

The Role of Alloresponsive Ly49⁺ NK Cells in Rat Islet Allograft Failure in the Presence and Absence of Cytomegalovirus

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There are still many factors to discover to explain the low success rates of islet allografts. In this study, we demonstrate that specific subpopulations of alloreactive NK cells may be involved in the failure of islet allografts. By performing allotransplantation in rats ($n=13$), we observed peripheral expansion and infiltration of alloreactive Ly49i2⁺ NK cells in the grafts. An effective strategy in rats to enhance the expansion of Ly49i2⁺ NK cells is performing a rat cytomegalovirus infection ($n=6$). Cytomegalovirus infection was associated with an early expansion of the Ly49i2⁺ NK cells and accelerated islet graft failure. The Ly49i2⁺ NK cells are both alloreactive and involved in virus clearance. The expansion of this subpopulation could not be blocked by cyclosporin A immunosuppression. Also alloreactive KLRH1⁺ NK cells infiltrated the grafts, but nonalloreactive NKR-P1B⁺ cells were not observed in the islet allografts. Perforin staining of the infiltrating NK cells demonstrated the cytotoxic capacity of these cells. Our data suggest a role for this NK subpopulation in rat islet allograft destruction.

Key words: Islets; Natural killer (NK) cells; Allotransplantation; Immunosuppression; Cytomegalovirus

INTRODUCTION

Large-scale clinical application of islet transplantation is hampered by a low long-term success rate. With the present approaches, only approximately 10% of the recipients are still normoglycemic and insulin independent at 5 years after transplantation (39). To improve long-term graft survival, it is important to identify the factors involved in success or failure of islet allografts.

In a recent rat study, we found high numbers of natural killer (NK) cells in failing rat islet allografts (42). This NK cell infiltration was enhanced after cytomegalovirus (CMV) infection. NK cells have not received much attention in islet allograft failure but have been suggested to be a factor involved in failure of human islets in the liver (23). A recent report shows this may not be restricted to the liver as both human and rodent islets carry the receptor for NK cell p46-related protein (NKP46), an NK cell receptor that after ligation recognizes pancreatic β -cells (18). In the autoimmune process leading to type 1 diabetes, evidence is accumulating that NK cells and natural killer T (NKT) cells play an important role in the destruction of β -cells (5,18). For destruction of β -cells in an

allograft setting, the role of NK cells is less clear, but as current immunosuppressive protocols applied in islet transplantation have no effect on NK cells, it might be an overlooked factor in the success or failure of islet grafts (18).

An important role for NK cells in allorecognition and graft rejection has been demonstrated in bone marrow (21,22,30,40) and solid organ transplantation (11,32,37). NK cells can be found in different subpopulations with distinct functions. In rodents, these NK cell subpopulations can be identified based on the differential expression of major histocompatibility complex I (MHC class I) recognizing killer cell lectin-like receptors (KLRs). Several KLR families are clustered together within the NK cell complex (NKC) and contain both inhibitory and activating family members. NK cells expressing a combination of the inhibitory Ly49i2, the activating Ly49s3, and the inhibitory KLR subfamily H, member 1 (KLRH1) are, in vitro, highly alloreactive toward allogeneic target cells expressing foreign MHC class I molecules (34–36). A functionally distinct population of rat NK cells expresses natural killer cell surface protein P1B (NKR-P1B), a receptor distinct from Ly49 receptors (25).

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These NKR-P1B-positive NK cells effectively lyse tumor target cells but fail to recognize allogeneic targets (25). It is unknown whether these subpopulations are involved in islet allograft rejection.

In the present study, we hypothesized that islet allograft failure is associated with selective expansion of alloreactive Ly49/KLRH1-expressing NK cell subpopulations and that this population can be enhanced by CMV. To test the hypothesis, we applied two approaches. First, we applied a rat allograft transplantation model in which immunosuppression was withdrawn after 10 days to allow gradual development of an allograft response to follow the kinetics of NK cell recruitment in the circulation. Second, we performed an infection with CMV in the rat recipient of islet allografts at 1 day after implantation. CMV is known to enhance alloreactivity against rodent islets (42) and is recognized for the effects on NK cells (1,12,38). We studied the association of recruitment of Ly49⁺ NK cells and survival of islet allografts. The NK cell subpopulations that are responsive to CMV are unknown. We studied, in both experiments, the Ly49i2-, Ly49s3-, KLRH1-, and NKR-P1B-expressing NK cell subpopulations, before and after the withdrawal of immunosuppression.

MATERIALS AND METHODS

Study Design

Streptozotocin (STZ)-treated diabetic male Albino Oxford (AO; *u-u-u* haplotype) rats received a renal subcapsular male Lewis (*l-l-l* haplotype) rat islet graft. AO rats are known to be high NK cell responders (26). Cyclosporin A (CsA) immunosuppression was applied but was withdrawn at 10 days after implantation. To study NK cell (sub) populations, blood was sampled from the tail vein 1 day before transplantation (Pre-Tx) and at days 7 and 13 after implantation, and just before graft failure. After sacrifice, the islet grafts were retrieved. Islet grafts were sectioned and stained to study infiltrating NK cell subpopulations. One day after transplantation, six AO rat islet graft recipients received a CMV infection, while seven of the recipients received a mock infection and served as controls. CMV-infected and mock-infected rats were transplanted pairwise or in triplicate (two CMV-infected recipients vs. one mock-infected control) to reduce batch-to-batch differences in islet quality as much as possible. All CMV-infected animals and three mock-infected controls were sacrificed after complete graft failure. Four paired mock-infected control animals were sacrificed after graft failure of the corresponding CMV-infected recipient.

Experimental Animals and Diabetes Induction

Inbred male AO rats (*u-u-u*, Harlan, Oss, the Netherlands) weighing 280–300 g served as islet recipients. As islet donors, inbred male Lewis rats (*l-l-l*) (Harlan) weighing 300–320 g were used. All animal experiments

were performed after receiving approval of the Institutional Animal Care Committee of the Groningen University.

Diabetes was induced in the recipient rats by injection of 75 mg/kg streptozotocin (Zonasar, Upjohn Co., Kalamazoo, MI, USA) via the tail vein. Only animals showing weight loss and two consecutive blood glucose measurements exceeding 20 mmol/L served as islet graft recipients.

Islet Isolation and Transplantation

Islets were isolated from pancreata of Lewis rats as previously described (7). The total islet volume obtained by the isolation procedure was determined by measuring the diameters of the islets in a 2% aliquot of the islet suspension (9).

Transplantation of 5- μ l islet grafts under the kidney capsule was performed immediately after the islet isolation procedure under isoflurane anesthesia. The transplanted endocrine graft volume of 5 μ l induces normoglycemia in 100% of noninfected recipients (48). Immunosuppression was applied for 10 days. For this, rats received 5 mg/kg cyclosporin A (CsA; Sandimmune, Novartis, Basel, Switzerland) subcutaneously on a daily basis, starting at the day of transplantation (42).

CMV Infection

Rats received a rat CMV (RCMV) infection by intraperitoneal injection of 2×10^5 plaque-forming units (pfus) of RCMV (Maastricht strain) at day 1 after islet transplantation isolated as previously described (4). The amount of infectious virus was determined by plaque assay (4). Virus-free homogenate was obtained by homogenization of salivary glands of irradiated, noninfected AO rats. This virus-free homogenate was injected as control and called a mock infection. Mock infection was established by intraperitoneal injection of virus-free salivary gland homogenate at day 1 after transplantation.

