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Intrathymic injection of anti-Fas monoclonal antibody prolongs murine non-vascularized cardiac allograft survival

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Abstract Fas is a pro-apoptotic molecule involved in activation-induced cell-death of T lymphocytes, central and peripheral tolerance and immune privilege. We sought to determine the role of Fas in the thymus after organ transplantation. Heterotopic non-vascularized heart transplants from C3H mice were placed in C57Bl/6 recipients. A hamster anti-mouse anti-Fas mAb (JO2) was injected in the thymus of allograft recipients at the time of transplant. Results were compared with intrathymic injections of hamster IgG (HIgG), anti-FasL, as well as surgical thymectomy or intraperitoneal or intravenous injections of JO2 as controls. Intrathymic injection of 5 μg JO2 induced massive thymocyte apoptosis and enhanced allograft survival compared to HIgG (median graft survival 16 days vs 12.5 days, respectively, $P=0.01$). The effect was receptor and ligand specific. Intraperitoneal or intravenous injections of JO2 did not prolong graft survival. Thymocyte apoptosis was confirmed in vitro, in vivo and in situ. In the thymus, double positive immature $CD4^+8^+$ thymocytes were most susceptible to Fas-induced apoptosis. Our data shows that specific modulation of Fas pathways in the thymus at the time of transplant improves modestly but significantly murine heterotopic non-vascularized cardiac allograft survival and is associated with apoptosis of immature $CD4^+8^+$ thymocytes.

Keywords Fas · Thymus · Thymocytes · Transplantation · Graft survival

Introduction

There are mutual interactions between the thymus and the allograft in organ transplantation. Intrathymic injections of donor-specific peptides [1], non-lymphoid [2] and lymphohematopoietic [3] cells prolong allograft survival. Conversely, it has been shown that lymphocytes recirculate through the thymus [4] and that accelerated rejection can trigger thymocyte apoptosis [5]. In the thymus, resident and bone marrow-derived dendritic cells play a central role in the education of thymocytes through the processes of positive and negative selection, where apoptosis is the principle mechanism of cell death [6].

Fas and Fas ligand (FasL) are proapoptotic molecules belonging to the TNF-R and TNF family, respectively [7]. Fas bearing cells undergo apoptosis following the interaction between Fas and its ligand. These molecules are involved in peripheral deletion of

activated mature T cells [8], elimination of malignant and infected target cells [9], and apoptosis of inflammatory cells at "immune privileged" sites including the eye and the testes [10, 11]. We have previously reported long-term donor specific unresponsiveness after intrathymic injection of donor antigens and showed that the mechanism may be deletional [3]. Since recipient thymocytes are reeducated to be tolerant to donor antigens, and Fas may trigger apoptosis in both positive and negative selection of thymocytes [12, 13], we sought to determine whether Fas apoptotic pathway plays a role in the thymus of allograft recipients early after transplant.

Materials and methods

Mice

Adult 6 to 8-week-old male C57Bl/6 (H-2^b) mice, and female C3H (H-2^k) late gestation-untimed pregnancies, were purchased from Harlan Sprague-Dawley (Indianapolis, Ind., USA). Male B6.MRL-Fas^{lpr} (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, Me., USA). Mice were maintained at the University of Wisconsin-Madison Medical School Animal Care Unit. Principles of laboratory animal care were followed and the Institutional Animal Care and Use Committee approved the procedures carried out.

Antibodies

Purified and FITC-conjugated hamster anti-mouse Fas mAb (JO2), purified and FITC-conjugated Hamster IgG (HIgG) mAb immunoglobulin isotype standard (anti-keyhole limpet hemocyanin (Ha4/8), purified mouse anti-mouse FasL mAb (K10), PE-conjugated rat anti-mouse anti-CD4 (GK1.5) and APC-conjugated rat anti-mouse anti-CD8 (Ly-2) were purchased from Pharmingen (San Diego, Calif., USA).

Transplant model

Heterotopic non-vascularized cardiac allografts from neonatal mice were placed in the ear pinna of adult recipient mice as described previously [14, 15]. Briefly, neonatal cardiac allografts were harvested during the first 24 h after birth. They were excised and separate ventricles were transplanted into a subcutaneous pocket of the pinna of the ear of recipient mice under anesthesia (etomidate 0.03–0.05 mg/g ip). Survival of cardiac allografts was followed daily by visualizing cardiac tissue contractions under an operating microscope. The day cardiac contractions ceased was determined to be the day of rejection and graft survival was expressed as Median Graft Survival (MGS).

Recipient conditioning at the time of transplant

Allograft recipients were matched for age (6–8 weeks) and divided in the following groups: no thymic manipulation; surgical thymectomy (ATX); intrathymic injection of either anti Fas mAb JO2 (2.5 μg, 5 μg, 10 μg) or HIgG (5 μg) or anti-FasL mAb K10 (5 μg); intravenous (iv) injection of JO2 (5 μg), and intraperitoneal (ip) injections of JO2 (5 μg). The narrow dose-response study was based on data from Ogasawara et al. showing that 100 μg JO2 ip was lethal in all treated animals [16]. The doses of all mAbs were adjusted to a total volume of 50 μl Phosphate Buffered Saline (PBS). Thymic manipulations, as well as iv and ip injections of mAbs were done 15 to 45 min prior to transplant.

