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ORIGINAL ARTICLE

Antibody-induced NKG2D blockade in a rat model of intraportal islet transplantation leads to a deleterious reaction

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SUMMARY

Intraportal islet transplantation is plagued by an acute destruction of transplanted islets. Amongst the first responders, NK cells and macrophages harbour an activating receptor, NKG2D, recognizing ligands expressed by stressed cells. We aimed to determine whether islet NKG2D ligand expression increases with culture time, and to analyse the impact of antibody-induced NKG2D blockade in islet transplantation. NKG2D-ligand expression was analysed in rat and human islets. Syngeneic marginal mass intraportal islet transplantations were performed in rats: control group, recipients transplanted with NKG2D-recombinant-treated islets (recombinant group), and recipients treated with a mouse anti-rat anti-NKG2D antibody and transplanted with recombinant-treated islets (antibody-recombinant group). Islets demonstrated increased gene expression of NKG2D ligands with culture time. Blockade of NKG2D on NK cells decreased *in vitro* cytotoxicity against islets. Recipients from the control and recombinant groups showed similar metabolic results; conversely, treatment with the antibody resulted in lower diabetes reversal. The antibody depleted circulating and liver NK cells in recipients, who displayed increased macrophage infiltration of recipient origin around the transplanted islets. *In vitro* blockade of NKG2D ligands had no impact on early graft function. Systemic treatment of recipients with an anti-NKG2D antibody was deleterious to the islet graft, possibly through an antibody-dependent cell-mediated cytotoxicity reaction.

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Key words
hypersensitivity type II, islet transplantation, liver, NKG2D

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Introduction

Over the last two decades, intraportal islet transplantation has become a viable therapeutic option for selected patients with type 1 diabetes. However, most recipients still need at least two sequential islet infusions in order to hope for insulin independence due to the acute early loss of islets [1]. One of the major causes for this loss is the instant blood-mediated inflammatory reaction (IBMIR), a massive, deleterious and aspecific reaction of the innate immune system to the contact of isolated pancreatic islets with blood [2]. It is mostly triggered by

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the expression of tissue factor on the islets, initiating the coagulation cascade, culminating in the formation of a thrombus and a dense immune cell infiltrate [3–5]. Macrophages and natural killer (NK) cells are amongst the first responders of the innate immune system in IBMIR [1]. Moreover, NK cells, be they resident in the liver, circulating or memory-like, are directly implicated in the loss of islets after intraportal allogeneic transplantation [6–8]. Almost all NK cells, CD8+ T cells, γδ T cells, NKT cells and activated macrophages express the activating natural killer group 2 member D (NKG2D) receptor [9]. NKG2D is a C-type lectin transmembrane glycoprotein that is mostly known for its role in tumour cell clearance [9]. It has also been implicated in the onset of type 1 diabetes mellitus [10,11] and in the rejection of cardiac allografts in mice [12]. We therefore identified NKG2D as a potential target for the prevention of early islet loss.

For logistic reasons, islets are often kept in culture for at least 2 days between isolation and transplantation. During this time, isolated islets suffer from hypoxia [13], which is further maintained after transplantation until a new vasculature develops [14,15]. The expression of NKG2D ligands [MHC-class I chain-related proteins (MICA, MICB), and UL-16 binding proteins (ULBP1-6) in humans; ribonucleic acid export 1-like (RAE1L), and similar to retinoic acid early transcript 1L (RRLT) in rats] increases in cells exposed to stress such as hypoxia, DNA damage, exposure to cytokines or stimulation by Toll-like receptors (TLR) [16]. We hypothesize that it is also the case in cultured islets, enhancing the impact of the NKG2D-expressing cells.

The aims of this paper were to determine whether cultured human and rat islets demonstrate increased NKG2D ligand expression and whether islet engraftment and function are improved by blocking the NKG2D ligands and/or receptor in recipients.

Methods

Animals

Pancreatic islets were isolated from either Lewis rats (Janvier Labs, Le Genest St Isle, France), or GFP+ LEW-Tg(CAG-EGFP)YsRrrc (RRRC, Columbia, MO, USA), weighing 200–220 g, and transplanted in Lewis rats (Janvier Labs) 145–160 g. Animals were cared for according to international guidelines on animal care. Ethical approval was obtained from the Geneva veterinary authorities (Licences GE/129/15, GE/96/16 and GE/57/17).

Pancreatectomy and islet isolation

Pancreatectomy, islet isolation and islet purification were performed as described previously [17]. After pancreas retrieval and digestion, islet purification was carried out with a continuous Optiprep (Axis-Shield, Dundee, Scotland) gradient [18]. Islets were stained with dithizone (Sigma-Aldrich, Buchs SG, Switzerland) and counted in absolute number and number of islet equivalents (IEQ) immediately after isolation and prior to transplantation. Islets were cultured for 5 days in an 11.1 mM glucose Dulbecco's modified Eagle medium (DMEM; Gibco, Paisley, UK) (islet DMEM), in a 37 °C, 5% CO_2, environment.

Human pancreatic islets

Non-transplantable human pancreatic islets were used for research purposes when no refusal was declared. Human islets were provided through the Juvenile Diabetes Research Foundation award 31-2008-416 (European Consortium for Islet Transplantation Islet for Basic Research programme), and ethical approval was obtained from the Geneva cantonal ethics committee for research (Licence 2017-01642).

RNA extraction and quantitative polymerase chain reaction of islets

Rat islets

400 IEQ lysates were stored at −80 °C in RLT buffer (Qiagen, Hilden, Germany) + β-mercaptoethanol (BioRad, Hercules, CA, USA) for batch analyses. RNA extraction (RNEasy Microkit; Qiagen) and cDNA synthesis (Qiagen) were performed according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using the MESA BLUE Mastermix for SYBR Assay/No ROX (Eurogentec, Liege, Belgium) and the following primers (Table 1 for the sequences): RPLP1 as the housekeeping gene; the rat NKG2D ligands RRLT, RAE1L; and the hypoxia-related genes hypoxia-inducible 1 alpha (Hif1a) (NM_024359.1; Qiagen).

Human islets

5000 IEQ lysates were stored at −80 °C. qPCR analysis was performed as described above with the following ready-for-use primers (Qiagen): RPLP0 as the housekeeping gene and the human NKG2D ligands MICA (NM_000247), MICB (NM_005931), ULBP1 (NM_025218), ULBP2 (NM_025217), ULBP3 (NM_024518). Results were

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Table 1. PCR sequences used for gene expression analyses in rat islets.

Gene	Sequence	Forward primer	Reverse primer
RPLP1	NM_001007604	5′-TCTCTGAGCTTGCCTGCATCTACT-3′	5′-CCTACATTGCAGATGAGGCTTCCA-3′
RRLT	FJ_971664	5′-TGACCCAGTGGATAGACCGATGTA-3′	5′-GGAAGCATGGTGACCCGTAAAGTG-3′
RAE1L	NM_001140975.1	5′-TGTGACCCAGTGGATAGACCTGTT-3′	5′-TGGCTGTGGTTAGAAAGCATGGTG-3′
IRP94	AF_077354	5′-GTCTGATGGCTCCAGCTCAAAAGT-3′	5′-GGCTGTTGCTCTTCAGTATGTGGT-3′

analysed with the $2^{-\Delta\Delta C_t}$ method and expressed as the fold changes compared to day 0.

Induction of diabetes, intraportal islet transplantation and recipient follow-up

Induction of diabetes was performed 4 days prior to transplantation. A single intraperitoneal dose of streptozotocin (Sigma-Aldrich; 80 mg/kg) was administered after a 6-h fasting period.

Three groups were studied: control group, recipients transplanted with untreated islets; recombinant group, recipients transplanted with islets previously treated with 0.25 μg/100 IEQ recombinant NKG2D (Recombinant Mouse NKG2D Fc Chimera Protein, R&D Systems, Minneapolis, MN, USA) and antibody-recombinant group, recipients receiving anti-NKG2D antibody injections and transplanted with islets treated with recombinant NKG2D. At days 0–5 after transplantation, recipients received 8 mg/kg intraperitoneal injections of either control IgG1 isotype (BioXCell, West Lebanon, NH, USA) or anti-NKG2D antibody (11D5F4). The antibody doses were chosen in accordance with a previously-published report [19]. Transplantations were performed according to a 'marginal mass' islet transplantation model, 5500 IEQ/kg in this experiment, with the same protocol described previously [20]. The experimental groups included six recipients in the control group and eight each in the recombinant and antibody-recombinant groups.