Graft Function and Sacrifice

The glucose concentration was determined using the Accu-Chek Sensor system (Roche, Mannheim, Germany). Transplantation was considered successful when nonfasting blood glucose concentrations reached levels below 10 mmol/L. Animals were sacrificed when animals demonstrated two blood glucose measurements exceeding 20 mmol/L in a period of 1 week.

After graft failure, pancreas, kidneys, salivary glands, spleens, and islet grafts were removed for histological examination. Islet grafts were removed and snap frozen in liquid nitrogen. Grafts were sectioned at 5 μ m and stored at -20°C until use.

Detection of Viral Infection

To confirm successful viral infection in the salivary glands, we performed PCR analysis for viral DNA and

did histological staining for RCMV early protein R44 as described below. All RCMV-infected animals had viral DNA and R44-positive cells within the salivary glands, showing successful systemic CMV infection.

RCMV PCR

To determine the presence of viral DNA in the salivary glands, DNA was extracted by lysing the tissue in SE buffer containing 75 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS) (all from Merck, Darmstadt Germany), and 200 µg/ml Proteinase K (Life Technologies, Bleiswijk, the Netherlands) at 55°C until all tissue was dissolved. DNA was extracted using chloroform–isopropanol extraction (Sigma-Aldrich). Subsequently, RCMV-specific (nested) PCR analysis was performed as described previously (3).

The forward primer sequence was 5'-AAGGGATCGATTTCGCCAGCCTCTACC-3', the reverse primer sequence was 5'-AAGGGATCCTGTCCGGTGTCCCCGTACAC-3'. The nested forward primer sequence was 5'-AAGGGATCCCCTCTGTTACTCCACCCTGC-3', the nested reverse primer sequence was 5'-TTCGGATCCACGCCGACCTCGGAGACCAG-3'. DNA from RCMV-infected rat embryonic fibroblasts served as a positive control. When samples were negative after the first-round PCR, we performed nested PCR. PCR products were visualized by ethidium bromide staining (Sigma-Aldrich, Zwijndrecht, the Netherlands) and agarose gel electrophoresis (Invitrogen/Life Technologies).

Detection of R44 Protein

The salivary glands were removed after the animals were sacrificed and snap frozen in liquid nitrogen. The salivary glands were sectioned at 5 µm, 10 min fixed in ice-cold acetone (Sigma-Aldrich), and air dried for histological examination. Endogenous peroxidase was blocked for 20 min in phosphate-buffered saline (PBS; Sigma-Aldrich) containing 0.15% hydrogen peroxide (Merck). Sections were incubated with mAb8 (1:8) (24) directed against RCMV R44 for 1 h. Subsequently, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG1 (Dako, Glostrup, Denmark) for 30 min. The whole procedure was performed at room temperature. Peroxidase activity was demonstrated by using 3,3'-diaminobenzidine tetra HCL (Sigma-Aldrich) containing hydrogen peroxide. In all control sections, PBS was used instead of the primary antibody. All control sections were consistently negative.

Flow Cytometry

Flow cytometry was performed on blood and splenocytes. Blood was collected at regular time intervals from the tail vein. A sample of 40 µl of blood was applied to count the total number of leukocytes per milliliter

of blood or splenocytes, using the Coulter Counter Z1 (Coulter, Hialeah, FL, USA). The remainder of the blood was diluted 1:1 with PBS and centrifuged for 20 min at 1,200×g at 4°C. Buffy coats were taken out, and erythrocytes were lysed using NH₄Cl (Sigma-Aldrich) for 10 min at 4°C. After centrifugation (1,200×g for 10 min at 4°C), leukocytes were resuspended in PBS containing 5% fetal calf serum (FCS; Gibco, Bleiswijk, the Netherlands) and 0.1% sodium azide (Sigma-Aldrich) and counted.

Splenocytes were isolated from the spleen according to standard methods (43) and were collected on the day of sacrifice. After washing, the erythrocytes were lysed using NH₄Cl for 10 min at 4°C. After centrifugation (1,200×g for 10 min at 4°C), splenocytes were resuspended in PBS containing 5% FCS and 0.1% sodium azide and counted.

For three-color flow cytometric analysis, 5×10⁵ cells (blood or splenocytes) were resuspended in PBS containing 5% FCS and 0.1% sodium azide (PBS/FCS/azide). All antibodies were obtained from BD Pharmingen (San Diego, CA, USA) unless otherwise specified. Primary antibodies used were peridinin chlorophyll (PerCP)-conjugated R73 (1:100) against rat T-cell receptor (TCR) αβ (cluster of differentiation 3; CD3), combined with phycoerythrin (PE)-conjugated anti-NKR-P1A (1:50), and combined with either biotin-conjugated STOK2 (1:100) against Ly49i2 (34), biotin-conjugated STOK6 (1:50) against Ly49s3 (26), biotin-conjugated DAR13 (1:100) against Ly49s3 (35), biotin-conjugated STOK9 (1:300) against KLRH1 (36), or biotin-conjugated STOK27 (1:100) against NKR-P1B (25). STOK6 and DAR13 (kind gifts of Dr. Vaage, Institute of Immunology, Rikshospitalet University Hospital, Oslo, Norway) both recognize Ly49s3 on AO NK cells (26). Blood NK cells were stained with STOK6 antibody. Splenocytes were stained with both STOK6 and DAR13. Similar percentages of NK cells positive for DAR13 and STOK6 confirmed that both antibodies recognize the same receptor. After washing in PBS/FCS/azide, allophycocyanine (APC)-conjugated streptavidin was added for 30 min. Cells were washed twice and resuspended in PBS/FCS/azide and analyzed by flow cytometry (FACS Calibur, Beckton Dickinson, San Jose, CA, USA). During flow cytometry, we counted at least 5×10⁴ lymphocytes, based on the forward and sidescatter pattern. Data were analyzed using Winlist 5.0 software (Verity Software House, Topsham, ME, USA). During analysis, lymphocytes were gated in the forward-sidescatter plot (Fig. 1A). This gate was then copied to a PerCP (TCR)-PE (NKR-P1A) plot (Fig. 1B), and NKR-P1A-positive/TCR-negative cells were defined as NK cells. A sample stained for NKR-P1A, TCR, and only streptavidin-APC was used to set the gate so that 99% of the cells were negative (Fig. 2A, control). This gate was then copied to the samples stained for and the percentage of cells positive for

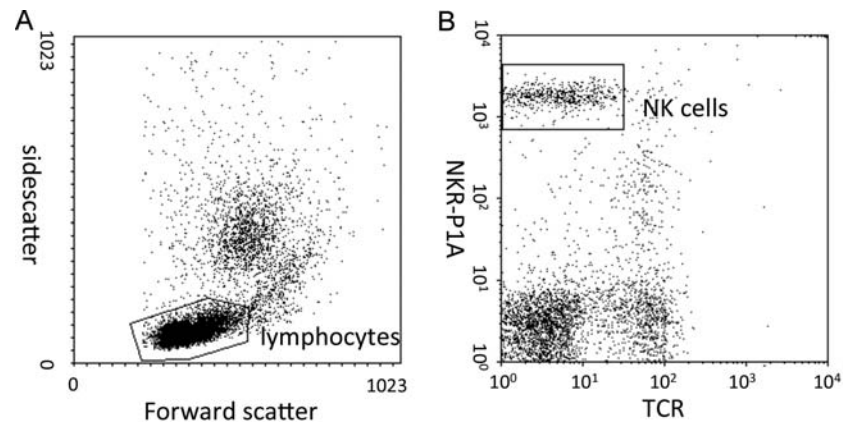


Figure 1. Gating strategy for the natural killer (NK) cells. Lymphocytes were gated in the forward sidescatter plot (A). This gate was then copied to a peridinin chlorophyll (T-cell receptor)–phycoerythrin (natural killer cell surface protein P1A) (PerCP [TCR]-PE [NKR-P1A]) plot (B), and NKR-P1A-positive/TCR-negative cells were defined as NK cells.

