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ADAP deficiency combined with costimulation blockade synergistically protects intestinal allografts

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adhesion and degranulation promoting adapter protein, costimulation blockade, intestinal transplantation, rejection.

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Summary

Adhesion and degranulation promoting adapter protein (ADAP) plays an important role in T cell activation. ADAP deficiency was recently found to prolong heart graft survival in mice. We investigated the role of ADAP in intestinal transplantation and the synergistic effect of ADAP deficiency and Costimulation blockade (CB). T cell proliferation and cytotoxic T lymphocyte (CTL) activity were determined. MHC mismatched intestinal allografts was transplanted heterotopically. Anti-CD40L antibody was applied to the recipient. Upon stimulation with allogenic dendritic cells (DC), ADAP-deficient (ADAP−/−) T cells displayed impaired proliferative responses compared with that of wild-type (WT) T cells. In contrast, the CTL activity in ADAP−/− mice was comparable with that of WT mice. Rejection of intestinal allografts was ameliorated, but not prevented in ADAP−/− mice. Although CB alone was not sufficient to mitigate the rejection, the combination of CB and ADAP deficiency profoundly inhibited rejection. This was accompanied by less infiltration and activation of host lymphocytes in the gut-associated lymphoid tissue of intestinal allografts. ADAP deficiency combined with CB protected the intestinal allografts synergistically. ADAP could be a novel target in the induction phase of the immune responses in organ transplantation.

Introduction

Intestinal transplantation (ITx) is the only alternative therapy for patients with irreversible intestinal failure, who have life-threatening total parental nutrition complications, e.g. TPN-related liver dysfunction and difficulty of central venous access. Clinical ITx was first performed in the 1960s but failed as a result of uncontrolled rejection and infection. Although there is an improvement in the outcome of ITx, because of the introduction of new immunosuppressants such as FK506, as well as refinements in surgical techniques and better postoperative care, the clinical results are not so satisfactory compared with that of other solid organ transplants, e.g. liver and renal transplantation. Refractory rejection remains the main obstacle to gain longer graft and patient survival. Therefore, it is critically needed to explore the immunological basis of ITx rejection and to develop more potent, specific treatment strategies against intestinal allograft rejection.

One member of the adapter protein family involved in T cell activation, adhesion and degranulation promoting adapter protein (ADAP), is a potential target for immunosuppression in intestinal transplantation. Its main function was clarified by two independent research groups by investigating T cell activation, proliferation, and integrin-

mediated adhesion *in vitro* [1,2]. ADAP positively couples the T cell receptor (TCR) signal to activation of integrins (e.g. leukocyte function associated antigen-1, LFA-1). As we have previously found that ADAP deficiency leads to prolongation of heart allograft survival in mice [3], we proceeded to investigate the role of ADAP in the most immunogenic solid organ transplantation model, which is intestinal transplantation.

Costimulation blockade using anti-CD40L monoclonal antibodies (mAb) is an effective protocol for tolerance induction in various transplant models [4,5]. In mouse ITx, however, it failed to prolong graft survival. The underlying mechanism has been attributed to the presence of refractory CD8+ T cells in these mice [6]. In this study, we aimed to test the *in vivo* role of ADAP in the alloimmune response in ITx. In addition, the potential synergistic effect of anti-CD40L mAb and ADAP deficiency in ITx was studied.

Materials and methods

Animals

ADAP deficient mice (ADAP−/−) on the C57BL/6 (B6, H-2b) background (B6.S129-P2-FYB^{tm1Kor}) with a targeted disruption of the ADAP (FYB, SLAP-130) locus were kindly provided by G. Koretzky (Abramson Family Cancer Research Center, Philadelphia, PA, USA). Mice were kept in the Central Animal Laboratory at Hannover Medical School. BALB/c (H-2d) and wild-type (WT) C57BL/6 mice were purchased from the Central Animal Laboratory at Hannover Medical School. All animals were maintained with controlled light/dark cycles, with free access to water and food, and were used at 8–12 weeks of age. All animal manipulations were carried out according to 'Principles of Laboratory animal care' published by NIH.

Bone marrow-derived dendritic cell differentiation and maturation

Bone marrow cells were harvested from tibiae and femurs of C57BL/6 and BALB/c female mice. Cells were seeded at 2×10^6 cells per Petri dish in the presence of 30 ng/ml of recombinant murine Granulocyte Monocyte-colony Stimulating Factor for 7 days. Immature dendritic cells were further matured in the presence of 30 ng/ml of TNFα from day 7 to 9. The extent of DC maturation achieved was routinely monitored by flow cytometry before the cells were used as stimulators in the mixed lymphocyte reaction and for the generation of cytolytic T cells to ensure that the cells obtained expressed characteristic markers of DC maturation (CD11c and high amounts of MHC class II and CD86).

