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Tissue-Resident NK Cells Mediate Ischemic Kidney Injury and Are Not Depleted by Anti–Asialo-GM1 Antibody

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NK cells are innate lymphoid cells important for immune surveillance, identifying and responding to stress, infection, and/or transformation. Whereas conventional NK (cNK) cells circulate systemically, many NK cells reside in tissues where they appear to be poised to locally regulate tissue function. In the present study, we tested the contribution of tissue-resident NK (trNK) cells to tissue homeostasis by studying ischemic injury in the mouse kidney. Parabiosis experiments demonstrate that the kidney contains a significant fraction of trNK cells under homeostatic conditions. Kidney trNK cells developed independent of NFIL3 and T-bet, and they expressed a distinct cell surface phenotype as compared with cNK cells. Among these, trNK cells had reduced asialo-GM1 (AsGM1) expression relative to cNK cells, a phenotype observed in trNK cells across multiple organs and mouse strains. Strikingly, anti–AsGM1 Ab treatment, commonly used as an NK cell–depleting regimen, resulted in a robust and selective depletion of cNKs, leaving trNKs largely intact. Using this differential depletion, we tested the relative contribution of cNK and trNK cells in ischemic kidney injury. Whereas anti–NK1.1 Ab effectively depleted both trNK and cNK cells and protected against ischemic/reperfusion injury, anti–AsGM1 Ab preferentially depleted cNK cells and failed to protect against injury. These data demonstrate unanticipated specificity of anti–AsGM1 Ab depletion on NK cell subsets and reveal a new approach to study the contributions of cNK and trNK cells in vivo. In total, these data demonstrate that trNK cells play a key role in modulating local responses to ischemic tissue injury in the kidney and potentially other organs. ***The Journal of Immunology*****, 2015, 195: 4973–4985.**

Natural killer cells are rapidly responding lymphoid cells that have a central role in local and systemic immune surveillance. NK cell recognition and activation are mediated through engagement of multiple activating and inhibitory receptors, broadly referred to as NKRs, with the relative engagement of inhibitory and activating receptors influencing whether a cell is protected from, or becomes a target for, NK cell destruction (1–5). The specificity and sensitivity of NK cell recognition are thought to be initially acquired in the bone marrow and further refined in the periphery, a process known as education and licensing (6–10). Upon activation, NK cells can profoundly shape the immune response via cognate and noncognate interactions, rapidly producing cytokines, chemokines, and/or directly inducing cell death (e.g., by perforin/granzyme-mediated apoptotic induction within target cells) (4, 11–14).

Genetic models have provided insights into NK cell development and their role as potent regulators of inflammation and immunity (15–21). However, given that many genetic models of NK cell deficiency are compounded by additional defects, studies often investigate the role of NK cells using Ab depletion methods. In vivo depletion studies of NK cells have been primarily done using 1) Abs to NK1.1, a glycoprotein expressed on NK cells and subsets of γδ T, NKT, and CD8 T cells in certain strains of mice; or 2) Abs to asialo-GM1 (AsGM1), a glycolipid highly expressed on NK cells, basophils, and subsets of γδ T, NKT, and CD8 T cells (22). Whereas anti–NK1.1 Ab depletion using the PK136 Ab clone works robustly in some strains of mice (e.g., C57BL/6J [B6], SJL, and NZB), this Ab does not work in other strains of mice due to allelic variation in the NKR-P1 gene that encodes NK1.1 (CD161) (23, 24). In contrast, anti–AsGM1 Ab robustly depletes NK cells across multiple strains of mice, including B6, BALB/c, DBA2, and 129S1/SvImJ (129) strains, and it remains in use to this day (22, 25, 26).

Although NK cells were once considered to be a functionally homogeneous lymphocyte population (e.g., in contrast to the diversity of specificities and functionalities of T cells), there is now clear evidence that NK cells have the capacity to discriminate between diverse targets and to respond with diverse functions (27–36). In attempts to better understand this NK cell diversity, NK cells

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Abbreviations used in this article: 129, 129S1/SvImJ; AKI, acute kidney injury; AsGM1, asialo-GM1; B6, C57BL/6J; CD1dKO, CD1d-deficient; cNK, conventional NK; GFR, glomerular filtration rate; IRI, ischemia/reperfusion injury; NOD, NOD/ShiLtJ; trNK, tissue-resident NK.

have been divided into subsets based on heterogeneous expression of various cell surface markers (e.g., TRAIL, Thy1, KLRG1, CCR2, CD11b, and CD27) or divergent functional properties (37–41). Recently, liver NK cells were shown to contain two distinct subsets, tissue-resident NK (trNK) cells and conventional NK (cNK), circulating cells, based on differences in trafficking and tissue retention (42). Notably, further studies demonstrated that beyond these differences in trafficking, trNK and cNK cells have differences in cytokine production, cell surface proteins involved in cell adhesion and NK cell recognition, and distinct developmental requirements (43). Whereas the contribution of trNK cells to shaping immune responses remains an active area of investigation, liver trNK cells are known to confer memory-like immune responses in a contact hypersensitivity model (42). The existence of both local and systemic NK cell reservoirs has significant implications in understanding the multifaceted role of NK cells in regulating local and systemic immune responses.

In this study, we sought to investigate the characteristics and contribution of trNK cells to local tissue injury in the context of ischemia/reperfusion injury (IRI). To study the role of NK cells in IRI, we used a mouse model of ischemic acute kidney injury (AKI), a common human pathology characterized by ischemia that can result from various etiologies, including diabetes, major surgery, sepsis, and drug toxicity (44). Although several previous studies have highlighted the importance of NK cells in ischemic AKI using patch-clamping models of AKI (45–47), the role of NK cells in ischemic AKI in the absence of intrarenal coagulation remain unknown. Notably, the dynamics and contributions of cNK and trNK cells in ischemic tissue injury remain undefined.