Ly49i2, Ly49s3, KLRH1, and NKR-P1B was determined within the NK cell population (Fig. 2B). Absolute cell numbers were calculated by multiplying percentages by the number of white blood cells.

Fluorescence Microscopy

Grafts were retrieved, snap frozen, sectioned, and stained for the presence of cells expressing NKR-P1A, NKp46 (41), and Ly49i2, Ly49s3, KLRH1, NKR-P1B, and perforin (all BD).

In short, islet graft sections were air dried and fixed in ice-cold acetone before starting the staining procedure. Endogenous avidin and biotin were blocked using a two-step avidin–biotin block kit (Dako). Primary antibodies were incubated for 1 h, and secondary antibodies were incubated for 30 min. The whole procedure was performed at room temperature in the dark. Primary monoclonal antibodies used were anti-NKR-P1A (1:50) and WEN23 (1:100) (kind gift from Erik Dissen, Rikshospitalet, Oslo, Norway) directed against NKp46, goat anti-perforin (1:50; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), biotin-conjugated STOK2 (1:100) against Ly49i2, biotin-conjugated DAR13 (1:100) against Ly49s3, biotin-conjugated STOK9 (1:300) against KLRH1, and biotin-conjugated STOK27 (1:100) against NKR-P1B. Secondary antibodies used were goat anti-mouse-tetrahromamine isothiocyanate (TRITC; 1:50; Dako) and goat anti-mouse biotin (1:100; Dako). Cy3-conjugated streptavidin (1:100) was used to determine the presence of NKp46-positive cells, and FITC-conjugated streptavidin (1:100) to determine the presence of Ly49/KLRH1-positive cells.

Finally, sections were counterstained with 2 μ g/ml 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA), mounted in Citifluor (Sigma-Aldrich), and analyzed by fluorescence microscopy (Leica Microsystems, Buffalo Grove, IL, USA). In all control sections,

PBS was used instead of the primary antibodies. All control sections were consistently negative.

Quantification of infiltrating NK cell subpopulations was assessed using a similar scoring system to those previously described (8,47). Five randomly selected technicians were asked to blindly score a large panel of stained graft sections of infected and noninfected animals on a semiquantitative scale. From these results, an average infiltration score was determined for the CMV-infected and MOCK-infected animals and tested for statistical significance. All sections were viewed first and subsequently graded, using a semiquantitative scoring system with five grades: 0, no positive cells, to 4, large amounts of positive cells.

Statistics

Values are expressed as mean \pm standard error of the mean (SEM) except where nonparametric, in which case median (range) was used. Normal distribution of the data was confirmed using the Kolmogorov–Smirnov test. Statistical comparisons were performed using the one-way ANOVA, subsequently followed by the two-sided Dunnett post hoc test that corrects for multiple comparisons (blood) or the two-sided Student's *t* test (spleen). Where no normal distribution could be demonstrated, we applied the nonparametric Mann–Whitney *U* test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Islet Allograft Failure Is Associated With the Recruitment of Cytotoxic, Allogeneic NK Cells

All recipients of an islet allograft became normoglycemic within 7 days after transplantation. After 10 days of complete immunosuppression, CsA was withdrawn, after which the allogeneic response developed. Graft failure

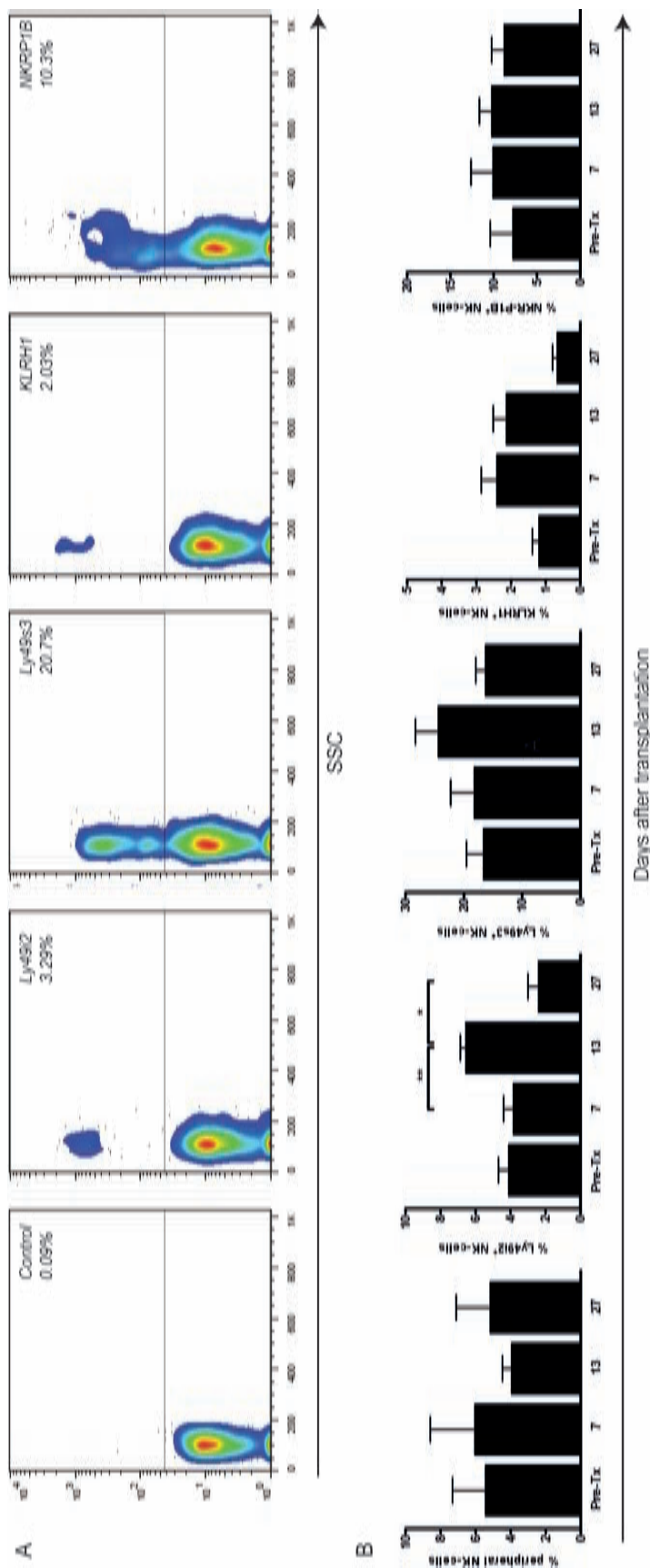


Figure 2. Blood lymphocytes were stained for the expression of the TCR and NKR-P1A. NKR-P1A-positive TCR-negative NK cells were gated, and the frequency of Ly49 inhibitory 2 (Ly49i2)-, Ly49 stimulatory 3 (Ly49s3)-, killer cell lectin-like receptor subfamily H, member 1 (KLRH1)-, and NKR-P1B-positive NK cells was determined based on the negative control stained with the streptavidin conjugate only (A). Islet allotransplantation promotes an increased proportion of blood Ly49i2⁺ NK cells. The percentage of Ly49i2-positive, Ly49s3-positive, KLRH1-positive, and NKR-P1B-positive cells was determined within the blood NK cell population before transplantation (Pre-Tx), at 7 days (7), at 13 days (13), and at 27 days (27) after transplantation (B). Results are presented as the mean \pm SEM for $n=7$ recipient at time points Pre-Tx to 13 and $n=3$ recipients at time point 27. Statistical significance was calculated using the one-way ANOVA, subsequently followed by the two-sided Dunnett post hoc test. * $p < 0.05$, ** $p < 0.01$.

was complete at 27.3 ± 0.3 days. The composition of the NK cell population in blood was studied 1 day before (Pre-Tx), at day 7, at day 13 (i.e., 3 days after stopping the administration of CsA), and after the allogeneic response (i.e., at the day of complete graft failure).