Mixed lymphocyte reaction

RPMI 1640 culture medium was used, with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids (BioWhittaker, Walkersville, MD, USA), 100 U/ml penicillin, 100 μg/ml gentamycin (Life Technologies, Grand Island, NY, USA), 5×10^{-5} M 2-ME (Sigma, St. Louis, MO, USA), and 10% fetal calf serum. Responder murine splenocytes were harvested from wild-type B6 and ADAP−/− mice, and red cells were lysed in ACK (Ammonium Chloride Potassium) lysing buffer (Biowhittaker, Walkersville, MD, USA). Triplicate wells containing 2×10^5 wild-type B6 and ADAP−/− responder cells were incubated with mature bone marrow-derived DC stimulator cells obtained from C57BL/6 and BALB/c mice, which were irradiated with 30 Gy, at 37 °C in a cell culture incubator containing 5% CO_2. Culture plates were pulsed on day 3 with 1 μCi of ^{3}[H] thymidine per well (Amersham Biosciences, Braunschweig, Germany) and the supernatants were harvested 12–18 h later using a Perkin Elmer harvester device. Finally, thymidine incorporation was counted on a Perkin Elmer counter and counts per minute were determined. The mean and standard deviation were calculated from 5 wells.

Cytotoxic T lymphocyte activity assay

MLR was established at day 0 using allogenic DCs as stimulator and effector cells. Responder cells were collected at day 5. Target lymphocytes from B6 or BALB/C were isolated and cultured in a medium containing 2 μg/ml of ConA for 2 days. Target cells were incubated with 3.7 MBq ^{51}Cr for 1 h and re-suspended to the concentration of 5×10^4/ml. A concentration of 100 μl of target cells was added to a 96-well plate and the effector cells were added from an effector/target ratio of 40:1 to 0.75:1 with a final volume of 200 μl. Plates were incubated for 4 h at 37 °C in a humidified 5% CO_2 incubator. Supernatants were harvested using the Skatron Supernatant Collection System (Skatron Instruments, Inc., Sterling, VA, USA) and counted in a gamma counter.

The percentage of cytotoxicity was calculated according to the following formula:

$$100 \times [\text{experimentalcpmrelease} - \text{spontaneouscpmrelease}] / [\text{maximalcpmrelease} - \text{spontaneouscpmrelease}]$$

Heterotopic intestinal transplantation

Mouse heterotopic ITx was performed in a modified technique from He *et al.* [6]. Intestinal allografts from BALB/c mice were transplanted to C57BL/6 wild-type

(WT) and ADAP−/− recipients. In brief, superior mesenteric artery and portal vein were anastomosed to the recipient's abdominal aorta and inferior vena cava, respectively. The distal end of the intestinal graft was anastomosed to the host jejunum. The proximal end of the graft was exteriorized as a stoma.

Experimental groups and treatment

Four groups were included in the study: group 1, untreated WT ($n = 12$); group 2, anti-CD40L mAb in WT recipients (clone MR-1, hamster IgG; BioXcell, Lebanon, NH, USA; 500 μg days 0, 2, 4, and 7 intraperitoneally; $n = 12$); group 3, untreated ADAP−/− ($n = 12$); and group 4, anti-CD40L mAb in ADAP−/− recipients ($n = 12$). Our previous experiments showed that day 14 postoperation is an optimal time point to detect the macro-morphology of intestinal allograft and day 6 postoperation is suitable for flow analysis [7]. Six mice in each group were killed on day 14 post-transplantation for histological analysis and the other six mice were used for flow cytometry analysis on day 6 post-transplantation.

Histological analysis

Recipient mice were killed by CO_2 inhalation. A 3-cm segment of the proximal portion of the graft was embedded in Tissue Tek freezing medium (Jung, Germany). Swiss rolls with the luminal side facing outwards were snap frozen, cross-sectioned, and kept at −20 °C until staining with hematoxylin-eosin. All slides were observed and analyzed blindly according to the histologic criteria described by He *et al.* [8,9]. Rejection grade was scored from 0 to 4: 0, no rejection; 1, minimal infiltrate, scattered apoptotic crypt cells, normal villi; 2, focal crypt destruction, modest infiltrate, and mild villous architectural distortion; 3, massive infiltration, diffuse crypt destruction, focal mucosal ulceration, and moderate vasculitis; 4, near-total mucosal sloughing, severe vasculitis.

