The Journal of Immunology

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J Immunol 2006; 177:6920-6929; ;
doi: 10.4049/jimmunol.177.10.6920
http://www.jimmunol.org/content/177/10/6920

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Double-Negative T Cells, Activated by Xenoantigen, Lyse Autologous B and T Cells Using a Perforin/Granzyme-Dependent, Fas-Fas Ligand-Independent Pathway[1]

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The ability to control the response of B cells is of particular interest in xenotransplantation as Ab-mediated hyperacute and acute xenograft rejection are major obstacles in achieving long-term graft survival. Regulatory T cells have been proven to play a very important role in the regulation of immune responses to self or non-self Ags. Previous studies have shown that TCR$\alpha\beta^+$CD3$^+$CD4$^-$CD8$^-$ (double-negative (DN)) T cells possess an immune regulatory function, capable of controlling antidonor T cell responses in allo- and xenotransplantation through Fas-Fas ligand interaction. In this study, we investigated the possibility that xenoreactive DNT cells suppress B cells. We found that DNT cells generated from wild-type C57BL/6 mice expressed B220 and CD25 after rat Ag stimulation. These xenoreactive B220$^+$CD25$^+$ DNT cells lysed activated, but not naive, B and T cells. This killing, which took place through cell-cell contact, required participation of adhesion molecules. Our results indicate that Fas ligand, TGF-β, TNF-α, and TCR-MHC recognition was not involved in DNT cell-mediated syngenic cell killing, but instead this killing was mediated by perforin and granzymes. The xenoreactive DNT cells expressed high levels of granzymes in comparison to allo- or xenoreactive CD8$^+$ T cells. Adoptive transfer of DNT cells in combination with early immune suppression by immunosuppressive analog of 15-deoxyspergualin, LF15-0195, significantly prolonged rat heart graft survival to 62.1 ± 13.9 days in mice recipients. In conclusion, this study suggests that xenoreactive DNT cells can control B and T cell responses in perforin/granzyme-dependent mechanisms. DNT cells may be valuable in controlling B and T cell responses in xenotransplantation. *The Journal of Immunology,* **2006, 177: 6920–6929.**

B cells, through the Abs they produce, play a major role in immune responses and are involved in the elimination of Ags, as well as the prevention of infections. Controlling B cell responses is one of the major challenges in Ab-mediated autoimmune diseases, as well as in allotransplantation and xenotransplantation. Although xenotransplantation offers the promise of an unlimited supply of donor organs, the body's robust immune attack against the xenograft is still a major hurdle. The mechanism of xenograft rejection in discordant models, such as pig to nonhuman primate, involves pre-existing, naturally occurring Abs that bind to Ags on the surface of vascular endothelial cells, resulting ultimately in vascular graft injury (1–4). In a concordant animal model, such as a rat-to-mouse model, where natural Abs are not present, graft-induced Abs also appear to be responsible for xenograft rejection. Indeed, in our previous studies, we have shown that B cell deficiency allows a rat heart graft to survive for a long time in the mouse (5).

While treatment with immunosuppressive drugs has shown substantial progress in allotransplantation, there are currently no immunosuppressive drugs capable of preventing xenograft rejection in a pig-to-nonhuman primate transplantation model (1, 6, 7). Furthermore, current immunosuppressive protocols that prevent xenograft rejection in nonhuman primates are too toxic for patient use. As a result, induction of transplant tolerance or permanent graft acceptance without long-term use of immunosuppression has become one of the major goals in xenotransplantation studies (1–3, 8).

Many studies have demonstrated that regulatory T (Treg)[3] cells play an important role in the regulation of immune responses to self or allogeneic Ags in numerous in vivo models of autoimmunity, inflammatory diseases and transplantation (9–13). Furthermore, several studies strongly suggest that tolerance may be achieved through the manipulation of Treg cells (12, 14, 15). However, most of these studies have focused only on allotransplantation so the role of Treg in xenotransplantation remains to be elucidated.

Treg cells can be of different subtypes. Although the majority of studies have focused on CD4$^+$CD25$^+$ Treg cells, the existence of immunosuppressive CD8$^+$, $\gamma\delta$-TCR, and NK$^+$ T cells has also been described previously (16, 17). In our recent studies, CD3$^+$CD4$^-$CD8$^-$ double-negative T (DNT) cells have also been found to possess immune regulatory functions in the development of transplant tolerance (18–21). For example, in one MHC class I Ag-mismatched allotransplantation model, mouse DNT cells specifically eliminated activated syngeneic antidonor CD8$^+$ T cells ex vivo through Fas ligand (FasL)-Fas interactions (18). The infusion of recipient-derived DNT cells, activated ex vivo, led to significantly prolonged survival of donor-specific skin and heart allografts in mice (18, 22). Furthermore, DNT cells appear to suppress

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Received for publication March 30, 2006. Accepted for publication August 25, 2006.

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[1] This study was supported by the Multi-Organ Transplant Program, the Lawson Health Research Institute at London Health Sciences Centre, and the Canada Institute for Health Research.

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[3] Abbreviations used in this paper: Treg, regulatory T; 7-AAD, 7-aminoactinomycin D; AVR, acute vascular rejection; DNT, CD3$^+$CD4$^-$CD8$^-$ double-negative T; FasL, Fas ligand.

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$CD4^+$ T cell activation (19, 23) and down-regulate $CD8^+$ T cell-mediated immune responses, which appear to subsequently inhibit autoimmune disease ex vivo and in vivo (24). Interestingly, DNT cells are able to regulate chlamydia infection in the genital track by eliminating $CD8^+$ T cells and suppressing the activation of $CD4^+$ T cells (25).

Several studies have demonstrated the involvement of Treg cells in xenograft survival. A treatment protocol that consisted of depleting $CD4^+$ and/or $CD8^+$ T cells has shown that $CD4^+$ T cells were implicated in tolerance following a rat-to-mouse xenograft (26, 27). In addition, a treatment regimen of donor lymphocyte infusion and $CD4^+$ T cell depletion resulted in increased DNT cells in the recipients' spleens. These DNT cells suppressed $CD4^+$ and $CD8^+$ T cell responses, which correlated with long-term xenograft survival (28, 29). Recent studies have reported that $CD4^+$ Treg cells can directly suppress B cells in lymphoid tissue (30), lyse Ag-presenting B cells through FasL-Fas interactions (31), as well as kill autologous target cells, including $CD4^+$ and $CD8^+$ T cells, $CD14^+$ monocytes, and dendritic cells via the perforin pathway (32). These findings indicate the possibility that Treg cells may be capable of inducing B cell tolerance. In this current study, we investigate this potential and show that DNT cells mediate syngeneic B cell death and thus can modulate antixenogeneic immune responses. Furthermore, we show that the killing is mediated by the release of perforin and granzymes.

Materials and Methods

Animals

C57BL/6 (B6, H-2^b), BALB/c (H-2^d), B6 perforin-deficient (perforin$^{-/-}$), FasL-deficient B6Tnfsf^{gld} (*gld*) mice, and Brown Norway (RT1^n) and Lewis rats (RT1^1) were purchased from The Jackson Laboratory and Charles River Laboratories. The animals described above were maintained in the animal facility at the University of Western Ontario using approved protocols and procedures.

Abs and reagents

At various time points after activation, DNT cells were characterized with fluorescent-conjugated mAbs that specifically recognize the $\alpha\beta$-TCR, CD3, CD4, CD8, CD25, CD28, CD62L, NK1.1, $\gamma\delta$-TCR, granzyme B, and perforin (eBioscience). For function assay, anti-CD2, CD48, LFA-1 (CD11a), CD138 (syndercan-1), CD40, IgG, ICAM-1, ICAM-2, rat MHC II (RT1B, clone OX-6), rat MHC I (RT1A, clone OX-18), mouse MHC II (I-A^b, clone 25-9-17), and mouse MHC I (H-$2k^b$/D^b, clone 28-8-6) (BD Biosciences) were used. Data were acquired and analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter). Neutralization Abs to FasL, TGF-β, and TNF-α were purchased from R&D Systems and Cedarlane Laboratories. Isotype controls used were mouse IgG1 (anti-rat, 1C7) and total mouse IgG1 (BD Pharmingen).

