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ORIGINAL ARTICLE

Role of resident macrophages in the immunologic response and smooth muscle dysfunction during acute allograft rejection after intestinal transplantation

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Summary

Resident muscularis macrophages initiate an inflammatory cascade during ischemia/reperfusion that is associated with dysmotility and the activation of immunologic processes. We hypothesized that these muscularis macrophages may also play a potential immunologic role for acute allograft rejection in intestinal transplantation. Orthotopic SBTx (BN-Lew) was performed without immunosuppression. Animals were sacrificed 7 days after SBTx. The role of resident macrophages was evaluated by transplantation of macrophage-depleted and gadolinium chloride-treated gut. Leukocyte infiltration was investigated in muscularis whole mounts by immunohistochemistry. Mediator mRNA expression was determined by Real-Time-RT-PCR. Apoptosis was evaluated by TUNEL. Smooth muscle contractility was assessed in a standard organ bath. In comparison to vehicle-treated grafts, macrophage-depleted grafts exhibited significantly lower mediator mRNA peak expression (IL-6, IL-2, IL-10, MCP-1, iNOS, TNFα, IFNγ, FasL), leukocyte infiltrates (ED1- and ED2 positive monocytes and macrophages, neutrophils, $CD4^+$ and $CD8^+$ lymphocytes), apoptosis rates and an improved histologic rejection grading. Vehicle-treated grafts showed a 77% decrease in smooth muscle contractility compared to naïve controls, while macrophage-depleted gut exhibited only a 51% decrease in contractile activity. Transplantation of macrophage-depleted gut attenuates the functionally relevant molecular and cellular immunologic response within the graft muscularis in acute allograft rejection. Resident macrophages participate in initiating these processes.

Introduction

The outcome of intestinal transplantation (ITx) in patients with gut dysfunction or short gut syndrome remains limited by tissue injuries such as ischemia/reperfusion, acute rejection and infection [1–5]. In order to improve the results of small bowel transplantation further, these problems have been addressed in various clinical and experimental studies. However, most studies have focused on the immunologic role of the intestinal mucosa or submucosa, whereas only the muscularis propria has been recognized as a motility organ with significant impact on long-term graft function [6]. Nevertheless, primary data exist, indicating that the process of intestinal transplantation initiates an inflammatory cascade within the grafts muscularis that successively leads to motoric graft dysfunction [7–12]. The underlying inflammation process is promoted by complex injuries to the intestinal muscularis during transplantation, such as surgical manipulation and handling during organ procurement, organ preservation and ischemia-reperfusion injury [9,13,14].

In this context, resident muscularis macrophages have been identified as one of the potential triggers for the initiation of the inflammatory cascade [8]. Macrophages within the intestinal muscle layer are inactive under physiologic conditions and are presenting a hidden line of defense. Recent studies have revealed that these resident macrophages are activated by various tissue trauma such as ischemia and reperfusion, laparotomy, surgical gut manipulation and sepsis [13,15–17]. This in turn leads to the release of various prototypic proinflammatory cytokines, chemokines and adhesion molecules with successive recruitment of leukcocytes into the muscularis. The co-expression of kinetically active mediators such as iNOS and COX-2 with the subsequent release of NO and prostanoids results in a massive inhibition of motoric gut function [9,16,18–20]. It is generally known that macrophages play an important role in alloantigen presentation and initiation of an immunologic response as effector cells that induce donor-specific cytotoxic responses during transplantation. It has already been shown in small bowel transplantation that systemic macrophage depletion in both the donor and the recipient ameliorates the severity of acute allograft rejection [21]. However, little is known about the specific role of intestinal muscularis macrophages and we hypothesized that these resident cells play a key role in initiating and promoting acute intestinal allograft rejection. Using an intestinal allotransplantation model with chemical macrophage depletion, our objectives were to determine the role of resident macrophages in initiating the immunologic response within the graft muscularis and to assess the impact of these macrophages on motoric allograft dysfunction in acute rejection.

Materials and methods

Animals

Inbred male Lewis rats weighing 180–200 g were obtained from Charles River WIGA GmbH (Sulzfeld, Germany). All experiments were performed in accordance with the federal law regarding the protection of animals. The principles of laboratory animal care (NIH Publication No.85-23, revised 1985) were followed. The animals were maintained on a 12-h light/dark cycle and provided with commercially available chow (Altromin, Lage, Germany) and tap water *ad libitum*.

Experimental Procedures

For the rat experiments, four different groups were defined and studied. In group one, allogeneic SBTx was performed between Brown-Norway rats (BN) and Lewis rats (BN ⇒ Lewis). In this group (AT 168 h depletion), the role of resident macrophages was analyzed by performing macrophage depletion and inhibition prior to recovery of organs. Based on pilot studies and results from the literature, we adopted and applied a model of macrophage depletion with liposomes encapsulating dichloromethylene diphosphonate (chlodronate, Cl_2MDP), which is known to induce apoptosis in macrophages [20,22–24]. This was combined with an additional selective macrophage inhibition by administration of gadolinium chloride ($GdCl_3$), a selective inhibitor of stretch-activated ion channels, which is regularly used for inhibition of Kupfer cell function in experimental liver transplantation [25,26]. Chlodronate liposomes were prepared according to the protocol of van Rooijen and Sanders and slowly injected intravenously under isoflurane gas anesthesia [23]. Rats were given Cl_2MDP liposomes (50 mg chlodronate/kg body weight) at 400 nm on days -4 and -2 (prior to recovery of organs) and alternate administration of $GdCl_3$ (10 mg/kg body weight) on days -3 and -1. This depletion and inactivation protocol has proved to be reliable. It is a well established standard in our laboratory and has recently been published [20,22]. In the second group (AT 168 h) also allogeneic SBTx was performed and the BN rats received liposomal phosphate-buffered saline (PBS) and alternate injections of normal saline as vehicle administration prior to transplantation as control for liposomally encapsulated Cl_2MDP and GdCl. In the third group (Control), only the naïve bowel of Brown Norway rats was procured, but neither transplanted nor exposed to any procedures. In the fourth group (control depletion), effect and efficacy of the above-described macrophage-depletion protocol was evaluated and controlled by implementation of the same protocol in additional BN rats with harvest of the bowel at the end of the protocol without transplantation and immunohistochemical staining for quantification of the resident ED2 positive macrophage network. In order to evaluate the effect of the combined treatment on the other investigated leukocyte populations (MPO+ neutrophils, ED1-positive monocytes and macrophages, CD4- and CD8 lymphocytes) additional immunohistochemical staining was also performed for these cells.

Immunosuppressive or immunomodulatory agents were not applied in any of the groups. To define the time course of gene expression within the graft muscularis, for functional studies, histopathologic analysis and grading, evaluation of apoptosis and immunohistochemical or immunohistologic analysis, animals were sacrificed at a specific time point 168 h after transplantation and reperfusion by isoflurane anesthesia inhalation overdose. This time point was chosen as in this high responder transplant model, a definitive acute rejection episode can be observed and monitored 7 days after transplantation without additional immunosuppression.