Isolation and preparation of cells in GALT

Digestion media:20 ml RPMI 1640 + 20% FCS + 4.8 mg Collagenase A (Roche Diagnostics GmbH, Germany).

Gradient Percoll I: 40% Percoll (Amersham Biosciences) and 60% PBS.

Gradient Percoll II: 70% Percoll and 30% PBS.

Graft mesenteric lymph nodes (MLNs) were mechanically dissociated in FACS buffer, filtered through a nylon strainer, and counted (Neubauer improved counting chamber). Nonviable cells were excluded from analysis on the basis of trypan blue staining. Peyer's Patches (PPs) were taken by curved-tip scissors and were prepared in a fashion identical to that of other lymph nodes. Graft infiltrating lymphocytes in the intestinal graft were harvested on day 6 after transplantation, as described by Pabst *et al.* [10]. To compare the absolute number of infiltrating cells among various groups, a defined length of the intestinal graft (12 cm) was obtained by cutting along the mesenteric side. The corresponding part of host gut was used as control. After dissection of the attached PPs, the intestine was washed gently with cold PBS. The intestine was split longitudinally, washed with cold PBS, minced, and digested in 50 ml digestion media (as previously described) at 37 °C for 90 min on a shaking machine. Thereafter, the supernatant was collected and centrifuged at 750 *g* for 20 min, the cells were suspended by Percoll I, and carefully moved to Percoll II as the upper layer. This was followed by 2000 rpm centrifugation for 20 min. The intermediate layer containing the lymphocytes was harvested and re-suspended in PBS/5% FCS for further analysis.

Flow cytometry

The following list of unlabeled and fluorochrome labeled antibodies was used in flow cytometry: CD4-PerCP (L3T4), CD4-PE (L3T4), CD4-FITC (L3T4), CD8-FITC (53–6.7), CD8β-PE (RmCD8-2), CD8-Bio (53–6.7), CD44-PE (IM7), Kb-FITC (AF6), CD62L-Bio (MEL-14), and streptavidin APC (Caltag Laboratories, Buckingham, UK). A concentration of 5×10^5 cells from each specimen was centrifuged at 200 *g* for 3 min, washed twice in FACS buffer, and incubated with primary mAbs for 20 min at 4 °C. The specimens incubated with biotinylated mAbs were subsequently stained with streptavidin APC. Following washing, all specimens were analyzed on a FACS Calibur TM (Becton Dickinson). Cell number and percentage of positively stained cells were calculated and compared using CellQuest Program software (BD bioscience, Heidelberg, Germany).

Statistical analysis

The rejection grades and cell percentages for different experimental groups were compared using independent-samples *t*-test. FACS data were compared by paired *t*-test. *P*-values of <0.05 were considered significant. Calculations were performed using SPSS 11.5 (Chicago, IL, USA).

Results

Proliferative activity of lymphocytes was impaired in ADAP−/− mice

The difference in spleen cell proliferation between WT and ADAP−/− mice was determined in a one-way mixed lymphocyte reaction (MLR). As shown in Fig. 1, the pro-

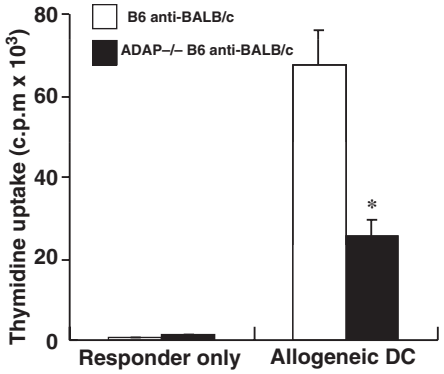

Figure 1 The thymidine incorporation amount (c.p.m.) of responder cells without or with allogenic DC, white column represents WT, and black column represents ADAP–/– mice. There is an evident proliferative defect in ADAP–/– mice compared with WT. The results shown are the mean ± standard deviation (SD) of triplicate samples from a representative experiment from four individual studies, $n = 5$ in each group. $*P < 0.01$.