Intracellular staining was performed using a fixation/permeabilization kit according to the manufacturer's protocol (BD Biosciences). The serine protease inhibitor mixture, consisting of 4-(2-aminoethyl) benzenesulfonylfluoride, aprotinin, elastinal, and Glu-Gly-Arg-chloromethyl ketone, was purchased from Calbiochem. LPS was purchased from Sigma-Aldrich.

Isolation of DNT cells, $CD4^+$ T cells, $CD8^+$ T cells, and B cells

Spleen and lymph node cells, obtained from B6 mice, were treated with anti-CD4 and anti-CD8 MACS beads (Miltenyi Biotec) to deplete $CD4^+$ and $CD8^+$ T cells. The remaining cells were added to anti-CD90 (Thy-1)-coated MACS beads (Miltenyi Biotec) to purify $CD4^-CD8^-$ T cells. The purity of naive DNT cells was monitored by flow cytometry, and TCR$\gamma\delta^-CD3^+CD4^-CD8^-NK1.1^-$ cells were >92% pure. Purified B6 DNT cells were stimulated with irradiated (2500 rad) BALB/c or Lewis rat spleen cells in RPMI 1640, supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM), 2-ME (0.5 mM), and 50 IU/ml IL-2. Viability and purity of the cells were monitored by flow cytometry. Further purification was performed if necessary. In vivo-activated DNT cells were obtained from spleen and lymph nodes of B6 mice that were immunized with rat spleen cells (30×10^6) and boosted once before purification. $CD19^+$ B cells, $CD138^+$ (syndercan-1) plasma cells, $CD4^+$ T cells, and $CD8^+$ T cells were purified by MACS beads, either directly from B6 spleen cells or from MLR cultures.

Cytotoxicity assays

Target cell death, resulting from coculture with DNT cells, was measured as reported previously (18). Briefly, DNT cells were stimulated with irradiated Lewis rat or BALB/c splenocytes for 4–5 days in the presence of IL-2 (50 IU/ml). Any contaminated $CD8^+$ T cells or $NK1.1^+$ cells were further depleted by anti-CD8 and anti-NK1.1 with MACS bead (Miltenyi Biotec). The purity of DNT cells was confirmed by anti-TCRβ, CD3, CD4, CD8, NK1.1, and TCR$\gamma\delta$, and TCRβ^+TCR$\gamma\delta^-CD3^+CD4^-CD8^-NK1.1^-$ cells were >94% pure before continuing with the experimental procedure.

Viable cells were harvested and seeded into 96-well microtiter plates in the presence of IL-2 (50 IU/ml) as effector cells. The naive B6 mice were immunized with rat or BALB/c spleen cells (20×10^6 i.v. and 10×10^6, i.p.), which were harvested and used for B cell purification 5–10 days later. B cells or $CD138^+$ (syndecan-1) plasma cells were purified by CD19- or CD138-positive selection on MACS beads column (Miltenyi Biotec) and were stimulated either by anti-CD40 (5 μg/ml) and anti-mouse IgG (1 μg/ml) or LPS (20 μg/ml) for 1–3 days. Activated B6 B cells or plasma cells were labeled with 10 μCi/ml [^{3}H]thymidine at 37°C overnight, washed, and then used as targets. In some experiments, B6 spleen cells were mixed with irradiated rat spleen cells plus IL-2 (50 IU/ml) for 4–6 days, and activated $CD4^+$ or $CD8^+$ T cells were purified by MACS beads and used as a target, as described above. After coculture with the effector cells at 37°C for 4–8 h, the cells were harvested and counted in a beta scintillation counter (PerkinElmer). Specific cell lysis was calculated using the following equation: percent specific killing = $(S - E)/S \times 100$, where E (experimental) is counts per minute of retained DNA in the presence of effector cells, and S (spontaneous) is counts per minute of retained DNA in the absence of effector cells.

To measure if cell-cell contact was necessary for DNT cell-mediated killing, cytotoxicity assays were conducted in a 0.2-μm Transwell in 24-well plates (BD Biosciences).

Cell apoptosis was confirmed with 7-aminoactinomycin D (7-AAD) staining, according to the manufacturer's protocol (BD Biosciences). Naive B cells were purified from the spleens of untreated B6 mice and were labeled with 0.5 μM CFSE (Molecular Probes). The CFSE-labeled B cells were mixed with DNT cells or cultured alone for various lengths of time. 7-AAD staining and flow cytometry were used to determine the percentage of the apoptotic B cells by gating on $CFSE^+$ cells.

Real-time PCR

After activation for 96 h in the presence of IL-2 and Ags, T cells were subjected to density centrifugation with Lympholyte-R (Cedarlane Laboratories) to remove stimulator cells. Total RNA was extracted with a spin column according to the manufacturer's protocol (Qiagen). cDNA pools were synthesized with the StrataScript First-Strand Synthesis System according to the manufacturer's protocol (Stratagene). Primers were designed using Primer Express, primer designing software from Applied Biosystems, and are listed in Table I. The granzyme gene sequences were obtained from the National Center for Biotechnology Information database (⟨www.ncbi.nlm.nih.gov⟩). Real-time quantitative PCR was performed on standardized quantities of cDNA using the Brilliant SYBR Green QPCR Master Mix kit, and amplified DNA products were generated and detected using the Mx4000 system (Stratagene). Each PCR amplification condition was set up in triplicate. Melting curve analyses and negative controls were used to ensure specificity of primers and PCR. β-actin amplification was used as the endogenous control. The normalized δ threshold cycle value and relative expression levels (2^{ddCt}) were calculated according to the manufacturer's protocol.

Confocal microscopy

DNT cells (1×10^5) were plated on 35-mm glass bottom, poly-L-lysine-precoated microwell dishes (MatTek) and incubated for 30 min at 37°C before the addition of 1×10^6 activated B cells. Dishes were washed twice with PBS plus 1% FCS and stained with anti-LFA (CD11a)-FITC and anti-CD3-Alexa Fluor (BD Pharmingen) for 30 min on ice, followed by anti-CD19, anti-ICAM-1, anti-MHC class I (H-$2k^b$), or anti-MHC class II (I-A^b; eBioscience). The image was captured with a LSM 510 meta confocal microscope (Carl Zeiss), using a ×63 objective and analyzed with the corresponding LSM software (Carl Zeiss).

Heart and skin transplantation

Intra-abdominal heterotopic cardiac transplantation was performed using 2-wk-old Lewis rats (25–30 g) as donors. Recipient mice were treated with

Table I. *Real-time PCR primers*

Gene	Sense	Antisense
Granzyme A	TGTGCTGGCGCTTTGATTG	GAACTTAGATCTCTTTCCCA
Granzyme B	CGATCAAGGATCAGCAGCC	CTGGGTCTTCTCCTGTTCT
Granzyme C	TTCCTGGTTCGAGACAAATTC	GCTTTAATAGCATGATGTCATTA
Granzyme D	GCAGATCATCCCTGTGGCAAAAGA	AGGAAGTGCACAACCTTAGTGAAGAT
Granzyme E	CTCCTGACCCTACTTCTGCCTCTT	CAAGAAGCCTCCACAGTATCTCCTA
Granzyme F	CGGGTGAAGCCAGGGCATGTT	GTTGAGCCTCTCGTAGGCAGGA
Granzyme G	GAGGCTTCTTGGTTCAAGATGAT	AATGTCATTGGTGCCATGCTTTC
Granzyme K	CATACTGAAATTATTGGAGGG	CGGCTGTTAGCACCCACTG
Granzyme M	AGGCAACAGATTTGAGACCC	TCTATCTAGCTTAAGCAGTGCC
Granzyme N	AGGAGATGGAGCAGAGGAGG	GTAGTCTTGAACCAGGAAGCCG
Perforin	GAAGACCTATCAGGACCAGTACAACTT	CAAGGTGGAGTGGAGGTTTTTG
FasL	GAAGGAACTGGCAGAACTCCG	CCCTGTTAAATGGGCCACACT
TNF-α	TCGAGTGACAAGCCCGTAGC	CTCAGCCACTCCAGCTGCTC
TGF-β	TGCTGCTTTCTCCCTCAACCT	CACTGCTTCCCGAATGTCTGA
β-Actin	CCAGCCTT CCTT CCT GGG TA	CTA GAA GCA TTT GCG GTG CA

the immunosuppressive analog of 15-deoxyspergualin, LF15-0195 (LF provided by Fournier Laboratories), at 2 mg/kg (dissolved in 0.9% sodium chloride) s.c. from day 0 to postoperative day 14. DNT cells were purified from B6 mice that had been immunized with rat spleen cells (3×10^7) 7–10 days earlier and boosted 1 day before purification. A total of 5×10^6 DNT cells was purified from immunized B6 mice and then i.v. adoptively transferred into a transplanted mouse on day 16. In some mice, DNT cells were transferred on day 16, 25, and 35 or day 16, 20, and 25 after transplantation. When cardiac impulses were no longer palpable, the grafts and blood were removed for ex vivo studies. The circulating donor-specific IgM and IgG levels in the recipient's serum (1/100 dilution) were evaluated by flow cytometry, using donor spleen cells as targeting cells. The increase fold of mean fluorescent intensity was calculated according to serum obtained from naive B6 mouse before transplantation. The detecting anti-mouse IgG-FITC and IgM-FITC were purchased from Caltag Laboratories.