The frequency of NK cells and NK cell subpopulations was determined in the blood of allogeneically transplanted rats by flow cytometry (Fig. 2A). The size of the total NK cell population in the blood did not significantly change (Fig. 2B), but there were clear differences in the subpopulations of circulating NK cells. The proportion of allogeneic NK cells expressing Ly49i2 increased preceding graft failure (day 13: $p < 0.01$), which returned, as expected, to baseline levels after complete rejection of the graft (day 27: $p < 0.05$) (Fig. 2B). Significant changes in the proportion of NK cells expressing Ly49s3, KLRH1, or NKR-P1B were not observed (Fig. 2B).

In the four mock-infected, control grafts that were sacrificed at the moment of graft failure of their CMV-infected counterpart, we found high numbers of NK cells. They scored a median of 2.5 (range 2–3) for infiltration.

Large numbers of both NKR-P1A⁺ (Fig. 3A) as well as NKp46⁺ cells (Fig. 3B) were found in the islet allografts [median 2.5 (range 2–3)]. NKp46 is a cytotoxicity receptor solely expressed on NK cells (27,28).

To confirm the cytotoxic potential of these cells, we performed double stainings for NKp46 and perforin (2). Infiltrating NK cells contained perforin granules (Fig. 3C, yellow arrows). Notably, infiltration of perforin-negative NK cells was also observed (Fig. 3C, red cells), suggesting that also noncytotoxic NK cells infiltrated the islet grafts.

Since NK cell alloreactivity can be attributed to specific subpopulations rather than the NK cell population as a whole, we determined the infiltration of alloresponsive NK cells expressing Ly49i2, Ly49s3, and KLRH1 (25,26,34–36), and nonalloresponsive NK cells expressing NKR-P1B (25). This was done by performing double staining for the NK cell marker NKR-P1A combined with antibodies directed against inhibitory Ly49i2, activating Ly49s3, and inhibitory KLRH1, as well as antibodies against NKR-P1B. Double staining using NKp46

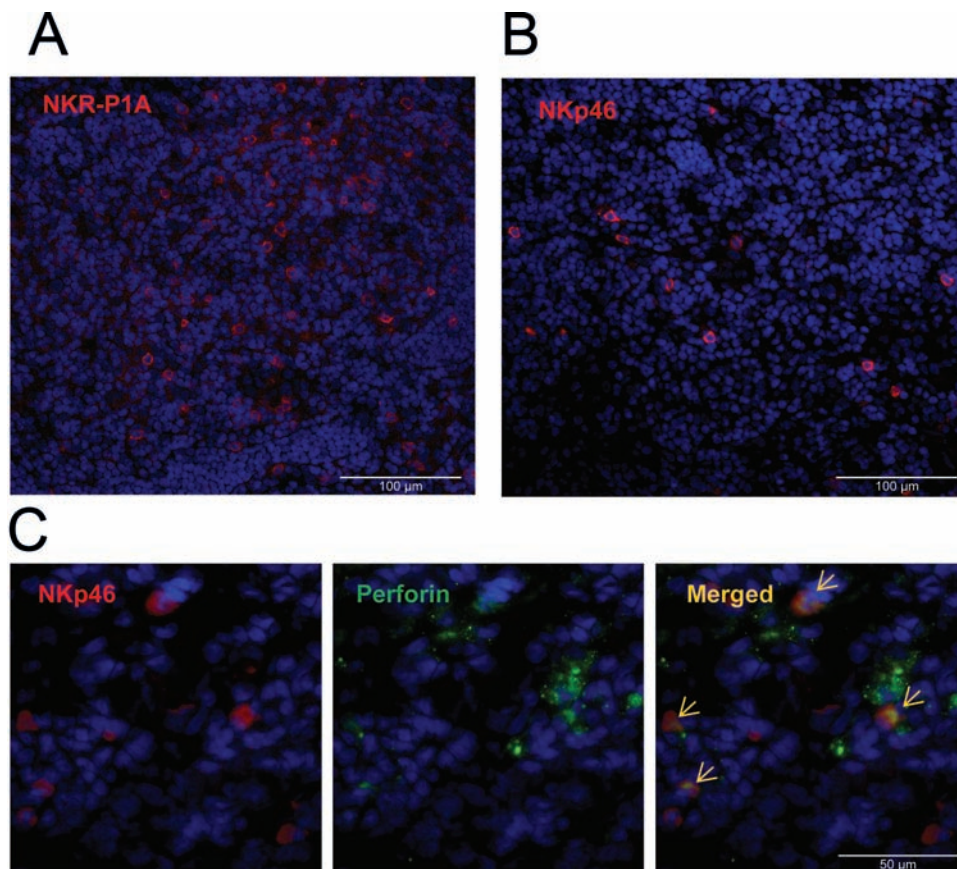


Figure 3. Cytotoxic NK cells in islet allografts from mock-infected rats on day 13 posttransplantation. Retrieved islet allografts were stained for the presence of NKR-P1A (A) and natural killer cell p46-related protein (NKp46) (B) positive cells (magnification: 20 \times). NKp46 (red) and perforin (green) staining demonstrates the presence of cytotoxic NK cells in the islet allografts (magnification: 40 \times) (C). Arrows show perforin-positive NK cells (i.e., double positive cells).

were not possible, since all antibodies required streptavidin conjugates.

The infiltration of specific alloreactive NK cell subpopulations was demonstrated by the presence of alloreactive Ly49i2⁺ (Fig. 4A), Ly49s3⁺ (Fig. 4B), and KLRH1⁺ (Fig. 4C), but not the nonalloresponsive NKR-P1B⁺ NK cells in the islet allografts (data not shown).

CMV Promotes the Recruitment of Cytotoxic NK Cells and Is Associated With Accelerated Islet Graft Failure

All recipients became normoglycemic within 7 days. The CMV-infected rats, however, showed complete graft

failure 10.6 ± 1.0 days after transplantation, which was significantly faster than that in the mock-infected control animals (23.2 ± 1.4 days; $p < 0.05$).