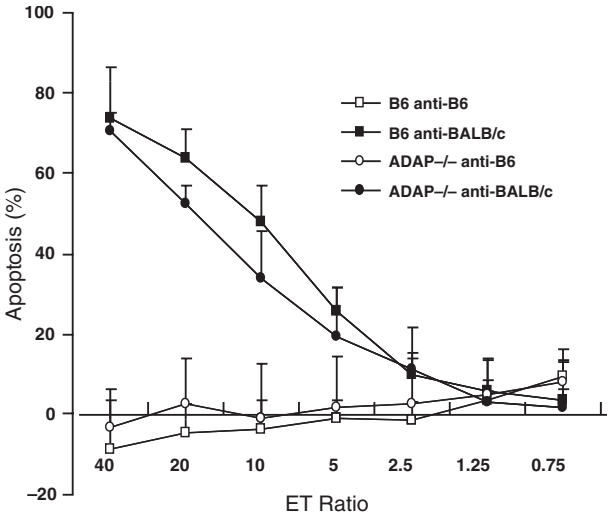

Figure 2 CTL activity in ADAP–/– mice was not impaired compared with that in WT mice. Splenic cells were cultured with irradiated (30 Gy) donor DC (at a ratio of 40:1) for 5 days, and used as effectors. CTL activity was determined in a Cr51 assay using BALB/c and B6 ConA blasts as donor-specific and syngeneic targets, respectively. Result is the representative of four independent experiments. White square: B6 anti-B6; white circle: ADAP–/– anti-B6; black square: B6 anti-BALB/c; black circle: ADAP–/– anti-BALB/c.

liferation of both WT and ADAP–/– responder cells without DC stimulation was negligible. After stimulation of allogenic DC, WT lymphocytes proliferated prominently, while the extent of thymidine incorporation of ADAP–/– lymphocytes was significantly decreased in comparison.

Intact CTL activity in ADAP–/– mice as well as WT mice

In contrast to the defect in proliferative ability, ADAP–/– mice can generate effective CTL after allogenic DC stimulation in comparison with WT mice. As shown in Fig. 2, there was no significant difference in the specific lysis of allogenic target cells between ADAP–/– and WT effector cells from a higher effector/target ratio (40:1) to a lower one (0.75:1).

Histological manifestation of intestinal allografts in the four groups

To investigate the rejection of small bowel allografts in ADAP–/– mice, BALB/c intestine was grafted into ADAP–/– and WT recipients. On day 14 after transplantation, the gross morphology of intestinal grafts of untreated WT recipients showed severe distension, massive caseous secretion, blank color, and evident hemorrhage of the PPs. In contrast, in the ADAP–/– recipients, we found an ameliorated gross morphology demonstrated as modest distension and caseous secretion, pale color, and no hemorrhage of the PPs (data not shown).

Graft histology of untreated WT mice showed clear evidence of rejection (mean rejection score 3.7 ± 0.2, $n = 6$): sloughing of the villi, severe mononuclear cell infiltration, loss of Goblet cells, and focal ulceration. In contrast, histology of intestinal allografts in untreated ADAP–/– mice revealed a modest cellular infiltration, focal crypt destruction, and mild villous architectural distortion (mean rejection score 2.6 ± 0.2, $n = 6$, $P < 0.01$ vs. WT control) (Fig. 3b). However, it showed that ADAP deficiency could not prevent rejection of ITx, but rather alleviated rejection severity compared with that in WT recipients. Anti-CD40L treatment in WT mice did not lead to any protection of the intestinal allograft on day 14 post-transplantation (Fig. 3e). Rejection score remained as high as that of untreated WT control (mean rejection score 3.5 ± 0.02, $n = 6$). Histology showed similar manifestation as that in untreated WT mice (Fig. 3d). These findings confirm that MR-1 alone was not efficient in prolonging intestinal graft survival. The same protocol of CD40-CD40L blockade applied in ADAP–/– recipients (Fig. 3c), but showed effective inhibition of ITx rejection with a lowest rejection score compared with that of untreated WT group (1.6 ± 0.2, $n = 6$, $P < 0.001$ vs. WT control). Histology demonstrated intact structure of intestinal villi and well-preserved goblet cells, very mild infiltration, and the absence of cryptitis comparable with syngeneic control (Fig. 3a).