At necropsy, tissue samples were processed for routine hematoxylin-phloxine-saffron staining. Criteria for xenograft rejection included the presence of vasculitis, infarction, lymphocytic infiltration, thrombosis, and hemorrhage.

DNT cells treated mice were receives full thickness (1×1 cm in size) rat skin graft on the late thoracic wall and then the graft will be secured with bandage. Skin graft was monitor daily after remove bandage on day 8 and a complete necrosis of the skin graft was defined as rejection.

Statistical analysis

The obtained data were compared using Student's *t* test. Values of $p < 0.05$ were referred to as a significant difference.

Results

Activation of DNT cells purified from wild-type B6 mice by xenoantigen

In this study, we first wanted to determine xenoreactivity of DNT cells. Approximately $5–8 \times 10^5$ $CD4^-CD8^-$ DNT cells were purified from each B6 mouse and stimulated with irradiated (2500 rad) Lewis rat spleen cells in the presence of IL-2. As shown in Fig. 1*a*, these DNT cells did not proliferate against rat xenoantigen. However, this unresponsiveness could be reversed using the addition of exogenous IL-2 (50 IU/ml). Activated B6 DNT cells express a high level of B220 and CD25 (Fig. 1*b*) but do not express detectable levels of FasL, CD28, CD40L, OX40, CTLA-4, or 4-1BB, which are molecules typically expressed on activated $CD4^+$ and $CD8^+$ T cells (data not shown). We also found that anti-LFA (CD11a) and anti-rat MHC class I (RT1A) or class II (RT1B) Abs blocked DNT cell proliferation, indicating that the mouse TCR-rat MHC interaction and adhesion molecules on DNT cells are necessary for DNT cell activation (Fig. 1*a*). An unexpected finding was that blockade of both rat MHC class I and class II molecules by Abs could inhibit proliferation of DNT cells reacted with rat APC. A similar blockade result was obtained when the activated DNT cells were restimulated with rat APC (data not shown).

To further substantiate DNT cell activity to xenoantigen, confocal microscopy was used to analyze the interaction of DNT and rat spleen cells. As indicated in Fig. 1*c*, DNT cells could form a classical immune synapse, i.e., TCR/CD3 and LFA-1 with target rat spleen cells.

Ex vivo- and in vivo-activated DNT cells can directly kill activated syngeneic B and T cells in a FasL-independent, but perforin/granzyme-dependent, manner

Acute xenograft rejection is mediated primarily by Ab-producing B cells. Thus, we speculated that B cell regulation by DNT cells might be useful in xenotransplantation. We first examined if xenoreactive $B220^+CD25^+$ DNT cells could target syngeneic B cells. B cells were purified from B6 mice that were immunized with Lewis rat spleen cells 7–10 days before and activated with plate-bound anti-mouse IgG and anti-CD40 Abs or LPS before being used as target cells. B6 DNT cells were activated by irradiated Lewis rat spleen cells in the presence of IL-2 for 4–6 days. The purity of DNT cells was confirmed by flow cytometry, and only $TCR\beta^+TCR\gamma\delta^-CD3^+CD4^-CD8^-NK1.1^-$ cells that were >94% pure were used as effectors (data not shown). As shown in Fig. 2*a*, xenoreactive DNT cells induced the death of activated B cells in a dose-dependent manner. However, naive DNT cells did not exhibit any cytotoxicity toward B cells (Fig. 2*a*). In addition, xenoreactive DNT cells did not attack naive B cells (Fig. 2*b*). These results suggested that activation of both effector and target cells is required for the killing. To prove that in vivo-activated DNT cells possess a similar immune suppressive function as demonstrated above, DNT cells were purified from B6 mice, which had been immunized with Lewis rat spleen cells 10 days before and boosted 1 day before purification. As shown in Fig. 2*a*, in vivo-activated DNT cells could kill activated $CD19^+$ B cells dose-dependently. These results suggest that the xenoreactive DNT cells can directly mediate B cells death.

Next, we examined if DNT cells could eliminate Ab-producing plasma cells. $CD138^+$ cells were purified from B6 mice that were immunized with Lewis rat spleen cells and used as targets. As shown in Fig. 2*a*, DNT cells could kill $CD138^+$ cells at similar levels as those seen in B cell killing. This result further suggested that DNT cells might control antidonor humoral responses.

The suppressive function of $CD4^+$ Treg cells can be mediated by expression of IL-10, either surface CTLA-4 or membrane-bound TGF-β (9, 33). In addition, recent studies have suggested

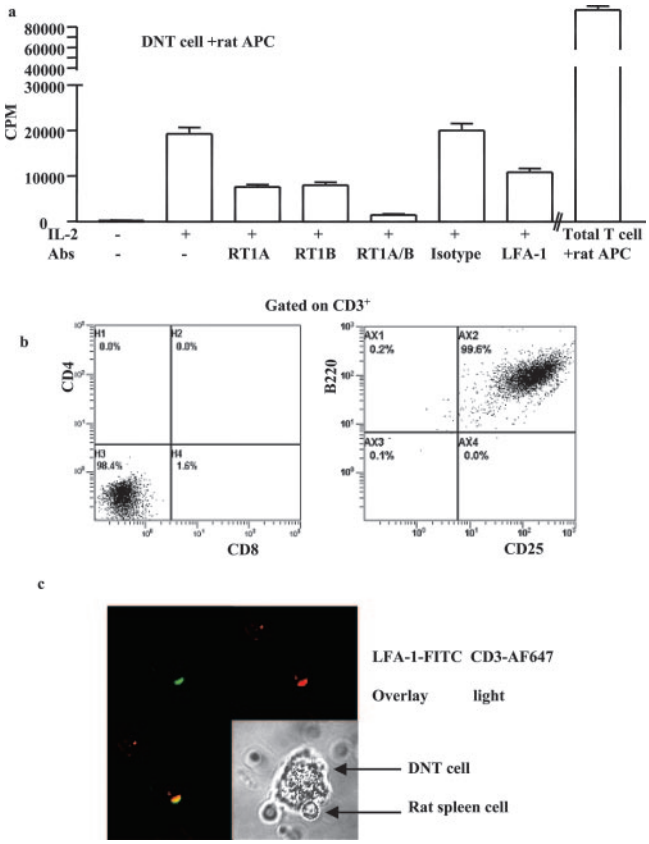

FIGURE 1. DNT cells could be activated by xenoantigen. *a*, DNT cell proliferation against xenoantigen in the presence of IL-2. The purified DNT cells (5×10^4/well) were stimulated with irradiated Lewis rat spleen cells (1×10^6/well) in RPMI 1640 and supplemented with 10% FCS with or without 50 IU/ml IL-2 or anti-rat MHC class I (RTA1, mouse IgG1, 5 μg/ml) or anti-rat MHC class II (RTB1, mouse IgG1, 5 μg/ml). The Ab isotype controls are mouse IgG1 anti-rat (1C7). The purified $CD90^+$ T cells (mixed $CD4^+$ and $CD8^+$ T cells, 5×10^4) were used as positive proliferation control. The proliferation was determined after being cultured for 4 days with incorporation of 10 μCi/ml [^{3}H]thymidine for the last 18 h. *b*, These activated DNT cells express B220 and CD25. The purity of activated DNT cells was confirmed with anti-CD3, anti-CD4, and anti-CD8 triple staining. The DNT and rat spleen cell mixture was stained with anti-LFA-1 (CD11a)-FITC and anti-CD3-Alexa Fluor 647 (BD Pharmingen). The image was captured with a LSM 510 meta confocal microscope (Carl Zeiss) using a ×63 objective. DNT cells could form a classical TCR/CD3 and LFA-1 synapse with target rat cells (*c*).

