NK cells do not mediate renal injury in murine adriamycin nephropathy

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In adriamycin nephropathy (AN), a model of chronic proteinuric renal injury, the absence of functional B and T cells with residual natural killer (NK) cells, and macrophages in severe combined immunodeficient (SCID) mice results in more severe disease than in immunocompetent mice. We have recently shown expression of the stimulatory NK cell molecule NKG2D and its ligand RAE-1 in the adriamycin (ADR) kidney. Therefore, we sought to determine the role of NK cells in AN. We used anti-asialo GM1 NK cell depletion in immunocompetent BALB/c mice with AN, and also compared AN in immunodeficient SCID mice and immunodeficient nonobese diabetic (NOD)-SCID mice (that have impaired NK cell function). The number of NK cells was increased in AN in BALB/c mice compared with normal controls. NK cell depletion or reduction of NK function in NOD-SCID mice did not affect the severity of disease. In both wild type and immunodeficient models, ADR upregulated RAE-1 in the kidney. High levels of Class I major histocompatibility complex molecules were found in both models of AN. In conclusion, NK cells do not play a significant role in AN.

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KEYWORDS: natural killer; MHC Class I molecule; NKG2D; RAE-1; adriamycin nephropathy; murine

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Adriamycin (ADR)-induced nephropathy is a robust model of human focal segmental glomerulosclerosis.[1,2] The severity of renal damage and dysfunction in human renal disease and animal models correlates with the degree of interstitial infiltration by inflammatory cells, predominantly T lymphocytes and macrophages.[3–5] Previous studies in our laboratory showed that depletion of CD8+ T cells protects while depletion of CD4+ T cells aggravates adriamycin nephropathy (AN) in BALB/c mice.[6,7] However, the role of other effector cells such as natural killer (NK) cells is unknown. The greater sensitivity of the severe combined immunodeficient (SCID) mouse to ADR, where NK cells are the predominant immune effector cell besides macrophages, suggests either that the cognate immune cells provide protection from injury as suggested by our CD4 depletion data or that the NK population together with macrophages in these mice exacerbates the renal injury. In glomerulonephritis, a possible involvement of NK cells was suggested by finding that local production of fractalkine and the presence of CD16-positive cells, which include NK cells, in the interstitial lesions of human crescentic glomerulonephritis.[8] However, evidence for cytotoxic injury by NK cell was not found in rat antiglomerular membrane glomerulonephritis[9] and cell-mediated glomerulonephritis.[10]

NK cells, a pivotal component of the innate immune system, are derived from haematopoietic stem cells, which have been identified in sites such as bone marrow, thymus, spleen, omentum, and liver in adults.[11] Under normal conditions, NK cells are mostly confined to peripheral blood, spleen, and bone marrow, but they can migrate to inflamed tissues in response to different chemoattractants.[12] NK cells recognize other cells which lack major histocompatibility complex (MHC) Class I expression via 'the missing self hypothesis' using a variety of inhibitory receptors that bind to Class I molecules; this leads to NK responses to both tumors and virally infected cells that downregulate their Class I expression.[13] NK cells release both inflammatory cytokines and cytotoxic molecules including perforin, granzyme, and tumor necrosis factor-related apoptosis-inducing ligand causing rapid injury to tissues not recognized as self.[14] More recently, molecules that function as activating receptors have

been described on NK cells. Specifically, NKG2D binds to a co-receptor DAP12 on the NK cell and is activated by a range of Class Ib MHC molecules including RAE-1 and H-60 in mice and MICA in humans that are upregulated by stress or danger. It is unclear whether the activating signal received through these interactions can override the inhibitory signals generated by inhibitory receptors interacting with Class I MHC molecules. The crucial importance of this pathway is demonstrated by its preservation across species and the use of molecular homologues of ligands by viruses to avoid killing.

In order to test the role of NK cells we have used two approaches. We first performed NK cell depletion in the standard model of AN using an antibody to glycolipid asialo GM1 or ganglio-n-tetraosylceramide.[15] This has been shown to be effective for NK depletion in tumor and transplantation animal models.[16–18] Secondly, we examined NK cell effects in isolation by comparing AN in the SCID and nonobese diabetic (NOD)-SCID mouse. The severity of AN was assessed in both the antibody depletion model and in immunodeficient NOD-SCID mice, where the NK cells are present but functionally inactive. We also measured expression of NK cell activating ligand RAE-1 and inhibitory ligand H-2k^d and the presence of the activating receptor NKG2D.