Intrathymic injections

Recipient mice were anesthetized with etomidate (0.03–0.05 mg/g ip). Under the light microscope, the skin over the cervical-sternal notch was incised and the proximal sternum divided for 5 mm and retracted, exposing the thymus. The upper segment of each thymus lobe was elevated and held by a pair of forceps while mAb suspensions were injected equally into both lobes (25 μl volume in each lobe) via a 30-gauge needle. After the intrathymic injection, the thymus became uniformly distended with no obvious leakage. The sternum and the skin were then sutured and the cardiac allograft was transplanted as described earlier.

Cell suspensions and cell culture conditions

Lymphoid cell suspensions from thymus, spleens, regional draining cervical lymph nodes and peripheral blood were prepared by standard techniques. Thymocytes and splenocytes were suspended in RPMI 1640 (GibcoBRL, Grand Island, N.Y., USA), supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 1% L-glutamine, 0.05% β-mercaptoethanol and 1% sodium pyruvate in 24-well flat bottomed plates. The final cell suspensions were at 5×10^6 cells/ml. Incubations were carried out for 6 and 24 h at 37°C under an atmosphere of 95% air: 5% CO_2, in media alone or in media with JO2 or HIgG standard isotype (1 μg/5×10^6 cells).

Flow cytometry

Single-cell suspensions from lymphoid organs of naive and JO2 treated adult male C57Bl/6 mice were studied with FITC-conjugated JO2, PE-conjugated anti-CD4, APC-conjugated anti-CD8 and isotype controls (1 μg/10^6 cells). Cells were incubated with the mAbs at 4°C for 30 min. They were then washed and resuspended in PBS

and 20 000 cells were analyzed by FACScan. DNA-binding fluorochromes propidium iodide (PI) and Bisbenzimide Hoechst No. 33342 trihydrochloride (Ho342) from Sigma (St. Louis, Mo., USA) were utilized as described [17, 18] to measure apoptotic and necrotic cells in the thymus, lymph nodes, spleen and peripheral blood lymphocytes (PBL) at time zero, 6 and 48 h after the *i.t.* inoculation of JO2 or HIgG ($n=3$). Lymphoid cell suspensions (5×10^6 cells/ml) were incubated with Ho342 (2 μg/ml) in complete RPMI 1640 containing 1% FCS at 37°C under an atmosphere of 95% air: 5% CO_2 for 10 min. To prevent further uptake of the dye, the cells were cooled at 4°C, centrifuged at 400 g for 5 min, resuspended in PBS containing PI (5 μg/ml), and examined by flow cytometry.

Histology and DNA fragmentation detection assays for apoptosis

Thymus tissue from healthy adult control animals and mice with intrathymic injection of either JO2 or HIgG was removed 6 h after injection. It was fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E) by a standard procedure or assayed for apoptosis by the Klenow-FragEL DNA fragmentation detection kit (Oncogene Research Products, Cambridge, Mass., USA) according to the manufacturer's instruction. Briefly, paraffin embedded tissue was deparaffinized and rehydrated by xylene and ethanol. Cells were then permeabilized by proteinase K and endogenous peroxidases were inactivated by H_2O_2 and methanol. Then the DNA fragment labeling was done either with deoxynucleotides alone (negative controls) or with deoxynucleotides and Klenow enzyme (the large fragment of *E. coli* DNA polymerase I). The labeling reaction was terminated by adding 0.5 M EDTA, pH 8, and labeled DNA was detected with a peroxidase streptavidin conjugate, 3, 3' diaminobenzidine and H_2O_2/urea. Finally, the tissue was methyl-green counterstained and examined under a light microscope. Apoptotic nuclei that exhibited a dark brown staining were counted in five 40× fields per specimen and the numbers were expressed as arithmetic means with standard deviations. Pictures were taken using a DAGE MTI DC-330 video camera attached to a Zeiss Axiovert 100TV microscope.

Statistical analysis

Student's *t* test and the non-parametric Mann–Whitney rank sum test (Sigma Stat Software, Jandel Scientific) were used when appropriate to analyze median graft survival, number of apoptotic nuclei and various cell population percentages. *P* values of 0.05 or less were considered significant.

Results

Fas expression in the lymphoid organs of 6-week-old C57Bl/6 mice

Fas expression was studied on single-cell suspensions from thymuses, spleens, lymph nodes and PBL in 6-week-old C57Bl/6 mice by flow cytometry as described above ($n=4$). Seventy percent of thymocytes, 20% of splenocytes, 15% of lymph node lymphocytes and 10% of peripheral blood lymphocytes expressed Fas on cell surface (Fig. 1).

