 3 have not enough activity to suppress the helper function of activated $CD4^+$ T cells in the induction of a B-cell response.

The production of DST-antibodies of IgG subclasses in splenectomized rats indicates that other SLOs can also respond to DST immunization and mainly produce IgG alloantibodies after quickly switching from IgM. This suggests that

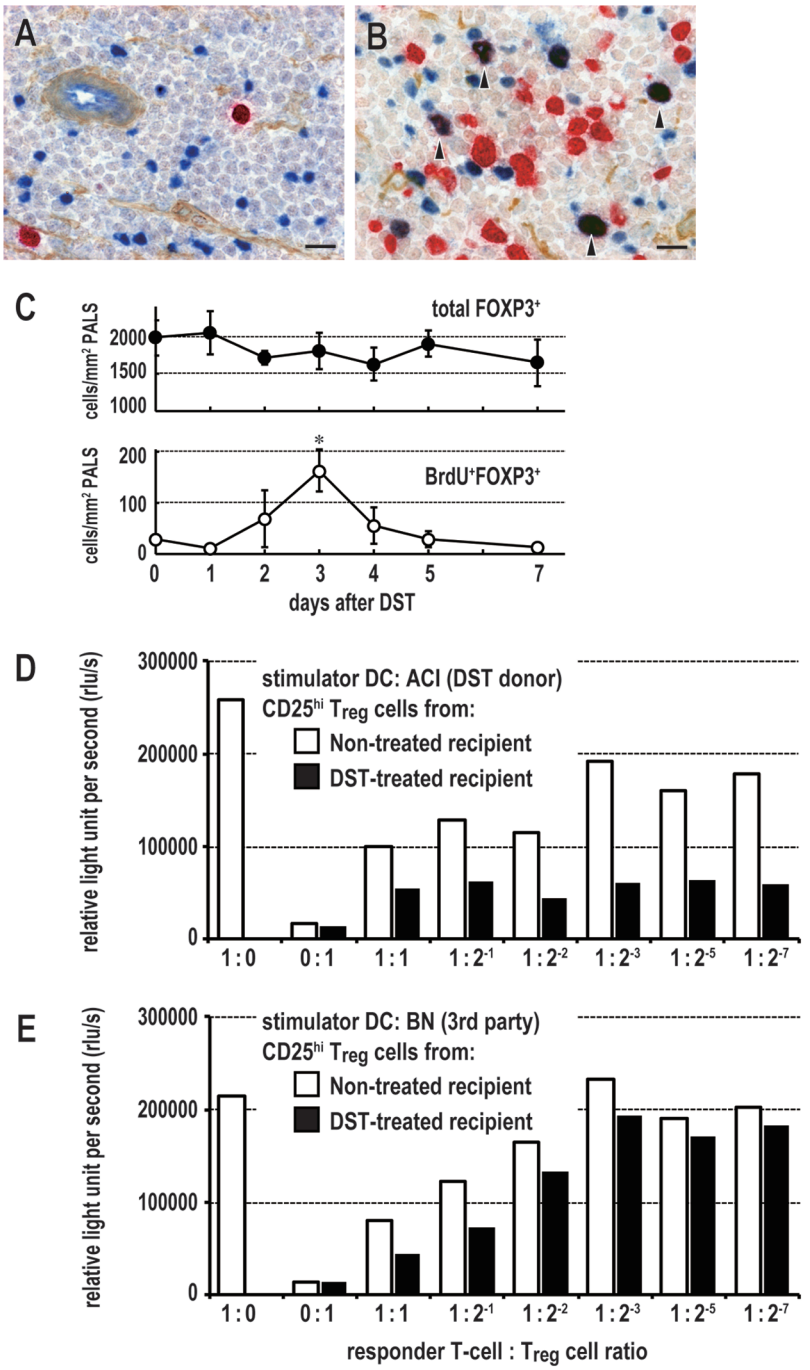


Fig. 8. Induction of the regulatory T-cell response by DST. (A, B) Triple immunostaining for FOXP3 (blue), BrdU (red) and type IV collagen (brown) in the splenic PALS in control rats (A) and day 3 after DST (B). The arrowhead in B indicates a $BrdU^+FOXP3^+$ cell (large dark purple nucleus) compared with the $BrdU^-FOXP3^+$ cell (small blue nucleus) in A. Scale bars = 20 μm. (C) Kinetics of total $FOXP3^+$ cells per mm^2 and $BrdU^+FOXP3^+$ cells per mm^2 in the PALS, indicating a significant increase in proliferating $FOXP3^+$ cells 3 days after DST (mean ± SD, $n = 3$ rats each, $^*P < 0.05$). (D, E) Donor-specific suppressive activity of recipient T_{reg} cells in mixed leukocyte culture. Different numbers of $CD4^+CD25^{hi}$ cells (1×10^5–$1/128 \times 10^5$) isolated from the recipient spleen 3 days after DST were added to the allogeneic mixed leukocyte cultures of donor DCs (1×10^5) and normal recipient $CD25^-CD4^+$ T cells (1×10^5). The proliferative response was estimated as relative light units (rlu) per second. Even $1/128 \times 10^5$ cells of ACI-DST-treated Lewis T_{reg} cells (closed square) could suppress the proliferative response of Lewis T cells (D), but BN-DST-treated Lewis T_{reg} cells (closed square) did not (E). In contrast, naturally occurring T_{reg} cells, sorted as $CD4^+CD25^{hi}$ cells from the untreated recipient spleen (open square, D and E), exhibited some inhibition only with high numbers of T_{reg} cells (1×10^5–$1/4 \times 10^5$). Representative data from three individual experiments are shown.