that chemokine metabolism (34), indirect tryptophan metabolism (35), FasL (31), and granzymes (36) could also be involved in the function of Treg cells. We and others (18, 24) have demonstrated that DNT cells can suppress $CD8^+$ T cells through the Fas-FasL interaction pathway. It has been reported that $CD4^+CD25^+$ T cells can lyse B cells by Fas-FasL interactions (31). Therefore, we were interested in examining the mechanism(s) DNT cells use to attack the B cells. Surprisingly, we found that DNT cell-mediated B cell death was not blocked by FasL-neutralizing Ab (Fig. 2*c*), implying a Fas-FasL-independent mechanism. In addition, anti-TGF-β- and anti-TNF-α-neutralizing Abs could not block DNT cell-mediated B cell killing, and CTLA-4 could not be detected by Ab staining after 24–96 h of activation (data not shown).

To further confirm that the Fas-FasL pathway was not involved in the observed B cell killing, we generated xenoreactive DNT cells from FasL-deficient *gld* B6 mice. We found that these *gld* DNT cells could kill B cells similar to that observed in the case of wild-type DNT cells (Fig. 2*c*). Interference with TCR-MHC interaction by the addition of anti-mouse MHC class I and II Abs had no effect on the B cell killings (Fig. 2*c*), thus suggesting that the killing was Ag nonspecific.

As these results indicate that DNT cells lyse the B cells by a FasL-independent mechanism, we were interested in investigating the potential role of perforin in DNT cell-mediated B cell killing. Thus, DNT cells, purified from B6 perforin$^{-/-}$ mice, were activated by irradiated Lewis rat spleen cells in the presence of IL-2 and then used as effector cells. As shown in Fig. 2*d*, perforin$^{-/-}$ DNT cells could not lyse target B cells, suggesting a perforin-dependent pathway for wild-type DNT cell-mediated B cell killing after xenoantigen activation. Interestingly, DNT cells were also capable of eliminating syngeneic $CD4^+$ and $CD8^+$ T cells while perforin$^{-/-}$ DNT cells lost this capacity, corroborating the idea of a perforin-dependent mechanism (Fig. 2*d*). This result further supports the previous finding that DNT cells can suppress $CD4^+$ and $CD8^+$ T cells after xenoantigen activation (28, 29).

To further investigate this killing mechanism, we used 7-AAD to detect DNA fragmentation, which is known to occur during apoptosis. We were able to detect target cell apoptosis using a cytotoxicity assay and identifying 7-AAD-positive populations (Fig. 2*e*, *i* and *ii*). To confirm the involvement of granzymes in the observed B cell killing, we used a serine protease inhibitor mixture (Calbiochem) to inhibit the activity of multiple granzymes. We found that the DNT cell-mediated apoptosis was greatly reduced with this inhibitor mixture (Fig. 2*eiii*). This result further confirmed the involvement of granzymes in DNT cell-mediated B cell killing. Similar results were seen when $CD4^+$ or $CD8^+$ T cells were used as targets (data not shown). Thus, our data show that xenoantigen-activated DNT cells use a perforin/granzyme-dependent pathway to attack syngeneic B and T cells rather than through Fas-FasL interactions.

Xenoreactive DNT cells express a high level of granzymes

Next, we determined if this syngeneic B cell killing is unique to xenoreactive DNT cells or if it could be mediated by other T cell subpopulations. Our results indicate that xenoreactive DNT cells exhibited a strong killing against syngeneic B cells, whereas $CD4^+$ and $CD8^+$ T cells that were activated by xeno- or alloantigens under the same conditions exhibited very little B cell killing (Fig. 3*a* and data not shown). Interestingly, alloreactive (BALB/c-stimulated) DNT cells exhibited weak killing toward activated syngeneic B cells compared with xenoantigen-activated DNT cells (Fig. 3*a*).

To further characterize the killing of B cells by granzyme-mediated cytotoxicity of DNT cells, we used real-time PCR to quantify the expression of genes involved in the cytolytic function. The relative quantification was based on serial dilutions of the sample and on the δ threshold value compared with B6 $CD8^+$ T cells, stimulated by alloantigens (BALB/c) under the same conditions as the DNT cell activations. Interestingly, DNT cells expressed higher levels of granzymes A and B after activation by rat or BALB/c cells compared with $CD8^+$ T cells (Fig. 3*b*). Xenoantigen-activated DNT cells express higher levels of granzymes (A and C) compared with alloreactive DNT cells and $CD8^+$ T cells (Fig. 3*b*). On the other hand, xenoantigen-activated DNT cells do not express significantly higher levels of perforin, FasL, TGF-β, and TNF-α compared with $CD8^+$ T cells (Fig. 3*c*). Intracellular staining using available anti-mouse perforin and granzyme B Abs further confirmed the expression of perforin and granzyme B in DNT cells (Fig. 3*d*). Next, we used light confocal microscopy to monitor the morphology of DNT cells. Purified B6 DNT or $CD8^+$ cells were activated ex vivo with irradiated Lewis rat spleen cells in addition to IL-2. Light microscopy revealed that there was