Non-fasting glycaemia and weight of recipients were assessed thrice weekly until day 30. Diabetes reversal was defined as two consecutive non-fasting glycaemia measures ≤11.1 mM (200 mg/dl).

Metabolic challenge of the islet grafts was performed via an intraperitoneal glucose tolerance test (IPGTT) was performed on recipients at day 30 after transplantation. Recipients received an intraperitoneal injection of 20% glucose solution (2 mg/kg). Glycaemia was measured at 0, 5, 10, 15, 30, 60, 90 and 120 min after injection.

Additional recipients of each group underwent islet transplantation in the caudate lobe for alternate analyses. Briefly, in addition to the standard procedure for intraportal islet transplantation, portal branches to the left, median and right liver lobes were transiently clamped. Islet transplantation was performed in the caudate lobe. The clamps were removed from the portal vein; the abdominal incision was closed, and rats were given analgesia as described previously. These recipients were sacrificed at day 1 or 7, and the caudate lobe was ligated and excised; a sample was frozen in Tissue-Tek® O.C.T™ compound (Sakura Finetek, Staufen, Germany) and stored at −80 °C, and the rest of the liver lobe was fixed in 10% formaldehyde. The remaining liver of animals sacrificed at day 7 was digested for flow cytometry analysis of immune cell phenotype as described in [21] apart from the perfusion, which was done through the portal vein. Isolated liver mononuclear cells were used for flow cytometry analysis (Table 2 for antibodies).

Flow cytometry analyses of recipient PBMCs and islet cells

Blood was sampled from the tail vein of all recipients in heparinized Pasteur pipettes at days 1, 4, 7, 14 and 30 after transplantation. 20 μl of whole blood was taken for peripheral blood mononuclear cell (PBMC) staining (Table 2 for antibodies); the blood was incubated with RBC lysis (Biolegend, San Diego, CA, USA). Flow cytometry was performed on the Attunes flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA), and results were analysed using FloJo (Treestar, Eysins, Switzerland). The remaining blood was centrifuged for 10 min at 9 000 *g* and 4 °C to allow for the separation of the serum from the blood cells. The serum was retrieved and stocked at −20 °C for batch analyses. Islets were dissociated with StemPro Accutase (Gibco) before incubation with antibodies and/or recombinant NKG2D (Table 2).

Statistical analyses

Statistical analyses were performed using PRISM 7 (GraphPad, San Diego, CA, USA). Mann–Whitney and Kruskal–Wallis tests were used where appropriate (as specified in the results section). Areas under the curve were analysed for IPGTT results. All *P*-values are two-tailed; significance was set at 5%. Results in charts are expressed as median

Table 2. Flow cytometry.

Antibody/protein	Clone	Fluorochrome	Target	Provider
Anti-CD161 (NKRP1)	10/78	FITC	NK cells	Biolegend
Anti-TCR	R73	APC	T cells	Biolegend
Anti-CD4	W3/25	APC-Cy7	CD4 cells	Biolegend
Anti-CD8	OX-8	PerCP	CD8 cells	Biolegend
Anti-hepatic sinusoidal endothelial cells	SE-1	FITC	LSEC	Novus
Anti-CD163	ED-2	Alexa 405	Macrophages	Novus
Anti-CD45	OX-1	PE	Myeloid cells	BD Biosciences
Anti-CD314 (NKG2D)	11D5F4	PE	NKG2D receptor	Thermo Fisher Scientific
Anti-CD103	OX62	Alexa 647	Dendritic cells	Biolegend
Anti-RT1B (MHC-II)	OX-6	PerCP	Antigen-presenting cells	Biolegend
Recombinant NKG2D Fc Chimera Protein			NKG2D ligands	R&D systems
Anti-human IgG	HP6017	PE	Human IgG (Recombinant)	Biolegend

Immunofluorescence, primary antibodies				
Antibody	Clone	Species	Target	Provider
Anti-CD161 (NKRP1)	10/78	Mouse	NK cells	Biolegend
Anti-CD68	ED-1	Mouse	Macrophages	Biorad
Anti-insulin	Polyclonal	Guinea pig	Beta cells	Thermo Fisher Scientific
Anti-beta catenin	Polyclonal	Rabbit	Hepatocytes	C2206 Sigma-Aldrich
Anti-NKG2D	Polyclonal	Rabbit	NG2D receptor	ab203353, Abcam
Anti-GFP	Polyclonal	Rabbit	GFP	ab6556, Abcam

Immunofluorescence, secondary antibodies			
Antibody	Species	Fluorochrome	Provider
Anti-guinea pig IgG (H + L)	Goat	Alexa 488	A11073, Thermo Fisher Scientific
Anti-mouse IgG	Goat	Alexa 555	Poly4053, Biolegend
Anti-rabbit IgG (H + L)	Goat	Alexa633	A21071, Thermo Fisher Scientific
Anti-rabbit IgG (H + L)	Donkey	Alexa555	A31572, Thermo Fisher Scientific
Anti-mouse IgG (H + L)	Donkey	Alexa 647	A31571, Thermo Fisher Scientific

± range. For all *ex vivo* analyses, one dot represents one sample from one animal/experiment. The complete methods can be found in Appendix S1.

Results

Islet mass and gene expression in rat islets

We observed an important decrease in the number of islets (in absolute number and number of IEQ) after 5 days of culture ($P < 0.0001$) (Fig. 1a). In parallel, the overall expression of NKG2D ligands increased with culture time (Day 0–2 $P = 0.04$; Day 0–5 $P = 0.003$; Day 2–5 $P = 0.40$) (Fig. 1b), as did the gene expression of individual ligands RRLT (Day 0–2 $P = 0.63$; Day 0–5 $P = 0.03$; Day 2–5 $P = 0.09$) and RAE1L (Day 0–2 $P = 0.02$; Day 0–5 $P = 0.03$; Day 2–5 $P = 0.78$; Fig. 1c). This was linked with hypoxia, as illustrated by increased expression of Hif1a (Fig. 1d). Finally, the flow cytometry analysis of NKG2D ligand protein expression on the surface of dissociated islet cells confirmed this increase over culture time (Fig. 1e).

Gene expression of NKG2D ligands in human islets

In order to strive for clinical relevance, we also analysed the gene expression of NKG2D ligands in human islets. The overall expression of NKG2D ligands in human islets increased early and further remained stable after 2 days of culture (Day 0–2 $P = 0.004$; Day 0–5 $P = 0.023$; Day 2–5 $P = 0.43$; Fig. 2). When analysed individually, we observed a similar trend in the expression of almost all NKG2D ligands, notably MICA, ULBP1 and ULBP2 (data not shown).