RESULTS

NK cell depletion

Repeated injections of anti-asialo GM1 serum significantly depleted NK cells in kidney as well as spleen in BALB/c mice with AN (Figures 1–3). Renal NK cells appeared to be more numerous in BALB/c mice with AN than controls, but the difference was not significant when assessed by fluorescence-activated cell scan (FACScan) (0.52 ± 0.18 versus $0.30 \pm 0.14\%$, $P = 0.11$). However, the number of NK cells in kidney, estimated by multiplying total number of interstitial CD45+ cells isolated by the percentage of NK cells within the CD45+ population, was significantly higher in AN BALB/c mice than in normal control ($13\,534 \pm 2984$ versus 3460 ± 1937, $P < 0.05$) (Figure 1e). Anti-asialo GM1 serum significantly depleted NK cells from kidney of mice with AN (0.21 ± 0.08 versus $0.52 \pm 0.18\%$ by FACScan, $P < 0.01$, $13\,534 \pm 2984$ versus 3819 ± 1081 in total number of NK cells per kidney, $P < 0.05$) (Figure 1). Anti-asialo GM1 serum also depleted NK cells in spleen of mice with AN (4.28 ± 0.67 versus $10.93 \pm 1.09\%$ by FACScan, $P < 0.01$) (Figures 2 and 3).

Renal functional injury

As expected, mice with AN had significantly worse renal function at 5 weeks than control, as assessed by urine protein (3.88 ± 0.72 versus 1.05 ± 0.27 mg/16 h, $P < 0.05$) and creatinine clearance (21.7 ± 9.1 versus 42.2 ± 15.9 μl/min, $P < 0.05$). Anti-asialo GM1 depletion of NK cells had no effect on any renal function parameter in mice with AN (Table 1).

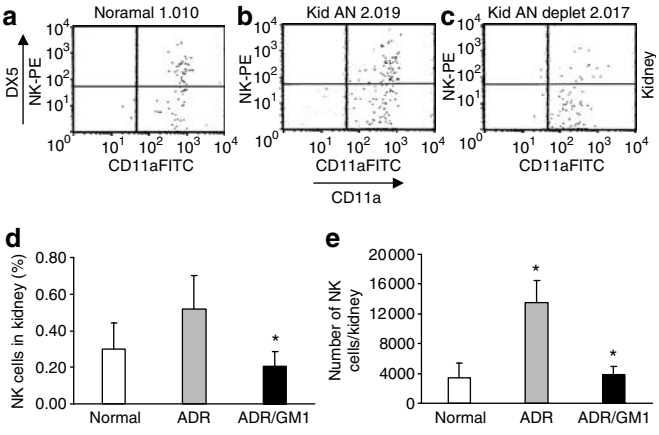

Figure 1 | NK cell depletion assessed by flow cytometry of kidney infiltrating NK cells. (**a–c**) Representative histograms of kidney infiltrating NK cells. (**d**) Percentage of NK cells in CD45+ gated population. (**a**) Normal BALB/c mice ($n = 4$), (**b**) AN mice ($n = 5$), and (**c**) AN mice with NK cells depleted by GM1 ($n = 5$). (**e**) Total number of NK cells per kidney estimated by multiplying total number of interstitial CD45+ cells isolated per kidney by the percentage of NK cells within the CD45+ population. *$P < 0.05$ versus ADR (analysis of variance).

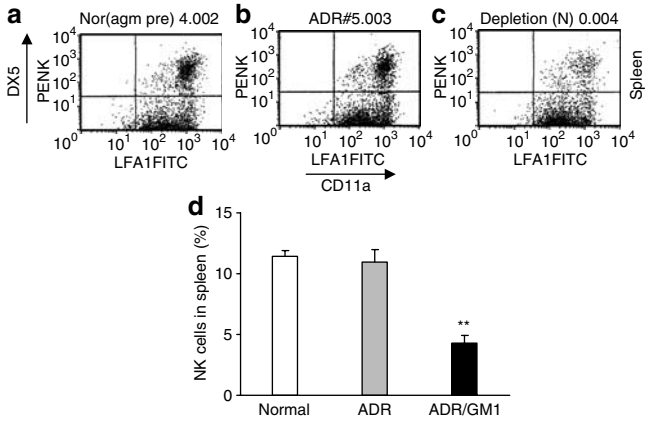

Figure 2 | NK cell depletion assessed by flow cytometry of splenic NK cells. (**a–c**) Representative histograms of splenic NK cells. (**d**) Percentage of NK cells in CD45+ gated population. (**a**) Normal BALB/c mice ($n = 4$), (**b**) AN mice ($n = 5$), and (**c**) AN mice with NK cells depleted by GM1 ($n = 5$). **$P < 0.01$ versus normal and ADR (analysis of variance).

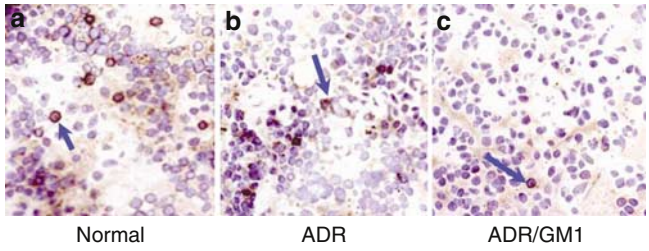

Figure 3 | (a–c) NK cell depletion assessed by immunohistochemical staining for NK cells in splenocytes. The arrows indicate NK cells located in the area of splenic pulp, where the membrane surfaces of NK cells are stained brown with GM1 antibody counterstained with hematoxylin. Original magnification $\times 400$.