a proliferative response of $CD4^+$ T cells and T_{reg} cells may also occur in other SLOs, as found in a preliminary study (Y. Kitazawa, unpublished observation). However, questions arise concerning the induction mechanism of these responses: which components of the donor blood, i.e. white blood cells, platelets, erythrocytes or blood plasma, are immunogenic in

the induction of the DST response, and how the responsible components induce them in the recipient SLOs. This is now under study and will be published soon.

The depleting effects of DST-antibodies suggest that donor passenger cells appearing in the recipient blood following organ transplantation (23) may be thoroughly eliminated by them. In this respect, we previously reported that after rat liver transplantation, graft-derived passenger DCs migrated to recipient SLOs via the blood or lymph and systemically induced a strong $CD8^+$ T-cell response through direct allorecognition, leading to graft rejection (7, 24). This suggests that following liver transplantation, DST-antibodies may deplete passenger DCs, resulting in suppression of the $CD8^+$ T-cell response in the recipient. In this context, the liver is resistant to antibody-mediated rejection relative to the heart (25), and that anti-donor MHC IgG is stronger than IgM in promoting graft rejection (26). Therefore, we consider that the tolerance-inducing effect of DST following liver transplantation may be at least partly due to IgM DST-antibodies via depletion of the graft-derived DCs without serious graft damage. A preliminary study showed that preoperative transfer of DST-primed sera as well as DST pre-treatments eliminated all passenger leukocytes including DCs, leading to the significant suppression of the $CD8^+$ T-cell response in the recipient SLOs with a significant delay of the graft rejection (H. Ueta *et al.*, manuscript in preparation).

The suppressive effects of DST-antibodies in an acute GvHD model may be due to a depletion of donor passenger T cells in the blood soon after transfer and of activated donor effector T cells in the early effector stage. Nakauchi *et al.* (27) reported that anti-donor class I HLA allele-specific mAbs selectively suppress GvHD following allogeneic hematopoietic stem cell transplantation in a humanized mouse model. Notably, the stem cells were relatively resistant to the mAb treatment and their hematopoietic activity recovered quickly. Thus, we suggest that DST-antibodies may also be applicable for the suppression of GvHD in patients following allogeneic hematopoietic stem cell transplantation or liver transplantation (18).

In organ transplantation after DST, the proliferation of T_{reg} cells sufficient to suppress rejection has been reported to take weeks (28). This implies a role of T_{reg} cells in the maintenance of tolerance rather than its induction. Therefore, the early stage after transplantation requires other immunosuppressive protocols, and DST-antibodies may be one of the candidates. Alternatively, a new method for further expansion of T_{reg} cells after DST prior to transplantation would facilitate clinical application of DST.

In conclusion, a single blood transfusion efficiently induces a helper T-cell-dependent anti-donor MHCI AFC response and donor-specific T_{reg} cell response, mainly in the spleen, most probably via indirect allorecognition by resident DCs. Other SLOs may also respond to DST. These antibodies and T_{reg} cells may be involved, at least partly, in the DST-induced suppression of allograft rejection.