Orthotopic small bowel transplantation (OSBTx) was performed between rats as previously described under isoflurane inhalation anesthesia [27].

Histopathologic analysis and apoptosis

For histopathologic evaluation and grading of rejection, intestinal tissue was embedded in 4% formalin-fixed paraffin, cut into 4-μm sections and stained with hematoxylin-eosin. The slides were blindly reviewed by two authors without knowledge of the experimental groups. The histologic criteria for grading of acute cellular rejection (ACR) included infiltration by a mixed mononuclear inflammatory cell population, extent of crypt injury, increase in the number of crypt apoptotic bodies and distortion of villous and crypt architecture. Grading of ACR included four grades as proposed and established in clinical intestinal transplantaion by Wu *et al.* [28]. Additionally, the histologic and morphological changes as well as infiltrating cell populations in the muscularis propria were observed and described as no standard histologic criteria concerning acute cellular rejection exist.

For evaluation and comparison of apoptotic cells in the muscularis propria and in the mucosa, the standard TUNEL method (TdT-mediated dUTP-X nick end labeling) was used on paraffin-embedded tissue (*In Situ* Cell Death Detection Kit, AP; Roche Biochemicals, Mannheim, Germany).

Functional studies

Mechanical *in vitro* activity of the mid-jejunum was evaluated postoperatively using smooth muscle strips of the circular muscularis as described previously for the jejunum (n = 5–7 each) [29]. After recording spontaneous contractility for 30 min, dose-response curves were generated using increasing doses of the muscarinic agonist bethanechol (1–300 μmol/l) for 10 min and intervening wash periods (KRB) of 10 min. The contractile response was recorded and analyzed as g/mm^2/s.

RNA extraction and quantification of gene expression

Extraction of RNA was performed as described previously [18,19,29,30]. The isolated muscularis was snap-frozen in liquid nitrogen and stored at −80 °C. Mediator mRNA expression in the small intestinal muscularis was analyzed using SYBR Green two-step real-time RT-PCR as previously described (n = 5–7) [31]. Total RNA extraction was performed using the RNeasy Mini extraction kit (Qiagen GmbH, Hilden, Germany) and contaminating DNA from the RNA preparations was eliminated using Ambion DNA-free (Ambion Ltd., Huntingdon, Cambridgeshire, UK). Aliquoted RNA (200 ng) was processed for complimentary DNA (cDNA) synthesis. Rat primer sequences were used as previously published (GAPDH, ICAM-1, iNOS and MCP-1) [31] or designed according to published sequences (IL-6, IL-2, IL-10, TNFα, IFNγ, FasL) using Primer Express software (Applied Biosystems Applera Deutschland GmbH, Darmstadt, Germany), purchased from Invitrogen GmbH (Karlsruhe, Germany). Sequences of the primers are depicted in Table 1. Each mediator-specific amplification was normalized to an endogenous control (GAPDH). The PCR reaction mixture was prepared using the SYBR Green PCR Core Reagents (Applied Biosystems Applera Deutschland GmbH). PCR conditions on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems Applera Deutschland GmbH, Darmstadt, Germany) were set as recommended by the manufacturer. Dissociation of the PCR products by a melting curve analysis protocol consistently showed specific single melting peaks for all used primer pairs. Relative quantification was performed using the comparative C_T method as described previously by Schmittgen *et al.* [32].

Table 1. Nucleotide sequences of oligonucleotide primers.

	Primer sequences (5′–3′)	
Target gene	Sense	Antisense
IL-6	GCCCTTCAGGAACAGCTATGA	TGTCAACAACATCAGTCCCAAGA
IL-2	CCCCATGATGCTCACGTTTA	ATTTTCCAGGCACTGAAGATGTTT
IL-10	CGACGCTGTCATCGATTTCTC	TCTTGGAGCTTATTAAAATCATTCTTC
IFNγ	AGTCTGAAGAACTATTTTAACTCAAGTAGC	CTGGCTCTCAAGTATTTTCGTGTTAC
TNFα	GGTGATCGGTCCCAACAAGGA	CACGCTGGCTCAGCCACTC
INOS	GGAGAGATTTTTCACGACACCC	CCATGCATAATTTGGACTTGCA
ICAM-1	CGTGGCGTCCATTTACACCT	TTAGGGCCTCCTCCTGAGC
MCP-1	CAGCCAGATGCAGTTAATGCC	AGCCGACTCATTGGGATCAT
FasL	AGGCTGTGGTTGGTGAACT	TTGGAATGGGGTTAGGAAT
GapDH	ACGGCACAGTCAAGGCTGAGA	CGCTCCTGGAAGATGGTGAT

Histochemistry and immunohistochemistry

Histochemical and immunohistochemical analysis was performed on whole mounts of the intestinal muscularis as described previously (n = 5–7) [15,31]. Leukocyte infiltrates were evaluated in the 'control depletion group' without transplantation and in the transplanted groups 168 h after reperfusion by myeloperoxidase (MPO) histochemistry [polymorphonuclear neutrophils (PMNs)], ED1 (monocytes and passenger macrophages) and ED2 (resident macrophages) immunohistochemistry as well as $CD4^+$ and $CD8^+$ immunohistochemistry (lymphocytes). MPO positive cells were detected using Hanker-Yates reagent (Polysciences Europe GmbH, Eppelheim, Germany). For immunohistochemistry, a mouse anti-rat-ED1-, ED2-, $CD4^+$ and $CD8^+$ antibodies were used as primaries. Muscularis whole mounts were incubated overnight at 4 °C in an ED1-, ED2-, CD4- and CD8-antibody solution (1:100), followed by washing thrice in 0.05 M PBS. Each specimen was then incubated with a Cy3 donkey-anti-mouse secondary antibody (1:500) and goat anti mouse-Alexa 568 (1:500) for 4 h at 4 °C and washing thrice in 0.05 M PBS. Secondary antibodies without ED1-, ED2-, CD4- and CD8-antibody pre-incubation were used in parallel in all staining procedures to ensure specificity. Leukocytes were counted in five randomly chosen areas in each specimen (three specimens per animal) at a magnification of 200×.

Drugs and solutions

A standard KRB was used as described previously [15,31]. Mouse-anti-rat-ED1, ED2, CD4 and CD8 antibodies were obtained from Serotec GmbH (Düsseldorf, Germany). Indocarbocyanine (Cy3)-conjugated donkey-anti-mouse antibody and goat anti mouse-Alexa 488 were purchased from Dianova GmbH (Hamburg, Germany) and Invitrogen.

Data analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using a Student's t-test and one-way ANOVA with a significance level of $P < 0.05$ followed by Dunnett's multiple comparison test of all manipulated probes versus dedicated controls.