JO2 induced apoptosis of thymocytes in vitro

Thymocytes from naive mice were incubated in vitro with media alone or media with either JO2 or HIgG standard isotype for 6 h and studied by flow cytometry after staining with PI and Ho342 to assess apoptosis. Four populations of cells were determined: live or $PI^-/Ho342^-$ (a), early apoptotic or $PI^-/Ho342^+$ (b), late apoptotic or $PI^+/Ho342^+$ (c) and necrotic or $PI^+/Ho342^-$ (d). Late apoptotic and necrotic cells together were defined as dead cells. There was no difference in the percentage of live, early apoptotic and dead cells between thymocytes incubated in media alone (75%, 13% and 12%, respectively, data not shown) or with HIgG (74%, 12% and 14%, respectively). Incubation with JO2 for 6 h dramatically increased the number of early apoptotic and dead cells (3.5- and 3-fold, respectively; Fig. 2a).

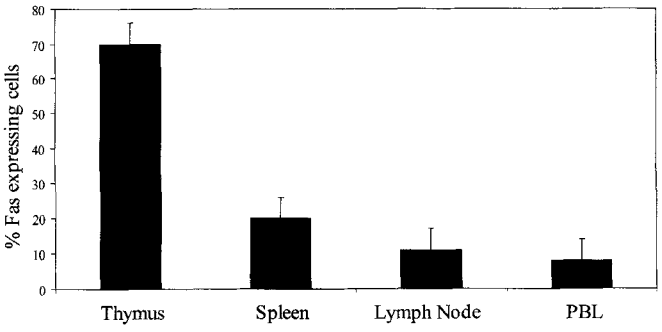
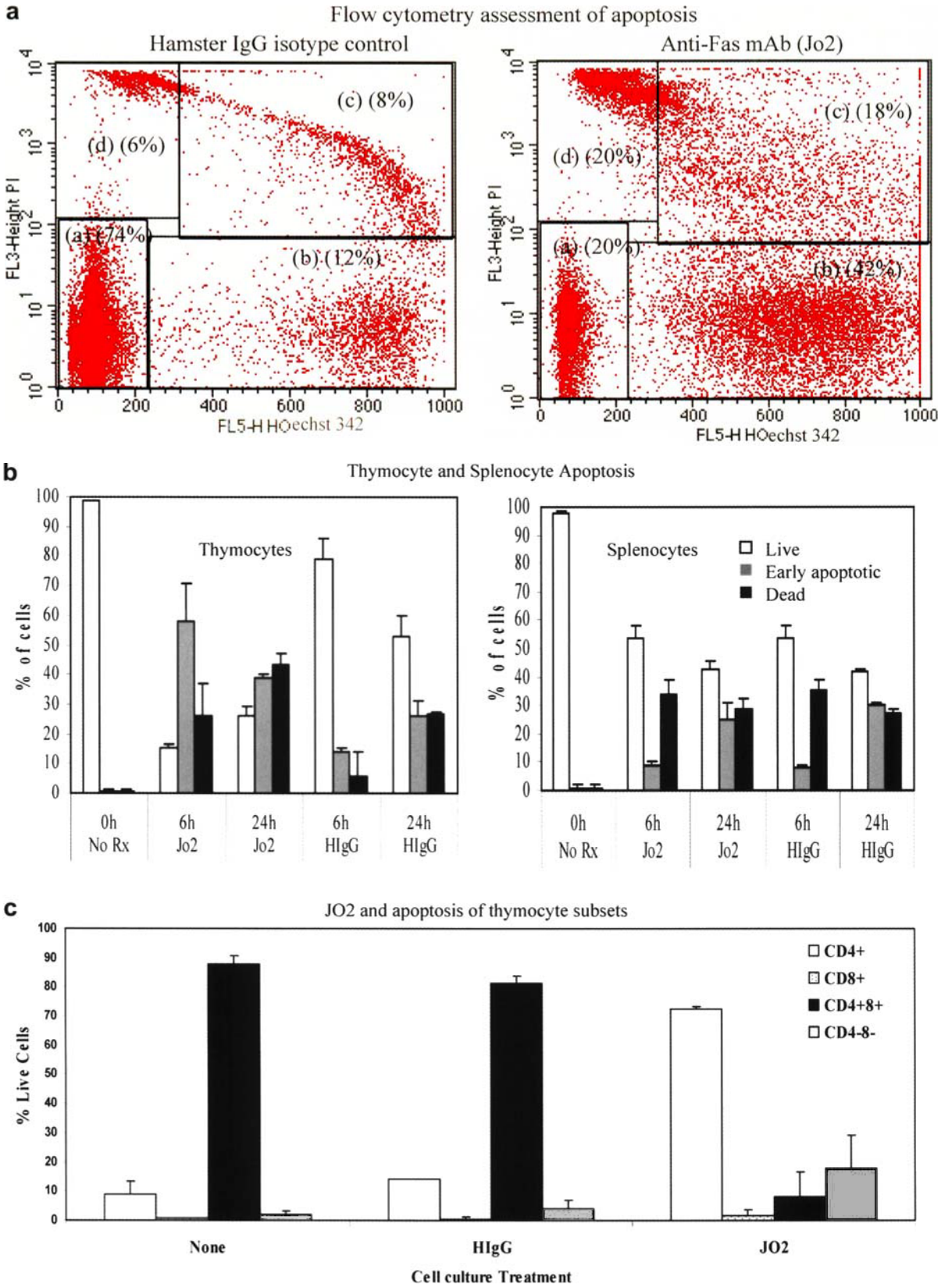