By studying trNK cells in the kidney, as well as in multiple other organs, we found that trNK cells have variable and reduced AsGM1 expression relative to cNK cells, such that trNK cells are relatively spared from depletion by anti–AsGM1 Abs. Using this knowledge, we demonstrate that trNK cells are a potent mediator of IRI. These data identify a new method to study trNK cells in vivo and define a new physiological role for trNK cells as a front-line player in the response to acute ischemic injury.

Materials and Methods

Mice

B6, NOD/ShiLtJ (NOD), BALB/c (BALB/cJ), 129, and CD1d-deficient (CD1dKO; formerly named B6.129S6-*Cd1d1/Cd1d2*^{tm1Spb}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and briefly housed in a specific pathogen-free colony at the University of Colorado, Anschutz Medical Campus. CD1dKO (a gift from Dr. Laurent Gapin, University of Colorado, Anschutz Medical Campus) (48), B6, and BALB/c mice were also obtained from breeding colonies at the University of Colorado, Anschutz Medical Campus and maintained in a pathogen-free facility and were of 8–12 wk of age for experimental use. Mice were age and sex matched for experiments, with male mice uniformly used for general screening and IRI studies. Female mice were solely used for the analysis of trNK cell distribution in multiple organs. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver, in accordance with the National Institutes of Health guidelines for use of live animals. The University of Colorado Denver, Anschutz Medical Campus is accredited by the American Association for the Accreditation of Laboratory Animal Care. *Tbet*$^{-/-}$ (formal gene name *Tbx21)* mice were purchased from The Jackson Laboratory and *NFIL-3*$^{-/-}$ mice (from Paul B. Rothman, Johns Hopkins University) (43, 49) were bred and housed in a pathogen-free facility, with procedures performed in accordance with the animal protocol approved by the Washington University School of Medicine Animal Studies Committee.

Induction of IRI

The hanging weight system was used as previously described (50). Briefly, mice were anesthetized with pentobarbital at 20 mg/kg and body temperature was regulated using an anal probe during the entire surgery. For sham surgery, mice were subjected to a right kidney nephrectomy followed by recovery under a heat lamp. To induce IRI, nephrectomy of the right kidney was done, after which the left kidney was exposed and connective and adrenal tissues were gently separated. Next, the kidney was placed in an acrylic cup and ischemia was induced for 30 min by placing 6-0 nylon suture under the renal artery and applying hanging weights, to occlude blood flow. Following 30 min of ischemia, hanging weights were removed and the kidney was allowed to reperfuse. Mice were given saline, sutured closed, and allowed to recover for the indicated time points of reperfusion (typically 4 and 24 h of reperfusion).

Analysis of renal function

To assess kidney function, glomerular filtration rate (GFR) was measured as previously described (50). Briefly, mice were anesthetized with pentobarbital at 20 mg/kg and kept warm to maintain their body temperature using an anal probe during the entire surgery. Once mice were fully anesthetized, a catheter was inserted into the jugular vein with a second catheter inserted into the bladder to collect urine. Next, inulin-FITC (Sigma-Aldrich) was infused at 800 μl per minute. Blood and urine were collected every 20 min to measure GFR as described (50, 51).

Parabiosis

Parabiosis surgery was performed as previously described (42, 43). Briefly, a longitudinal skin incision was made on the flanks of both C57BL/6NCr (Ly5.2) and B6-Ly5.1/Cr age-and weight-matched female mice. Their elbows and knees were joined with dissolvable sutures and the incision was closed with wound clips. Postoperative care included administration of buprenorphine (Buprenex) for pain control and 5% dextrose and 0.9% sodium chloride for fluid replenishment. Nutritional gel packs were provided in each cage and Sulfatrim (sulfamethoxazole/trimethoprim) was included in the drinking water for the duration of the experiment. Mice were sacrificed and analyzed 14 d after surgery. The procedure was approved by the Animal Studies Committee at Washington University (St. Louis, MO).