In vitro NKG2D receptor blockade and NK cell cytotoxicity against islets

To assess whether blocking NKG2D signalling might play a role in islet immune destruction, an *in vitro* cytotoxic assay was performed. As previously reported in

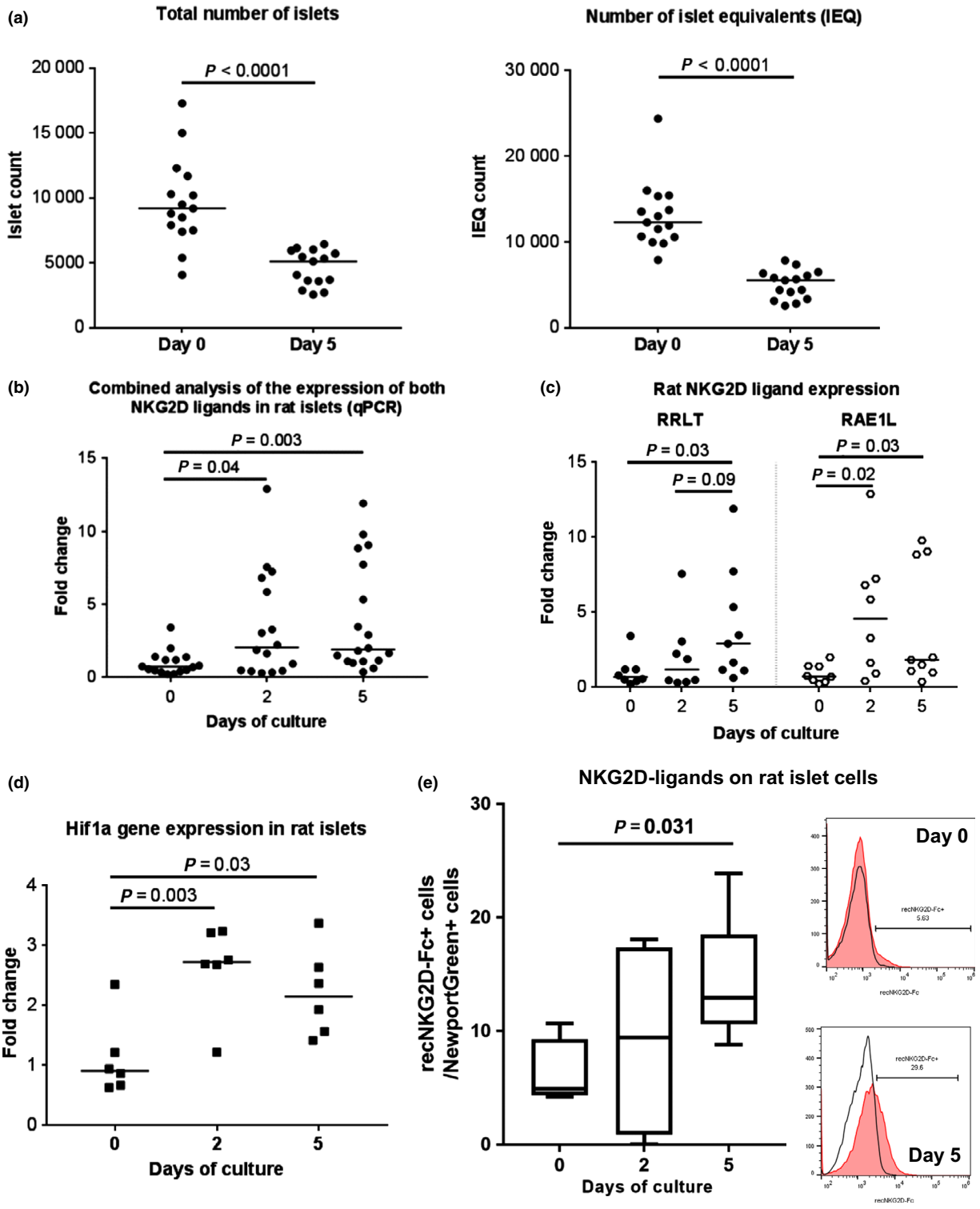

Figure 1 Islet mass and gene expression analyses in rat islets. (a) Left panel: number of islets, Day 0–5 $P < 0.0001$. Right panel: number of islet equivalents (IEQ), Day 0–5 $P < 0.0001$. (b) Combined analysis of the gene expression of both rat NKG2D ligands, RRLT + RAE1L (qPCR). Day 0–2 $P = 0.04$; Day 0–5 $P = 0.003$; Day 2–5 $P = 0.40$. (c) Individual analysis of rat NKG2D-ligand gene expression (qPCR). RRLT: Day 0–2 $P = 0.63$; Day 0–5 $P = 0.03$; Day 2–5 $P = 0.09$. RAE1L: Day 0–2 $P = 0.02$; Day 0–5 $P = 0.03$; Day 2–5 $P = 0.78$. (d) Hif1a gene expression in rat islets (qPCR). Day 0–2 $P = 0.003$; Day 0–5 $P = 0.03$; Day 2–5 $P = 0.39$. (e) NKG2D ligands expression on the membrane of islets cells at days 0, 2 and 5 of culture. Representative flow cytometry histograms: Day 0 = 5.83% cells, Day 5 = 29.6% cells harbouring NKG2D ligands on their surface.

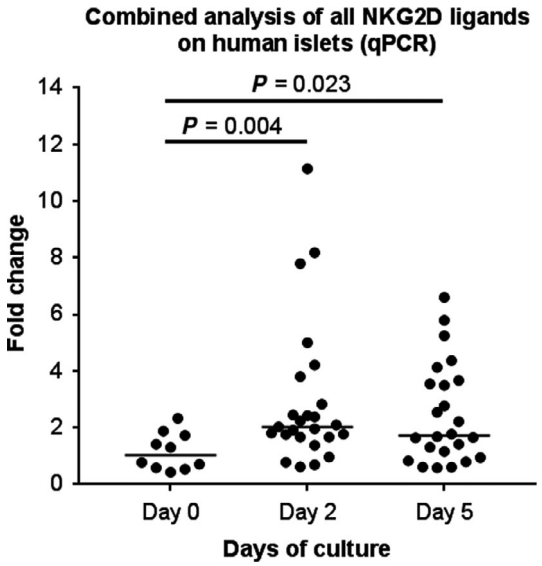

Figure 2 Combined analysis of the gene expression of all NKG2D ligands in human islets (qPCR): Day 0–2 $P = 0.004$; Day 0–5 $P = 0.023$; Day 2–5 $P = 0.43$.

the literature [22,23], the mouse anti-rat NKG2D antibody (clone 11D5F4) had a blocking effect, inducing a decrease in NK-cell-mediated cytotoxicity against the YAC-1 tumour cell line ($P = 0.031$; Fig. 3, left panel). In a syngeneic co-culture of Lewis NK cells with Lewis pancreatic islets (in culture since 5 days), the same anti-NKG2D antibody induced a decrease in NK-cell-mediated cytotoxicity ($P = 0.031$; Fig. 3, right panel).

Islet transplantation and *in vivo* analyses

In order to assess whether islet engraftment and function might be improved by blockade of the NKG2D receptor/NKG2D ligands interaction, islet transplantation was performed in the portal vein of recipients with a control isotype (Mouse IgG1) treatment (control), treatment of the islets with an NKG2D-recombinant protein (recombinant), or with recipient treatment with an anti-NKG2D antibody and treatment of the islets with an NKG2D-recombinant protein (antibody-recombinant). The antibody-recombinant group was designed in order to maximize the blocking of the receptor/ligands interactions.

Diabetes reversal and metabolic effects

When diabetic rats were transplanted with a marginal mass of 5500 IEQ/kg, diabetes was reversed in 66.6% of control recipients and 62.5% of recipients transplanted with islets pre-treated with an NKG2D-recombinant protein (recombinant group). However, only 25% of the recipients transplanted with pre-treated islets and treated *in vivo* with the anti-NKG2D antibody (antibody-recombinant group) reversed diabetes ($P = 0.07$ compared to the control and recombinant groups; Fig. 4a). Furthermore, the overall blood glucose level and weight gain were better in the control group compared to the other two groups (Fig. 4b,c). All three