We observed an increased number of NK cells in the blood of CMV-infected recipients compared to the mock-infected controls ($p < 0.001$) (Fig. 5A). This elevation was already observed as soon as 7 days after implantation (i.e., under complete CsA immunosuppression). CMV infection induced an increased proportion of Ly49i2⁺ NK cells (day 7: $p < 0.05$). The proportion of Ly49i2⁺ NK cells was further increased on the day of graft failure (day 13 vs. pre-Tx: $p < 0.05$). In addition, 7 days

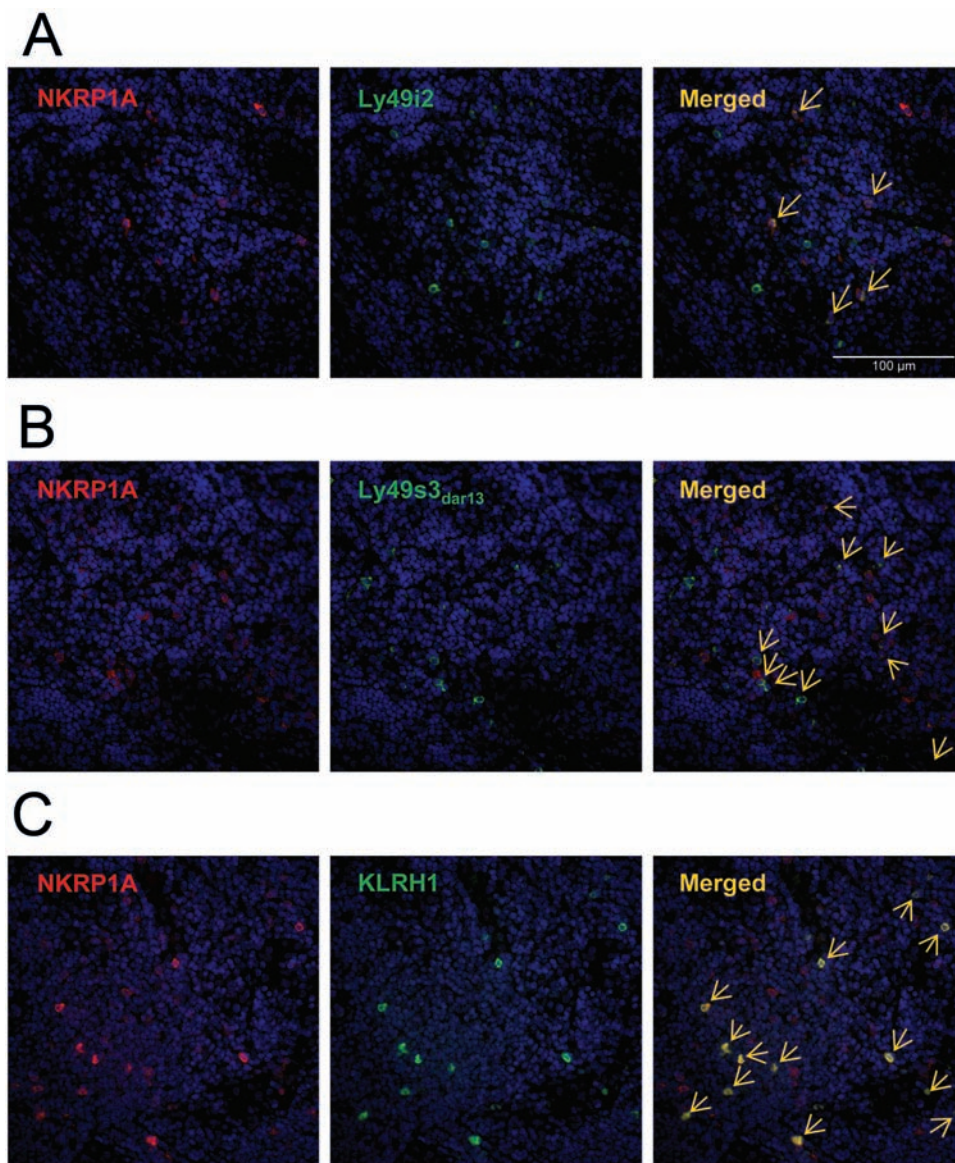


Figure 4. NK cell subpopulations in islet allografts from mock-infected control rats on day 13 posttransplant. Retrieved islet allografts were stained for the presence of NKR-P1A (red) and Ly49i2 (A), Ly49s3 (B), or KLRH1 (C) (all green) positive cells. Double positive cells are depicted by the yellow arrows.

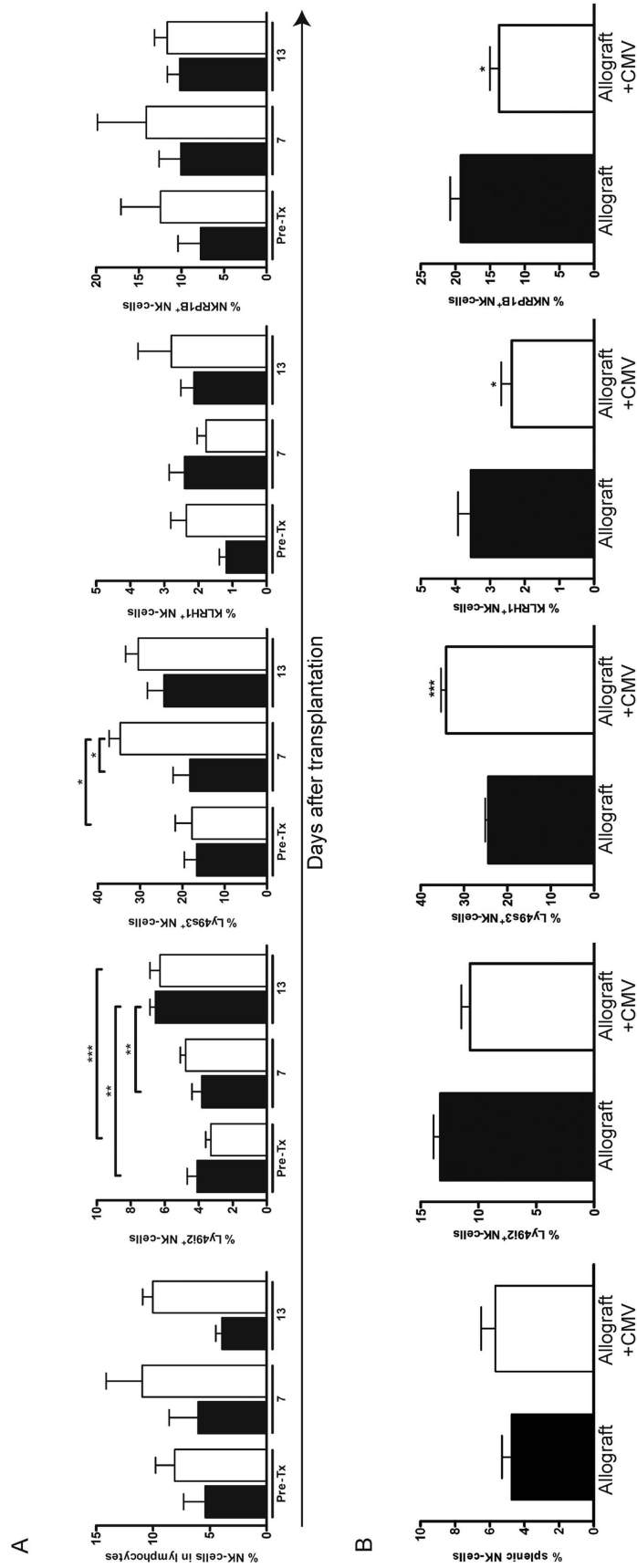


Figure 5. Ly49i2⁺ and Ly49s3⁺ NK cells in mock-infected controls and in CMV-infected rat recipients. The percentage of circulating NK cells within the total lymphocyte population was determined in blood (A) or in the spleen (B). In blood, the percentage of Ly49i2-positive, Ly49s3-positive, KLRH1-positive, and NKR-P1B-positive cells was determined before transplantation (pre-Tx), 7 days (7), and 13 days after transplantation. Black bars represent mock-infected control recipients and open bars the CMV-infected recipients. In the spleen (B), we only quantified the NK cell population at the day of sacrifice. Results are presented as mean ± SEM of seven recipients of noninfected controls and for six CMV-infected recipients. Statistical significance within groups was calculated using the one-way ANOVA, subsequently followed by the two-sided Dunnett post hoc test (A) or the two-sided Student *t* test (B). **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

after transplantation, the proportion of Ly49s3⁺ NK cells almost doubled in CMV-infected recipients (day 7 vs. pre-Tx and CMV vs. mock-infected control; $p < 0.05$). Significant changes in the KLRH1⁺ or NKR-P1B⁺ NK cell population were not observed. Thus, preceding islet graft failure, CMV-infected allograft recipients demonstrated an altered composition of the peripheral NK cell population, which is characterized by skewing toward alloresponsive Ly49i2⁺ and Ly49s3⁺ NK cells.