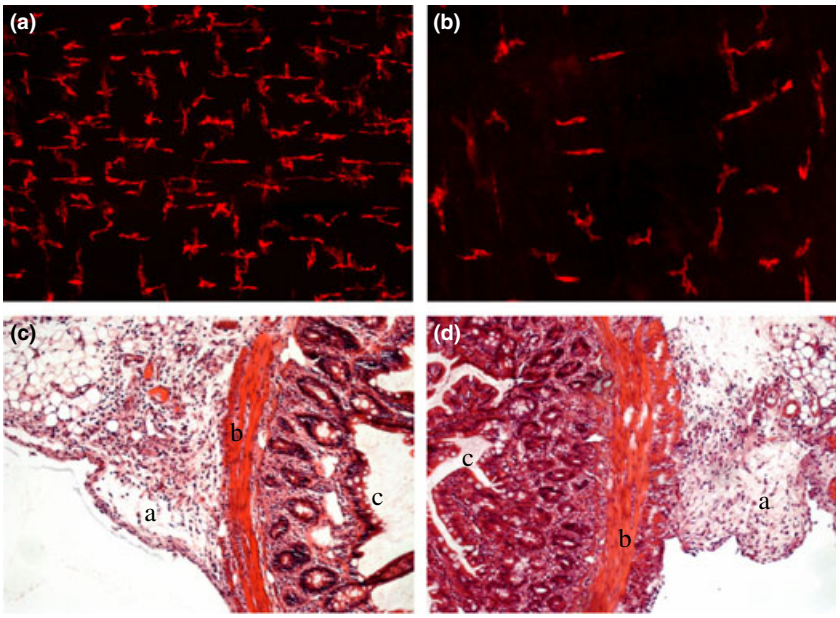

Figure 1 Panel a–d: Immuno-fluorescent staining of muscularis whole mounts demonstrating the amount of ED2-positive macrophages in the intestinal muscularis of naïve control bowel (a) and chlodronate liposomes and $GdCL_3$ pretreated bowel (b) (original magnification ×100). Panel c and d: Histologic sections of the rat small bowel wall of the different transplanted groups 168 h after reperfusion. Allogeneic transplanted vehicle-treated control grafts (Panel c) revealing moderate to severe ACR with the typical histopathologic criteria, as there are severe mucosal destruction and localized ulcerations (c), deterioration and disintegration of muscularis propria (b) and intense inflammatory and lymph cellular infiltration of the adjacent mesentery (a). In contrast, the macrophage-depleted gut (Panel d) revealed only mild to moderate ACR with a better preserved architectural structure of the muscularis propria (b), a milder localized inflammatory infiltrate in the adjacent mesentery (a) and a better preserved mucosa without ulcerations or erosions (c). (Hematoxylin-eosin; original magnification ×100).

Results

Distribution of resident muscularis macrophages

In the control group's whole mounts of naive rats, we observed a dense network of ED2 positive resident macrophages (59.3 ± 4.3 cells at 200×; Fig. 1; Panel a). Intravenous pre-treatment with 400 nm Cl_2MDP-liposomes and $GdCl_3$ caused a significant macrophage-depletion of 86%, compared to controls (8.3 ± 1.7; Fig. 1, Panel b). The remaining muscularis macrophages showed the following morphological changes: the dendrites were short; the cells appeared more oblong-shaped, the overall cell volume appeared to be smaller and margins of the cell were less distinct and defined. The combined treatment did not significantly alter the number of constitutively present MPO^+ neutrophils, ED1-positive cells, $CD4^+$ and $CD8^+$ lymphocytes in comparison to naïve controls, as depicted in the specific histograms of the different figures under the term 'control depletion'.

Histopathologic grading of rejection

In allogeneic transplanted vehicle-pretreated animals, moderate to severe ACR with typical histopathologic criteria was observed at 168 h of reperfusion. The muscularis propria presented with an infiltration of blastic or activated lymphocytes and showed a thinning and disintegration of the muscle cell layers. Furthermore, severe infiltration of leukocytes and vasculitis with intimal or transmural arteritis was observed in the adjacent mesentery. The mucosa revealed increases in the number of crypt apoptotic bodies (10–14 per ten crypts) with sometimes confluent apoptosis, progressive distortion of villous and crypt architecture as well as superficial erosions and sometimes even mucosal ulcerations (Fig. 1; Panel c). In contrast, macrophage-depleted allografts showed only mild-to-moderate ACR with an attenuated inflammatory infiltrate, a better preserved architectural structure (muscularis propria, lamina propria, adjacent mesentery) without mucosal ulcerations or erosions) (Fig. 1; Panel d) and presented with reduced crypt epithelial apoptosis (6–8 per ten crypts).

Apoptosis within the muscularis propria

As depicted in the histogram of Fig. 2, the number of apoptotic cells increased significantly at 168 h in vehicle-treated allografts (Fig. 2c and 2d) compared to depleted allografts and to naïve controls (Fig. 2a and 2b) (control: 2.1 ± 0.9, 168 h vehicle: 8.7 ± 1.1, depletion: 6.5 ± 0.5 signals/100 μm^2 at 200× magnification). The evaluation of FasL mRNA expression revealed a similar result. Whereas the graft muscularis of the vehicle-treated animals showed a 22.4-fold increase in mRNA expression at the time of ACR after 168 h, the macrophage-depleted gut exhibited a significant lower increase of only 13.9-fold in FasL mRNA expression (Fig. 2).

Molecular mediator expression within the muscularis

Inflammatory gene mRNAs were measured in a time course study. Gene expression was normalized on normal intestinal naïve control tissue. As calculated by the comparative C_T method and illustrated in Fig. 3, acute cellular rejection in vehicle-treated animals, compared to naïve controls, resulted in a significant expression of the prototypic inflammatory mediators IL-6 (139.8-fold), IL-2 (10.8-fold), TNFα (46.6-fold), INFγ (406.7-fold) and the anti-inflammatory cytokine IL-10 (179.3-fold). In comparison, the macrophage-depleted gut expressed a significantly alleviated mRNA upregulation of the above mentioned mediators (IL-6: 81.9-fold, IL-2: 3.1-fold, TNFα: 23.6-fold, INFγ: 157.8-fold, IL-10: 86.2-fold).

Compared to controls, both transplanted groups showed a moderate but significant mRNA expression of ICAM-1 after 168 h, albeit without any statistical difference in between the transplanted groups (Fig. 3). A strong and significant MCP-1 mRNA upregulation was observed in the transplanted grafts compared to controls (168 h vehicle: 42.4-fold; 168 h depletion: 20.6-fold). Macrophage-depleted allografts expressed a significantly lower MCP-1 mRNA upregulation.

iNOS was massively upregulated in the muscularis of both allogeneic transplanted groups compared to control grafts at the time of ACR after 168 h (168 h vehicle: 634.4-fold; 168 h depletion:164.0-fold). As expressed in Fig. 3, the depleted grafts again expressed a highly significant lower iNOS mRNA upregulation in comparison to the vehicle-treated grafts.