Flow cytometric cell analysis

To quantify the number of NK and NKT cells, mice were perfused with PBS and kidneys were harvested and minced through a 70-μm filter. Digested tissue was washed with RPMI 1640 and then lymphocytes were purified using a 36% Percoll gradient (GE Healthcare). Cell were stained in FACS buffer (PBS, 0.2% FBS, 0.01% sodium azide), anti-FcR blockade CD16/32, and anti-CD3 PE-Cy7 (145-2C11), anti-NK1.1 PerCP-Cy5.5 (PK136), anti-NKp46 (29A1.4), and anti-CD45 (30-F11). NK cells were defined as $CD45^+CD3^-NK1.1^+$ events, with NKT cells defined as $CD45^+CD3^+$ $NK1.1^+$ events. For studies analyzing trNK cells, mice were perfused with 15 ml saline (excluding parabiotic studies and analysis of *Tbet*$^{-/-}$ and *NFIL-3*$^{-/-}$ mice), after which kidney, liver, lung, pancreas, and uterus were harvested, minced, and mechanically disrupted, followed by a 1-h enzymatic digestion in collagenase D (1 mg/ml, Roche) at 37°C. Cells were then washed in complete RPMI 1640 (10% FCS, 1% penicillin/streptomycin). Cells were then stained with a mixture of fluorescently-labeled Abs from eBioscience: anti-CD3 eFluor 450 (145-2C11), anti-CD19 eFluor 450 (ebio1D3), anti–MHC class II eFluor 450 (MS/114.15.2), anti-CD45 allophycocyanin–eFluor 780 (30-F11), and anti-NK1.1 PerCP-Cy5.5 (PK136); or from BioLegend: anti-NKp46 PE or PE-Cy7 (29A1.4), anti-CD49b PE-Cy7 (DX5), anti-CD49b (HMα2), anti-TRAIL PE, anti-CD44 FITC, anti-CD160 PE, anti-CD49a PE or allophycocyanin, and anti-AsGM1 Alexa Fluor 647 (poly21460); or from BD Pharmingen: anti-CD49a (Ha31/8). NK cells were identified as lymphocytes (by forward scatter and side scatter) that were $CD45^+$, $Lineage^-$ ($CD3^-CD19^-$MHC class II^-), and either $NK1.1^+$ or $NKp46^+$. trNK and cNK cells were identified as either $CD49a^+$ or $DX5^+$, respectively. Anti–AsGM1 Ab staining was done using 0.35 μg Ab per stain for 30 min. In a subset of studies, cNK cells were defined as NK cells that expressed $CD49b^+CD49a^-$ using the HMα2 clone, instead of the CD49b-reactive DX5 clone. Note that both Abs recognize CD49b, although the DX5 clone may be less sensitive than HMα2 for cells that express lower levels of CD49b (52). For NK, trNK versus cNK, and NKT cell composition after IgG or NK1.1 treatment, mice were treated with either IgG control Ab or NK1.1 Ab on days −3 and −1. On day 0, mice were then subjected to the hanging weight system for 30 min to induce IRI. After 1 d of reperfusion, kidney was harvested and lymphocytes were purified and analyzed using flow cytometry after staining as noted above, with NKp46 being the NK cell marker. For parabiotic studies, cells were stained for CD45.1 (clone A20) and CD45.2 (clone 104) (both from eBioscience). Gating strategies for flow cytometric analysis are indicated in the figure legends. Samples were analyzed using a BD LSR II flow cytometer.

NK cell depletion

For NK1.1 cell–depleting experiments, B6 and CD1d knockout mice were treated with either 200 μg control anti-mouse IgG2a (Bio X Cell C1.18.4, mouse IgG2a isotype control) or anti-NK1.1 Ab (Bio X Cell PK136) on days −3 and −1. For AsGM1-depleting experiments, B6 mice were treated with 800 μg of either rabbit IgG (SouthernBiotech) or AsGM1 Ab (Wako Pure Chemical Industries) on day −1. On day 0, mice were then subjected to the hanging weight system for 30 min to induce IRI. After 1 d of reperfusion, kidney function was measured by GFR. NK cell depletion was verified by measuring NK cell percentages ($CD45^+CD3^-NKp46^+$).

Detection of cleaved caspase-3

B6 mice were treated with IgG or NK1.1 or AsGM1 Abs as above. Mice that received IgG or NK1.1 Abs were exposed to sham (−I) or ischemic (+I) surgery and allowed to recover for 4 h. AsGM1-treated mice only received ischemic surgery (+I). At 4 h reperfusion, kidneys were harvested, fixed in 4% paraformaldehyde, and paraffin embedded. Five-micrometer-thick paraffin sections were deparaffinized, and Ags were unmasked and immunohistochemically stained for cleaved caspase-3 (Cell Signaling Technology, Danvers, MA; rabbit polyclonal, catalog no. 9661, 1:1000 in TBST plus 1% BSA [w/v]). Sections required Ag retrieval in BORG solution (Biocare Medical, Concord, CA; high pH; 10 min at 110°C) in the Decloaking Chamber (Biocare Medical). All incubations were performed on the autostainer (Benchmark XT; Ventana Medical Systems/Roche, Tucson, AZ) at an operating temperature of 37°C. Primary Ab was incubated for 32 min and detected with a modified iVIEW DAB detection kit (Ventana Medical Systems/Roche). The iVIEW secondary Ab was replaced with a species-specific polymer (rabbit ImmPress; full strength; Vector Laboratories, Burlingame, CA; 8 min) and streptavidin-HRP was replaced with diluted rabbit ImmPress (1:2 dilution in PBS [pH 7.6]; 8 min). All sections were counterstained in Harris hematoxylin for 2 min, blued in 1% ammonium hydroxide (v/v), dehydrated in graded alcohols, cleared in xylene, and coverglass mounted using synthetic resin. Quantitation of caspase-3 immunopositive cells was determined by gathering the numerical total across the entire kidney section, at a ×20 (0.26 mm^2) magnification. Representative images were chosen based on the ratio of the number of caspase-3^+ cells in ischemic groups versus sham group. Differences between anti-NK1.1 and anti-AsGM1 groups were corroborated by a second, independent investigator who was blinded to the experimental groups.

Statistical analysis

Data analysis and plotting were done using Prism 5 (GraphPad Software). Flow cytometric data were analyzed using FlowJo version 10 (Tree Star), with data displayed as high-resolution pseudocolor dot plots showing outliers, plotted on a biexponential axis. Statistical analyses were performed using Prism 5, with an unpaired Student *t* test or one-way ANOVA with a Bonferroni posttest correction for multiple comparisons. Experimental values were considered to be statistically different when $p < 0.05$.