Table 1 | Body weight and renal function of normal BALB/c mice and BALB/c mice with AN treated or not with GM1 at 5 weeks

	Normal (n=6)	ADR (n=7)	ADR/GM1 (n=7)
Body wt (g)	24.0±3.4	20.1±2.6	21.1±3.6
Serum creatinine (μmol/l)	22.0±9.9	31.6±7.4	28.7±5.4
Serum albumin (g/l)	25.2±1.8	22.4±3.8	24.8±2.1
Urine protein (mg/16 h)	1.05±0.27	3.88±0.72*	4.86±0.63*
Urine creatinine (mmol/16 h)	2.21±0.80	0.60±0.19**	0.76±0.16**
Creatinine clearance (μl/min)	42.2±15.9	21.7±9.1*	15.2±4.5*

*$P<0.05$, **$P<0.01$ versus normal control.
ADR, adriamycin.

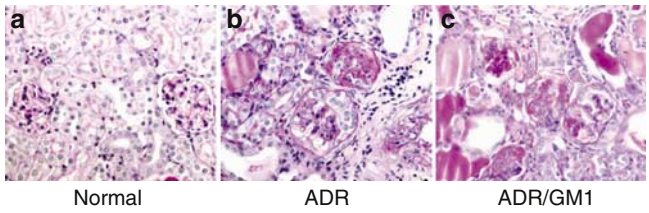

Figure 4 | Histology of kidney sections of normal and ADR BALB/c mice treated or not with GM1. (**a**) Kidney of normal BALB/c mice. (**b**) Kidney of BALB/c mice with AN. (**c**) Kidney of AN mice treated with GM1. The results of morphometric analysis of the histological changes are summarized in Table 2. Original magnification ×400.

Renal structural injury

The renal structural injury induced by ADR was consistent with our previous studies.[2] In particular, there was a reduction in tubular cell height, and an increase in tubular luminal diameter, glomerulosclerosis, and interstitial volume in comparison to control (Figure 4, Table 2). However, there was no effect of NK depletion with anti-asialo GM1 on any renal pathological parameter in mice with AN (Table 2).

Table 2 | Morphometric analysis of BALB/c mice treated with PBS (control), ADR or ADR and GM-1

	Normal (n=6)	ADR (n=7)	ADR/GM1 (n=7)
Tubular diameter (μm)	28.3±0.4	30.9±0.3*	31.3±2.3*
Tubular cell height (μm)	10.4±0.2	8.9±0.5*	9.1±0.4*
Glomerulosclerosis (%)	0	14.8±5.2***	11.3±2.2***
Interstitial volume (%)	7.5±0.6	47.1±1.9***	41.6±2.0***

*$P<0.05$, ***$P<0.001$ versus normal control.
PBS, phosphate-buffered saline; ADR, adriamycin.

AN in SCID and NOD-SCID mice

In order to investigate further whether NK cells are involved in the renal injury induced by ADR, SCID and NOD-SCID mice were treated with a single low dose (6.5 mg/kg) injection of ADR. Histological analysis demonstrated that ADR induced severe renal injury in both T/B cell deficient mice (SCID) and T/B cell plus NK cell deficient mice (Figure 5). Morphometric analysis also demonstrated that ADR led to similar renal injury in SCID or NOD-SCID mice (Table 3). Furthermore, both SCID and NOD-SCID mice exposed to low-dose ADR showed similar degrees of pathological injury in glomeruli, tubules, and interstitium to BALB/c mice (with intact T, B, and NK cells) treated with high-dose ADR with or without anti-asialo GM1 (Figures 4 and 5 and Tables 2 and 3), indicating that immunodeficient mice (SCID and NOD-SCID) are more sensitive to ADR than immunocompetent mice.

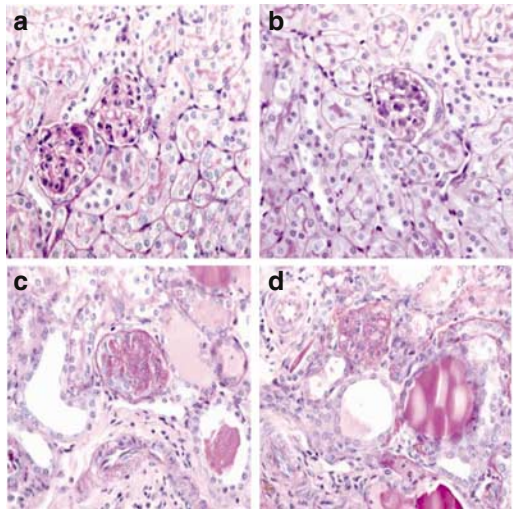

Figure 5 | Histology of kidney sections of normal and ADR SCID and NOD-SCID mice. (**a**) Kidney of normal SCID mice, (**b**) kidney of normal NOD-SCID mice, (**c**) kidney of AN SCID mice, and (**d**) kidney of AN NOD-SCID mice. The results of morphometric analysis of the histological changes are summarized in Table 3. Original magnification ×400.