Fig. 1 Fas expression on single-cell suspensions from thymus, spleen, lymph nodes and PBL. Thymocytes, splenocytes, lymph node lymphocytes and PBL were prepared from 6-week-old C57Bl/6 mice ($n=4$). Cells were directly labeled by FITC-conjugated JO2 or hamster IgG isotype control and analyzed by flow cytometry. Approximately 70% of thymocytes, 20% of splenocytes, 15% of lymph node lymphocytes and 10% of peripheral blood lymphocytes expressed Fas on their surface


a
Flow cytometry assessment of apoptosis
Hamster IgG isotype control
Anti-Fas mAb (Jo2)
(c) (8%)
(d) (6%)
(a) (74%)
(b) (12%)
(c) (18%)
(d) (20%)
(a) (20%)
(b) (42%)
FL3-Height PI
FL5-H HOechst 342
b
Thymocyte and Splenocyte Apoptosis
Thymocytes
Splenocytes
Live
Early apoptotic
Dead
% of cells
0h No Rx
6h Jo2
24h Jo2
6h HIgG
24h HIgG
c
JO2 and apoptosis of thymocyte subsets
CD4+
CD8+
CD4+8+
CD4-8-
% Live Cells
None
HIgG
JO2
Cell culture Treatment

◀

Fig. 2a–c Apoptosis of thymocytes and splenocytes incubated with anti-Fas mAb in vitro. **a** Thymocytes from naive mice were incubated in vitro with hamster IgG (HIgG) standard isotype or JO2 for 6 h (*a*). Apoptotic cells were studied by flow cytometry in a two-parameter setting including PI and Ho342. Four populations of cells were determined: live or $PI^-/Ho342^-$ (*a*), early apoptotic or $PI^-/Ho342^+$ (*b*), late apoptotic or $PI^+/Ho342^+$ (*c*) and necrotic or $PI^+/Ho342$ (*d*). Late apoptotic and necrotic cells together were defined as dead cells. **b** Thymocytes or splenocytes were cultured in the presence of either HIgG or JO2 for 0 h, 6 h, and 24 h, and flow cytometry was done to assess apoptosis as indicated above. *White*, *gray* and *black bars* represent the percentages of live, early apoptotic and dead cells, respectively. **c** Percentage of live thymocytes after treatment with JO2 or HIgG. Single-cell suspensions from the thymuses of 6- to 8-week-old C57BL6 male mice were studied by flow cytometry with PE-conjugated anti-CD4, APC conjugated anti-CD8, PI and Ho342 before and after incubation with JO2 or HIgG for 24 h. *White*, *white and black dots*, and *black and gray bars* represent $CD4^+$, $CD8^+$, $CD4^+8^+$ and $CD4^-8^-$ cells, respectively. *No Rx* no treatment

A kinetic study of thymocytes and splenocytes at 0 h, 6 h, and 24 h was carried out with cells cultured in media alone, or media with JO2 or HIgG standard isotype. In thymocytes incubated with media alone, there were 79 ± 7% live, 14 ± 2% early apoptotic, and 6 ± 8% dead cells at 6 h, and 53 ± 7% live, 26 ± 5% early apoptotic and 27 ± 1% dead cells at 24 h. Similar results were obtained when thymocytes were cultured with HIgG (Fig. 2b). Splenocyte cultures with media contained 54 ± 4% live, 8 ± 1% early apoptotic and 35 ± 4% dead cells at 6 h, and 42 ± 1% live, 30 ± 1% early apoptotic and 27 ± 2% dead cells at 24 h (Fig. 2b). JO2 increased the number of apoptotic thymocytes compared to HIgG at 6 h (58 ± 13% vs 14 ± 2%, $P < 0.005$) and 24 h (39 ± 1% vs 26 ± 5%, $P < 0.05$). However, we did not observe increased apoptosis in splenocytes after 6 h or 24 h incubation with HIgG (Fig. 2b).

Selective apoptosis of $CD4^+8^+$ thymocytes by JO2

To determine which thymocyte subset was most susceptible to JO2-induced cell death, single-cell suspensions from the thymus of C57Bl/6 male mice were studied by flow cytometry with PE-conjugated anti-CD4, APC conjugated anti-CD8, PI and Ho342 before and after incubation with JO2 or HIgG for 24 h. The percentage of live double positive $CD4^+8^+$ thymocytes dramatically decreased from 82 ± 2% to 8 ± 8% after treatment with JO2 (Fig. 2c). Most remaining live thymocytes after JO2 treatment were single positive $CD4^+8^-$ cells.