Results

Kidney cNK cells preferentially express AsGM1 compared with trNK cells

Recently, two subsets of NK cells were identified in the liver based on either tissue-retention (trNK) versus systemic circulation (cNK) (42). Further studies showed that these subsets could be identified as $CD49a^+DX5^-$ (trNK) versus $DX5^+CD49a^-$ (cNK) in the liver as well as in multiple other organs (42, 43). Although trNK cells were identified in multiple organs, the presence of trNK cells in the kidney has not been reported. To test this, we analyzed NK cells in the kidney and identified both $CD49a^+DX5^-$ and $CD49a^-DX5^+$ NK subsets (Fig. 1A). In the unmanipulated kidney, $CD49a^+$ NK cells were a minor but readily detectable fraction (15–20%) of the NK cell pool (Fig. 1B). To demonstrate that $CD49a^+$ NK cells in the kidney were tissue-resident, we analyzed these populations in parabiotic pairs of mice. This analysis demonstrated that $CD49a^+$ NK cells were present only in the host and not the other parabiont, indicating that $CD49a^+$ NK cells did not systemically circulate between the two parabionts (Fig. 1C, 1D). In contrast, $DX5^+CD49a^-$ NK cells were detectable in both parabionts, indicating that $DX5^+$ NK cells were capable of systemic circulation, exchanging these cells between the two parabionts in the kidney (Fig. 1C, 1D). The percentage chimerism in the parabiotic mice was determined by the expression of CD45.1 and CD45.2 in the spleen (Fig. 1E). These data demonstrate that the kidney contains both $CD49a^+$ trNK cells and $DX5^+$ cNK cells.

trNK cells and cNK cells have differential expression of multiple proteins (42, 43). To investigate the cell surface phenotype of these two subsets in the kidney, we performed an analysis of proteins involved in NK cell activation and trafficking. These studies showed that in the kidney, trNK cells had uniform increased expression of CD44 (an activation and adhesion protein), CD160 (an activation-associated protein), and TRAIL (an apoptosis-inducing cell surface protein) relative to cNK cells, with a subset of trNK cells also expressing the chemokine receptor CCR2 (Fig. 1F). In contrast, trNK cells had uniformly low expression of CD62L (an adhesion protein) relative to cNK cells (Fig. 1F). trNK cells also had reduced expression of two inhibitory receptors relative to cNK cells, uniformly lacking expression of KLRG1 with a prominent fraction of trNK cells that also failed to express CD244 (Fig. 1F). Strikingly, kidney trNK cells had reduced expression of AsGM1 (Fig. 1G), a glycolipid moiety that has been a target for Ab-mediated depletion of NK cells in vivo (53). These data identify that trNK cells have a more activated phenotype with potentially reduced susceptibility to inhibitory signals compared with cNK cells. They further indicate that trNK cells express variable levels of AsGM1 and may be differentially susceptible to AsGM1 Ab-mediated depletion in vivo.

Kidney trNK cells develop independent of NFIL3 and T-bet

We next sought to determine the developmental requirements for kidney trNK cells. To do this, we investigated the relative frequency and abundance of trNK and cNK cell subsets in the kidney under baseline conditions, in mice deficient in either NFIL3, which is required for cNK cell development (54, 55), or T-bet (Tbx21), which contributes to NK cell maturation (38) and is required for the development of liver and skin but not uterine trNK cells (43). Kidney trNK cells were present at or above levels found in B6 mice both by total cell number and by frequency in NFIL3-deficient mice (Fig. 2A, 2B). Conversely, cNK cells were largely absent from the kidney in NFIL3-deficient mice, consistent with previous reports identifying NFIL3 as an essential regulator of cNK cell development (54, 55). trNK cells were also present at a normal frequency in T-bet–deficient mice, although T-bet–deficient mice had reduced numbers of NK cells, with ∼50% the number of trNK cells and only ∼20% the number of cNK cells relative to B6 mice (Fig. 2A, 2B). AsGM1 was expressed at lower levels in trNK cells relative to cNK cells in both NFIL3-deficient and T-bet–deficient mice, consistent with AsGM1 expression levels observed in trNK cells in wild-type mice (Fig. 2C, 2D). Whereas differential AsGM1 expression was observed between trNK and cNK cells in all strains of mice, NFIL3-deficient mice have reduced AsGM1 expression in cNK cells and fewer $AsGM1^{low}$ trNK cells than in wild-type mice (Fig. 2C, 2D). These studies show that kidney trNK cells develop independent of *NFIL3* and *Tbet*.

AsGM1 is preferentially expressed on cNK cells compared with trNK cells across multiple strains and organs

Given the broad use of AsGM1 NK cell–depleting regimen on various strains of mice, we sought to determine whether preferential expression of AsGM1 on cNK cells was exclusive to the kidney and B6 mice. To determine whether trNK cells and cNK