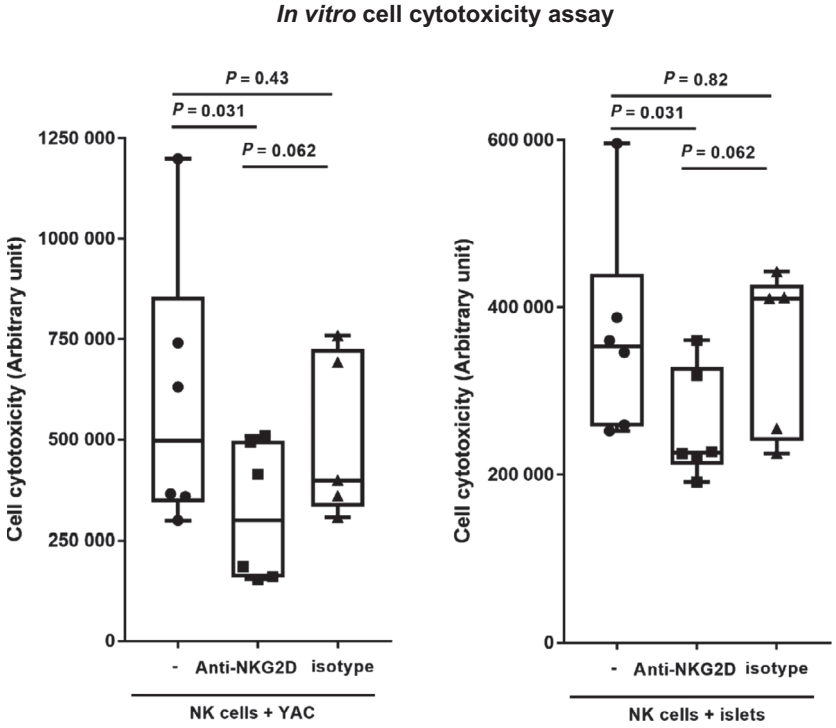

Figure 3 *In vitro* cell cytotoxicity assay ($n = 5$). Left panel: decrease in NK-cell-mediated cytotoxicity against YAC cells after addition of the anti-NKG2D antibody ($P = 0.03$ compared to untreated cells, $P = 0.01$ compared to cells treated with an IgG1 isotype). Right panel: decrease in NK-cell-mediated cytotoxicity against syngeneic islets after addition of the anti-NKG2D antibody ($P = 0.031$ compared to untreated cells).

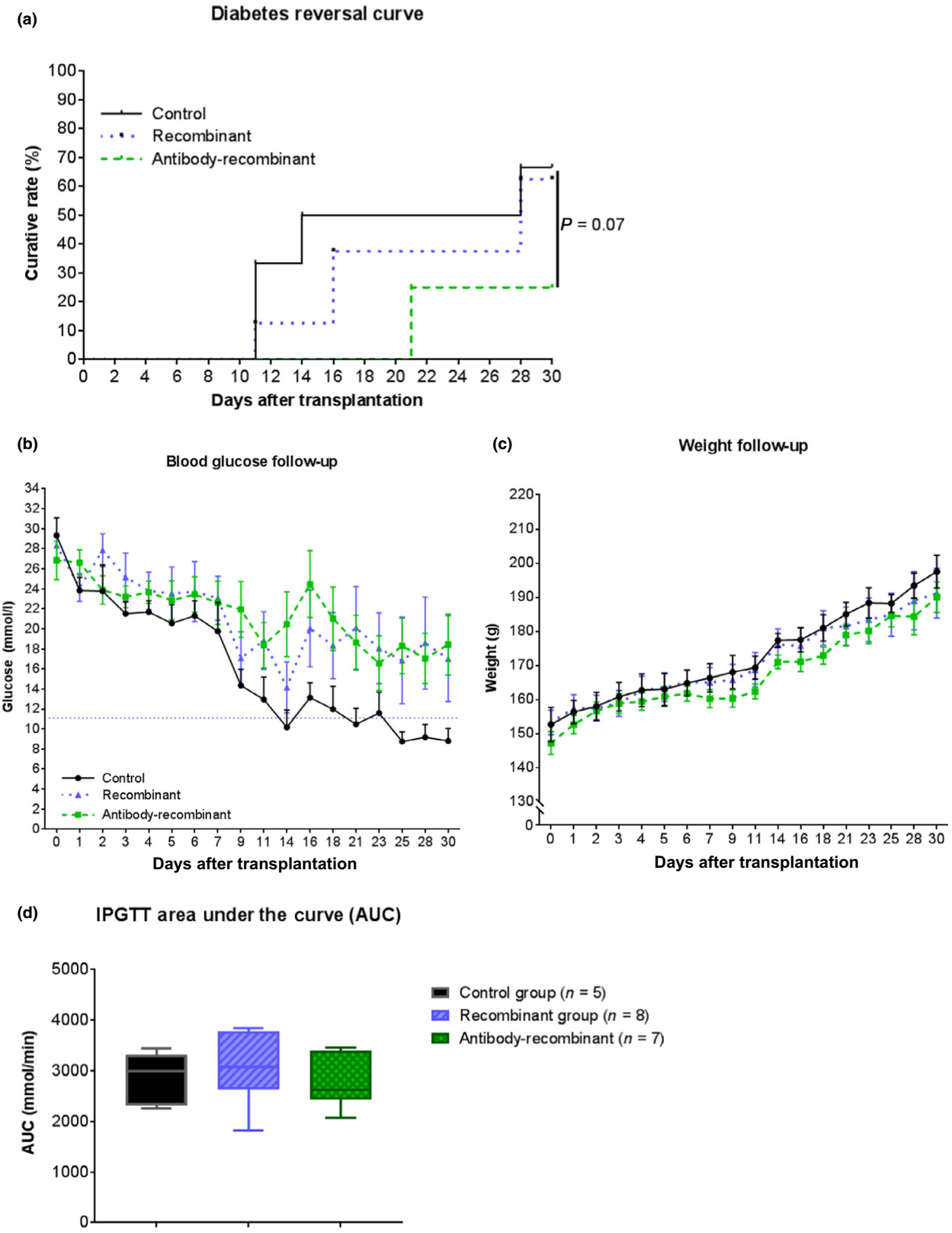

Figure 4 Diabetes reversal, glucose and weight follow-up and metabolic control in rats. (a) Diabetes reversal. Only 25% of the antibody-recombinant group reversed diabetes ($P = 0.07$ compared to the control and recombinant groups. (b) Glucose follow-up in recipients. (c) Weight follow-up in recipients. (d) Area under the curve of the intraperitoneal glucose tolerance test (IPGTT) at day 30 after transplantation. Control vs recombinant $P = 0.39$; control vs antibody-recombinant $P = 0.83$; recombinant vs antibody-recombinant $P = 0.23$

groups showed similar metabolic response during an intraperitoneal glucose tolerance test (IPGTT) 30 days after transplantation (Fig. 4d). One recipient from the control group and one recipient from the antibody-recombinant group were excluded from the metabolic analysis due to the accidental injection of glucose in the caecum.

In vivo effect of the NKG2D antibody

In order to have a more comprehensive analysis of the results following islet transplantation, immune cell phenotypes were assessed in the blood and in the liver.

Circulating immune cells. The analysis of PBMC phenotypes showed an efficient NKG2D blockade by the anti-NKG2D antibody on the NK cells of antibody-recombinant recipients (Fig. 5a), and on CD4+ and CD8+ T cells (not shown). This effect was associated with a decrease in the overall lymphocyte count (not shown), explained by a depletion of NK cells by the antibody, appearing as early as day 1 after islet transplantation (Fig. 5b). This depleting effect was maintained at day 14 after transplantation (between days 1 and 14, $P = 0.57$; between days 7 and 14, $P = 0.17$; *Kruskal–Wallis*). Moreover, we observed an increase in the proportion of circulating NK cells in the control and recombinant groups between day 1 and day 7 ($P = 0.02$ and $P = 0.007$, respectively, *Kruskal–Wallis*) and between day 1 and day 14 in the recombinant group ($P = 0.002$, *Kruskal–Wallis*).

We did not observe any depletion of the T-cell subsets in the anti-NKG2D antibody-treated recipients.

Resident liver immune cells. Immuno-histological analyses of recipient intra-hepatic islets confirmed efficient NKG2D blockade on the immune cells surrounding the islets after *in vivo* anti-NKG2D treatment as early as day 1 after transplantation (Fig. S1). As the NK cell depletion in the blood reached its peak at day 7 after transplantation, we performed flow cytometry analysis of resident liver immune cells at this time-point. A sustained blockade of NKG2D on the CD8+ T cells (not shown), NK cells and macrophages was found in the antibody-recombinant recipients (Fig. 6a). Furthermore, quantitative analysis of resident liver immune cells at day 7 showed a sustained decrease in NK cell presence (normalized as a NK cell to T-cell ratio) and an increase in macrophage infiltration [normalized as a macrophage to liver sinusoidal endothelial cell (LSEC) ratio] in the antibody-recombinant group compared to the control group (Fig. 6b). Immuno-fluorescent analyses of liver samples found that, in a syngeneic context, the most represented immune cells around the islets are NK cells and macrophages, further illustrating the need to modulate the potential negative impact of these immune cells on the graft. As with the flow cytometry analysis of resident liver immune cells, we observed increased macrophage infiltration surrounding islets in the antibody-recombinant group (Fig. 6c). There was minimal CD8+ T-cell infiltration around the transplanted islets (data not shown).