Leukocyte infiltrates

Immunohistochemical analysis revealed significant muscularis infiltration with $CD4^+$ and $CD8^+$ positive lymphocytes in vehicle-pretreated muscularis of allografts at the time of ACR after 168 h reperfusion (CD4: 150.1 ± 9.0 cells/field 200×; CD8: 59.8 ± 15.2) compared to naïve controls (CD4: 26.01 ± 5.5; CD8: 10.5 ± 3.4). In contrast, depleted grafts (CD4: 59.1.1 ± 5.5; CD8: 29.8 ± 3.5) exhibited significantly lower extravasation and infiltration of lymphocytes compared to vehicle-treated grafts (Fig. 4).

Evaluation of ED1-positive cells showed also severe infiltration of monocytes and macrophages in the muscularis

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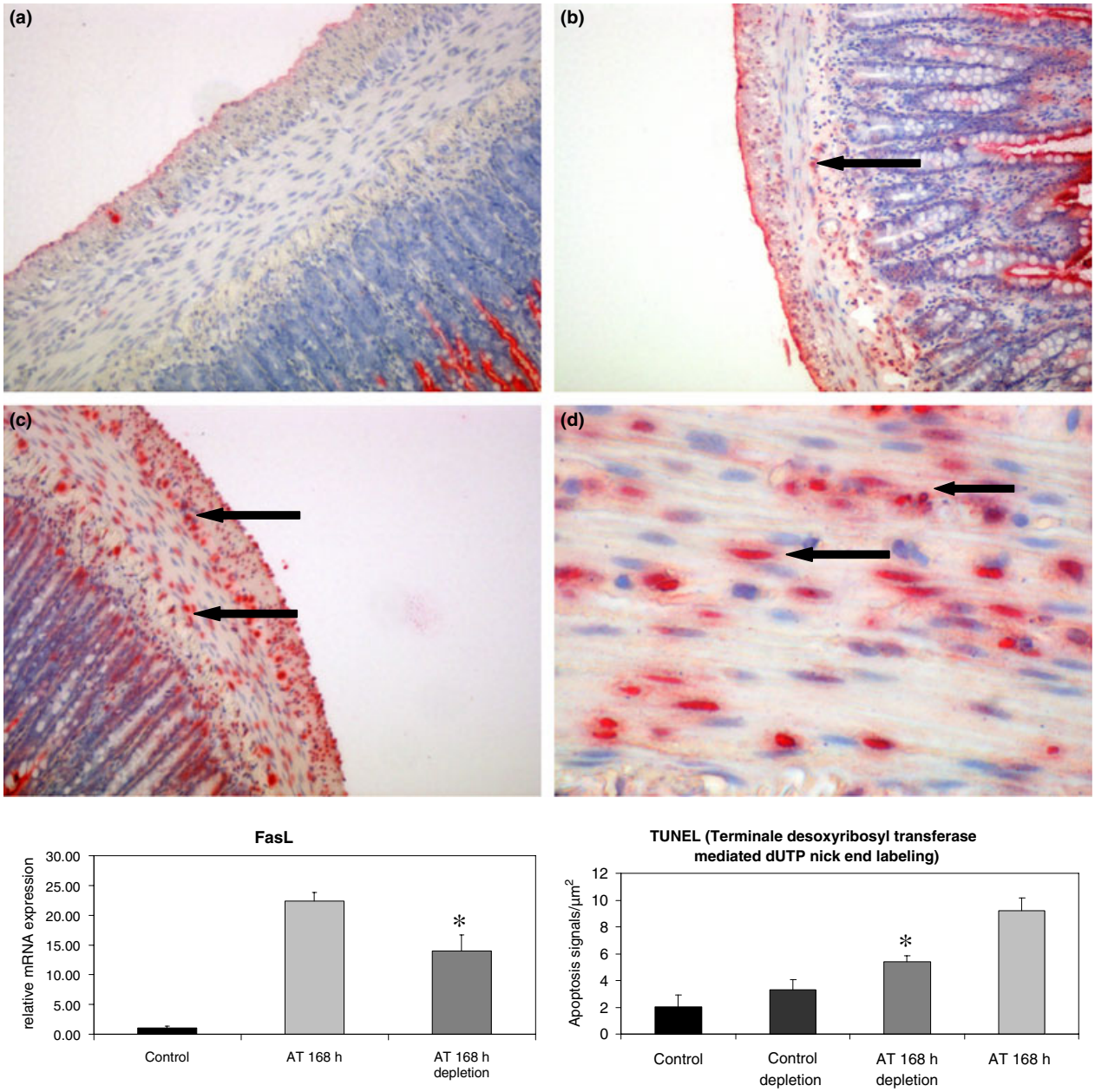

Figure 2 Panel a–d: TUNEL-labeling of histologic sections of the rat small bowel wall of a naïve control graft (a), a macrophage-depleted transplanted graft (b) and a vehicle-treated transplanted graft (c and d) after 168 h of reperfusion during acute rejection. In comparison to the control small bowel, the rejecting small bowel grafts of both transplanted groups exhibit a significant increase of apoptotic smooth muscle cells and neurons of the myenteric plexus (small arrows) in the muscularis propria (TUNEL labeling (TdT-mediated dUTP-X nick end labeling); original magnification ×100 – a, b and c; original magnification ×400 – d). Histograms showing FasL mRNA expression in the muscularis propria and the number of positive apoptotic signals of the muscularis in the naïve control, depletion control (not transplanted), vehicle-treated and depleted transplanted grafts after 168 h of reperfusion (n = 5–7) as direct and indirect evaluation criteria of apoptosis. (*P < 0.05 AT-168 h depletion vs. AT-168 h vehicle; depletion control versus control = n.s.) Data are expressed as mean ± SEM.

of vehicle-pretreated grafts (168 h Vehicle: 201.4 ± 9.9) at the time of ACR 168 h after transplantation in contrast to naïve control muscularis (Control: 31.0 ± 2.6) (Fig. 5). Comparing the two transplanted groups, the muscularis of the depleted grafts (168 h depletion: 104.8 ± 6.9) showed significantly less infiltration with ED1-positive cells when compared to the vehicle-treated grafts during ACR.