The total numbers of splenic NK cells were not increased when compared to mock-infected controls. Just as observed in blood, the splenic NK cells demonstrate skewing toward alloreactive Ly49s3⁺ NK cell subpopulations in response to CMV infection ($p < 0.001$) (Fig. 5B). In the spleen, the percentage of Ly49i2⁺ NK cells was not different between the CMV-infected recipients and mock-infected controls. In contrast to peripheral blood NK cells, the frequency of KLRH1⁺ ($p < 0.05$) and NKR-P1B⁺ ($p < 0.05$) NK cells was decreased in CMV-infected recipients compared to the mock-infected controls.

In the grafts, we found high numbers of infiltrating NK cells, as illustrated by both the expression of NKR-P1A (Fig. 6A) (median 3.5; range 3–4), as well as NKp46 (Fig. 6B) (median 3.5; range 3–4). We stained the tissue sections of the islet allografts of CMV-infected recipients for NK cells expressing Ly49i2, Ly49s3, KLRH1, or NKR-P1B. Similar to the islet grafts of noninfected recipients, NK cells expressing Ly49i2 (Fig. 7A), Ly49s3 (Fig. 7B), and KLRH1 (Fig. 7C), but not NKR-P1B (not shown), were detected in the islet grafts. Notably, the infiltration of both allogeneic Ly49i2⁻ and Ly49s3⁻ expressing NK cells was increased in the islet grafts of CMV-infected recipients compared to the mock-infected control animals (Table 1).

DISCUSSION

In the present study, we show that during the process of islet allograft failure, islet allografts are infiltrated by Ly49/KLRH1⁺ NK cells. When islet transplantation was combined with CMV infection, we observed an association between the expansion of circulating Ly49i2⁺ and Ly49s3⁺ NK cells and islet graft failure. The absence of expansion or infiltration of NKR-P1B⁺ NK cells suggests that alloreactive NK cell responses are mediated by specific Ly49⁺ or KLRH1⁺ subpopulations, rather than by the NK cell population as a whole.

The infiltration of large numbers of NKR-P1A and NKp46-positive cells into the allografts demonstrates that the infiltrating Ly49⁺ and KLRH1⁺ cells are NK cells and not NKT cells. Furthermore, the infiltration of perforin-containing NK cells demonstrates that the infiltrating Ly49/KLRH1⁺ NK cells are involved in proinflammatory, rejection-inducing responses (2), rather than tolerance-inducing responses (19,28,29). However, the presence of perforin-negative NK cells in the islet allografts suggests that also other NK effector functions, such as proinflammatory cytokine production as shown in our previous studies (42,43) and others (32,33), may contribute to islet graft failure. We observed increased frequencies in the blood and in the spleen together with increased frequencies in the graft in the CMV-infected animals compared to the mock-infected controls. This suggests that these subpopulations respond to the infection and the allograft and contribute to graft failure.

Many *in vitro* studies demonstrate the high alloreactive potential of rat Ly49⁺ NK cells (25,35,36), but up until now, *in vivo* studies never focused specifically on Ly49⁺ NK cell subpopulations. It has been shown in mice, however, that murine Ly49-expressing NK cell

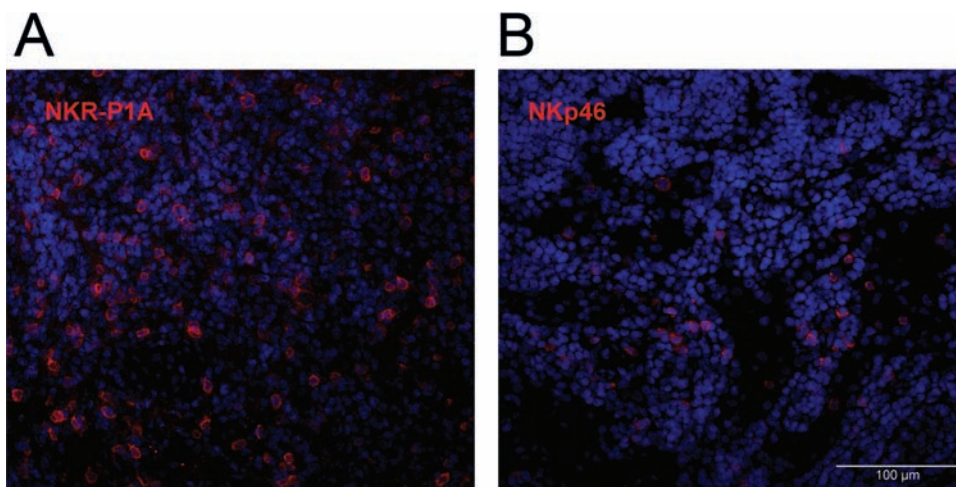


Figure 6. NK cells in rat islet allografts of CMV-infected recipients at graft failure on day 13 posttransplantation. Retrieved islet allografts of CMV-infected recipients were stained for the presence of NKR-P1A (A) and NKp46 (B) positive cells (magnification: 20 \times).