As it has been suggested that recipient NK cells may play a protective role by destroying the donor immune cells present in the organs at time of transplantation [24,25], we assessed the immune cells present in islets. The macrophages surrounding the transplanted islets are of recipient origin: *in vitro* Lewis islet analysis found that, after 5 days of culture, there was a very little proportion of intrinsic macrophages left (median 0.25%, $n = 3$; Fig. 7a). This was further confirmed by transplanting islets isolated from GFP+ Lewis rats into non-GFP rats; 24 h after transplantation, the macrophages surrounding the islets are not GFP+-stained and therefore of recipient origin (Fig. 7b). In our syngeneic model, these data suggest that the deleterious effect observed is not due to the absence of NK cells and their role in the destruction of donor antigen-presenting cells.

Observed effects of the NKG2D antibody on islet recipients

The observed detrimental effect of the anti-NKG2D antibody was not linked to a hepatotoxic side effect associated with an acute NK cell lysis, as alanine transaminase (ALT) levels remained similar between groups (Fig. S2). However, the administration of the anti-NKG2D antibody seemed to trigger an immuno-allergic reaction, as testified by skin hyperaemia appearing as early as 3 days after the start of treatment in all antibody-recombinant recipients (Fig. S3). Skin histological analysis at day 4 after anti-NKG2D antibody treatment found epidermal hyperplasia, keratinocyte necrosis and increased infiltration of neutrophils and eosinophils in the dermis of all treated animals (Fig. 8a). Serum cytokine analysis found an increase in IP-10, which is secreted in response to Interferon-γ, and an increase in cytokines associated with activated macrophages (CXCL-1, IL-1ß and MIP-1) in the anti-NKG2D antibody-treated group (Fig. 8b). This cytokine burst might be the cause of the increased macrophage infiltration in the liver of anti-NKG2D antibody-treated animals.

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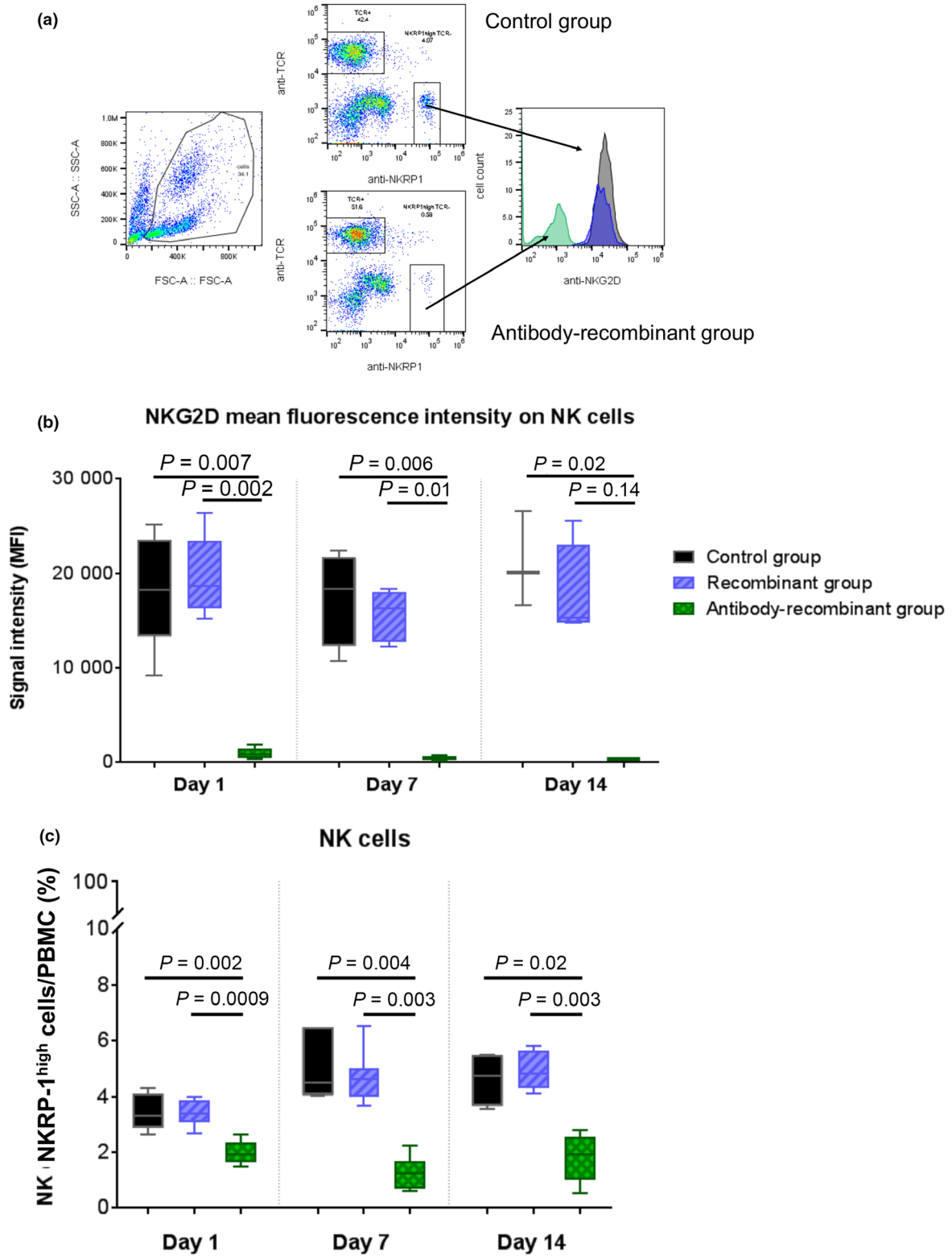

Figure 5 Peripheral blood mononuclear cells phenotype at days 1, 7 and 14 after transplantation. (a) Gating strategy. (b) Saturation of the NKG2D receptor on NK cells by the anti-NKG2D antibody. (c) Percentage of NK cells to total PBMC. Day 1 – Control n = 6, Recombinant n = 8, Antibody-recombinant n = 8; Day 7 – Control n = 5, Recombinant n = 7, Antibody-recombinant n = 6; Day 14 – Control n = 4, Recombinant n = 5, Antibody-recombinant n = 6.