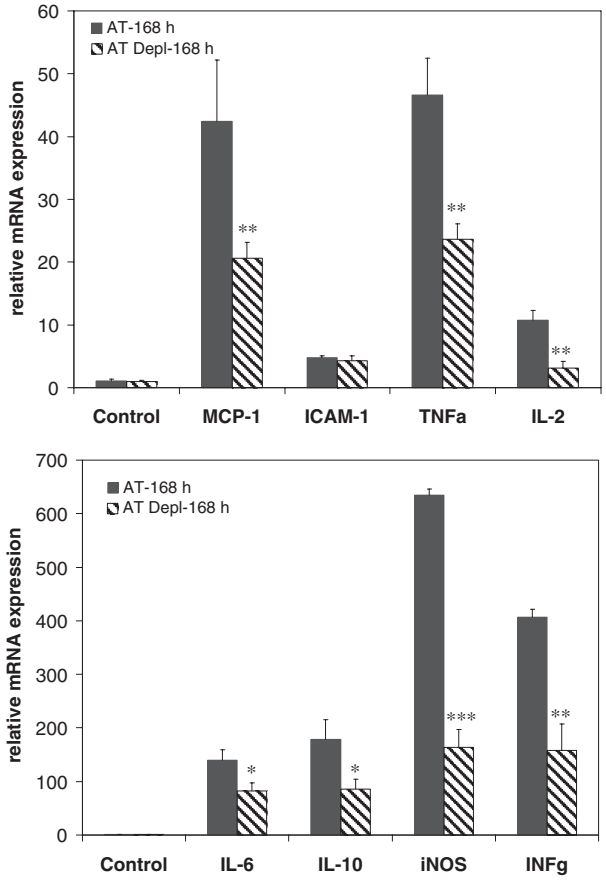

Figure 3 Results of Real-time RT-PCR analysis comparing relative mRNA expression of the adhesion molecules, cytokines and mediators MCP-1, ICAM-1, TNFα, IL-2, IL-6, IL-10, iNOS and INFγ and of vehicle-treated transplanted and depleted transplanted grafts after 168 h of reperfusion (n = 5–7). (*P < 0.05; **P < 0.01; ***P < 0.001 AT-168 h depletion vs. AT-168 h vehicle.) Data are expressed as relative mean ± SEM against normalized controls.

Immunohistochemical staining of ED2-positive macrophages revealed again a highly significant reduction in counted cell numbers in the muscularis of depleted grafts (168 h depletion: 74.5 ± 4.4) in comparison to vehicle-treated grafts (168 h vehicle: 162.4 ± 11.3)168 h after transplantation. While the vehicle-treated grafts revealed severe increases in overall cell numbers in comparison to the naïve control muscularis (Control: 59.3 ± 4.3), the number of ED2-positive macrophages in the depleted transplanted group reconstituted to levels of the naïve control group during the 168 h of reperfusion in comparison to the control depletion group before transplantation (control depletion: 8.3 ± 1.7).

As expressed by the histogram and the micrographs of Fig. 6, at 168 h of reperfusion a significant infiltration with MPO-positive neutrophils was observed in muscularis whole mounts of allogeneic transplanted grafts, compared to controls (control: 18.0 ± 4.3, 168 h Vehicle: 81.6 ± 9.3, 168 h depletion: 47.4 ± 3.2). Depletion of macrophages resulted again in a significantly reduced extravasation of these neutrophils into the muscularis in comparison to vehicle-treated grafts.

Evaluation of gastrointestinal motility

Representative bethanechol-stimulated dose-response curves of circular muscle contractile activity are depicted in Fig. 7. As illustrated, vehicle-pretreated allografts showed a massive 77% reduction in smooth muscle contractile activity at 168 h of reperfusion under stimulation with 100 µmol/l bethanechol (1.03 ± 0.22 g/mm^2/s) in comparison to controls (4.43 ± 0.51 g/mm^2/s). In contrast, macrophage-depleted grafts exhibited only a 55% decrease (2.21 ± 0.39 g/mm^2/s) of smooth-muscle contractility compared to controls. The difference in contractile activity between vehicle-pretreated allografts and macrophage- depleted allografts was statistically significant (P = 0.017).

Discussion

The previous observation in human as well as in rodent intestinal transplantations, that the muscularis propria participates in the development of early molecular and cellular inflammatory responses within the intestinal graft following transplantation, has led to the belief that the muscularis propria is a bowel compartment capable of inducing inflammatory and immunologic processes [8,9]. This is emphasized by the fact that the muscularis is also host of various cell types with a high inflammatory and immunologic potential [15]. The dense and extensive network of resident macrophages in particular has been identified as a potent source for the release of prototypic proinflammatory and kinetically active mediators [16,18,20,33,34]. The immunologic potential of these phagocytes as antigen-presenting effector cells has been supported in previous studies [21]. In the present set of experiments, we could demonstrate that reducing the number of resident macrophages in the donor muscularis and restricting the functional activity of residual cells result in a distinct alleviation of the immunologic and inflammatory responses in the muscularis propria, usually observed during acute allograft rejection. Consequently, the alleviated inflammatory cascade results in a significant improvement of allograft smooth muscle dysfunction.

In accordance with previous observations, the pharmacologic treatment with 400 nm chlodronate liposomes did not result in a complete depletion of resident ED2-positive muscularis macrophages [20,22]. Nevertheless, we