NK activating receptor NKG2D and its ligand RAE-1 are upregulated by AN

The NK cell activating receptor NKG2D and its ligand RAE-1 were upregulated in AN (Figure 6a). NKG2D is expressed on a variety of cells including $\gamma\delta$ T cells, CD8 T cells, NK cells, and macrophages. Baseline NKG2D expression was higher in SCID mice than BALB/c mice ($P<0.05$) consistent with higher levels of NK cells, but was not altered by AN consistent with the histology showing limited NK cell infiltration (Figure 6a). RAE-1 was upregulated in both BALB/c and SCID mice with AN analyzed by reverse transcriptase real-time PCR assay (Figure 6b), indicating increased level of the activation signal for NK cells after ADR.

MHC Class I molecule expression is also upregulated by ADR

The MHC Class I molecule identified by anti-H2K^d using flow cytometry was found expressed on non-haematopoietic (CD45−) renal cells in both BALB/c and SCID mice. ADR significantly increased the level of MHC Class I molecule expression on non-haematopoietic (CD45−) renal cells in both BALB/c and SCID mice (Figure 7).

Table 3 | Morphometric analyses and renal function of SCID and NOD-SCID mice treated or not with ADR

	SCID mice		NOD-SCID mice	
	Normal (*n*=6)	ADR (*n*=6)	Normal (*n*=6)	ADR (*n*=6)
Tubular diameter (μm)	30.6±1.4	34.7±1.3*	32.7±1.5	34.6±3.8
Tubular cell height (μm)	9.5±0.7	7.9±0.7*	9.8±0.2	8.6±0.1*
Glomerulosclerosis (%)	0	10.5±2.4***	0	10.8±5.6***
Interstitial volume (%)	9.5±0.6	41.1±14.5**	7.5±5.7	36.8±16.7**
Urine protein (mg/16 h)	0.74±0.32	3.20±0.43***	0.87±0.27	3.20±0.30***
Creatinine clearance (μl/min)	48.1±13.4	35.5±15.2	46.3±0.5	33.2±9.5*

*$P<0.05$, **$P<0.01$, ***$P<0.001$ versus normal. There were no difference between SCID ADR and NOD-SCID treated with ADR.
SCID-NOD, severe combined immunodeficient-nonobese diabetic.

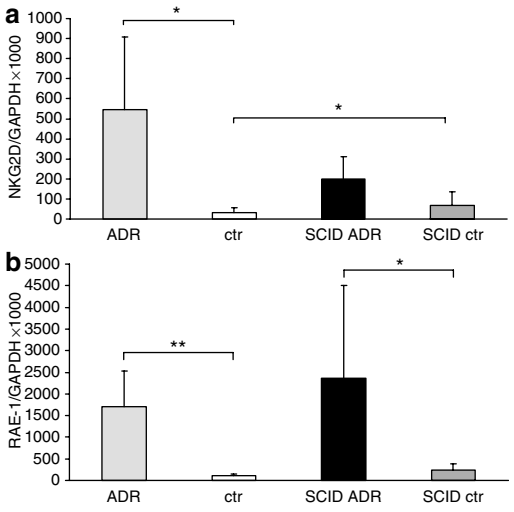

Figure 6 | NK cell activation receptor NKG2D (a) and its ligand RAE-1 (b) expression by reverse transcriptase real-time PCR assay in AN ($n=4$ for each group). The results presented were normalized against glyceraldehyde-3-phosphate dehydrogenase. Data are means±s.d. *$P<0.05$, **$P<0.02$ (rank-sum test). Ctr = control.

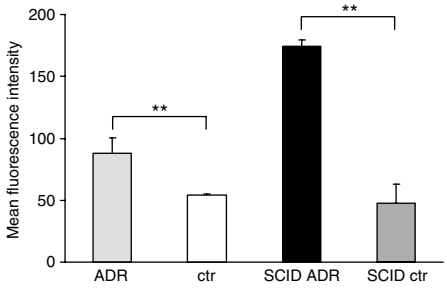

Figure 7 | Flow cytometry analysis of MHC Class I molecule expression by kidney tubular cells ($n=4$ for each group). The tubular $H2K^d$ positive cells were analyzed by gating at CD45− population. Data are means±s.d. of each group. **$P<0.01$ (Student's *t*-test).