JO2 prolonged cardiac allograft survival

Based on the sensitivity of thymocytes to JO2-mediated apoptosis and the previous observation of donor specific

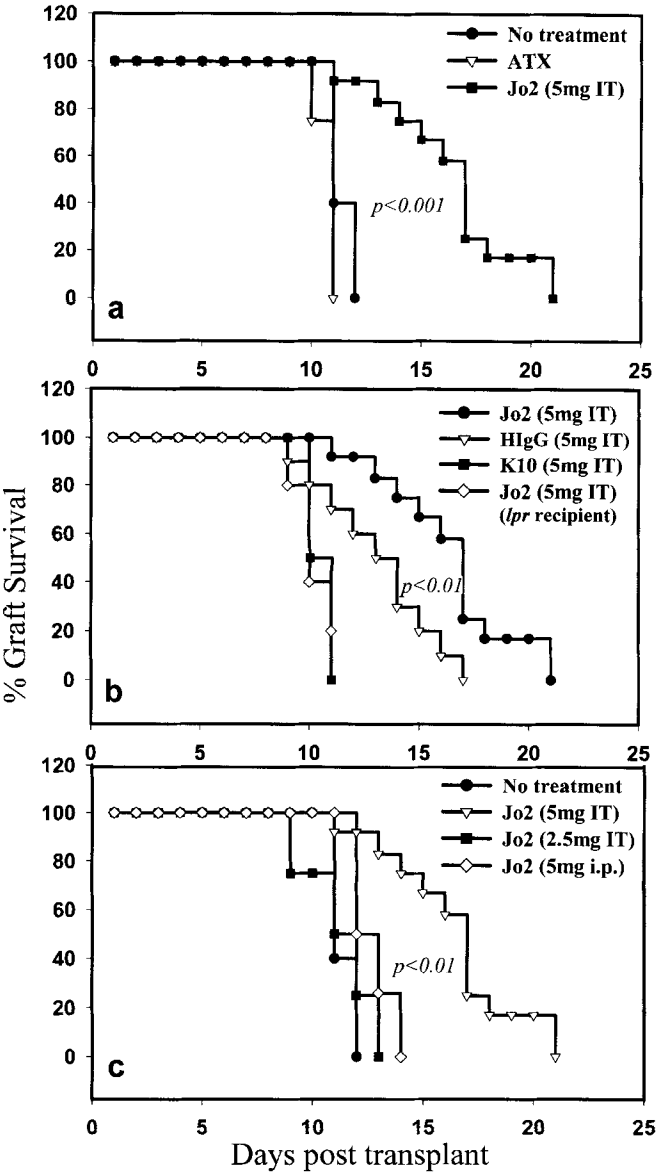


Fig. 3a–c Intrathymic injection of JO2 (5 μg) prolonged graft survival. **a** Intrathymic injection of JO2 5 μg prolonged graft survival (*black squares*, $n = 12$, MGS = 16 days) compared to surgical thymectomy (*white triangles*, $n = 4$, MGS = 10 days, $P = 0.001$) or no treatment (*black circles*, $n = 4$, MGS = 10 d, $P = 0.001$). **b** Intrathymic injection of JO2 5 μg prolonged graft survival compared to the hamster IgG isotype control group (*HIgG 5 μg*, *white triangles*, $n = 10$, MGS = 12.5 days, $P = 0.01$), *Lpr* (Fas-deficient) mice (*white diamonds*, $n = 4$, MGS = 9 days, $P = 0.001$) and intrathymic injection of anti-FasL (*K10 5 μg*, *black squares*, $n = 4$, $P = 0.001$). **c** Intrathymic injection of JO2 5 μg prolonged graft survival compared to a lower dose of 2.5 μg (*JO2 2.5 μg*, *black squares*, $n = 4$, MGS = 10 days, $P = 0.001$) and 5 μg intraperitoneal injections (*JO2 5 μg ip*, *white diamonds*, $n = 4$, MGS = 11.5 d, $P = 0.01$). All animals that received intrathymic injections of JO2 10 μg, and 3/7 of those receiving JO2 5 μg ip died from hepatic failure

unresponsiveness by thymic manipulation [3] we asked whether JO2-induced apoptosis in the thymus could prolong allograft survival. We found that intrathymic

injection of 5 μg JO2 delayed graft rejection ($n=12$, MGS = 16 days) compared to no thymic manipulation ($n=4$, MGS = 10 days, $P=0.001$; Fig. 3a). Most thymocytes present Fas on their surface. To evaluate the possibility that intrathymic JO2 injection resulted in a "chemical thymectomy", we performed surgical thymectomy (ATX, $n=4$) at the time of transplant. The median graft survival in this group was similar to the untreated group (10 days) and significantly shorter compared to the JO2 treated group ($P=0.001$, Fig. 3a).