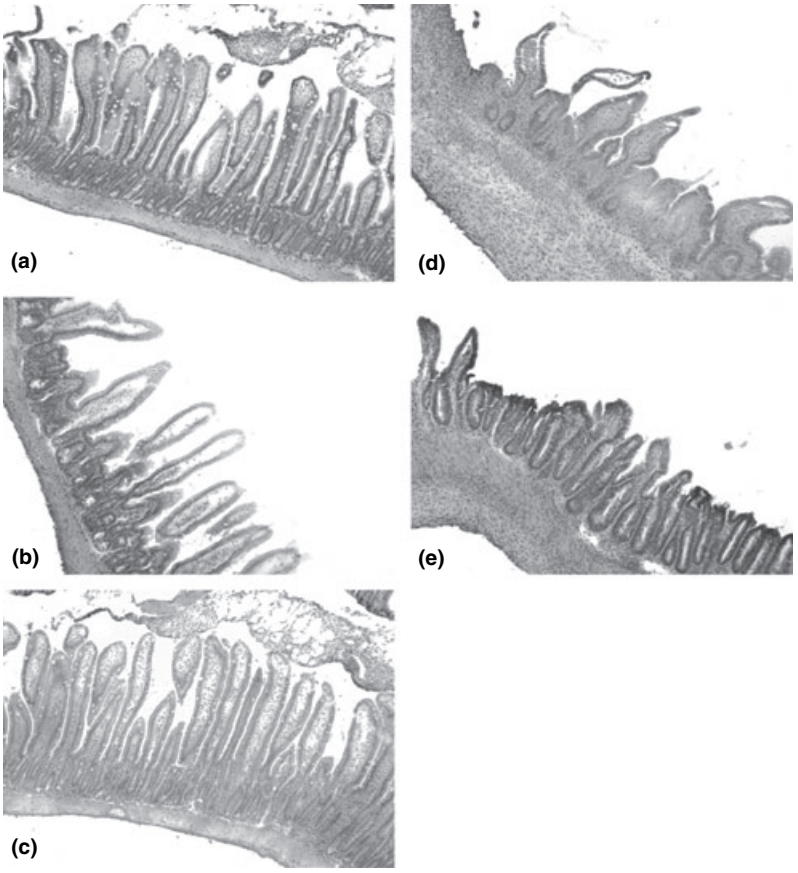

Figure 3 Histologic appearance of intestinal allografts on day 14 after transplantation. (a) syngeneic control group, (b) Untreated ADAP–/– group, (c) MR-1 in ADAP–/– recipient. (d) Untreated WT group (e) MR-1 in WT recipient. Magnification: 100×.

Less infiltration and activation of host lymphocytes in graft MLNs in ADAP−/− recipients

We compared the differences in host lymphocyte infiltration and activation in the four groups by analyzing the lymphocytes within GALT: graft mesenteric lymph nodes (MLNs), Peyer's Patches (PPs), and graft infiltrating lymphocytes (GILs), at day 6 after transplantation. On day 6 post-transplantation, the majority of donor lymphocytes (>60%) had been replaced by host lymphocytes in untreated and MR-1-treated WT mice, while there was an increased persistence of donor cells in MR-1-treated

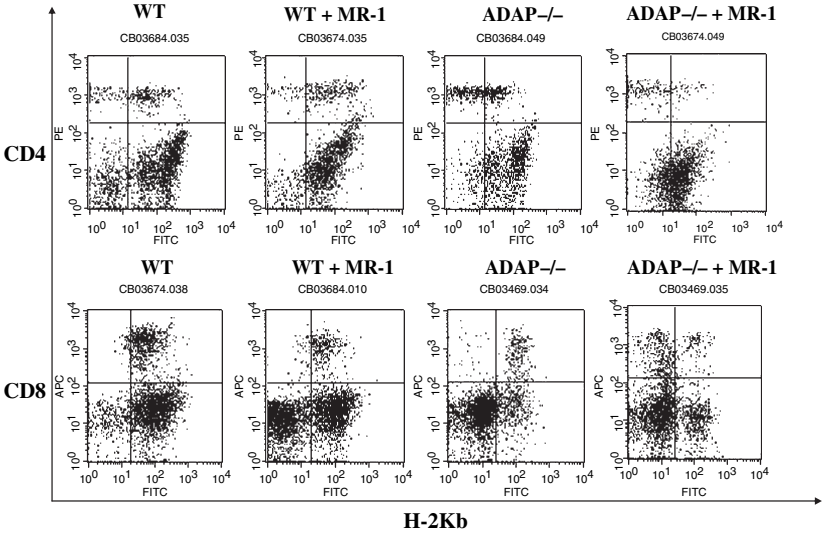

Figure 4 Host (H-2K^b positive) CD4 and CD8 cell infiltration in MLN of intestinal grafts. Less host CD4 and CD8 in graft MLN of MR-1-treated ADAP–/– mice and more donor lymphocytes were preserved.