DISCUSSION

Effector cells of the innate immune response play a crucial role in initiating T cell activation.[19–22] NK cells are capable of rapid secretion of cytokines such as IFN-γ, and may act as effector cells.[23–27] Our previous studies have demonstrated the involvement of CD4+ and CD8+ T cells in the ADR induced nephropathy in mice.[6,7] Depletion of CD8+ T cells protected against AN, suggesting CD8+ T cells are effectors in this model of progressive chronic renal disease.[6] The worsening of disease in SCID mice suggested that NK cells might be pathogenic either directly or through enhanced activation of other effector cells. Whereas the number of NK cells in kidney, analyzed by FACScan, was increased in AN BALB/c mice compared to control, depletion of NK cells from peripheral blood as well as ADR treated kidney had no effect in wild-type mice. Moreover, in immunodeficient SCID mice the additional functional impairment of NK cells (in NOD-SCID) had no effect on disease.

Despite the expression of NK cell activating receptors on NK cells and the expression and upregulation of its ligand RAE-1 in the AN kidney, significant NK cell mediated damage was not found in either the wild-type or immunodeficient mice. The enhanced NKG2D expression in wild-type mice with AN is consistent with its expression on other effector cells such as CD8 T cells, $\gamma\delta$ T cells, and macrophages.[28] The absence of enhancement of NKG2D expression in the kidney in AN in immunodeficient mice suggests that the populations expressing NKG2D are likely to be CD8 or $\gamma\delta$ T cells, which are not present in the immunodeficient mice, rather than macrophages which are present and activated in both models of AN.

RAE-1 is a molecule upregulated by stress and is found expressed in malignancy and infection. More recently, it has been shown to be expressed in autoimmune disease, in the pancreas of mouse models of diabetes where blockade of its interaction with NKG2D limits disease.[29] Similarly, its human homologue has been shown to activate CD8 T cells in coeliac disease.[30] However, while it may play a role in activating other immune cells in AN, it does not appear to activate the NK cell subset.

NK cell activation is controlled by a precise balance between inhibitory and activating signals. While inhibitory signals had been the explanation of the 'missing self' hypothesis of activation with identification of the inhibitory Ly49 receptors, this has changed with the recognition of activating receptors.[31,32] Activation of NK cells can be achieved when activation receptors are properly engaged by specific ligands expressed on target cells and the inhibition by MHC Class I molecule is missing or weak.[31] Expression of the

NKG2D ligand was found to induce antitumor activity even to tumors expressing Class I MHC.[33] A recent study exploring the balance of inhibitory and activating signals showed that the NKG2D receptor mediating NK cell activation was regulated by inhibitory Ly49 receptors upon recognition of MHC Class I molecules expressed on target cells.[34] An increased expression of the MHC Class I molecule H-2k^d is found in the AN kidney in keeping with an inhibitory role in NK cell activation. Thus, we speculate that the absence of NK cell activation through NKG2D by its ligand RAE-1 could possibly be overridden by the inhibitory signal of MHC Class I molecules expressed in AN. This speculation could be confirmed by blocking the interaction between increased MHC Class I molecules and their receptors on NK cells. It also suggests that the greater severity of AN in SCID mice is due to loss of protective or regulatory immune responses and not because of enhanced NK cell mediated damage. Thus, the absence of NK cell mediated injury in SCID mice suggests a role for other cells in the innate system such as macrophages and neutrophils. The success of AN model in SCID and NOD-SCID mice suggested a pathogenic role of macrophages. The pathogenic role of macrophage has been confirmed in another study from our laboratory using macrophage depletion.[35]

In conclusion, AN in wild-type and immunodeficient mice demonstrates that NK cells do not play a significant role in AN. An increased expression of NK inhibitory signals from the Class I MHC molecules in diseased kidney in the presence of NK activation signals NKG2D and RAE-1 might explain why NK cells are not involved in mediating renal injury in murine AN.

MATERIALS AND METHODS

Animals

BALB/c, SCID, and NOD-SCID mice were bred at the Animal House of Westmead Hospital. All animals were maintained under controlled conditions of light/dark exposure and constant temperature/humidity. The experiments were performed in strict accordance with protocols approved by the Animal Ethics Committee of the University of Sydney and Western Sydney Area Health Service.

Treatment of animals

Male mice, 7 to 8 weeks old and 20–25 g of body weight were randomly distributed among experimental groups. Normal control mice were treated with isotonic saline ($n = 6$). Adriamycin (ADR, doxorubicin hydrochloride; Pharmacia & Upjohn Pty Ltd, Perth, Australia) was administered by a single tail vein injection ($n = 7$). GM1/ADR mice ($n = 7$) were pretreated with 40 μl/mouse of anti-asialo-GM1 serum (GM1) in order to deplete NK cells, and then 36 h later 10 mg/kg of ADR was injected. Further, the same volume of GM1 was injected every 5 days until mice were killed at week 5. In another experiment, SCID mice (which lack functioning T and B cells) and NOD-SCID mice (which lack functioning NK, T, and B cells) were treated with a single low dose of ADR (6.5 mg/Kg)($n = 6$) or nothing (control) ($n = 6$). In these immunodeficient mice, a lower dose of ADR was used to produce the same degree of injury as in immunocompetent mice.