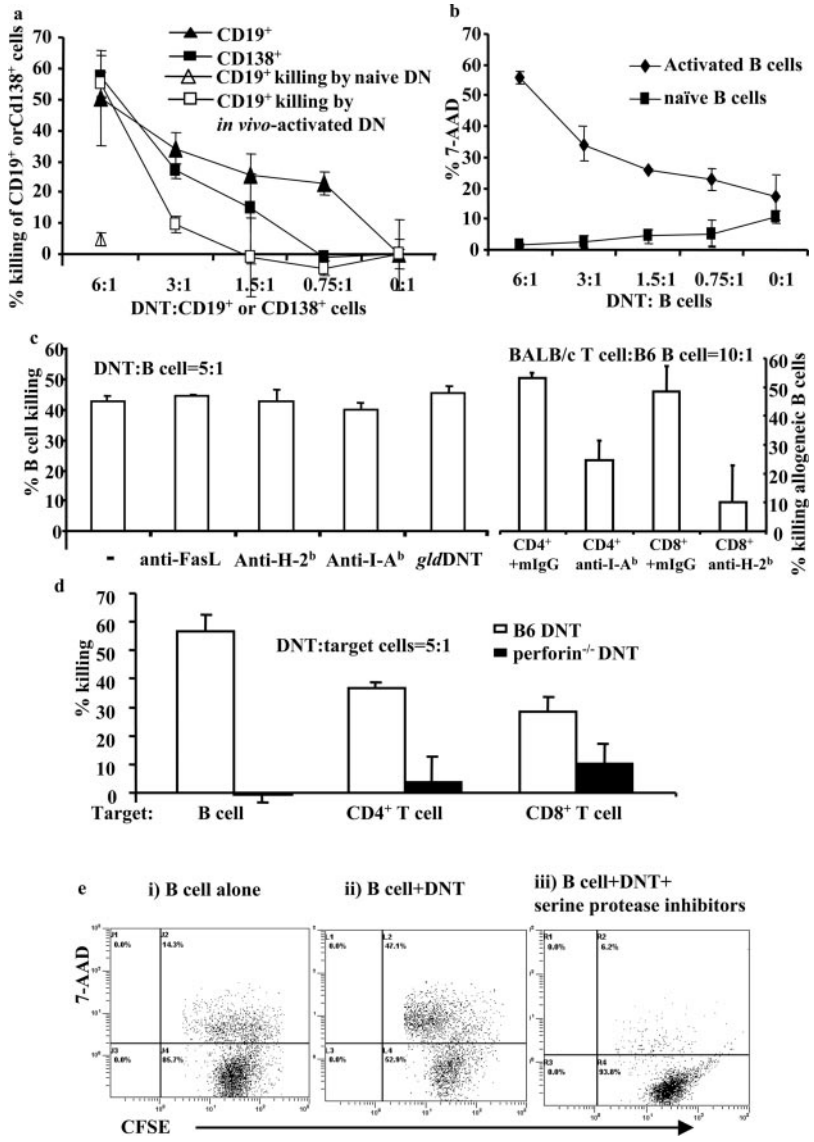

FIGURE 2. DNT cells directly lyse activated, but not naive, syngeneic B cells in perforin/granzyme-dependent pathway, independent of Fas-FasL interaction. The naive B6 mice were immunized with Lewis rat spleen cells 5–8 days before being used for B cell purification. B cells or plasma cells (2×10^4/well), purified by CD19- or CD138-positive selection (Miltenyi Biotec), were activated by anti-CD40 (5 μg/ml) and anti-mouse IgG (1 μg/ml) for 48 h and labeled with 10 μCi/ml [^{3}H]thymidine. DNT cells were purified from naive B6 mice or from B6 mice that were immunized by rat spleen cells and boosted once (in vivo activated) 1 day before purification. The purified B6 DNT cells were either used directly or activated by Lewis rat spleen cells with the addition of IL-2 (50 IU/ml) and then used as effectors. After effector and B cells were cocultured for 6–8 h, the cells were harvested and counted in a beta scintillation counter. Specific cell lysis was calculated according to the equation described in *Materials and Methods*. *a*, The dose-dependent killing of activated B cells (CD19$^+$) and plasma cells (CD138$^+$) by DNT cells after 8 h coculture. Activated DNT cells (ex vivo or in vivo) or naive DNT cells were used as effector cells. *b*, DNT cells only target activated B cells but not naive B cells. CD19$^+$ B cells (2×10^4/well) were activated by LPS for 48 h and labeled with CFSE (Invitrogen Life Technologies). Naive B cells were purified from the spleens of untreated B6 mice and labeled with CFSE. The CFSE-labeled B cells were cultured alone or mixed with DNT cells in the presence of IL-2 (50 IU/ml) for 8 h. 7-AAD staining was performed to determine the percentage of the apoptotic B cells by gating on the CFSE$^+$ cells. *c*, DNT cell-mediated B cell death is independent of Fas-FasL and TCR-murine MHC interaction. The DNT cells were purified from wild-type B6 or FasL-deficient *gld* mice and were activated by irradiated Lewis rat spleen cells and IL-2 (50 IU/ml). Target B cells or B6 DNT cells were preincubated with anti-I-A^b (10 μg/ml) and anti-H-2D^b/K^b (10 μg/ml) or anti-FasL-neutralizing Ab (10 μg/ml), respectively, for 30 min before being mixed together. Both B6 and *gld* DNT cells exhibit strong syngeneic B cell killing (E:T = 5:1), with or without anti-FasL and anti-H-2b or anti-I-Ab. To confirm the functions of anti-H-2^b and anti-I-Ab, CD4$^+$ and CD8$^+$ T cells were purified from BALB/c mice and activated by irradiated B6 spleen cells for 4–7 days before being used as effector cells. Allogeneic B6 B cells were activated by LPS and labeled by 3H before being used as target cells. Anti-H-K^b/D^b (5 μg/ml) and anti-I-A^b (5 μg/ml) were added into the mixture of effector and target cells. The percentage killing of target cells was calculated as described in *Materials and Methods* for DNT cell-mediated B cell killing. *d*, Cytotoxicity of DNT cells to syngeneic B cells is perforin dependent. The DNT cells, purified from perforin$^{-/-}$ mice, were activated by irradiated Lewis rat spleen cells in presence of IL-2 and then used as effector cells. B6 spleen cells were mixed with irradiated rat spleen cells plus IL-2 (50 IU/ml) for 5–7 days, and activated CD4$^+$ or CD8$^+$ T cell were purified. No cytotoxicity to either B or T cells was found in perforin$^{-/-}$ DNT cells after being cocultured with activated B or CD4$^+$ or CD8$^+$ T cells. *e*, DNT cells that kill target B cells need granzymes. DNT cells and CFSE-labeled B cells (5:1) were incubated in the presence of IL-2 (50 IU/ml) for 6–8 h, followed by staining with 7-AAD. Apoptosis of target B cells was determined by 7-AAD-positive and gated on the CFSE-labeled B cells. DNT cells were preincubated with serine protease inhibitor mixture (1/100 dilution) to block granzymes for 30 min before mixing with B cells. All data represented were repeated in three independent experiments.

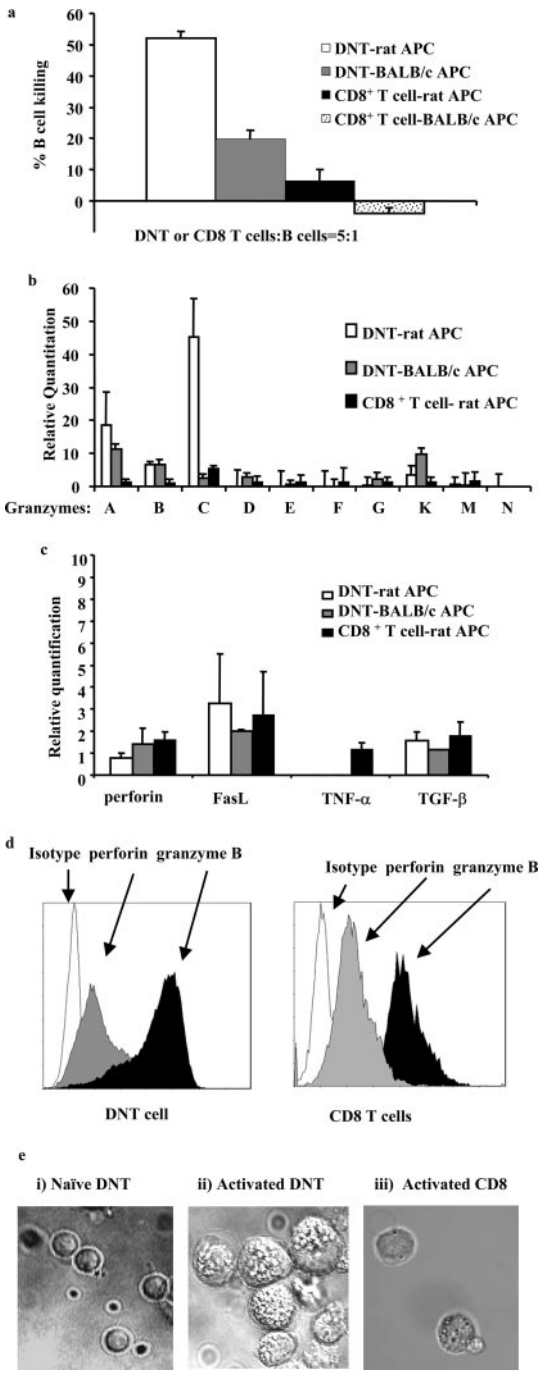

FIGURE 3. Xenoreactive DNT cells express a high level of granzymes. *a*, A cytotoxicity assay was performed as described in Fig. 2 and in *Materials and Methods*. The xenoreactive (anti-rat) DNT cells exhibit strong syngeneic B cell killing (E:T = 5:1, 8-h coculture) in comparison to alloreactive DNT cells and activated $CD8^+$ T cells. Similar results were obtained after repeating the experiment three times. *b*, Real-time PCR to quantify the expression level of killing related genes. DNT cells and $CD8^+$ T cells were purified and activated as described in *Materials and Methods* and Fig. 2. Total RNA was extracted from the DNT cells and $CD8^+$ T cells after activation by rat or BALB/c Ags in presence of IL-2 for 4 days. cDNA synthesis and real-time PCR were performed according to manufacturer's protocol. The relative quantification is calculated based on the normalized δ threshold cycle value according to the manufacturer's protocol (2^{ddCt}). Real-time PCR was repeated using same cDNA pool. The endogenous control is β-actin, and the relative quantification was compared with B6 $CD8^+$ T cells, activated by BALB/c spleen cells. Granzymes A to N (*b*) and perforin, FasL, TNF-α, and TGF-β were analyzed (*c*). Expression of perforin and granzyme B in DNT cells was confirmed by intracellular staining. $CD8^+$ T cells are used positive control (*d*). *e*, DNT cells exhibit abundant cytoplasmic granules after activation (*i* and *ii*) compared with $CD8^+$ T cells activated in the same condition (*iii*). The image was captured with a LSM 510 meta confocal microscope (Carl Zeiss) using a ×63 objective.

marked increases the size and granularity of DNT cells upon xenoantigen activation (Fig. 3*e*, *i* and *ii*). Indeed, DNT cells exhibited much higher granularity compared with naive DNT or activated $CD8^+$ T cells. Taken together, our data indicate that DNT cells express a high level of granzymes after xenoantigen activation.