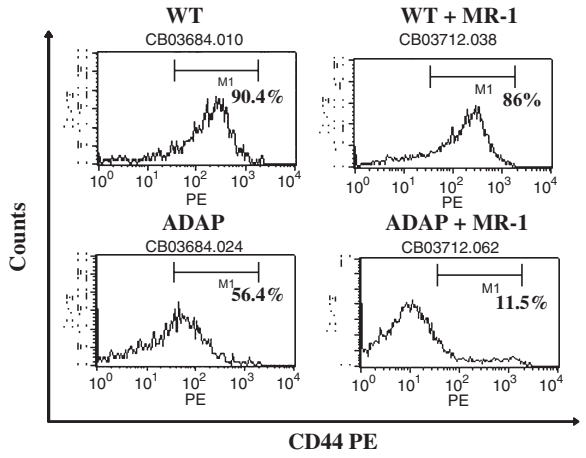

Figure 5 Activation state of host CD8 cells in graft MLN in the four groups. The proportion of CD44^{high} cells was decreased in MR-1-treated ADAP–/– mice.

ADAP–/– mice. The percentage of H-2K^{b}-positive host CD4 cells in MLNs was around 10% in groups 1, 2, and 3, while in group 4 it was only 2.5%. Similar to host CD4 cells, host CD8 cells in group 4 were less frequent compared with that in the other groups (Fig. 4). Besides the reduced infiltration of host CD4 and CD8 cells in MLNs, the activation of host CD8 cells in groups 3 and 4 was decreased (Fig. 5). Four of six mice in group 3 and group 4 each showed a decrease in the number of cells expressing the activation marker CD44 at high levels (CD44^{high}). Five of six mice in group 1 and all in group 2 showed a proportion of CD44^{high} cells greater than 50% (ranging from 54.9% to 95.1%).

Less CD62L^{-}/CD62L^{+} ratio in PPs host cells in ADAP–/– recipients

In PPs the infiltration of host CD4 and CD8 cells was similar in all groups. There was no difference in the frequency of host CD4CD44^{high} or CD8CD44^{high} cells between the four groups. Comparing the expression of L-selectin (CD62L), a marker of naive lymphocytes, in the four groups, the CD62L^{low} /CD62L^{high} ratio in host CD4 and CD8 cells was found to be lower in groups 3 and 4 (Table 1), indicating a higher proportion of naive host T cells infiltrating graft PPs of ADAP–/– recipients.

We further examined the proportion of CD44^{high} CD62L^{low} and CD44^{high} CD62L^{high} T cells in PPs and the result showed a lower proportion of CD44^{high} CD62L^{low} T cells and a higher proportion of CD44^{high} CD62L^{high} in ADAP–/– recipients (Fig. 6). This result indicates that in ADAP–/– recipients, less host effector memory T cells infiltrated the PPs.

Table 1. The ratio of CD62L^{low}/CD62L^{high} in host CD4 and CD8 cells in PPs.

Group	1	2	3	4
Combination and manipulation	BALB/c to WT	BALB/c to WT+MR1	BALB/c to ADAP–/–	BALB/c to ADAP–/– +MR1
Host CD4	5.4 ± 6.9	3.8 ± 1.9	1.5 ± 0.8*	0.8 ± 0.08*, **
Host CD8	11.8 ± 16.4	15.5 ± 21.5	2.6 ± 1.7*	0.8 ± 0.45*, **

*$P < 0.05$ versus group 1.
**$P < 0.05$ versus group 3.

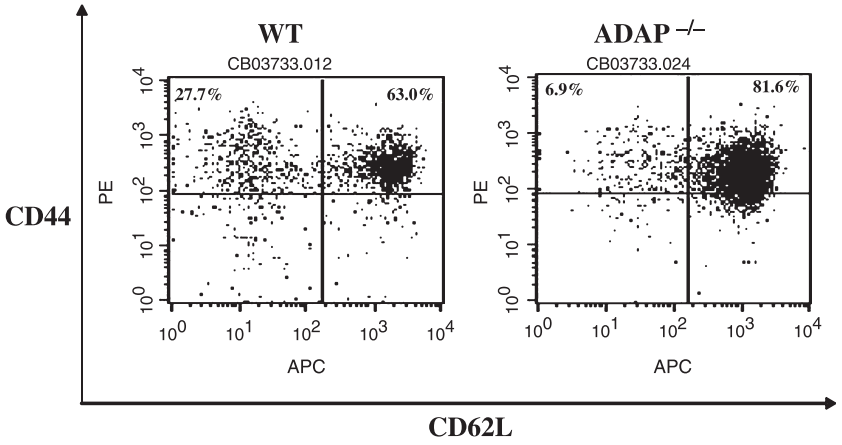

Figure 6 Effector and central memory cells in graft PPs on day 6 after transplantation. Less effector memory cells and more central memory cells were found in ADAP–/– PPs.