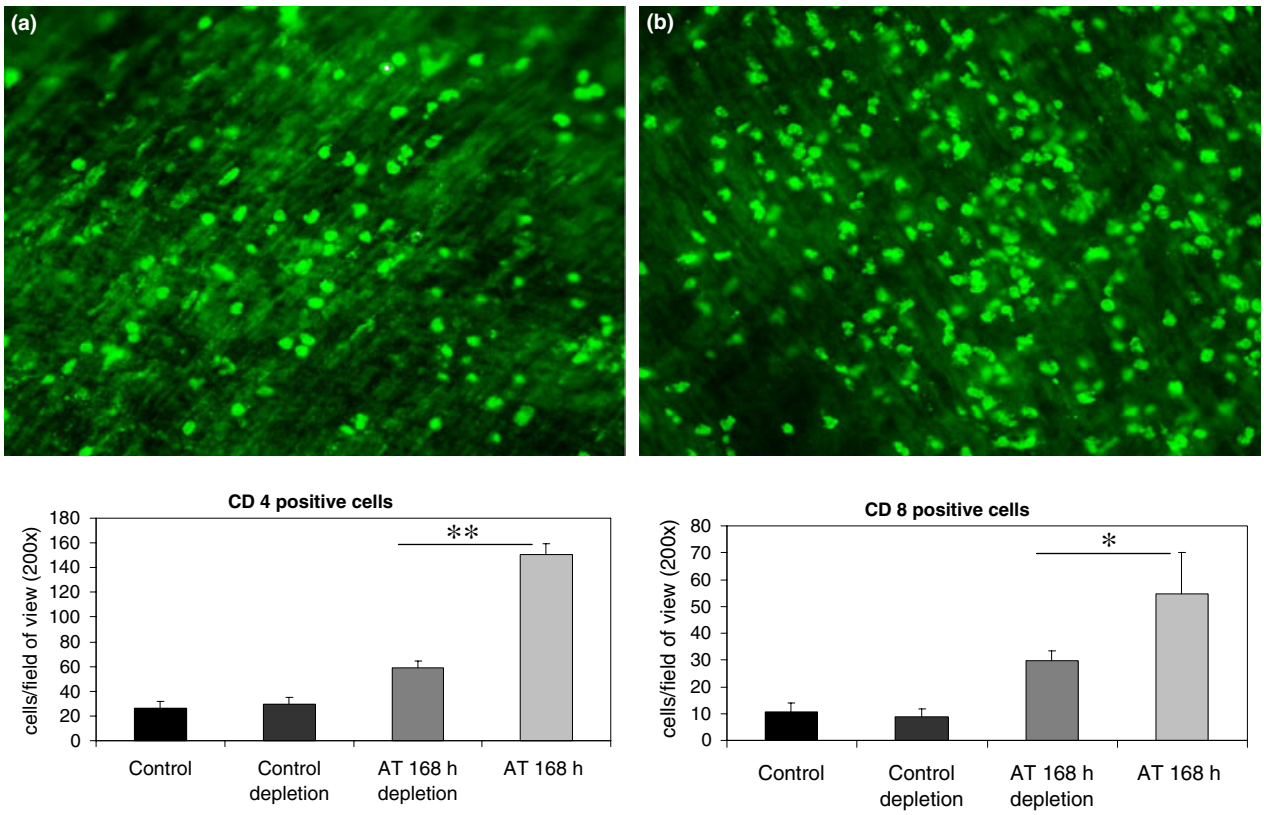

Figure 4 Panel a and b. Immuno-fluorescent staining of muscularis whole mounts demonstrating the infiltration of $CD4^+$ lymphocytes in the intestinal graft muscularis of depleted grafts (a) and vehicle-treated grafts (b) after 168 h of reperfusion at the time of acute cellular rejection (magnification 200×). Histograms quantifying and comparing the number of infiltrating $CD4^+$ and $CD8^+$ lymphocytes in the muscularis of the control, depletion control (not transplanted), vehicle-treated and depleted transplanted grafts after 168 h of reperfusion (n = 5–7). (*P < 0.05; **P < 0.01 AT-168 h depletion vs. AT-168 h vehicle.) Data are expressed as mean ± SEM.

reported an 86% depletion rate of resident macrophages after combined chlodronate liposome treatment. Also, histologic evaluation revealed a severe alteration of the remaining cells. We further inactivated these remaining macrophage cells by gadolinium chloride. The efficacy of this protocol has already been proven in former studies of our group, where we could demonstrate that the expression of macrophage activation markers can be totally inhibited by the combined treatment with chlodronate and gadolinium chloride [20,22].

As a major consequence of resident donor macrophage depletion, we observed a significant reduction of infiltrating leukocytes within the allograft muscularis at day 7 after transplantation, including neutrophils, monocytes and passenger macrophages as well as $CD4^+$ and $CD8^+$ lymphocytes in comparison to untreated animals. Nevertheless, compared to naïve control or depleted control gut without transplantation, the mentioned cell populations are also significantly increased during ACR 7 days after transplantation. As we did not perform additional bone marrow transplantation, it is most likely that the observed reconstitution (ED2-positive cells, maturation of ED1-positive cells) or increase of the other different leukocytes is now predominantly of recipient origin. Therefore it can be hypothesized, that most of the inflammation and gene expression may come from recipient host defense mechanisms by the innate immune system which is more and more recognized to be involved in all major aspects of transplantation like acute and chronic rejection and also adaptive immune responses [35,36].

These infiltrating leukocytes are known to be associated with inflammation processes and have been identified to play an essential role in the initiation and manifestation of immunologic responses during acute rejection in intestinal transplantation [28,37–43]. However, one might presume that the combined chlodronate and gadolinium treatment has also a depletion effect on the recruited leukocytes. In this context, it should be mentioned that phagocytosis is a precondition for an effective delivery of a toxic quantity of chlodronate into cells. Only actively

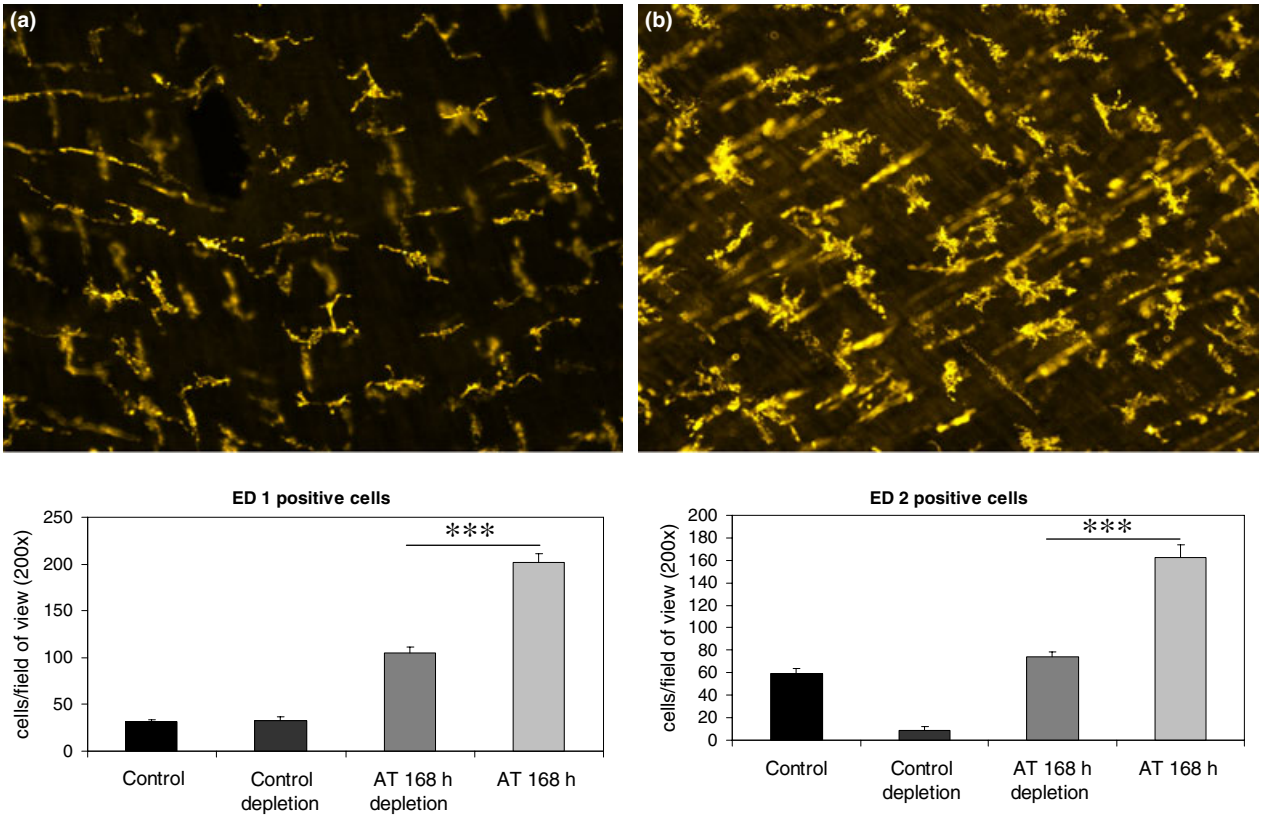

Figure 5 Panel a and b. Immunofluorescent staining of muscularis whole mounts demonstrating the amount of ED2-positive macrophages in the intestinal graft muscularis of depleted grafts (a) and vehicle-treated grafts (b) after 168 h of reperfusion at the time of acute cellular rejection (magnification 200×). Histograms quantifying and comparing the number of infiltrating ED1-positive monocytes and macrophages as well as ED2-positive resident macrophages in the muscularis of the control, depletion control (not transplanted), vehicle-treated and depleted transplanted grafts after 168 h of reperfusion (n = 5–7). (***P < 0.001 AT-168 h depletion vs. AT-168 h vehicle.) Data are expressed as mean ± SEM.