DNT cell-mediated B cell death requires cell-to-cell contact and formation of an unconventional immunological synapse

In general, cytotoxic T and NK cells target infected or activated cells through Ag recognition and adhesion molecules. In those cases, killing involves the formation of a mature immunological synapse between the effector cell and the target cell to ensure the transfer of various cytotoxic molecules from the effector to the target cells (37, 38). This synapse forms a tight molecular environment, ensured by TCR-peptide-MHC (NK cells and its receptor) interaction, and by the engagement of adhesion molecules. Our data suggest that, in the case of DNT cell-mediated B cell killing, the formation of an immunological synapse is a distinctive event because the TCR of DNT cells did not engage with syngeneic MHC molecules on the B cells, as shown in anti-mouse-MHC I and II Ab-blocking experiments (Fig. 2*c*).

Thus, we investigated if cell activation and cell-cell contact were necessary for the cytotoxicity mediated by DNT cells. As shown in Fig. 4*a*, DNT cell-mediated killing requires cell-to-cell contact, as demonstrated in the Transwell experiments (Fig. 4*a*). Next, we determined which molecule was involved in the DNT cell-B cell interaction. We used anti-LFA-1 (CD11a), anti-CD2, anti-ICAM-1, and anti-ICAM-2 in an attempt block DNT cell-B cell interaction. We found that blocking either LFA-1 or ICAM-1/2 partially decreased the cytotoxicity of DNT cells, suggesting that the interaction between LFA-1 and ICAM-1/2 plays an important role in DNT cell cytotoxicity to syngeneic B cells (Fig. 4*b*). However, a similar Ab blockade did not have any affect on DNT cell-mediated syngeneic $CD4^+$ T cell death (data not shown), suggesting that other molecules are likely involved in DNT cell-$CD4^+$ T cell interactions.

Next, we used confocal microscopy to examine the morphology of the cytotoxic interaction of DNT and B cells. Confocal microscopy indicated that a partial synapse was formed between activated DNT cells and B cells where ICAM-1 had accumulated, but LFA-1 did not show a significant accumulation at the contact site (Fig. 4*ci*). In addition, TCR/CD3 on DNT cells did not form a mature distinguishable synapse with $CD19^+$ B cells (Fig. 4*cii*). Our data indicate that DNT cells need cell to cell contact to ensure their cytotoxicity, but there is no clear classical immunological synapse formed between the effector T cells and target B cells.

Adoptive transfer of DNT cells prolongs vascular xenograft survival

Finally, we further tested the ability of DNT cells to suppress antixenograft immune responses in vivo after adoptive transfer. Because adoptive transfer of DNT cells alone had no effect on xenograft survival (data not shown), we combined DNT cell transfer with an immunosuppressive treatment using LF. We first treated B6 mice recipients receiving a Lewis rat cardiac xenograft with 2 mg/kg LF from day 0 to day 14. DNT cells were purified from B6 mice that are immunized with rat spleen cells (3×10^7)

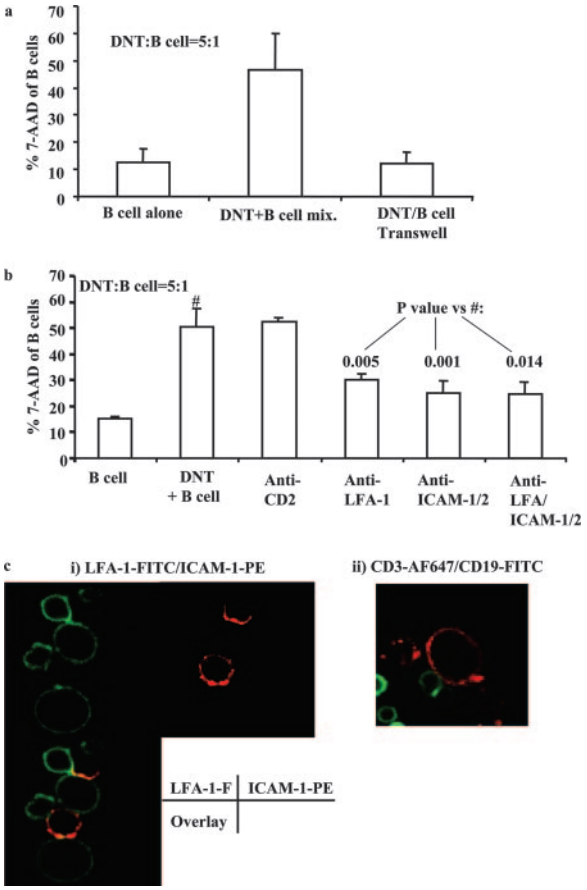

FIGURE 4. DNT cells target syngeneic activated B cells through cell-cell contact mechanisms. DNT cells and B cells were purified and activated as described in *Materials and Methods* and Fig. 2. DNT cells and the CFSE-labeled/activated B cells (5:1) were incubated in the same well or separated by a Transwell membrane (0.2-μm filter) with the addition of IL-2 (50 IU/ml) for 8 h before being stained with 7-AAD (*a*). The effector DNT cells were preincubated with anti-CD2, anti-LFA-1, and anti-ICAM-1/2 for 30 min, followed by an 8-h incubation with target B cells and 7-AAD staining to determine B cell apoptosis (*b*). Statistics (Student's *t* test) were evaluated by comparing the Ab-treated group with the untreated mixture of DNT cells and B cells. *c*, DNT and B cells did not form a classical immunosynapse. Synapse of DNT and B cells was formed as described in the *Materials and Methods*. The DNT cell and B cell mixture was stained with either anti-LFA-1 (CD11a)-FITC, ICAM-1-PE, or CD19-FITC after coincubating and fixing. The formation of synapse between DNT cells and B cells was analyzed by the LSM 510 meta confocal microscope using a ×63 objective and analyzed with the corresponding LSM 510 software (Carl Zeiss). *i*, Anti-LFA-1-FITC and anti-ICAM-1 PE. *ii*, Anti-CD3-Alexa Fluor 647 and anti-CD19-FITC.