Table 2. Absolute number ($\times 10^4$) of host CD4 and CD8 cells infiltrating intestinal grafts.

Group	1	2	3	4
Combination and manipulation	BALB/c to WT	BALB/c to WT+MR1	BALB/c to ADAP−/−	BALB/c to ADAP−/− +MR1
Host CD4	61.5 ± 40	24.2 ± 8.7	19.5 ± 13.2*	21.3 ± 5.9*
Host CD8	352.2 ± 273.7	126.8 ± 14.1	107.9 ± 38.1	74.0 ± 10.3*

*$P < 0.05$ versus group 1.

Less host CD4 and CD8 T cells infiltrate intestinal allografts of ADAP−/− recipients

The absolute number of host CD4 and CD8 cells in intestinal grafts in groups 3 and 4 was significantly decreased compared with those in group 1 (Table 2).

Discussion

Among all solid organ transplants, acute and long-term graft survival of ITx are far from being satisfactory compared with those of renal and liver transplants. Recent data show that graft survival of ITx was 54% at 5 years and 42% at 10 years [11], and that acute rejection had a significant negative impact on graft survival [12]. ITx has been resistant to conventional immunosuppressive strategies [13] and the clinical application of tolerance induction protocol is limited in spite of its positive result in mouse models [14]. Understanding the mechanism of acute rejection and investigating the treatment strategies in ITx deserve special attention.

The integrin-mediated cell-cell/cell-matrix adhesion is an important process leading to the activation and infiltration of recipient cells into the allograft. Particularly, in the gastrointestinal tract, this binding involves a unique expression pattern of adhesion molecules that mediate the extravasation and retention of lymphoid cells in a tissue-selective fashion [15,16]. The main physiological function of ADAP is to regulate the integrin activation and its mediated cellular adhesion. So, theoretically the cellular adhesion induced by integrin and its ligand is impaired in ADAP−/− mice. This could explain partly the reduced rejection severity of ITx in ADAP−/− recipients in this study. Kellersmann *et al.* [17] reported that after allogeneic ITx, there was a marked increase in α4β7 integrin expression on recipient lymphocytes. The application of mAb against β7 integrins significantly prolonged graft survival [17]. It was also demonstrated that antibodies against αLβ2 [18] ameliorated the rejection of ITx. More recently, Ihara Y *et al.* showed that antibody against mucosal addressin cell adhesion molecule-1, the ligand of β7 integrin, prolonged intestinal allograft survival in a rat model [19]. Wang *et al.* [20] reported that using an oligonucleotide termed M12 to interfere with the ADAP coupling and signaling inhibited the T cell activation, which indicated the potential pharmacological inhibition of ADAP in future.

The mechanism of the protective effect of ADAP deficiency in ITx was studied *in vitro* and *in vivo*. We found that the T cell proliferative activity induced by allogenic dendritic cells in ADAP−/− recipients was impaired, while the cytotoxic function of effector cells in ADAP−/− recipients was comparable with that of wild-type mice. In a previous study by our group, we found the same result in a murine model of heart transplantation [3]. The discrimination between the induction and effector phase of rejection was confirmed again in this study and may explain why we only observed a marginal protection of intestinal allografts in ADAP−/− recipients. In spite of the defect in proliferation, the effector cells were eventually generated and mediated the target allograft destruction.

Another protective mechanism of intestinal allografts in ADAP−/− recipients was that in graft PPs we found a lower proportion of $CD44^{high}$ $CD62L^{low}$ T cells compared with that in WT mice. This indicates that in ADAP−/− recipients less host effector memory T cells infiltrated the PPs. It has been shown that there is quite a lot of cross reactivity to different allo-MHC determinants in alloantigen-specific T cells. It has also been reported that pathogens such as viruses, bacteria, and parasites have common antigen determinants shared with MHC peptides [21]. It is comprehensible that we saw a high proportion of memory T cells in PPs of WT recipients, because the intestine encounters more antigens than any other part of the body, and it was shown that antigen is taken up and then presented to T cells in PPs [22].