phagocyting cells (mainly mature macrophages and to a much lesser extent immature dendritic cells) are able to take up chlodronate. The leukocyte populations, which are predominantly involved in the observed immunologic response during allogenic transplantation and acute rejection, like donor and recipient T cells, donor dendritic cells and immature, bone-marrow derived infiltrating recipient cells are not affected by this treatment and not inhibited in their immunologic potential and responsiveness [20,35,44–46]. Therefore, the number of cell types that are possibly affected by the treatment is very limited. Kalff *et al.* [15] determined the number of different resident immuno-competent cells within the normal gut muscularis (ED1 positive macrophages, PMN, mast cells, T cells, NK cells and DC cells). The authors demonstrated that the number of these cell types within the muscularis is much less compared to ED2-positive resident macrophages. In addition, Fryer *et al.* [21] showed that a single injection of chlodronate liposomes leads to different macrophage-depletion levels in the liver, mesenteric lymph nodes, spleen, and small bowel, but has no effect on donor dendritic cells of the small bowel. Furthermore, by applying the depletion protocol only to donors in our present set of experiments, systemic recipient leukocytes are not affected. However, Fryer *et al.* were able to show that depletion of only recipient macrophages also led to a significantly milder rejection and that depletion of both donor- and recipient macrophages led to an improved graft survival by 4 days without the additional use of immunosuppresssion [21]. Based on these data, there is evidence that resident donor macrophages as well as recipient passenger macrophages play an important role in the final manifestation of acute rejection in intestinal transplantation. However, we believe that resident donor macrophages within the allograft muscularis significantly participate in initiating inflammation and inducing the early immunologic processes of the rejection process.

In this context it is important to state that the macrophage-depletion and inactivation protocol most likely also affects resident donor leukocyte populations capable

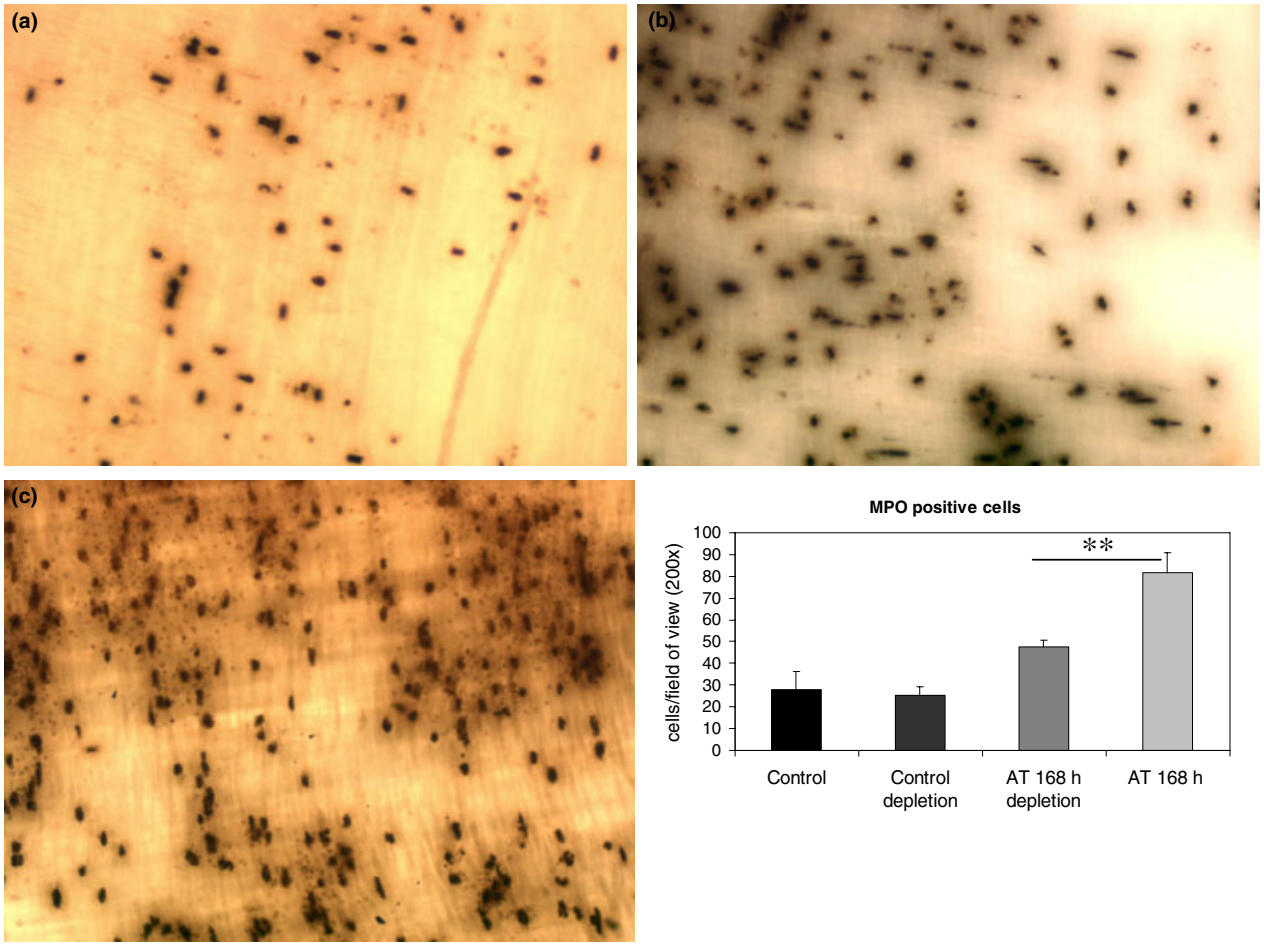

Figure 6 Panel a–c. Myeloperoxidase (MPO) staining of muscularis whole mounts demonstrating the infiltration of polymorphonuclear neutrophils (PMNs) in the intestinal graft muscularis of the control animals (a), of the allogeneic transplanted depleted grafts (b) and vehicle-treated grafts (c) after 168 h of reperfusion (magnification 100×). Histogram quantifying and comparing the number of recruited and infiltrating MPO positive polymorphnuclear neutrophils in the muscularis of the control, depletion control (not transplanted), depleted grafts and vehicle-treated grafts at the investigated time point (n = 5–7). (**P < 0.01 AT-168 h depletion vs. AT-168 h vehicle.) Data are expressed as mean ± SEM.