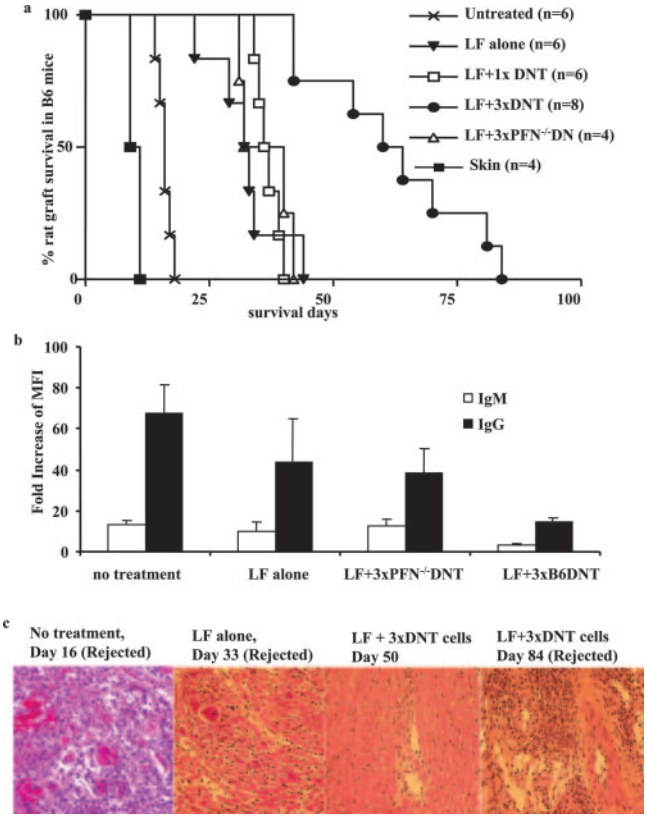

FIGURE 5. Adoptive transfer of DNT cells prolonged heart xenograft survival. *a*, B6 mice recipients were treated with 2 mg/kg LF from day −1 to day 14 after receiving rat heart transplants. DNT cells (5×10^6) were purified from B6 or perforin$^{-/-}$ (PFN$^{-/-}$) mice that were immunized by rat spleen cells (3×10^7) 10 days before and boosted once 1 day before purification and then i.v. adoptive transferred into transplanted mice on day 16. Multiple DNT cell transfer was performed on days 16, 25, and 35 or day 16, 20, and 25, after transplantation. Four DNT cells and LF-cotreated mice received rat skin graft transplantation after last time DNT cell transfer. *b*, The antidonor IgM and IgG was inhibited significantly in DNT cell-treated recipients (IgG: $p < 0.05$, IgM: $p < 0.01$, compared with 3× PFN$^{-/-}$ DNT cells or LF-treated alone group, Student's *t* test). Increase ratio of antidonor mean fluorescence intensity (MFI) was calculated according to sera obtained from naive B6 mice before transplantation. *c*, Representative microscopic photos of a Lewis rat heart in C57BL/6 mouse after routine hematoxylin-phloxine-saffron staining. LF treatment alone delayed heart graft rejection but still showed AVR, whereas a mouse treated with 3× DNT cells and LF had no sign of AVR 50 days after transplantation. The mouse receiving 3× DNT cells and LF treatment rejected the graft on day 84 and massive graft-infiltrating cells were observed (*right panel*). Original magnification, ×400.

7–10 days earlier. A total of 5×10^6 DNT cells was then adoptively transferred into each transplanted mouse on day 16 after transplantation. Results are shown in Fig. 5*a*. Treatment with LF alone significantly prolonged vascular xenograft survival (mean survival in days = 32.3 ± 6.6 vs 16 ± 1.4 in nontreated mice, $n = 6$, $p < 0.001$, Student's *t* test). Single DNT cell adoptive transfer in combination with LF treatment, however, did not further improve heart xenograft survival (survival in days = 35.7 ± 3.6, $n = 6$, $p > 0.1$) compared with LF treatment alone (32.3 days). In contrast, with multiple DNT cell transfer on days 16, 25, and 35 in combination with day 0 to day 14 of LF treatment, heart xenograft survival was significantly prolonged (62.1 ± 13.9 days, $n = 8$, $p < 0.001$, compared with LF-treated alone). Adoptive transfer of 3× DNT cells, purified from rat cell-immunized B6 perforin$^{-/-}$ mice, combined with LF did not prolong heart graft survival, compared with LF-treated alone mice (36.2 ± 5.5 days, $n = 4$, $p < 0.003$), indicating that DNT cells need perforin to perform their suppressive function in vivo. The antidonor IgM and IgG levels were inhibited significantly in the mice treated with multiple DNT cells adoptive transfer in combination with LF treatment (Fig. 5*b*, IgG: $p < 0.05$, IgM: $p < 0.01$, Student's *t* test, compared with LF-treated alone), which is coincided with the delayed Ab-mediated acute vascular heart graft rejection (AVR) (62.1 days vs 32.3 days, Fig. 5*a*). Interestingly, LF-treated alone or LF plus 3× DNT transfer failed to prolong xenoskin graft survival, and all rat skin grafts were rejected within 11 days (10 ± 1.2 days or data not shown, Fig. 5*a*) (5), suggesting that DNT cells can significantly suppress the level of anti-donor Ab and delay heart rejection, but not sufficient to eliminate antidonor cellular responses.

Taken together, these results indicate that adoptive transfer of DNT cells can suppress anti-donor Ab levels and prolong heart graft survival. Routine histopathology results also supported this notion with representative microscopic photos of a Lewis heart in C57BL/6 mouse (Fig. 5*c*). A mouse that received LF treatment alone showed AVR on day 33, whereas a mouse treated with LF plus multiple adoptive transfer of DNT cells had no sign of AVR 50 days after rat heart transplantation. However, all heart grafts in mice treated with LF and multiple DNT cell transfers were rejected, and massive graft-infiltrating cells were observed (Fig. 5*c*, *right panel*, day 84). Our results indicate that adoptive transfer of DNT cells can limit antidonor B cell responses and convert AVR.

Discussion

In this study, we have demonstrated that xenoantigen-activated DNT cells can eliminate syngeneic B and T cells ex vivo, and adoptive transfer of DNT cells can significantly prolong xenograft survival. We have identified a different mechanism of xenoreactive DNT cell-mediated immune regulation that is dependent on perforin/granzymes, but independent of the Fas-FasL interaction (18, 24). This finding is consistent with the high expression level of granzymes in xenoreactive DNT cells compared with those in $CD8^+$ T cells. Taken together, our data indicate that DNT cells can mediate immune regulation in xenotransplantation by targeting B and T cells.

Our finding that activation of DNT cells could be inhibited by Abs against either rat MHC I or II molecules in the presence of IL-2, implying that the interaction between TCR on DNT cells and xeno-MHC molecules is Ag nonspecific. This notion is supported by crystallographic data showing that $CD8^+$ T cell xenoreactivity is not based on a molecular mimicry (39). It is unlikely that the observed blocking with anti-rat MHC II Ab is due to cross-reactivity of anti-rat MHC II Ab (RT1B, clone OX-6; BD Biosciences) with the rat MHC I molecule, as we did not witness any positive staining by applying this Ab on rat $CD8^+$ T cells whereas rat $CD8^+$ T cells exhibit positive staining with the anti-MHC I Ab (RT1A, clone OX-18; BD Biosciences) (data not shown). However, the inhibition of DNT cell proliferation by anti-rat MHC I and II molecules is still unresolved. Possible mechanisms involved may be that the TCR of DNT cells cross-reacting with both rat MHC I and II molecules or that two different TCR repertoires exist on DNT cells that recognize either rat MHC I molecules ($CD8^+$ T cell like) or rat MHC II molecules ($CD4^+$ T cell like). We are currently investigating these possible mechanisms involved.

Treg cell-mediated immune regulation has been documented in a wide range of studies. To ensure the suppression of the target cells, cell to cell contact is necessary for Treg cell activity, although studies have demonstrated that this contact does not depend on Ag presentation by the target cells (33). Likewise, the TCR on DNT cells does not engage with MHC molecules on syngeneic B cells to lyse the activated B cells (Fig. 2*c*). It has been demonstrated that $CD8^+$ T cells and alloreactive DNT cells from mice and humans can acquire MHC class I molecules from target cells and thus allow the engagement of DNT cells with syngeneic T cells (18, 40, 41). In our study, the blocking of mouse MHC I and II molecules by Abs did not influence the killing ability of DNT cells (Fig. 2*c*; data not shown), indicating an Ag-nonspecific targeting. In addition, the DNT cells were activated directly by xenoantigens (rat) that were not primed by syngeneic APC. Thus, the targeting of B cells by DNT cells occurs through a bystander killing mechanism. Although Ag recognition was not needed for the DNT cell-medicated B cell killing, DNT cells only attacked activated, but not naive, B cells (Fig. 2*b*). Furthermore, DNT cells needed to be activated by rat cells to target B cells because naive DNT cells did not exhibit cytotoxicity toward activated B cells (Fig. 2*a*). Therefore, we would expect that an Ag-dependent activation would be necessary for the cytotoxicity of DNT cells after xenotransplantation.