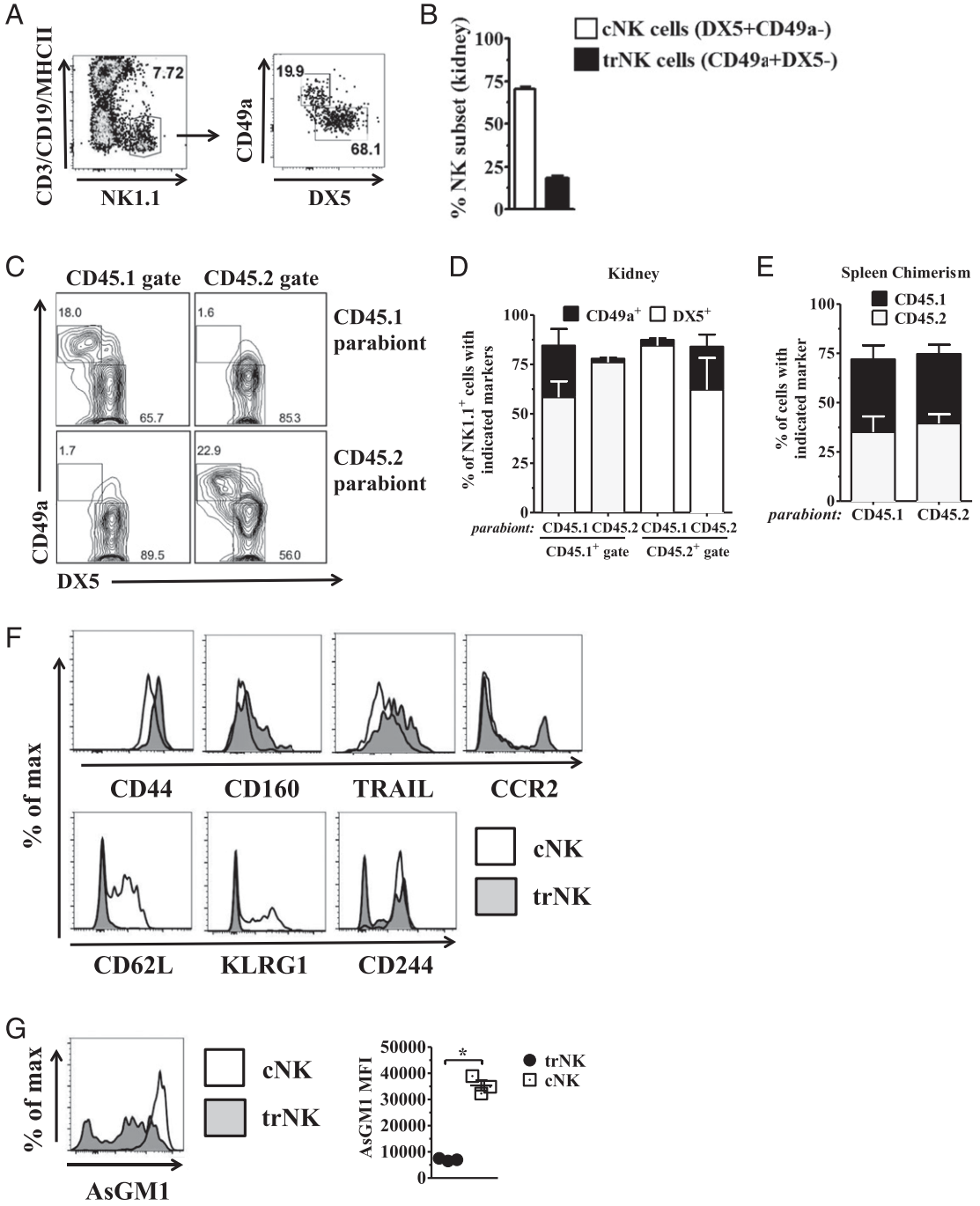

FIGURE 1. The kidney contains both cNK and trNK cells characterized by differential AsGM1 expression. B6 mice were perfused with PBS, and kidney tissue was analyzed for the presence of $CD49a^+$ trNK versus $DX5^+$ cNK cells. (**A**) Representative flow cytometric analysis to identify NK cells ($NK1.1^+$ $CD3^-CD19^-$MHC class II^-) that were either trNK cells ($CD49a^+DX5^-$) or cNK cells ($DX5^+CD49a^-$). (**B**) Percentage distribution of trNK and cNK cell subsets in the kidney. (**C**–**E**) Negligible exchange of kidney $CD49a^+DX5^-$ NK cells in parabiotic mice. WT B6 (CD45.2) mice were parabiosed to congenic B6 (CD45.1) mice. At day 14 after surgery, the spleen and kidney were harvested and flow cytometry was performed. (C) Representative contour plots of the kidney gated on live $CD3^-CD19^-NK1.1^+$ cells followed by a CD45.1 gate (*left panels*) and CD45.2 (*right panels*) as indicated for each parabiont. The percentages of $CD49a^+DX5^-$ and $CD49a^-DX5^+$ cells are indicated in the dot plots. (D) The percentages of $CD49a^+DX5^-$ and $DX5^+CD49a^-$ cells in the kidney from (C) are shown in the stacked bar graph, which represents eight parabiotic pairs done three independent times. (E) The chimerism in the parabiotic pairs is measured by the percentage of CD45.1 and CD45.2 expression in the spleen depicted in the stacked bar graph. (**F**) Representative expression levels of cell surface proteins comparing trNK (shaded gray) and cNK (open black line) cells, defined as in (A). (**G**) Representative histogram of AsGM1 expression for trNK and cNK cell subsets, with quantification of mean fluorescence intensity (MFI) on the right. Data depict mean ± SEM, with each data set containing data from three to five mice per group from independent experiments. $*p < 0.05$ as determined by unpaired *t* test.

cells had differential expression of AsGM1 in additional tissues and genetic backgrounds, we analyzed these NK cell subsets in different strains of mice using NKp46, a common marker for NK cells (56). Notably, NKp46 is interchangeable with NK1.1 when identifying trNK and cNK cell subsets in the kidney of B6 mice (Supplemental Fig. 1).

When we analyzed the relative frequency of cNK and trNK cells across common inbred strains of mice (B6, NOD, BALB/c, and