Even though our results revealed a relative protection against rejection of intestinal allografts in ADAP−/− mice, the average rejection score was higher than 2, which means that there was morphologic injury to the villi, while the ADAP deficiency prolonged the heart graft survival quite a lot [3]. It is not surprising if the high immunogenicity and heavy lymphoid contents of the intestinal grafts are considered as risks of rejection. Our research group has previously shown that MR-1, the anti-CD40L antibody, combined with FTY720 inhibited intestinal allograft rejection much more effectively than the monotherapy of FTY720 or anti-CD40L antibody [7]. We applied the same antibody of MR-1 to the ADAP−/− recipient to

test if there would be a synergistic effects against rejection. The result was that MR-1 combined with ADAP deficiency inhibited acute rejection of intestinal allografts efficiently. We found less migration of host CD4 and CD8 cells to graft MLN and less activation of host CD8 cells in ADAP−/− recipients treated by MR-1. This is what we expected, because LFA-1 plays an important role in the infiltration of lymphocytes into lymph nodes, and the ADAP−/− recipient we used did have a defect in LFA-1 activation. Our results show that the infiltration and activation of host cells in graft MLN clearly correlated with rejection severity. Similar results were reported by Kanokogi [23], who showed that donor lymphocytes in graft MLNs were rapidly replaced by host-derived lymphocytes after transplantation, while our result showed less infiltration of host-derived cells and higher persistence of donor cells in MR-1-treated ADAP−/− mice, which accounted for the observed beneficial effects.

In contrast to MLN, we did not find a difference in host cell infiltration in PPs between ADAP−/− and WT recipients. Our group also reported that FTY plus MR-1 leads to reduced host cell infiltration in MLN but not in PPs [7]. PPs seem to be more resistant to immune manipulation than MLN, and it is also interesting to note that PPs are essential for triggering the alloimmune responses in acute GVHD [24]. However, the ratio of host $CD62L^{low}$ (memory)/$CD62L^{high}$ (naive) in PPs was lower in ADAP−/− recipients with or without MR-1 treatment, indicating a higher frequency of naïve cells, presumably as a result of an activation defect of ADAP−/− T cells.

Blockade of the CD40/CD40L costimulatory pathway has been confirmed as an efficient way for tolerance induction in many animal models, but not in mouse ITx. Our results in WT recipients treated with MR-1 demonstrated no protection against rejection, while in ADAP−/− recipients, MR-1 provided an effective inhibition of intestinal allograft rejection in the histological analysis. In ADAP−/− recipients treated with MR-1, there was less infiltration of host CD4 and CD8 T cells in the intestinal grafts. The donor/host ratio was much higher compared with that in the other three groups, which is consistent with the previous results of our group [7]. As we know, costimulation blockade (CB) mainly affects CD4 T cells and, therefore, is quite effective in the models in which CD4 T cells dominate the rejection response. However, CB is not sufficient when CD8 T cells can mount rejection independently, such as in intestinal transplantation. As CD8 T cells have been considered as the major obstacle for tolerance induction in ITx, and ADAP deficiency, on the other hand, could have a more pronounced effect on CD8 T cells rather than CD4 T cells via LFA-1 inactivation, one may speculate that a combination of CB and ADAP inactivation could exhibit synergistic effects in strongly immunogenic transplant models. Some recent reports have focused on the synergistic effect of a combination of targeting LFA-1 and CD40/CD40L blockade. Lunsford *et al.* [25] showed that combined treatment of anti-LFA-1 antibody and MR-1 induced long-term graft survival of hepatocellular allografts in the majority of recipients in CD4 knockout mice. They illustrated that CD8 T cell activation and development of alloreactive CD8+ cytotoxic T cell effectors were interfered with. Another study by the same group showed that targeting of LFA-1 synergizes with CD40/CD40L blockade for suppression of both CD4- and CD8-dependent rejection [26]. In addition to the histological results, our flow data have in part demonstrated the synergistic effect compared to that in ADAP deficiency or MR-1 treatment alone. No evident decrease of host CD8 T cells occurred, while in ADAP−/− recipients treated with MR-1, the proportion of host CD8 T cells in the intestinal graft was reduced significantly. So the fact that ADAP deficiency and CD40L blockade influence distinct subpopulations of T cells could explain the additive effect of the combined treatment, because CD4 T cells and CD8 T cells play independent roles in the acute rejection of ITx [27–29]. In summary, we have shown that in ADAP-deficient mice, rejection of intestinal allograft was ameliorated. This effect was related to a defect in T cell activation, proliferation, and graft infiltration. ADAP deficiency combined with costimulation blockade synergistically protected the intestinal allograft against rejection. ADAP might constitute an interesting target to be selectively blocked in early phase of T cell activation events after organ transplantation.

Authorship

JT: performed study and wrote the paper. JIRB: performed study. OP: performed study. DR: performed study. RF: contributed important reagents. JB: collected data. MWH: designed study.

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