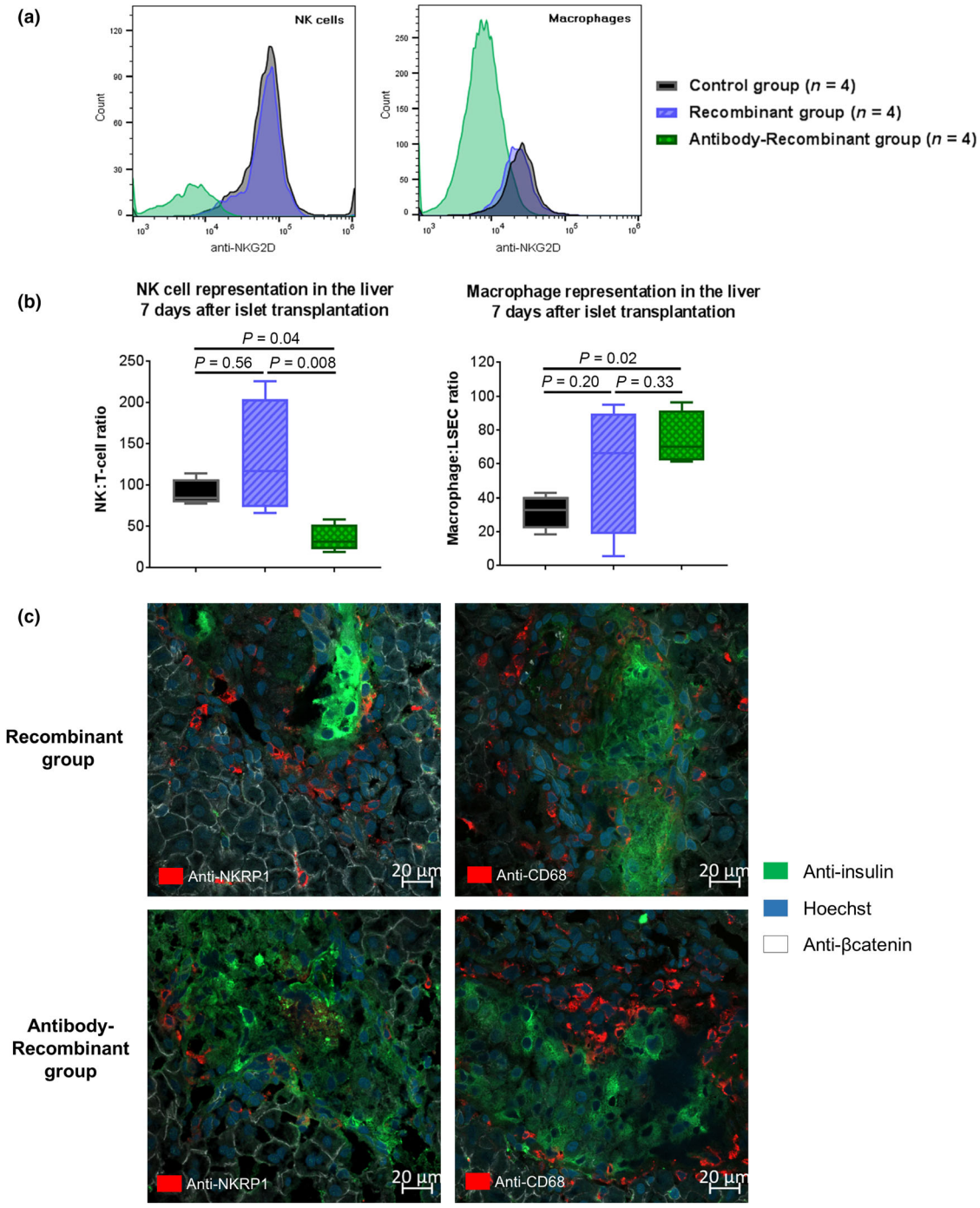

Figure 6 Flow cytometry analyses of resident liver immune cells at day 7. (a) NKG2D expression on liver NK cells (Left panel: control MFI = 57 101.5; recombinant MFI = 52 881; antibody-recombinant MFI = 3241) and macrophages (Right panel: control MFI = 13 625.5; recombinant MFI = 8316; antibody-recombinant MFI = 576; n = 2). *Black: control recipients; Purple: recombinant recipients; Green: antibody-recombinant recipients.* (b) NK cell and macrophage representation in the liver. Left panel: NK cell depletion in the liver in antibody-treated recipients (P = 0.04 compared to controls, and P = 0.008 compared to the recombinant group). Right panel: Increased macrophage infiltration in the liver in antibody-treated recipients compared to controls (P = 0.02). (c) Analysis of macrophage and NK cell presence around the transplanted islets in the recombinant only and the antibody-recombinant groups. Decreased number of NK cells (left panel) and increased macrophage infiltrate (right panel) around the islets of antibody-recombinant recipients at day 1.

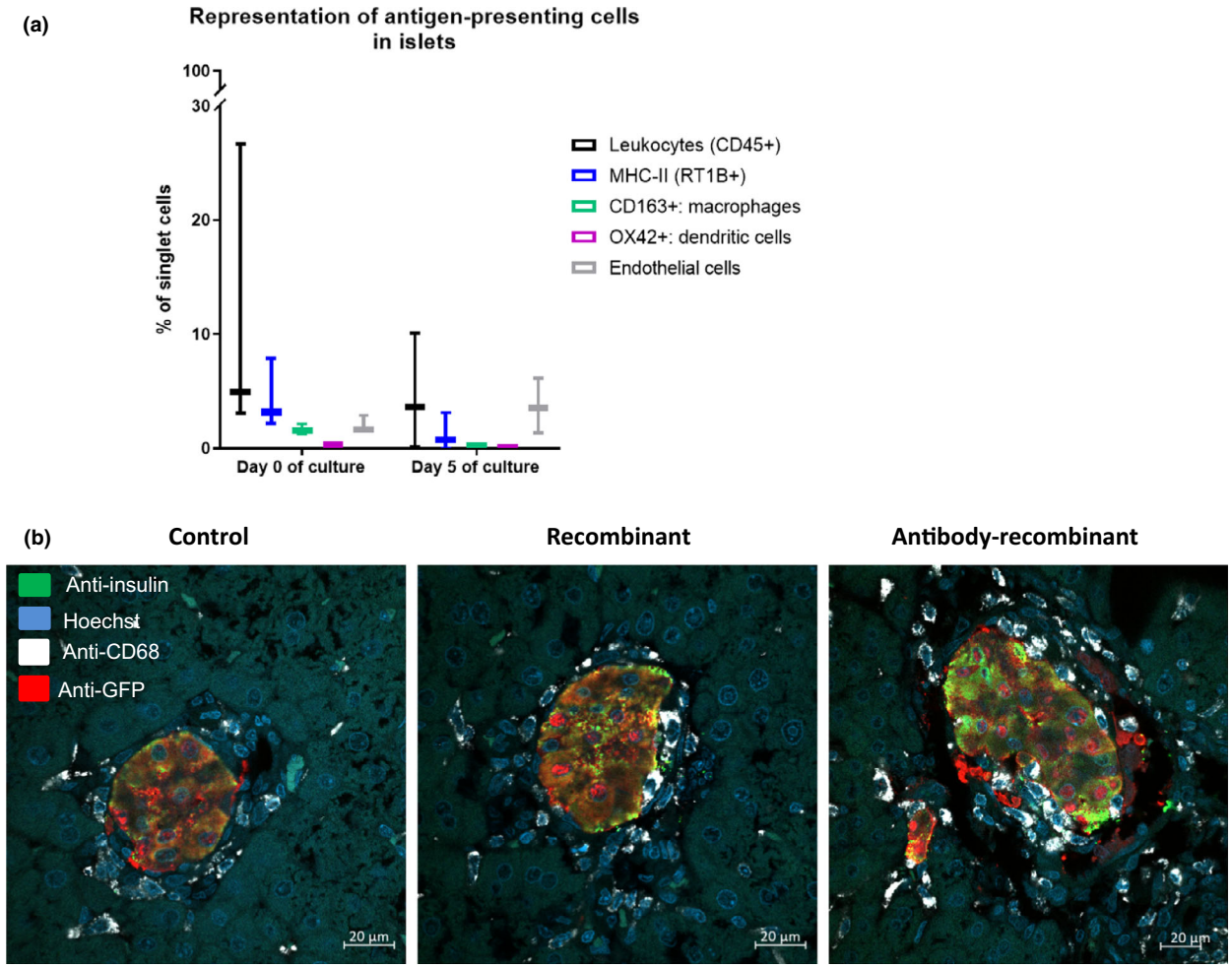

Figure 7 Immune cells in islets. (a) Representation of resident immune cells in rat islets (n = 3) with culture time. Overall decrease in all myeloid cells after 5 days of culture, notably macrophages, with a relative increase in endothelial cell representation. (b) Analysis of infiltrating macrophage origin in non-GFP recipients one day after transplantation with GFP+ islets.

Discussion

Human and rat islet expression of NKG2D ligands increases during culture in the presence of hypoxia-mediated cellular stress.