Classically, cytotoxic T or NK cells target infected or activated cells. TCR-MHC recognition and the engagement of adhesion molecules ensure that the immunological synapse forms a tight molecular cluster environment for the internalization of various cytolytic molecules into target cells (37, 38). This process ensures that the cytotoxicity is specifically targeted toward infected, and not normal, cells. However, perforin-independent granzyme-dependent cytotoxicity has occurred in other studies when granzymes bound directly to several surface molecules on target cells, becoming internalized and causing target cell death (42–44). Interestingly, recent studies have suggested that $CD4^+CD25^+$ Treg cells can use the perforin pathway to cause autologous cell death (32). However, $CD4^+$ Treg cells have also been found to lyse target cells through a perforin-independent, granzyme-dependent mechanism (45). Similarly, $CD4^+$ Treg cells kill allogeneic tumor cell lines in a TCR-MHC-independent manner (36). In the case of DNT cells, cytotoxicity to autologous B cells can be achieved without TCR-MHC recognition, as demonstrated in our study (Fig. 2*c*). However, the adhesion molecule LFA-1 is required for the cytotoxicity of DNT cells (Fig. 4*b*).

Our results from confocal microscopy studies indicate the lack of a conventional immunological synapse between DNT cells and B cells (Fig. 4*c*). In contrast, we did observe an accumulation of ICAM-1 at the contact site between DNT cells and B cells, whereas only a low level of LFA-1 was had accumulated on the interface of cell contact (Fig. 4*ci*). Whether other molecules are involved in the DNT cell-B cell interaction remains to be determined. Interestingly, a recent study has supported the notion that neither the formation of a stable and mature immunological synapse nor complete signaling are required for T cell cytotoxicity (46), and $CD8^+$ T cells can form an Ag-independent ring junction with APC (47). In our study, the engagement of cell adhesion molecules, including LFA-1 and its ligand ICAM-1, is nonetheless needed to ensure B cell killings in the Ab-blocking experiments (Fig. 4*b*). On the other hand, anti-CD2, anti-LFA-1, and ICAM-1 failed to block DNT-mediated $CD4^+$ T cell death (data not shown).

The suppressive function of $CD4^+$ Treg cells has been shown previously to be mediated by IL-10, CTLA-4, and/or membrane-bound TGF-β (33). In addition, recent studies have suggested that chemokine metabolism (34), indirect tryptophan metabolism (35), FasL (31), and granzymes (36) could also be involved in the function of Treg cells. In previous studies, alloreactive DNT cells suppressed $CD8^+$ T cells through the Fas-FasL interaction (18, 19, 24). However, DNT cells can also suppress $CD4^+$ T cells through a Fas-independent mechanism involving the inhibition of IL-2 production in $CD4^+$ T cells (23). Interestingly, in our study, the xenoantigen-activated DNT cells target both B and T cells by a perforin/granzyme-dependent pathway, rather than FasL-dependent pathway (Fig. 2, *c–e*). This difference might be caused by the activation pattern of DNT cells by xenoantigen. There are several nonconventional features involved in this activation. First, stimulation of DNT cells requires murine TCR-rat MHC I or II molecule interaction (Fig. 1*a*) (48). Second, we would expect that other stimulatory signals should be involved in DNT cell activation by xenoantigen, given the absence of classical stimulatory factors such as CD4, CD8, CD28, CD40L, 4-1BB, ICOS, and OX40 on DNT cells in our staining study.

We have demonstrated that xenoreactive DNT cells possess a strong B cell lysis ability in comparison to $CD8^+$ T cells and

alloreactive DNT cells. When we compared the expression levels of adhesion molecules, including LFA-1, CD2 and CD48, by immune staining, we could not find a significant difference among xenoreactive DNT cells, alloreactive DNT cells, and $CD8^+$ T cells (data not shown). However, the results from real-time PCR suggest that DNT cells, activated by either xeno- or alloantigens, express higher levels of granzymes A and B, in comparison to either allo- or xenoreactive $CD8^+$ T cells (Fig. 3*b*). Levels of granzyme C were particularly higher but only in xenoreactive DNT cells. Indeed, a high granular formation is seen in DNT cells compared with $CD8^+$ T cells after activation (Fig. 3*e*). It is possible that the exceptional high levels of granzyme expression are responsible for the bystander B cell killings that we observed, and this may correlate with the differential killing capacity of the DNT and $CD8^+$ T cells (Fig. 3*a*). The mechanism for this elevated level of granzyme expression in DNT cells after encountering xenoantigens is not clear and is worthy of further investigation. Interestingly, differential granzyme (A and B) expression was also seen in $CD4^+CD25^+$ Treg cells (32, 36), suggesting that $CD4^+$ Treg cells could also use either perforin and/or granzymes-dependent pathways to cause target cell death.

Although granzymes cause rapid loss of membrane integrity and DNA damage in target cells, features of cell-death pathways, induced by granzymes, are quite different. Granzyme B initiates cascades of intracellular caspase-dependent cell death signaling and mitochondrial damage that ultimately lead to DNA fragmentation (37, 38). Granzyme A causes cell death in a caspase-independent pathway by targeting the mitochondrial and the endoplasmic reticulum-associated complex, called the SET complex, subsequently leading to DNA damage (37, 38, 49). Granzyme A destroys the nuclear envelope and renders the DNA vulnerable for degradation by targeting histones. It has been suggested that granzymes A and B play a dominant role in T cell-mediated cytotoxicity (37, 38). As DNT cells express higher levels of granzymes A and B after Ag activation, so we would expect that DNT cells to use these two proteases with the help of perforin to target activated B cells. A significantly higher level of granzyme C was found in xenoreactive DNT cells (Fig. 3*b*). It has been shown that granzyme C induces a caspase-independent cell death, but this is distinct from granzyme A. It causes marked mitochondrial swelling and cytochrome *c* release without further caspase activation (50). Even though our data indicate that granzymes are involved in the cytotoxicity of DNT cells, we do not know if DNT cell-mediated B and T cell killing results from effects of multiple granzymes or were mediated by only certain granzymes. It will be interesting to establish a system to distinguish between the different uses of granzymes in DNT cell-mediated B and T cell killing.

Currently, the major barrier for xenotransplantation is Ab-mediated AVR. Therefore, the first goal of xenotransplantation is to establish B cell tolerance by either eliminating or regulating antidonor B cell responses. However, DNT cells alone do not prolong vascular xenograft survival after adoptive transfer (data not shown), whereas LF treatment alone had a modest effect on graft survival (32.3 ± 6.6 vs 16 ± 1.4 days in nontreated mice; Fig. 5*a*). Interestingly, multiple transfers of DNT cells, combined with early LF treatment, significantly prolonged vascular xenograft survival to 62.1 ± 13.9 days (Fig. 5*a*) and significantly inhibited both antidonor IgM and IgG levels (Fig. 5*b*). The finding that DNT cells can significantly delay heart rejection through suppression of antidonor Abs is supported by the histopathological observations in the mice received adoptively transferred DNT cells. These mice showed no sign of AVR up to 50 days after rat heart transplantation, whereas those with LF treatment alone or with no treatment showed strong AVR (Fig. 5*c*). However, rat skin graft survival was not prolonged after adoptive transfer of DNT cells (Fig. 5*a*), suggesting that cellular immunoresponsiveness was not efficiently suppressed. However, in this study, it is not known whether DNT cells directly kill B and T cells or if they have indirect effects in vivo.

It is necessary to develop a new strategy to increase efficacy of DNT cells in vivo in the long term because adoptive transfer of DNT cells did not induce tolerance in the recipients. We compared the proportion of DNT cells at the terminal day in the different treatment groups and no significant difference was found (DNT/$CD3^+ = 9 \pm 2.3\%$ in spleens of LF treatment alone vs $9.6 \pm 2.5\%$ in 3× DNT cells and LF treatment, $p > 0.3$). This suggests that survival and function of DNT cells after adoptive transfer were not efficient and that a proper environment in recipients is necessary for maintaining DNT cells. It has been suggested that IL-2 treatment is critical for $CD4^+CD25^+$ Treg cell homeostasis in both cancer patients and the mouse model (51). However, implementation of IL-2 therapy may be detrimental by provoking antidonor immune responses and accelerate graft rejection. Further investigations are needed to optimize the conditions for maintaining DNT cells in vivo, which will play a vital role for ultimate success of adoptive transfer of DNT cells to achieve tolerance induction after xenotransplantation.

Acknowledgments

We thank Dr. Gill Strejan for interesting discussion and critical reading of the manuscript; Drs. Antony Jevnikar and David White for interesting discussion of results; Dr. Winnie Liu for pathology staining; and Dr. Jin Su for assistance in confocal microscopy.

Disclosures

The authors have no financial conflict of interest.

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