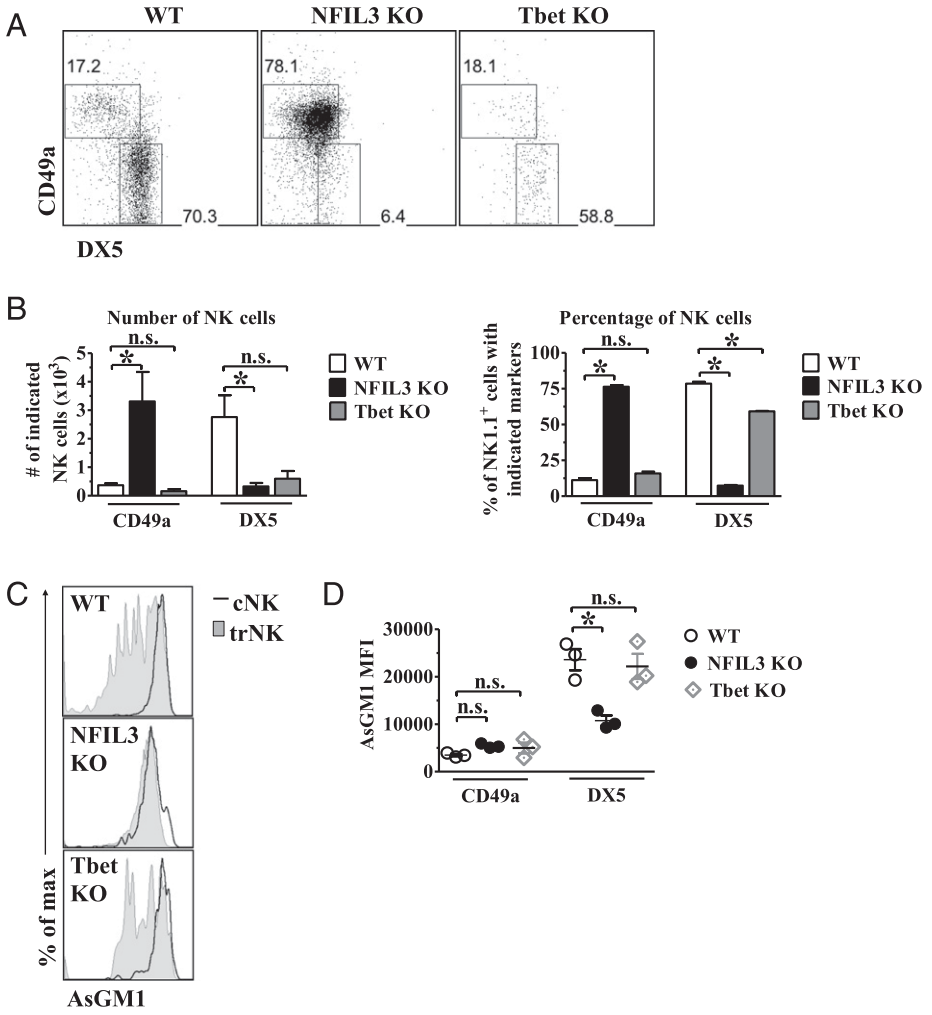

FIGURE 2. Kidney CD49a$^+$DX5$^-$ NK cells develop independent of NFIL3 and T-bet. The kidney was isolated from WT, NFIL3 KO, and T-bet KO mice, stained, and analyzed by flow cytometry. (**A**) Representative dot plots were gated on live CD45$^+$CD3$^-$CD19$^-$NK1.1$^+$ and show the percentage of CD49a and DX5 expression. (**B**) The bar graphs depict the number (*left panel*) and percentage (*right panel*) of live CD45$^+$CD3$^-$CD19$^-$NK1.1$^+$ that express CD49a and DX5 in the kidney of WT, NFIL3 KO, and T-bet KO mice. (**C**) The histograms indicate the expression of AsGM1 on gated CD45$^+$CD3$^-$CD19$^-$NK1.1$^+$CD49a$^+$ (shaded gray histogram, trNK) and CD45$^+$CD3$^-$CD19$^-$NK1.1$^+$DX5$^+$ (black line histogram, cNK). (**D**) The graph represents the mean fluorescence intensity (MFI) of AsGM1 on CD49a$^+$ and DX5$^+$ on NK cells of WT (○), NFIL3 KO (●), and T-bet KO (◇) mice. Experiments were performed three independent times. *$p < 0.05$ as determined by unpaired *t* test between trNK and cNK cells within the same genotype. KO, knockout.

129), we found that trNK frequency was relatively comparable across different backgrounds, with NOD mice showing a slightly increased trNK frequency in some organs (Fig. 3A). Whereas certain organs consistently had low frequencies of trNK cells (spleen and lung), other organs uniformly had elevated frequencies of trNKs (liver, kidney, and pancreas) regardless of background (Fig. 3A, Supplemental Fig. 1B, 1C). The frequency of uterine trNK cells varied dramatically between different strains of mice, with BALB/c and 129 strains of mice having low frequencies of trNK cells compared with B6 and NOD mice (Fig. 3A).

Next, we analyzed the expression of AsGM1 on cNK and trNK cells in various organs and genetic backgrounds. cNK cells uniformly expressed high levels of AsGM1 across all organs analyzed, regardless of strain (Fig. 3B, Supplemental Fig. 1D). In contrast, trNK cells had differential expression of AsGM1, with most trNK cells expressing only low levels of AsGM1 in the kidney, spleen, liver, and lung (Fig. 3B). Whereas a subset of trNK cells had comparable expression of AsGM1 relative to cNKs (e.g., in the liver of B6 mice), the only instance where trNK cells uniformly expressed high levels of AsGM1 comparable to cNK cells was in the uterus of some strains of mice (B6 and 129) (Fig. 3B). Analysis of NK cell subsets using AsGM1 as a primary parameter relative to either CD49a or DX5 further identified CD49a$^+$ trNK cells with low, intermediate, and high levels of AsGM1 expression, varying by organ and genetic background (Supplemental Fig. 2). Taken together, these data show that cNK cells uniformly express high levels of AsGM1 and that trNK cells contain a phenotypically heterogeneous population characterized by variable, but reduced, expression of AsGM1 across multiple organs and strains of mice.