A group of 10 control recipient mice received 5 μg purified HIgG mAb immunoglobulin isotype standard (anti-Keyhole Limpet Hemocyanin, clone Ha4/8) intrathymically. There was no significant prolongation of allograft survival in this group compared to the control untreated animals (MGS = 12.5 vs 10 days, P = NS) and graft survival was significantly shorter in this group compared to JO2 treated recipients (MGS = 12.5 days vs 16 days, $P=0.01$; Fig. 3b). The blockade of Fas-FasL interactions by injecting 5 μg anti-FasL (K10) mAb ($n=4$) did not modify the timing of graft rejection (MGS = 10 days). To determine whether the observed effect was receptor-specific, B6.MRL Fas^{lpr}mice matched for age received intrathymic injections of JO2 at the time of transplant. *Lpr* is a loss-of-function mutation in the gene encoding Fas resulting from the insertion of an early transposable element (ETn) into intron 2 of the Fas gene [7]. Graft survival in this group was comparable to the untreated control mice (MGS = 9 and 10 days, respectively; Fig. 3b).

To determine dose-responsiveness, a narrow dose-response study was done by injecting 2.5 μg ($n=4$) and 10 μg ($n=5$) JO2 in the thymuses of transplant recipient mice. Delayed allograft rejection was only observed in the 5 μg group; as the 2.5 μg dose had no effect on graft outcome (median survival: 10 days) (Fig. 3c) and all animals receiving the 10 μg dose died within 16 h after the injection. At autopsy, livers of these animals displayed characteristics of fulminant hemorrhagic hepatitis as described previously [16] and thymuses showed extensive cortical and medullary apoptosis and necrosis (data not shown). However, livers from the 5 μg JO2 group showed normal histology (data not shown) and thymuses showed areas of apoptosis. To determine the specific role of the thymus in our observations, 5 μg JO2 were injected iv ($n=4$) and ip ($n=7$) at the time of transplant. All iv injections and 43% of ip injections caused lethal liver failure in the recipients. Moreover, surviving animals did not exhibit prolonged graft survival ($n=4$, MGS = 11.5; Fig. 3c).

In vivo response of lymphoid organs to intrathymic injection of JO2

The effect of JO2 on lymphoid organs including the thymus, cervical lymph nodes and spleen in vivo was studied by flow cytometry, 6 h and 48 h after intrathymic injections of JO2. Only the 48 h data from JO2 treated animals are shown (Fig. 4). In the JO2 group, the percentage of live thymocytes dropped from 99% to 65 ± 16% ($P=0.01$) 6 h after the injection and remained at 64 ± 10% up to 48 h (Fig. 4). In the HIgG group, there was a transient reduction of live thymocytes (99% to 83 ± 12%, P = NS) at 6 h, which nearly resolved (92 ± 3%) at 48 h. In contrast, the percentage of live splenocytes and lymphocytes at 48 h was 90 ± 9% and 75 ± 7%, respectively, similar between JO2 and HIgG treated animals and mildly reduced compared to thymocytes from JO2 treated animals.

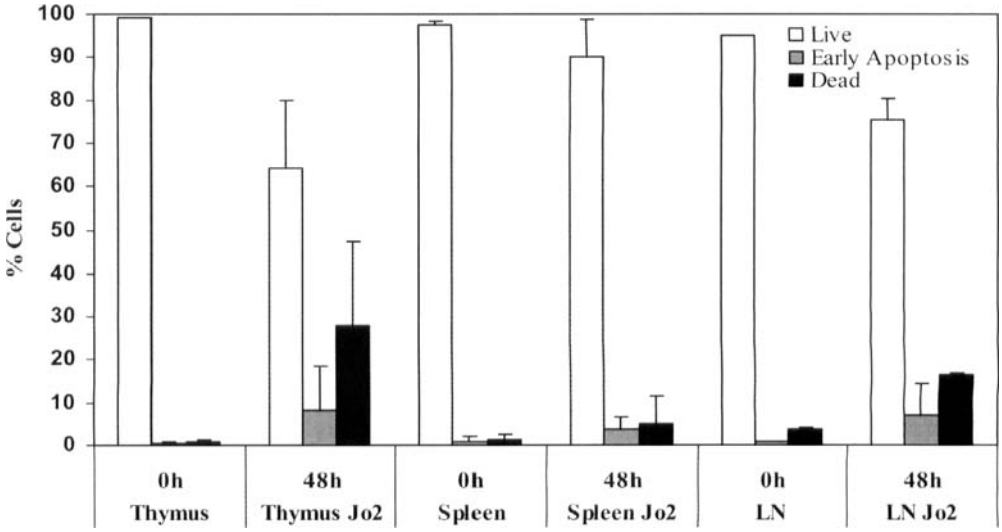


Fig. 4 The effect of JO2 on thymocytes, lymph node lymphocytes and splenocytes in vivo. Cell populations were studied by flow cytometry on single-cell suspensions from the thymus, cervical lymph nodes and spleen ($n=3$ for each group) at time zero (healthy non-transplanted animals) and 48 h after transplantation (hamster IgG or JO2 treated animals). The analysis was done on double-stained (Ho342 and PI) lymphocytes and results are shown as bar graphs showing the percentage of cells in each category. *White*, *gray* and *black bars* represent live, early apoptotic and dead cells, respectively