Reports on solid organ transplantation have demonstrated that kidney, heart and pancreas graft rejection are linked with increased NKG2D ligand expression in humans and in mice. In the present experiment, blockade of the NKG2D ligands on islets whilst in culture did not change outcome after transplantation in the recombinant group compared to the control group. This absence of effect may be related to a continued expression of the NKG2D ligands by the islets, as they suffer from major hypoxia until at least day 7–10 after transplantation. This would therefore annihilate any beneficial effect of *in vitro* NKG2D blockade prior to transplantation. Another explanation would be the recognition of the human Fc part on the NKG2D recombinant by rat Fcγ receptors leading to phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and harmful events on islets. The best hope for lasting blockade of NKG2D ligands on transplanted islets would therefore logically be *in vivo* administration of NKG2D recombinant receptor without the Fc fragment. This is not, however, a viable long-term option for clinical practice. Indeed, organisms rely heavily on the recognition of NKG2D ligands by NKG2D in order to suppress tumour growth by eliminating cells with damaged DNA [26,27]. A major factor to cancer development is the evasion of recognition by NK cells by shedding the NKG2D ligands from the cell surface [28]. We can therefore imagine that prolonged *in vivo* blockade of NKG2D ligands would lead to unchecked tumour growth.

Increased mRNA levels of NKG2D and numbers of NKG2D-expressing cells were shown to be implicated in acute and chronic kidney graft rejection [29]. However, blocking NKG2D has had variable and conflicting

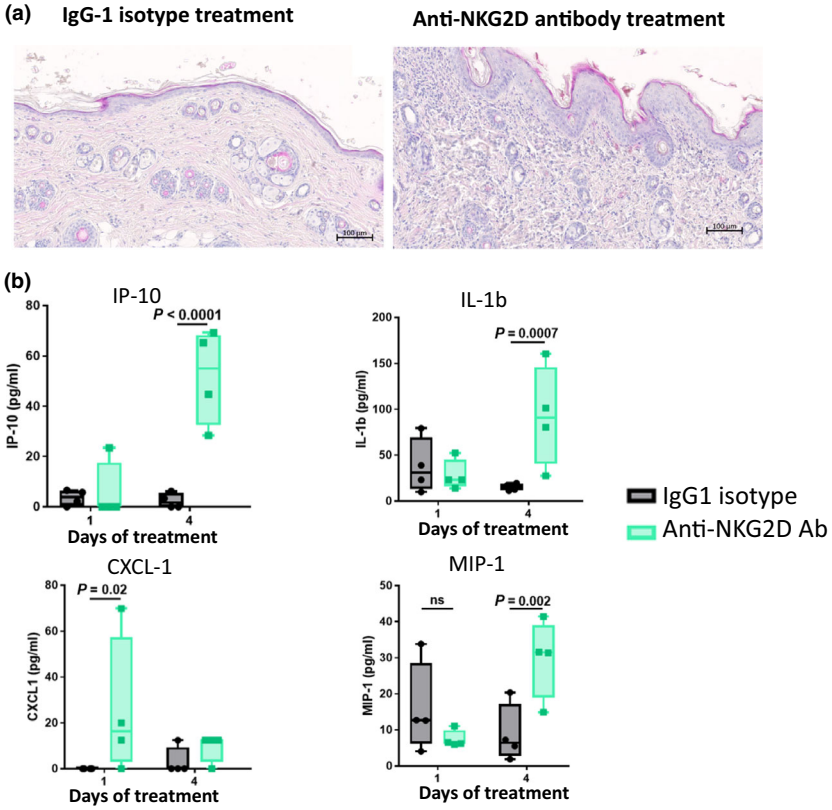

Figure 8 Effects of the antibody on recipients. (a) Skin biopsy histology at day 4 of treatment in IgG1 isotype- and anti-NKG2D antibody-treated animals. Right panel: Anti-NKG2D antibody-treated animal. Epidermal hyperplasia, keratinocyte necrosis and increased infiltration of neutrophils and eosinophils in the dermis. (b) Selected cytokines with significant differences from multiplex cytokine analysis in IgG1 isotype- or anti-NKG2D antibody-treated animals at day 4. IP-10: secreted in response to Interferon-γ, increase in the anti-NKG2D group at day 4 ($P < 0.0001$). Cytokines associated with macrophage activation: CXCL-1 – increased in the anti-NKG2D group at day 1 ($P = 0.02$); IL-1ß – increased in the anti-NKG2D group at day 4 ($P = 0.0007$); MIP-1 – increased in the anti-NKG2D group at day 4 ($P = 0.002$).

outcomes in solid organ transplantation. Past reports have observed NKG2D blockade to prolong heart [12,30] and skin [31] allograft survival, whereas a more recent report has shown that it accelerated rejection of cardiac allografts through increased NK cell infiltration [24]. A single report on allogeneic islet transplantation showed no improvement of graft survival with *in vivo* NKG2D blockade alone, but showed a synergistic effect of NKG2D blockade along with cytotoxic T-lymphocyte associated protein 4 (CTLA-4) blockade [19]. However, this experiment was performed under the kidney capsule, where IBMIR does not occur, and the microenvironment differs vastly from that of the liver.

In this syngeneic experiment, the intraperitoneal administration of the only existing mouse anti-rat NKG2D antibody (clone 11D5F4) effectively blocked the NKG2D receptor on the surface of NK cells (as previously demonstrated in the literature and through the cytotoxicity assay against YAC cells), but also had the major side effect of long-term depletion of circulating NK cells *in vivo*. This is the first time that the *in vivo* effect of this clone is reported in rats. Where we expected this to have a positive effect on transplant outcome, it had a surprisingly deleterious effect on intraportally transplanted islets, with minimal diabetes reversal in this group, increased macrophage infiltration in the liver and around the transplanted islets and increased levels of circulating cytokines associated with macrophage activation. These observations are consistent with a report concluding that NK cells are required for islet graft tolerance [32], notably through perforin-dependent killing of donor antigen-presenting cells. However, we demonstrated that at time of transplantation, almost no donor-originating antigen-presenting cells (MHC-II+ cells, macrophages and dendritic cells) were present in the islets. Hence, the deleterious effect observed after depletion of NK cells cannot, in our model, be explained by the protective role thought to be conferred by the destruction of donor cells. Another report observed that depletion of NK cells increased cardiac allograft rejection and increased, along with NKG2D depletion, macrophage infiltration in the graft [33]. It has previously been reported that NK cells modulate immune cell response, where NK cell depletion increases T-cell response to infection and allogeneic transplantation [34–37]; moreover, a recent report has suggested that NK cells are essential in inducing B-cell-dependent tolerance to islet grafts [38]. The disadvantage in our model is the NK cell depletion induced by NKG2D blockade; we can therefore not conclude as to whether our observed results are due to NKG2D blockade or to the absence of NK cells. However, based on

the above-mentioned reports, we can speculate that NK cell depletion is most probably mediated by macrophage activation, according to an antibody-dependent cell-mediated cytotoxicity with an unbridled inflammatory response by macrophages, *o*r that the repeated injection of a mouse-originated antibody induces the production of secondary anti-mouse antibodies. This last hypothesis, however, seems not to be implicated in the adverse reaction observed in antibody-treated recipients, as the control recipients receiving mouse IgG1 did not demonstrate any such reaction and the duration of antibody administration is too short to induce anti-mouse-directed antibodies. Intraportal islet transplantation in a rat, and not a mouse, model better approximates the intricate responses observed in humans. However, it has the disadvantage to limit the number of investigations as the tools are less developed in rats models. In our model, blockade of the NKG2D receptor and/or NK cell depletion led to an increase in circulating pro-inflammatory cytokines. This cytokine burst might be the cause of the increased macrophage infiltration in the liver of anti-NKG2D antibody-treated animals, thus reflecting the crucial role of the liver microenvironment in the context of islet transplantation.

Overall, *in vitro* blockade of the NKG2D ligands prior to transplantation did not have an effect on islet graft function. However, with our antibody administration scheme, NKG2D blockade in recipients was deleterious to the intraportally transplanted islets, most probably through macrophage activation in a type II hypersensitivity reaction. The next step of investigations would be the use of anti-NKG2D Fab'2 or chimeric anti-NKG2D antibodies in order to avoid the detrimental side effects. Indeed, we have assessed that the blocking of NKG2D with Fab'2 was enough to inhibit the in vitro cytotoxicity and to block the NKG2D receptor in vivo without NK cell depletion (Fig. S4). Further studies will be needed in order to finally demonstrate the impact of the NKG2D blockade in islet transplantation.