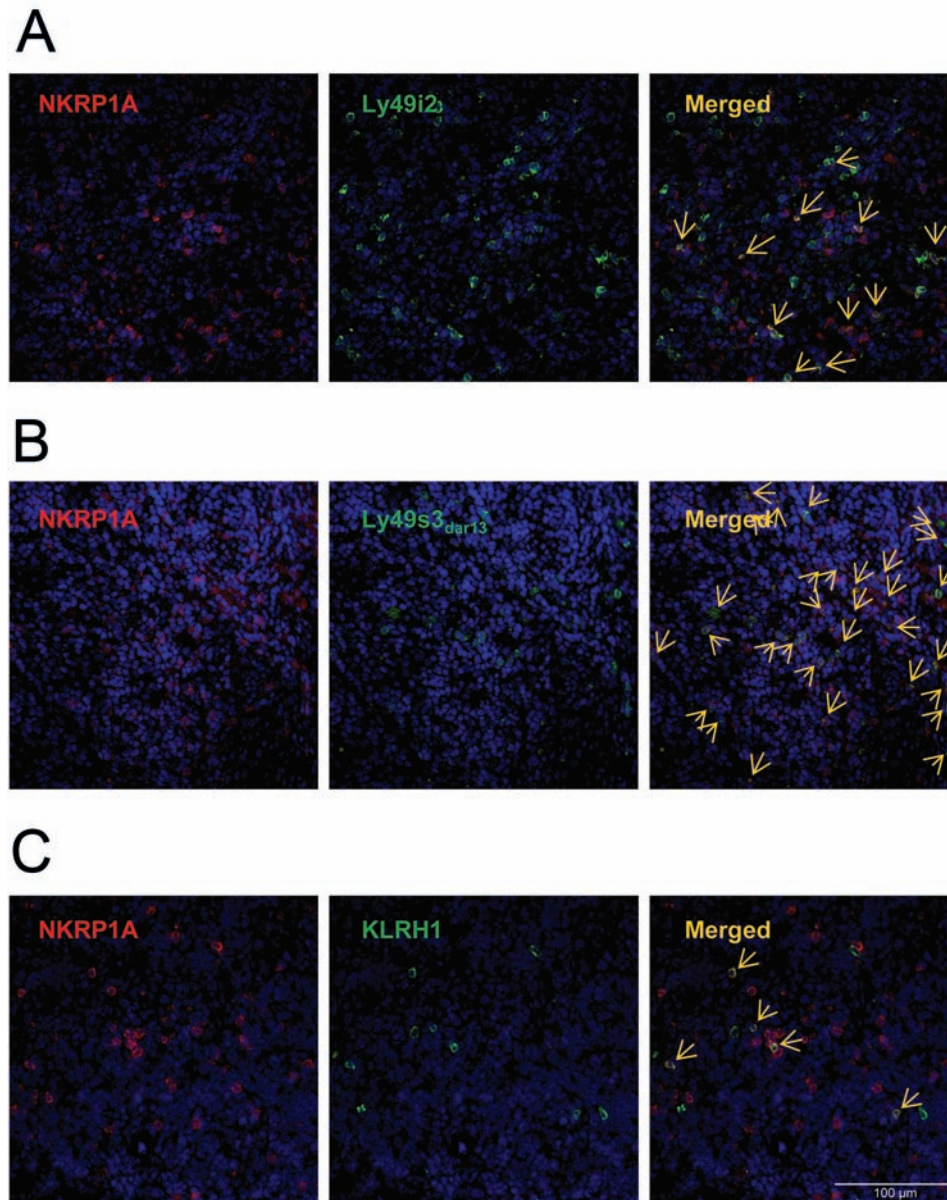


Figure 7. NK cell subpopulations infiltrating rat islet allografts of CMV-infected recipients on day 13 posttransplantation. Retrieved islet allografts of CMV-infected recipients were stained for the presence of NKR-P1A (red) and Ly49i2 (A), Ly49s3 (B), or KLRH1 (C) (all green) positive cells. Double positive cells are depicted by the yellow arrows.

Table 1. Natural Killer (NK) Cell Subset Infiltration in Islet Grafts

Receptor	Allografts Median (Range)	Allografts and CMV Median (Range)	<i>p</i> Value
Ly49i2	2 (1–2)	3 (3–4)	0.0498 (*)
Ly49s3	1 (1–2)	3.5 (2.5–4)	0.0498 (*)
KLRH1	2 (2–2.5)	3 (2.5–3)	0.07

All sections were viewed first and subsequently graded using a semi-quantitative scoring system with five grades: 0, no positive cells, to 4, large amounts of positive cells. CMV infection enhanced the influx of Ly49 inhibitory 2 (Ly49i2) and ly49 stimulatory 3 (Ly49s3) NK cells in the graft, while the presence of killer cell lectin-like receptor subfamily H, member 1 (KLRH1) NK cells was not significantly altered.

subpopulations are alloresponsive in allogeneic bone marrow rejection (14,15), solid organ rejection (32), and in graft-versus-host disease (GVHD) (31). As Ly49⁺ NK cell populations are not suppressed by immunosuppression, we feel that our data show that it is advisable to study its role in human islet graft failure.

It might be argued that the role of T-cells should be studied in addition to that of NK cells. However, T-cells have been studied in great detail already (42). Owing to constraint in blood supply and experimental materials, we focused, in this study, on NK cells as their influence

on the outcome of islet grafts has not been characterized. The Ly49s3⁺ NK cells are probably the NK cells that are directly responsible for allograft failure. This subpopulation has been shown to have allogeneic responsiveness *in vitro* (26) and in bone marrow rejection *in vivo* (14,15). Ly49s3⁺ NK cells are highly dependent on activating Ly49 receptors, rather than on inhibitory Ly49 receptors. Moreover, islets are known to upregulate the expression of stress-related, nonclassical MHC I ligands for activating NK cell receptors after isolation and transplantation (18,20). Taken together, infiltration of Ly49s3⁺ NK cells suggests that besides lack of RT-1A (classical MHC class I molecule)-mediated inhibition to NK receptors, activation of NK cells via nonclassical MHC IB (RT-1CE/N/M)-Ly49s3 interactions may be of particular importance to islet allograft recognition and destruction.

Also, it may be argued that graft failure may be accelerated by direct infection of islet cells. However, in a previous study, we showed that in both rat islets (44) and human islets (43), CMV infects a small portion of not more than 5% of the cells. This infection degree cannot explain graft failure. However all islet cells in the graft had enhanced expression of proinflammatory molecules such as MHC-I, intercellular adhesion molecule-1 (ICAM-1), and lymphocyte function-associated antigen 3 (LFA-3). This was caused by enhanced secretion of interleukin 6 (IL-6), IL-8, IL-15, and monocyte chemoattractant protein 1 (MCP-1) by the CMV-infected islet cells (43). We therefore concluded that islets have an enhanced immunogenicity after CMV infection.

The expansion of the Ly49i2 subpopulation is something we did not expect. The Ly49i2 is a nonfunctional receptor in AO rats (34). Therefore, the responses in the Ly49i2⁺ NK population can only be interpreted as an innocent bystander effect. Since Ly49 receptors are expressed on overlapping subpopulations (26,35), the Ly49i2 response is most likely the result of NK cell activation through an unidentified Ly49 receptor, enriched within the Ly49i2⁺ NK cell population. The Ly49i2⁺ NK cell infiltration may also be responsible for the infiltration of KLRH1⁺ NK cells. Although KLRH1 is expressed on highly alloreactive NK cells, the direct involvement of KLRH1 in alloreactivity seems unlikely, since alloreactivity *in vitro* is not directly mediated through KLRH1, but through coexpressed Ly49 receptors (36). Therefore, also the infiltration of KLRH1⁺ NK cells in the islet allografts is most likely an innocent bystander response and a direct consequence of infiltration of Ly49-expressing alloreactive NK cells.

CMV enhanced the infiltration of Ly49i2⁺ and Ly49s3⁺ NK cells and was associated with shorter graft survival times. This is expected, as after CMV infection, splenic antiviral NK cell responses are induced for the clearance of virally infected cells and restraining viral spread (27).

Although we did not observe an expansion of the total splenic NK cell compartment after CMV infection, we observed an increased proportion of Ly49s3⁺ NK cells in the graft after CMV infection. Several lines of evidence point to the involvement of specific NK cell subpopulations in the restraint of CMV. In human renal graft recipients, the degree of CMV infection is inversely correlated to the number of activating killer cell Ig-like receptors (KIR) expressed (46). In mice, the activating receptor Ly49H is known to recognize m157, a MCMV protein, and protects the mice from the early stages of MCMV infection (45). It is not known whether Ly49s3 can recognize RCMV, but it could explain the infiltration of Ly49s3⁺ NK cells in the CMV-infected recipients.