AsGM1 Ab treatment preferentially depletes cNK cells in B6 and BALB/c mice and enhances the relative frequency of trNK cells

Anti–AsGM1 Abs have been commonly used to deplete NK cells across diverse genetic backgrounds. Our data indicate that although AsGM1 is uniformly expressed at a high level in cNK cells, it is variably expressed in trNK cells, with many trNK cells expressing low levels of AsGM1. We next sought to determine whether the presence of AsGM1^{low} trNK cells may reduce the susceptibility of trNK cells to be depleted by AsGM1 Ab treatment in B6 mice. AsGM1 Ab treatment resulted in a pronounced decrease in the frequency and number of total NK cells across multiple organs, consistent with the overall higher frequency of cNK cells relative to trNK cells (Fig. 4A, 4B, Supplemental Fig. 3A). Whereas AsGM1 treatment reduced the number of both cNK and trNK cells (Supplemental Fig. 3B), the magnitude of depletion between cNK cells and trNK cells profoundly differed. Indeed, when we measured the relative frequency of cNK cells and trNK cells, we found that AsGM1 Ab treatment resulted in a pronounced shift in the relative frequency of cNK cells and trNK cells within the kidney, liver, lung, and spleen (Fig. 4A, 4B). These data indicate that in B6 mice trNK cells have reduced susceptibility to AsGM1-based Ab depletion.

Whereas AsGM1 Ab depletion is used in B6 mice, it is often used as a method to validate results obtained by NK1.1 Ab depletion. In contrast, certain strains of mice (e.g., BALB/c) do not express the

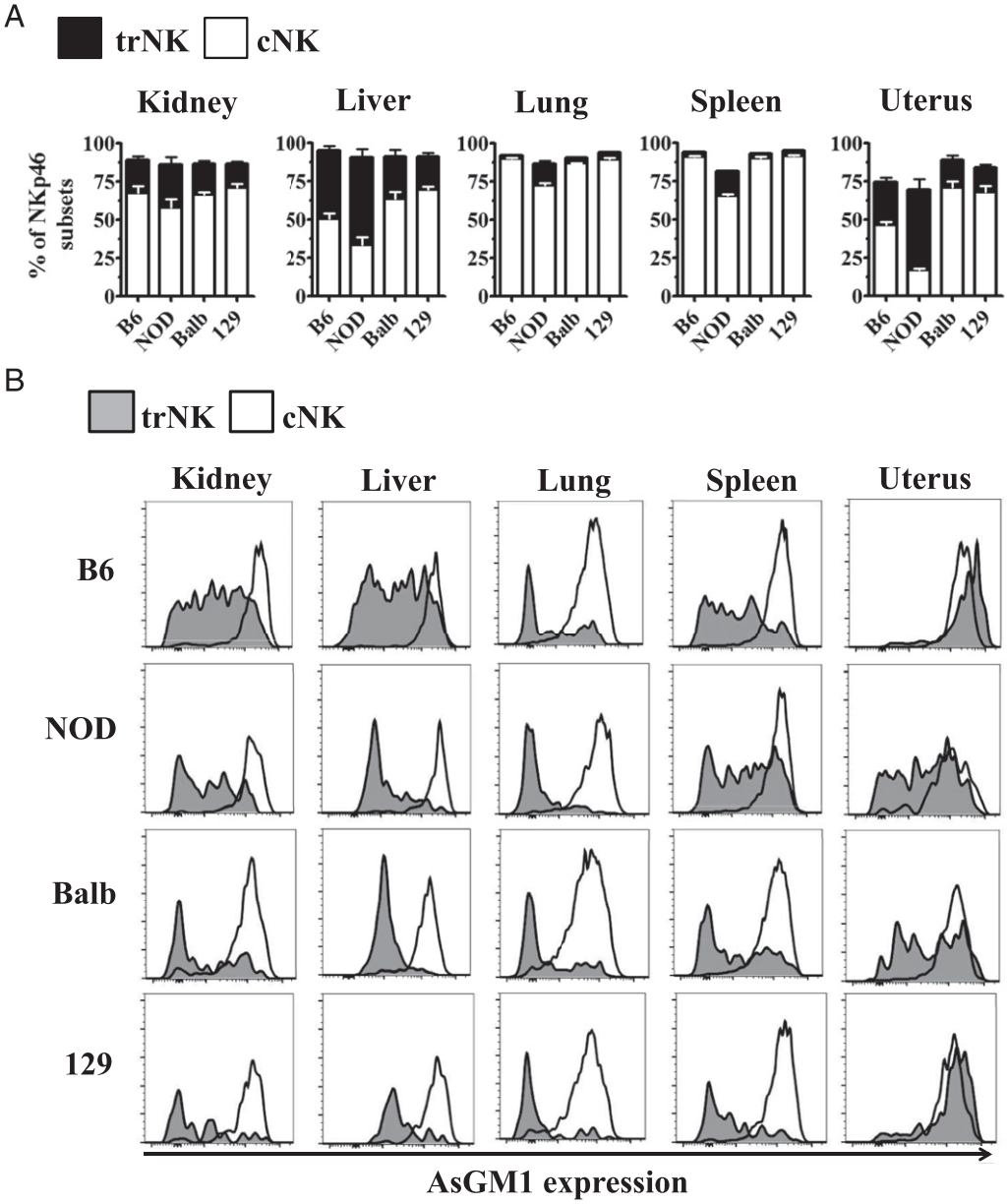

FIGURE 3. cNK and trNK cells have differential expression of AsGM1 across multiple organs and genetic backgrounds. B6, NOD, BALB/c, and 129 mice were analyzed for the frequency of trNK and cNK cell subsets and the AsGM1 expression in these subsets. (**A**) Frequency of trNK and cNK cells was determined in multiple organs in various strains based on NK cells, defined as $CD3^{-}CD19^{-}NKp46^{+}$. (**B**) AsGM1 expression in trNK (shaded gray) and cNK (open black line) cells, with organ analyzed indicated at the top of each column, and genetic background indicated at the left of each row. Each data set has three to five mice per group from independent experiments.