Assessment of NK cell depletion

NK cell levels were assessed by flow cytometric analysis of kidney infiltrating cells and spleen cells from normal control ($n = 4$), ADR ($n = 5$) and ADR/GM1 ($n = 5$) BALB/c mice. The kidney infiltrating cells and splenocytes were prepared by Lymphoprep™ (NYCOMED PHARMA AS, Oslo, Norway). The kidney infiltrating cell suspension was made by mashing kidneys, which have been perfused with warm saline to remove blood cells before collection, through a 70 μM cell strainer (BD Falcon™, Bedford, MA, USA) with RPMI-1640. The splenocyte suspension was made by mashing spleen between two frosted glass plates and reconstituting in phosphate-buffered saline. The cells were collected from the interface of gradient centrifugation and washed three times with fluorescence-activated cell-sorter staining buffer (Dulbecco's phosphate-buffered saline with 2% fetal calf serum/0.2% NaN_3), and 10^6 cell aliquots were incubated with Fc blocking anti-CD16/CD32 at 1 μl per 10^6 cells diluted in 100 μl staining buffer for 10 min on ice. Then the cells were washed twice and stained with fluorescence *in situ* hibridization-conjugated antibody for CD11a, PE-conjugated antibody for NK cells (DX5), and PE-Cy5-conjugated antibody for mouse CD45, incubated on ice for 30 min, then washed three times, resuspended in staining buffer and analyzed by three-color flow cytometry using a FACScan and Cell Quest software (Becton Dickinson, San Jose, CA, USA). The cells were gated on CD45+ cells for analysis of CD11a+/DX5+ NK cells. Respective isotype controls: PE Rat IgM isotype control, PE-Cy5 rat IgG2b isotype control, and fluorescence *in situ* hibridization Rat IgG2a isotype control, were used for determination of background fluorescence levels. An estimation of number of NK cells in kidney was performed by multiplying the total number of interstitial CD45+ cells by the percentage of NK cells determined by FACS.

Sample collection and tissue preparation

At 34 days after ADR treatment (day 0), 16-h-urine was collected using a metabolic cage on the day before killing, with access to water only. Urinary volumes of all animals were recorded. At day 35, blood samples were collected using cardiac puncture and the kidney and spleen were harvested. Half of the organs were fixed in 10% neutral-buffered formalin solution and embedded in paraffin, and the other half were frozen using liquid nitrogen and stored at −80°C.

Real time–polymerase chain reaction

Reverse transcription. Total RNA was extracted from kidneys using Trizol (Gibco BRL, Drand Island, NY, USA) according to the manufacturer's recommendations. cDNA was synthesized by using random hexamer primers (Promega, Madison, WI, USA) and a M-MuLV reverse transcriptase kit (Gibco BRL, Drand Island, NY, USA) following standard protocols.

Real-time PCR. Specific primers for mouse NKG2D (sense 5′-caattcgattcacccttaacacatt-3′; antisense, 5′-gccacagtagcccctctcttgag-3′) and Taqman probe (FAM-tcagccagtattgtgc-MGB) and primers for RAE-1 (sense 5′-atgaattcttaaagcagtccaaggaa-3′; antisense 5′-ggaagtt gatgtaagctgggtgata-3′) and Taqman probe (FAM-agccaagatcaacctc-MGB) were obtained from PE Applied Biosystems. Primers and a probe for glyceraldehyde-3-phosphate dehydrogenase as a house keeping gene control were used as previously described.[36] cDNA was amplified with specific primers in Universal Master Mix (PE Applied Biosystems, Foster City, CA, USA) on an ABI 7700 instrument (Applied Biosystems), according to the manufacturer's instructions. The cycling conditions for real-time were 50°C for 2 min and 95°C

for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed using the Sequence Detector v1.9 Analysis Software (Applied Biosystems).

MHC Class I molecule expression by flow cytometry

Cell suspension of kidney cortex was prepared by mashing through 70 μM cell strainer as described, followed by lysis of red cells, then stained with PE-Cy5-conjugated antibody for mouse CD45 and PE-conjugated anti-mouse H2k^d for MHC Class I molecule, and analyzed by two-color flow cytometry using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). The cells were gated on CD45 negative population for tubular H2k^d expression, excluding dead cells by propidium iodide (PI, BD) staining.