The CMV-induced selective infiltration of highly alloreactive Ly49s3⁺ effector NK cells may explain the enhanced alloreactivity after CMV infection that we observed in a previous study (42) and in the present study. We found that the expansion is splenic and not only local. Splenic NK cells serve as a reservoir of mature effector NK cells, which are rapidly recruited to sites of inflammation, such as the site of transplantation (16). Although NK cells can be activated locally, without the need for activation in secondary lymphoid organs, the peripheral activation of NK cells enhances NK cell proliferation, cytolytic capacities, and cytokine production (6,10,16). In this way, CMV infection may simultaneously enhance both antiviral and alloreactive NK cells that allow allogeneic interactions and exacerbation of allograft rejection.

The present study confirms our hypothesis that islet allograft failure is associated with selective expansion of alloreactive Ly49/KLRH1⁺ NK-cell subpopulations. These cells infiltrated the grafts. Enhancement of the alloreactive subpopulations by CMV infection resulted in accelerated islet allograft failure. Depletion of this population has been considered but is lethal in rats when combined with immunosuppression and CMV. In spite of this limitation, our current approach of applying CMV to enhance, instead of delete, the population gives us mechanistic insight. The mechanism is probably as follows: peripheral and local activation of Ly49/KLRH1⁺ NK cells by alloreactive components can efficiently induce expansion of NK cell effector functions by sensing the lack of "self" MHC I ligands for the inhibitory receptors and/or the presence of MHC I ligands for activating Ly49 receptors. This may lead to graft destruction (Fig. 8A). CMV infection was associated with an even more pronounced infiltration by Ly49/KLRH1⁺ NK cells and accelerated allograft rejection. This effect of CMV infection may be caused by two processes. First, CMV infection favors the peripheral expansion of Ly49s3⁺ NK cells in addition to Ly49i2⁺ NK cells, which not only restrains CMV infections, but also enhances NK cell effector functions and NK cell interactions with allogeneic target cells. Second,

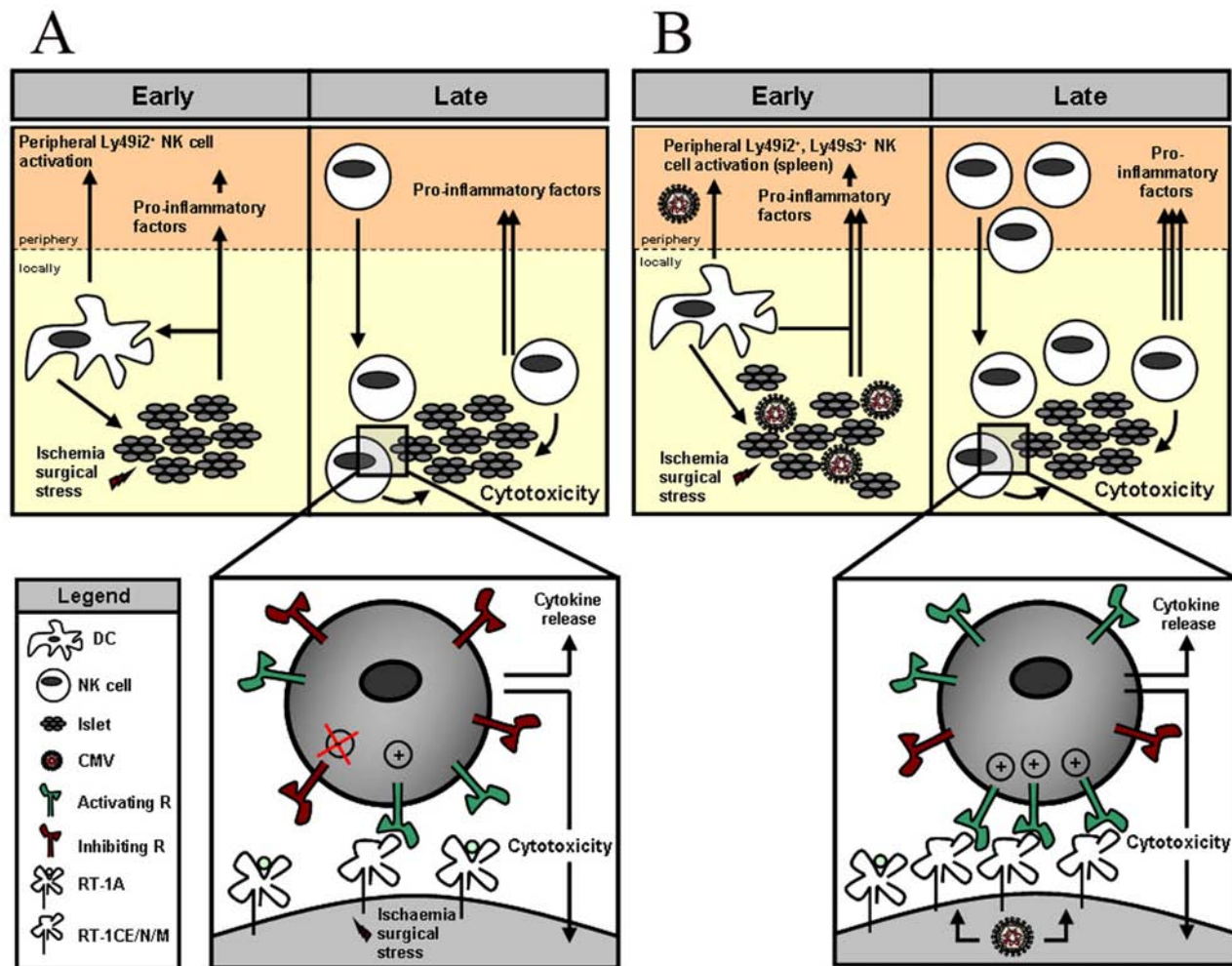


Figure 8. Contribution of Ly49⁺ NK cells to islet allograft failure. Ischemia and surgical stress may lead to production of proinflammatory factors and the upregulation of nonclassical MHC I molecules in the islet graft. Subsequently, peripheral (via dendritic cells; DCs) and local allogeneic NK cell activation may occur due to production of proinflammatory cytokines and lack of inhibitory “self” MHC I molecules and/or the expression of activating nonclassical MHC I molecules on islet allografts. Activation of NK effector functions may directly lead to cytotoxicity toward the allograft and/or abundant production of proinflammatory cytokines, further exacerbating inflammation (A). CMV infection may accelerate the rejection process by increasing the stress-induced release of proinflammatory factors and nonclassical MHC I molecules on the islet graft, and stimulating the peripheral expansion of allogeneic NK cell subpopulations in the spleen. Increased numbers of circulating alloresponsive NK cells, combined with enhanced inflammation in the islet allograft may lead to increased NK cell attraction and infiltration, and accelerated islet allograft destruction (B). RT-1A=classical MHC class I molecule; RT-1CE/N/M=nonclassical MHC class IB molecule.

since CMV infection is able to induce MHC complex class I chain-related gene A or B (MICA or MICB) expression on infected human cells (17), a CMV-driven enhanced expression of Ly49s3 ligands, that is, either RT-1CE/N/M proteins or viral proteins directly (45) may enhance graft allogenicity and NK–target cell interactions, subsequently leading to graft destruction (Figure 8B).

The findings from the present study may have implications for human islet transplantation. The currently applied immunosuppressives are unable to suppress NK cell function (49) or might even enhance NK cell

cytotoxicity (50). Therefore, also in humans, we feel the role of KIR-expressing NK cell subpopulations in islet graft failure needs further attention. The same goes for the role of CMV in islet graft failure. Although the role of CMV is clinically still a subject of debate (13), our data warrant caution in interpretation of the effects of CMV as we show that it can have unexpected effects on cell populations involved in allogeneic responses.

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