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References

1 Welsh, K. L. and Batchelor, J. R. 1978. Assays for antibodies against histocompatibility antigens. In Weir, D. M., ed., *Handbook of Experimental Immunology*, 3rd edn, p. 35.1. Blackwell Scientific Publications, Oxford, UK.
2 Iniotaki-Theodoraki, A. 2001. The role of HLA class I and class II antibodies in renal transplantation. *Nephrol. Dial. Transplant.* 16(Suppl. 6):150.
3 Josien, R., Heslan, M., Brouard, S., Soulillou, J. P. and Cuturi, M. C. 1998. Critical requirement for graft passenger leukocytes in allograft tolerance induced by donor blood transfusion. *Blood* 92:4539.
4 Jovičić, S., Ležaić, V., Simonović, R. and Djukanović, L. 2011. Beneficial effects of donor-specific transfusion on renal allograft outcome. *Clin. Transplant.* 25:317.
5 Siemionow, M. and Agaoglu, G. 2005. Role of blood transfusion in transplantation: a review. *J. Reconstr. Microsurg.* 21:555.
6 Lechler, R., Ng, W. F. and Steinman, R. M. 2001. Dendritic cells in transplantation–friend or foe? *Immunity* 14:357.
7 Ueta, H., Shi, C., Miyanari, N. *et al.* 2008. Systemic transmigration of allosensitizing donor dendritic cells to host secondary lymphoid organs after rat liver transplantation. *Hepatology* 47:1352.
8 Claassen, E., Kors, N. and Van Rooijen, N. 1986. Influence of carriers on the development and localization of anti-trinitrophenyl antibody-forming cells in the murine spleen. *Eur. J. Immunol.* 16:271.
9 Matsuno, K., Ezaki, T. and Kotani, M. 1989. Splenic outer periarterial lymphoid sheath (PALS): an immunoproliferative microenvironment constituted by antigen-laden marginal metallophils and ED2-positive macrophages in the rat. *Cell Tissue Res.* 257:459.
10 Kitazawa, Y., Ueta, H., Hünig, T., Sawanobori, Y. and Matsuno, K. 2015. A novel multicolor immunostaining method using ethynyl deoxyuridine for analysis of in situ immunoproliferative response. *Histochem. Cell Biol.* 144:195.
11 Hontsu, S., Yoneyama, H., Ueha, S. *et al.* 2004. Visualization of naturally occurring Foxp3+ regulatory T cells in normal and tumor-bearing mice. *Int. Immunopharmacol.* 4:1785.
12 Ford, W. L. and Hunt, S. V. 1973. The preparation and labelling of lymphocytes. In Weir, D. M., ed., *Handbook of experimental immunology*, 2nd edn, p. 23.1. Blackwell Scientific Publications, Oxford, UK.
13 Zhou, S., Ueta, H., Xu, X. D., Shi, C. and Matsuno, K. 2008. Predominant donor CD103+CD8+ T cell infiltration into the gut epithelium during acute GvHD: a role of gut lymph nodes. *Int. Immunol.* 20:385.
14 Rolstad, B., Williams, A. F. and Ford, W. L. 1974. The alloantibody response to a strong transplantation antigen (Ag-B). Quantitative aspects and thymus dependence of the response. *Transplantation* 17:416.
15 Rada, C., Lorenzi, R., Powis, S. J., van den Bogaerde, J., Parham, P. and Howard, J. C. 1990. Concerted evolution of class I genes in the major histocompatibility complex of murine rodents. *Proc. Natl Acad. Sci. USA* 87:2167.
16 Saiki, T., Ezaki, T., Ogawa, M. and Matsuno, K. 2001. Trafficking of host- and donor-derived dendritic cells in rat cardiac transplantation: allosensitization in the spleen and hepatic nodes. *Transplantation* 71:1806.
17 Xu, X. D., Ueta, H., Zhou, S. *et al.* 2008. Trafficking of recirculating lymphocytes in the rat liver: rapid transmigration into the portal area and then to the hepatic lymph. *Liver Int.* 28:319.

18 Yu, E., Ueta, H., Kimura, H., Kitazawa, Y., Sawanobori, Y. and Matsuno, K. 2017. Graft-versus-host disease following liver transplantation: development of a high-incidence rat model and a selective prevention method. *Am. J. Transplant.* 17:979.
19 Miyatake, T., Sato, K., Takigami, K. *et al.* 1998. Complement-fixing elicited antibodies are a major component in the pathogenesis of xenograft rejection. *J. Immunol.* 160:4114.
20 D'Orsogna, L. J., Roelen, D. L., Doxiadis, I. I. and Claas, F. H. 2012. TCR cross-reactivity and allorecognition: new insights into the immunogenetics of allorecognition. *Immunogenetics* 64:77.
21 Kelly, L. M., Pereira, J. P., Yi, T., Xu, Y. and Cyster, J. G. 2011. EBI2 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and nonlymphoid tissues. *J. Immunol.* 187:3026.
22 Saiki, T., Ezaki, T., Ogawa, M., Maeda, K., Yagita, H. and Matsuno, K. 2001. *In vivo* roles of donor and host dendritic cells in allogeneic immune response: cluster formation with host proliferating T cells. *J. Leukoc. Biol.* 69:705.
23 Wood, K. J. 2003. Passenger leukocytes and microchimerism: what role in tolerance induction? *Transplantation* 75(9 Suppl.):17S.
24 Yu, B., Ueta, H., Kitazawa, Y. *et al.* 2012. Two immunogenic passenger dendritic cell subsets in the rat liver have distinct trafficking patterns and radiosensitivities. *Hepatology* 56:1532.
25 Furuya, T., Murase, N., Nakamura, K. *et al.* 1992. Preformed lymphocytotoxic antibodies: the effects of class, titer and specificity on liver vs. heart allografts. *Hepatology* 16:1415.
26 Callaghan, C. J., Rouhani, F. J., Negus, M. C. *et al.* 2007. Abrogation of antibody-mediated allograft rejection by regulatory CD4 T cells with indirect allospecificity. *J. Immunol.* 178:2221.
27 Nakauchi, Y., Yamazaki, S., Napier, S. C. *et al.* 2015. Effective treatment against severe graft-versus-host disease with allele-specific anti-HLA monoclonal antibody in a humanized mouse model. *Exp. Hematol.* 43:79.
28 Kitade, H., Kawai, M., Rutgeerts, O. *et al.* 2005. Early presence of regulatory cells in transplanted rats rendered tolerant by donor-specific blood transfusion. *J. Immunol.* 175:4963.