Authorship

VD: designed and performed the experiments, analysed the data and wrote the paper. SL designed the experiments, analysed the data and wrote the paper, crucial scientific input. QG, FS and AK-Q: collected the data, performed sample analysis. CT: crucial scientific input and editing of the paper. GK: crucial scientific input. VL, LAO and AP: crucial revision and editing of the paper.

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Conflicts of interest

The authors have declared no conflicts of interest.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Analysis of NKG2D expression around transplanted islets.

Figure S2. Analysis of alanine aminotransferase (ALT) in all three groups at days 1, 7 and 14 after transplantation.

Figure S3. Macroscopic skin differences in IgG1-treated and NKG2D antibody-treated animals.

Figure S4. Production and use of anti-NKG2D Fab'2 fragment in NKG2D blockade.

Appendix S1. Monoclonal antibody production, cytotoxicity assays, immunofluorescence, liver enzymes and cytokine multiplex.

REFERENCES

1. Delaune V, Berney T, Lacotte S, Toso C. Intraportal islet transplantation: the impact of the liver microenvironment. *Transpl Int* 2017; **30**: 227.
2. Bennet W, Groth C-G, Larsson R, Nilsson B, Korsgren O. Isolated human

islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Ups J Med Sci* 2000; **1**: 125.
3. Sever CE, Demetris AJ, Zeng Y, *et al*. Islet cell allotransplantation in diabetic patients. Histologic findings in four adults simultaneously receiving kidney or liver transplants. *Am J Pathol* 1992; **140**: 1255.
4. Johansson H, Lukinius A, Moberg L, *et al*. Tissue factor produced by the endocrine cells of the islets of langerhans is associated with a negative outcome of clinical islet transplantation. *Diabetes* 2005; **1**: 1755.
5. Lamblin A, Tournoys A, Gmyr V, *et al*. Activation de la coagulation par la greffe intraportale d'ilots de Langerhans chez le porc*. *Ann Chir* 2001; **126**: 743.
6. Ishiyama K, Rawson J, Omori K, Mullen Y. Liver natural killer cells play a role in the destruction of islets after intraportal transplantation. *Transplantation* 2011; **15**: 952.
7. Saeki Y, Ishiyama K, Ishida N, Tanaka Y, Ohdan H. Role of natural killer cells in the innate immune system after intraportal islet transplantation in mice. *Transplant Proc* 2017; **49**: 139.
8. Saeki Y, Ishiyama K, Ishida N, Tanaka Y, Ohdan H. Memory-like liver natural killer cells are responsible for islet destruction in secondary islet transplantation. *Sci Rep* 2019; **31**: 1022.
9. Spear P, Wu M-R, Sentman M-L, Sentman CL. NKG2D ligands as therapeutic targets. *Cancer Immun Arch* 2013; **13**: 8.
10. Ogasawara K, Hamerman JA, Ehrlich LR, *et al*. NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity* 2004; **20**: 757.
11. Markiewicz MA, Wise EL, Buchwald ZS, *et al*. RAE1ε ligand expressed on pancreatic islets recruits NKG2D receptor-expressing cytotoxic T cells independent of T cell receptor recognition. *Immunity* 2012; **27**: 132.
12. Kim J, Chang CK, Hayden T, *et al*. The activating immunoreceptor NKG2D and its ligands are involved in allograft transplant rejection. *J Immunol* 2007; **15**: 6416.
13. Giuliani M, Moritz W, Bodmer E, *et al*. Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. *Cell Transplant* 2005; **14**: 67.
14. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K. Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* 1989; **38** (Suppl. 1): 199.
15. Jansson L, Carlsson P-O. Graft vascular function after transplantation of pancreatic islets. *Diabetologia* 2002; **45**: 749.
16. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. *Annu Rev Immunol* 2013; **31**: 413.
17. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP. An improved method for isolation of mouse pancreatic islets. *Transplantation* 1985; **40**: 437.
18. van der Burg MPM, Graham JM. Iodixanol density gradient preparation in university of wisconsin solution for porcine islet purification. *ScientificWorldJournal* 2003; **1**: 1154.
19. Pawlick R, Gala-Lopez B, Pepper AR, McCall M, Ziff O, Shapiro AMJ. The combination of anti-NKG2D and CTLA-4 Ig therapy prolongs islet allograft survival in a murine model. *Am J Transplant* 2014; **14**: 2367.
20. Delaune V, Lacotte S, Gex Q, *et al*. Effects of remote ischaemic preconditioning on intraportal islet transplantation in a rat model. *Transpl Int* 2019; **32**: 323.
21. Lacotte S, Slits F, Orci LA, *et al*. Impact of myeloid-derived suppressor cell on Kupffer cells from mouse livers with hepatocellular carcinoma. *Oncoimmunology* 2016; **5**: e1234565.
22. Lacotte S, Oldani G, Slits F, *et al*. Alloimmune activation promotes anti-cancer cytotoxicity after rat liver transplantation. *PLoS ONE* 2014; **9**: e91515.
23. Zhuo M, Fujiki M, Wang M, *et al*. Identification of the rat NKG2D ligands, RAE1L and RRLT, and their role in allograft rejection. *Eur J Immunol* 2010; **40**: 1748.
24. Fabritius C, Ritschl PV, Resch T, *et al*. Deletion of the activating NK cell receptor NKG2D accelerates rejection of cardiac allografts. *Am J Transplant* 2017; **17**: 3199.
25. Harper IG, Ali JM, Harper SJF, *et al*. Augmentation of recipient adaptive alloimmunity by donor passenger lymphocytes within the transplant. *Cell Rep* 2016; **10**: 1214.
26. Lanier LL. NKG2D receptor and its ligands in host defense. *Cancer Immunol Res* 2015; **3**: 575.
27. Sagiv A, Burton DGA, Moshayev Z, *et al*. NKG2D ligands mediate immunosurveillance of senescent cells. *Aging* 2016; **13**: 328.
28. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* 2002; **419**: 734.
29. Seiler M, Brabcova I, Viklicky O, *et al*. Heightened expression of the cytotoxicity receptor NKG2D correlates with acute and chronic nephropathy after kidney transplantation. *Am J Transplant* 2007; **7**: 423.
30. Chen H, Xia J, Zhang L, *et al*. NKG2D blockade attenuated cardiac allograft vasculopathy in a mouse model of cardiac transplantation. *Clin Exp Immunol* 2013; **173**: 544.
31. Ito A, Shimura H, Nitahara A, *et al*. NK cells contribute to the skin graft rejection promoted by CD4+ T cells activated through the indirect allorecognition pathway. *Int Immunol* 2008; **20**: 1343.
32. Beilke JN, Kuhl NR, Van Kaer L, Gill RG. NK cells promote islet allograft tolerance via a perforin-dependent mechanism. *Nat Med* 2005; **11**: 1059.
33. Van der Touw W, Burrell B, Lal G, Bromberg JS. NK cells are required for costimulatory blockade induced tolerance to vascularized allografts. *Transplantation* 2012; **94**: 575.
34. Yu G, Xu X, Vu MD, Kilpatrick ED, Li XC. NK cells promote transplant tolerance by killing donor antigen-presenting cells. *J Exp Med* 2006; **7**: 1851.
35. Laffont S, Seillet C, Ortaldo J, Coudert JD, Guéry J-C. Natural killer cells recruited into lymph nodes inhibit alloreactive T-cell activation through perforin-mediated killing of donor allogeneic dendritic cells. *Blood* 2008; **1**: 661.
36. Zecher D, Li Q, Oberbarnscheidt MH, *et al*. NK cells delay allograft rejection in lymphopenic hosts by downregulating the homeostatic proliferation of CD8+ T cells. *J Immunol Baltim Md 1950* 2010; **15**: 6649.
37. Lang PA, Lang KS, Xu HC, *et al*. Natural killer cell activation enhances immune pathology and promotes chronic infection by limiting CD8+ T-cell immunity. *Proc Natl Acad Sci USA* 2012; **24**: 1210.
38. Schuetz C, Lee KM, Scott R, *et al*. Regulatory B cell-dependent islet transplant tolerance is also natural killer cell dependent. *Am J Transplant* 2017; **17**: 1656.