of performing phagocytosis (macrophages, immature dendritic cells) within other transplanted small bowel compartments as there is the mucosa, including the Peyer's patches, the submucosa and the mesentery with mesenteric lymph nodes. It is conceivable that these other intestinal layers will also display reduced molecular and cellular inflammatory and immunoregulatory responses after macrophage-depletion and therefore these layers of course also significantly participate in the induction of the various early inflammatory and immunologic events leading to acute cellular rejection. This is emphasized by the fact that these compartments are important sites of cellular sensitization after small intestinal transplantation because of the preference of recipient leukocyte trafficking for peripheral and central donor lymphoid tissues and *vice versa*. In order to further clarify the extent to which and the point of time following the transplantation after which the different compartments participate in the induction of events leading to rejection, further experiments have to be performed directly comparing the different layers at well-defined closely set time-points by various molecular and cellular diagnostic methods.

The alleviated inflammatory and immunologic cellular response within the depleted intestinal allograft muscularis has to be interpreted as a result of the altered molecular expression profile of immunologic mediators, such as MCP-1 and ICAM-1. These mediators have been reported to be involved in allograft rejection for several organs [47–51]. Concurrent to the significantly inhibited mRNA expression of MCP-1 in the depleted animals, we demonstrated a significantly reduced infiltration in MPO-positive neutrophils and monocytes as well as ED1-positive monocytes. Furthermore, IL-6 and TNFα, predomi-

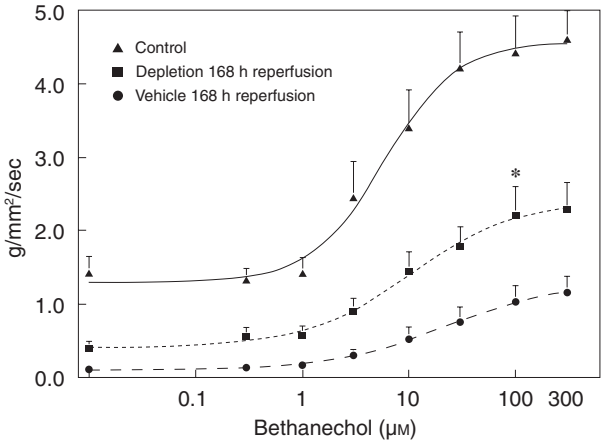

Figure 7 Bethanechol dose-response curves of smooth muscle contractile activity of depleted and vehicle-treated intestinal grafts at 168 h of reperfusion compared to non-transplant controls (n = 5–7 per group). Data are expressed as mean ± SEM. (*P < 0.05 AT-168 h depletion compared to AT-168 h vehicle under the stimulation with 100 μM/l Bethanechol.)

nantly expressed by macrophages and T cells that have also been associated with intestinal allograft rejection showed a significantly reduced expression profile in the depleted grafts [33,52–55] We also investigated the expression of IL-2 and INF γ, which are well known to be associated with immunoregulatory processes, T-cell proliferation and activation and induction of apoptosis, in the setting of transplantation, allograft rejection and tolerance [30,55–57]. The observation of a strong and significant reduction in mRNA expression of these mediators correlates well with the diminished infiltration of $CD4^+$ and $CD8^+$ lymphocytes in the depleted grafts, confirming the fact that IL-2 stimulates growth, differentiation and survival of cytotoxic T-cells ($CD8^+$) and that INFγ is expressed by TH1 helper cells, NK cells and $CD8^+$ cells and that it activates APCs (macrophages and dendritic cells) and $CD4^+$ cells. We also evaluated apoptosis by apoptotic body count and mRNA expression of FasL in the muscularis and the mucosa as apoptotic body count is one of the key factors in monitoring the onset or severity of acute rejection episodes. The depleted grafts revealed a significant reduction in apoptotic body counts and FasL mRNA expression. On account of the overall improvement of histopathologic rejection criteria, the severity of acute rejection could therefore be downgraded by one degree.

The *in vitro* contractility measurements clearly demonstrate that rejection leads to a massive inhibition of smooth muscle contractile activity of the graft. This motoric dysfunction is significantly improved by depletion of resident donor macrophages, most likely on account of the reduced mRNA expression of the mediator iNOS as well as improved cellular and histopathologic factors affecting contractility. Considering its potentiality as a liberated agent capable of impairing intestinal motility, we investigated the kinase iNOS, which has been identified as one of the important suppressors of rodent postoperative intestinal smooth muscle function by synthesizing NO [18,34,58]. Nitric oxide is produced during ischemia-reperfusion injury, has a promotional effect on acute and chronic rejection and is one of the most important suppressors of postoperative intestinal smooth muscle contractility [18,34,58–60]. In the small bowel, particularly ED2-positive resident macrophages, ED1-positive passenger macrophages and glial cells of the enteric plexus have been shown to be a major source of iNOS expression with successive impairment of smooth muscle function in models of postoperatively induced surgical ileus [18,20,34,61]. In addition to the functionally relevant iNOS over expression in the allograft muscularis, the morphologic changes in rejected grafts, caused by the destructive immunity, such as thinning and disruption of the muscularis, muscle cell edema and massive infiltration with different leukocyte populations, result in a mechanical disintegration of the tissue and subsequently in further impairment of motoric graft function. In the depleted grafts, these destructive histopathologic changes were prevented and the allograft smooth muscle contractile activity was significantly improved.

In conclusion, the present data demonstrate that acute allograft rejection after intestinal transplantation is associated with a complex molecular and cellular inflammatory response within the graft muscularis. Depletion and inactivation of donor resident macrophages leads to a significant alleviation of infiltration, recruitment and activation of leukocytes with reduced coexpression of various immunoregulatory and kinetically active mediators in the muscularis propria. Additionally, a decline in apoptosis and a reduction of tissue disintegration of the muscularis were observed. This in turn results in a significant improvement of smooth muscle contractile activity that is usually impaired during acute allograft rejection. The results emphasize that macrophages are likely to be an important early source for the induction of multiple inflammatory and immunologic events that may participate in the early induction of acute allograft rejection. This suggests that the extensive network of resident muscularis macrophages might be a promising target for future treatment strategies in acute rejection.

Authorship

NS, KA-E, JCK, AH, AT: designed research study in the paper. NS, KT, MVW, AT: performed research for the

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paper. SW and RT: contributed important reagents for the trials referred in the paper. MVW, RT, JCK: analyzed the data in the paper. KA-E, AT: wrote the paper. TP: collected the data to the paper.

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