Histology

The methods for histochemistry were as described previously.[2] In brief, slides of sectioned kidney were stained with each of periodic acid-Schiff, hematoxylin–eosin[37] and Masson's trichrome. Glomerulosclerosis was evaluated in periodic acid-Schiff-stained sections, while cellular infiltration of glomeruli and interstitium was assessed by counting nuclei in hematoxylin–eosin-stained sections. Interstitial fibrosis was assessed in sections stained with Masson's trichrome.

Morphometric analysis

Renal morphology was analyzed using Optimas image analysis system (Optimas Corporation, Seattle, WA, USA). Glomerular surface area, glomerular capillary area, mesangial matrix area, number of nuclei per glomerulus, interstitial volume, tubular diameter, and tubule cell height was assessed as described previously.[2] Glomerulosclerosis was quantitated using the percentage increase in relative mesangial matrix area (periodic acid-Schiff-positive area within the glomerulus divided by the glomerular capillary area, magnification $\times 400$),[38] while the degree of interstitial expansion was quantitated using the relative interstitial volume on periodic acid-Schiff-stained sections. Tubular atrophy was defined as the presence of dilated tubules with cellular degeneration (low cell height and absence of brush border) and thickened, wrinkled basement membrane. All mean values were calculated from each of 20 glomeruli or tubules or 10 random cortical regions per section, as described previously.[2]

Immunohistochemistry

Immunohistochemical analysis was performed using previously described methods.[2] In short, tissues were incubated in Tris-buffered saline (0.8% Tris-HCl (Sigma) and 0.9% NaCl (Sigma), pH 7.6) for 5 min and incubated in blocking reagent (Tris-buffered saline + 10% (vol/vol) normal rabbit serum; Dako) for 15 min. Incubation with relevant primary antibodies was carried out for 2 h at room temperature. Control cryostat sections were incubated at the same time with control antibody (normal rat immunoglobulin). After rinsing twice in Tris-buffered saline for 5 min each, sections were incubated with secondary antibodies for 30 min at room temperature. This was followed by rinsing in Tris-buffered saline and incubation with ABC complex (Zymed Laboratories Inc., San Francisco, CA, USA) for 30 min; 3,3-diaminobenzidine substrate-chromogen solution (Dako) was applied for less than 5 min and then washed thoroughly in distilled water. Slides were counterstained with hematoxylin (Sigma), dehydrated, and cover-slipped. Positive immunoreactivity resulted in brown staining of the cell membrane. Normal nuclei and cytoplasm were stained lightly. For assessment of interstitial infiltration, positively stained interstitial cells were counted from five random cortical fields (magnification $\times 400$) in each section, and the numbers were averaged for each field.

Renal function

Total urinary protein was quantitated using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The concentration of albumin and creatinine in plasma and urine were determined in duplicate using a BM/Hitachi 747 analyzer (Tokyo, Japan) at the Institute of Clinical Pathology and Medical Research, Westmead Hospital. Renal function was assessed by creatinine clearance (C_{Cr}) according to the clearance formula, $C_{Cr} = (U_{Cr} \times V)/P_{Cr}$, where U_{Cr} is the concentration of creatinine in urine, P_{Cr} is the concentration of creatinine in plasma, and V is the urine flow rate in milliliters per minute.

Statistical analysis

Data are presented as mean $\pm$ s.d. Data were compared using one-way analysis of variance, followed by multiple pair-wise comparison according to the Newman–Keuls test, Student's t-test, and nonparametric rank test. A P-value of <0.05 was considered significant.

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REFERENCES

1. Chen A, Sheu LF, Ho YS *et al.* Experimental focal segmental glomerulosclerosis in mice. *Nephron* 1998; **78**: 440–452.
2. Wang Y, Wang YP, Tay YC *et al.* Progressive adriamycin nephropathy in mice: sequence of histologic and immunohistochemical events. *Kidney Int* 2000; **58**: 1797–1804.
3. Nath KA. Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* 1992; **20**: 1–17.
4. Nikolic-Paterson DJ, Lan HY, Hill PA *et al.* Macrophages in renal injury. *Kidney Int Suppl* 1994; **45**: S79–S82.
5. Markovic-Lipkovski J, Muller CA, Risler T *et al.* Association of glomerular and interstitial mononuclear leukocytes with different forms of glomerulonephritis. *Nephrol Dial Transplant* 1990; **5**: 10–17.
6. Wang Y, Wang YP, Tay YC *et al.* Role of CD8(+) cells in the progression of murine adriamycin nephropathy. *Kidney Int* 2001; **59**: 941–949.
7. Wang Y, Feng X, Bao S *et al.* Depletion of CD4(+) T cells aggravates glomerular and interstitial injury in murine adriamycin nephropathy. *Kidney Int* 2001; **59**: 975–984.
8. Furuichi K, Wada T, Iwata Y *et al.* Upregulation of fractalkine in human crescentic glomerulonephritis. *Nephron* 2001; **87**: 314–320.
9. Huang XR, Tipping PG, Apostolopoulos J *et al.* Mechanisms of T cell-induced glomerular injury in anti-glomerular basement membrane (GBM) glomerulonephritis in rats. *Clin Exp Immunol* 1997; **109**: 134–142.
10. Kitching AR, Tipping PG, Kurimoto M *et al.* IL-18 has IL-12-independent effects in delayed-type hypersensitivity: studies in cell-mediated crescentic glomerulonephritis. *J Immunol* 2000; **165**: 4649–4657.
11. Godin I, Cumano A. The hare and the tortoise: an embryonic haematopoietic race. *Nat Rev Immunol* 2002; **2**: 593–604.
12. Moretta A, Bottino C, Mingari MC *et al.* What is a natural killer cell? *Nat Immunol* 2002; **3**: 6–8.
13. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* 1990; **11**: 237–244.
14. Biron CA, Nguyen KB, Pien GC *et al.* Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 1999; **17**: 189–220.
15. Kasai M, Iwamori M, Nagai Y *et al.* A glycolipid on the surface of mouse natural killer cells. *Eur J Immunol* 1980; **10**: 175–180.
16. Kondo T, Morita K, Watarai Y *et al.* Early increased chemokine expression and production in murine allogeneic skin grafts is mediated by natural killer cells. *Transplantation* 2000; **69**: 969–977.