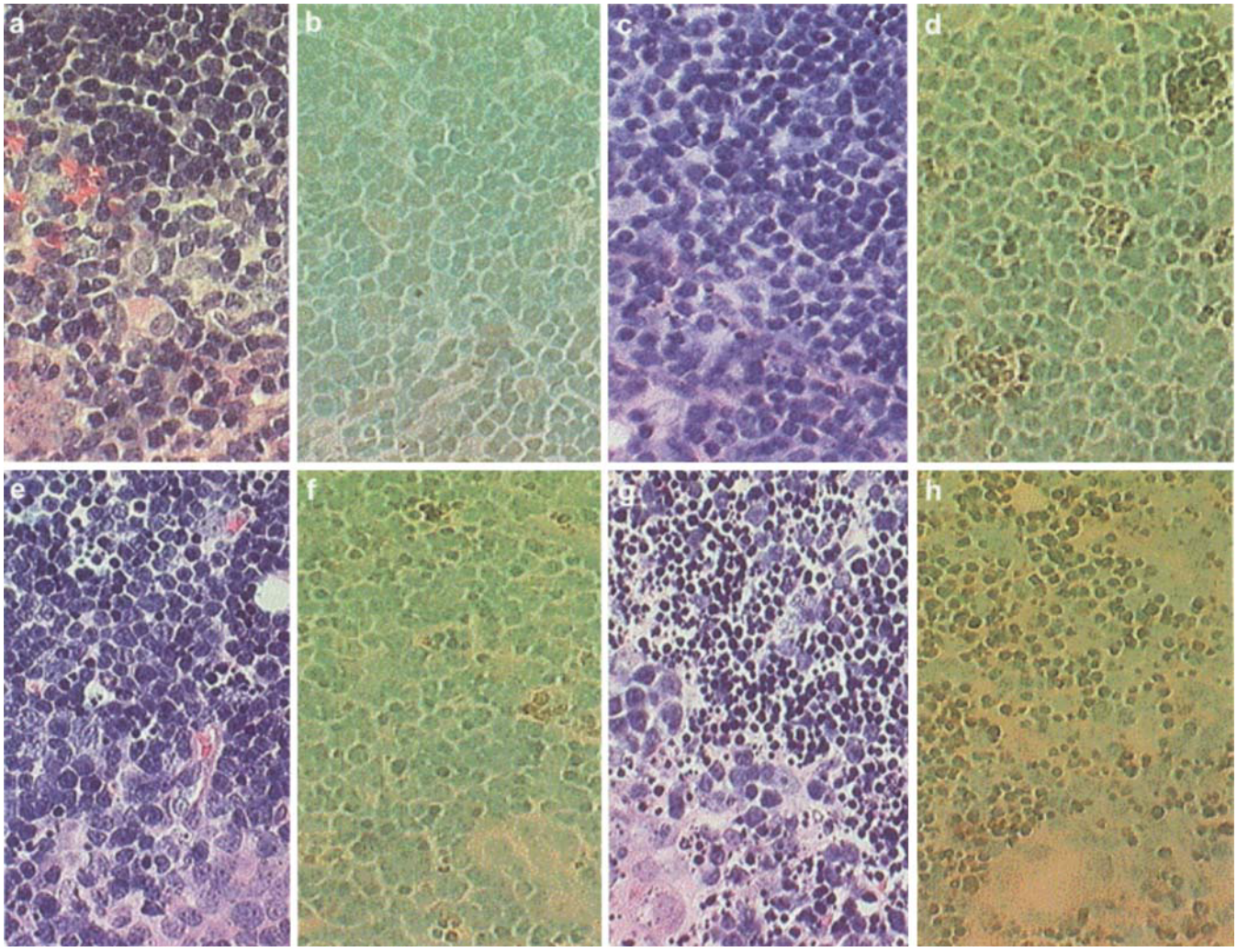
Thymic apoptosis following intrathymic injection of JO2

A DNA fragmentation detection assay (FragEL) was used to study apoptosis in the thymuses. Mice undergoing transplantation surgery without i.t. injection had more apoptotic nuclei in their thymuses than the naïve, adult animals matched for age (110 ± 20 vs 3 ± 1, respectively, $P < 0.001$). Similar results were obtained when HIgG was injected i.t. and compared to the control (113 ± 15 vs 3 ± 1, respectively, $P < 0.001$). However, this observation was not specific as we observed comparable findings 6 h after syngeneic transplantation and/or general anesthesia (data not shown). JO2 treated animals showed more apoptotic cells within the thymus than the the HIgG group (446 ± 24 vs 113 ± 15, respectively, $P < 0.001$) confirming the apoptotic effect of JO2 in situ (Fig. 5).