17. Barlozzari T, Reynolds CW, Herberman RB. *In vivo* role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM1-treated rats. *J Immunol* 1983; **131**: 1024–1027.
18. Wang M, Ellison CA, Gartner JG *et al.* Natural killer cell depletion fails to influence initial CD4 T cell commitment *in vivo* in exogenous antigen-stimulated cytokine and antibody responses. *J Immunol* 1998; **160**: 1098–1105.
19. Romagnani S. Induction of TH1 and TH2 responses: a key role for the 'natural' immune response? *Immunol Today* 1992; **13**: 379–381.
20. Bogen SA, Fogelman I, Abbas AK. Analysis of IL-2, IL-4, and IFN-gamma-producing cells *in situ* during immune responses to protein antigens. *J Immunol* 1993; **150**: 4197–4205.
21. Scharton-Kersten T, Scott P. The role of the innate immune response in Th1 cell development following *Leishmania* major infection. *J Leukoc Biol* 1995; **57**: 515–522.
22. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. *Science* 1996; **272**: 50–53.
23. Trinchieri G. Recognition of major histocompatibility complex class I antigens by natural killer cells. *J Exp Med* 1994; **180**: 417–421.
24. Dunn PL, North RJ. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. *Infect Immun* 1991; **59**: 2892–2900.
25. Bancroft GJ, Schreiber RD, Unanue ER. Natural immunity: a T-cell-independent pathway of macrophage activation, defined in the scid mouse. *Immunol Rev* 1991; **124**: 5–24.
26. Scharton TM, Scott P. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. *J Exp Med* 1993; **178**: 567–577.
27. Zhang T, Kawakami K, Qureshi MH *et al.* Interleukin-12 (IL-12) and IL-18 synergistically induce the fungicidal activity of murine peritoneal exudate cells against *Cryptococcus neoformans* through production of gamma interferon by natural killer cells. *Infect Immun* 1997; **65**: 3594–3599.
28. Diefenbach A, Jamieson AM, Liu SD *et al.* Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol* 2000; **1**: 119–126.
29. Ogasawara K, Hamerman JA, Ehrlich LR *et al.* NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity* 2004; **20**: 757–767.
30. Meresse B, Chen Z, Ciszewski C *et al.* Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* 2004; **21**: 357–366.
31. Moretta L, Bottino C, Pende D *et al.* Different checkpoints in human NK-cell activation. *Trends Immunol* 2004; **25**: 670–676.
32. Dimasi N, Moretta L, Biassoni R. Structure of the Ly49 family of natural killer (NK) cell receptors and their interaction with MHC class I molecules. *Immunol Res* 2004; **30**: 95–104.
33. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor *in vivo*. *Proc Natl Acad Sci USA* 2001; **98**: 11521–11526.
34. Regunathan J, Chen Y, Wang D *et al.* NKG2D receptor-mediated NK cell function is regulated by inhibitory Ly49 receptors. *Blood* 2005; **105**: 233–240.
35. Wang YP, Tay YC, Spicer T *et al.* Partial depletion of macrophages by ED7 reduces renal injury in Adriamycin nephropathy. *Nephrol* 2005; **10**: 470–477.
36. Yin JL, Shackel NA, Zekry A *et al.* Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. *Immunol Cell Biol* 2001; **79**: 213–221.
37. Kim TW, Hung CF, Ling M *et al.* Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. *J Clin Invest* 2003; **112**: 109–117.
38. Vleming LJ, Baelde JJ, Westendorp RG *et al.* The glomerular deposition of PAS positive material correlates with renal function in human kidney diseases. *Clin Nephrol* 1997; **47**: 158–167.