Fig. 5a–h Thymus of non-vascularized cardiac allograft recipients 6 h after transplantation (immunohistochemistry). We studied thymuses of healthy animals that had not undergone transplantation (**a, b**), transplanted mice without intrathymic injection (**c, d**), and allograft recipients that had undergone intrathymic injections with 5 μg of either hamster IgG isotype control (HIgG) (**e, f**) or anti-Fas mAb JO2 (**g, h**) at the time of transplant. Sections (5 μm, 40× enlargement) were stained either with H&E by standard procedure (**a, c, e, g**) or assayed for apoptosis by the Klenow-FragEL DNA fragmentation detection kit (**b, d, f, h**). Apoptotic nuclei are stained *dark brown* with the FragEL kit

Discussion

Our data demonstrates that intrathymic injection of JO2 at the time of transplant prolongs allograft survival in a murine non-vascularized heterotopic cardiac transplant model. We observed massive apoptosis in the thymus of treated animals and significant apoptosis of immature double-positive $CD4^+8^+$ thymocytes when cells were cultured with JO2 in vitro. These findings confirm previous reports that showed selective apoptosis of $CD4^+8^+$ thymocytes by JO2, with nearly no effect on



mature single-positive cells [19, 20]. Cortical immature $CD4^+8^+$ thymocytes terminate their maturation process as they migrate through the medulla and become single positive $CD4^+8^-$ or $CD4^-8^+$ cells through a process described as positive and negative selection [21]. Furthermore, studies by Kurasawa and Kishimoto have independently demonstrated that Fas-induced apoptosis is involved in both positive and negative selection processes, suggesting a role for this apoptotic pathway in the differentiation and maturation of thymocytes [12, 13]. Although $CD4^+8^+$ thymocytes were the main focus of our study, we believe that the prolongation of graft survival in the experimental settings could be explained by three possible mechanisms. First, the non-specific deletion of immature $CD4^+8^+$ thymocytes by JO2 may reduce the pool of future alloreactive lymphocytes and/or delete these cells during their maturation process in the thymus through additive or independent effects. Second, JO2 may delete activated lymphocytes that are derived from the allograft and recirculate through the thymus [4]. Finally, $CD4^+25^+$ immunoregulatory T cells, although not the target of our study, might have contributed to the observed improvement of graft survival. $CD4^+25^+$ regulatory cells have suppressor properties and have been involved in autoimmune diseases and more recently, in the induction of transplant tolerance [22]. JO2 treatment might have enhanced their suppressor function [23]. Further in vivo characterization of these interactions could confirm this hypothesis.

In vivo thymocytes showed a more selective apoptotic response to JO2 compared to splenocytes and lymph node lymphocytes. This observation was even more pronounced in vitro. As described earlier, the expression of Fas on the surface of thymocytes was significantly higher than the other two cell populations. Furthermore, insufficient amounts of physiological survival factors in cell culture media and different distribution patterns of JO2 in vitro compared to in vivo could contribute to the observed differences in the apoptotic response to JO2. Blocking the Fas-FasL pathway by an anti-FasL mAb (K10) did not improve graft survival. We believe that prolongation of graft survival was due to the pro-apoptotic effects of JO2, whereas K10 prevents FasL induced apoptosis by binding to FasL. The therapeutic window with intrathymic injections of JO2 was narrow: a 10 μg-dose was hepatotoxic and lethal and a 2.5 μg-dose did not prolong graft survival. Lethal effects of JO2 have been shown when the drug was given systemically [16]. However, the intrathymic utilization of JO2 has not been reported and our data shows that the safety profile of JO2 is better with local, thymic injections where small doses are used and efforts are made to minimize systemic circulation of the drug. Moreover, other hamster anti-mouse Fas mAbs (RK-8) with better safety profiles have been described [24] that could potentially yield similar results with less toxicity.

In clinical and experimental transplantation, one often utilizes systemic induction immunosuppressive therapies to prolong graft survival or induce tolerance. Our data suggest that a similar approach can be used locally, by disrupting the maturation of $CD4^+8^+$ thymocytes through the Fas system. However, whether the effects observed here in this non-vascularized transplant model could apply to a large animal or human vascularized transplant model is not known. Further, young transplant recipients might benefit more from this approach as the thymus probably plays a more important role in younger individuals. In conclusion, intrathymic injection of anti-Fas mAb at the time of transplant prolongs allograft survival modestly but significantly, and is associated with the apoptotic deletion of immature double positive $CD4^+8^+$ thymocytes. Studies investigating the role of Fas-FasL in the thymus in allograft rejection could involve the local use of molecules less toxic than JO2, and address the role of $CD4^+CD25^+$ immunoregulatory cells.

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