PK136-reactive NK1.1 allele, such that AsGM1 Ab is the primary method to test the role for NK cells in vivo (23). Given that BALB/c mice also contain $AsGM1^{low}$ trNK cells, we next tested the selectivity of AsGM1 Ab depletion on cNK and trNK cells in this strain. AsGM1 Ab treatment again resulted in a large reduction in the frequency of NK cells and an overall decrease in cNK and trNK cell numbers (Supplemental Fig. 3A, 3C), with AsGM1 Ab-treated mice showing a notable skewing in the relative frequency of trNK cells in the liver, lung, and spleen (Fig. 4C, 4D). In total, these data indicate that AsGM1 Ab treatment preferentially depletes cNK cells, resulting in a relative sparing of trNK cells across multiple organs and two common inbred strains of mice.

trNK cells and cNK cells in IRI of the kidney

NK cells have been implicated in IRI in the kidney, but the role of trNK and cNK cells is unclear (45–47). We sought to characterize the dynamics of trNK cells and cNK cells during ischemic injury in the kidney by employing the hanging weight system of IRI, a well-established model of AKI (50). In mice subjected to 30 min of ischemia, kidney function as measured by GFR rapidly declined within 4 h after reperfusion, with maximal loss of kidney function occurring by 24 h (Fig. 5A). We next determined the total number of NK cells at 4, 8, and 24 h after reperfusion and found that there were no significant differences in the total number of NK cells present in the kidney following this acute ischemic injury (Fig. 5B). Given that NK cell numbers did not change dramatically, we next determined whether there were changes in the relative distribution of trNK and cNK cells or their activation phenotype during this acute time course. At 4 and 24 h after reperfusion, there were no changes in the relative distribution of trNK and cNK cells in the kidney (Fig. 5C, 5E). Furthermore, when we analyzed the activation status of trNK cells and cNK

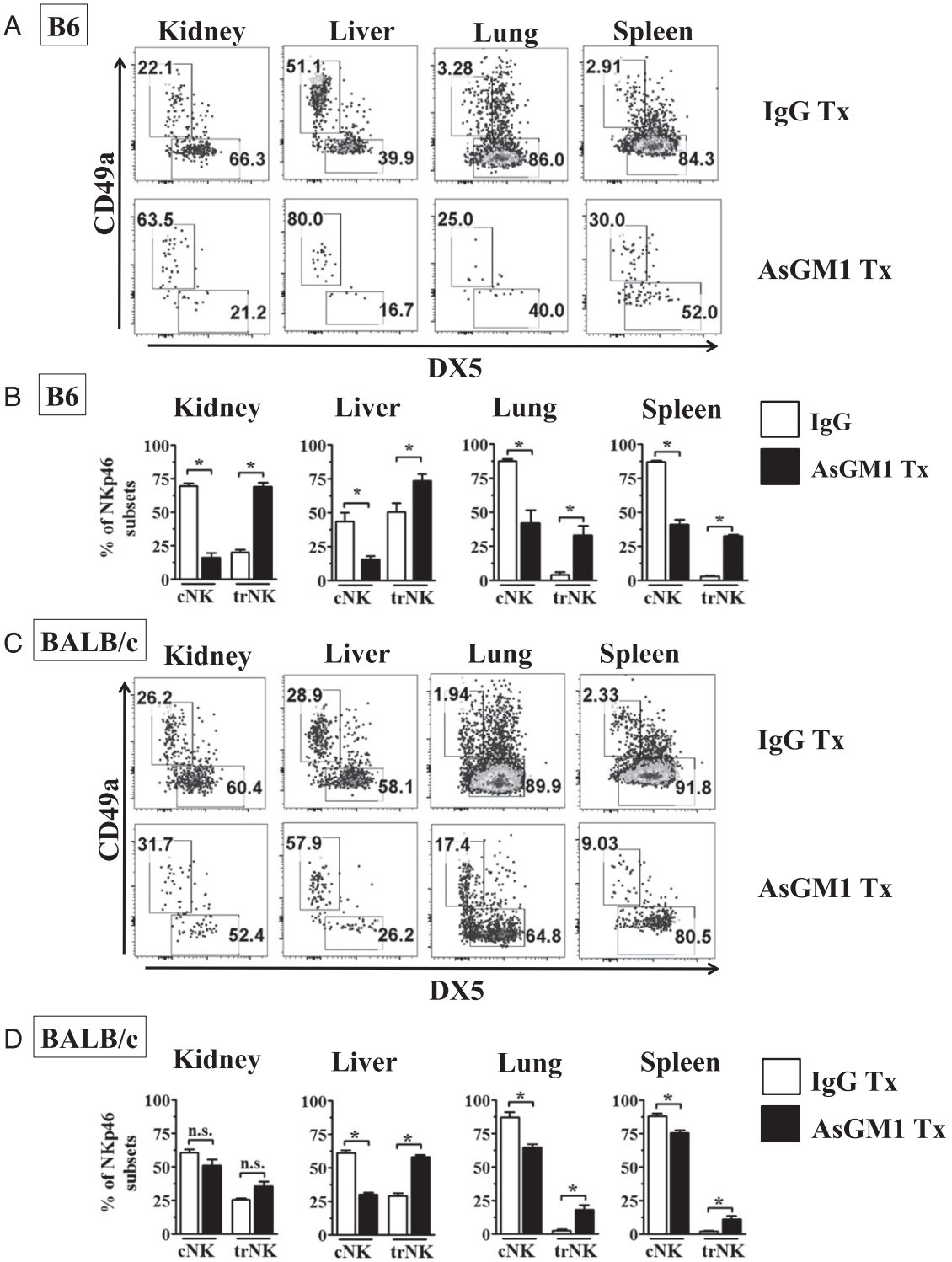

FIGURE 4. AsGM1 Ab treatment preferentially depletes cNK cells in B6 and BALB/c mice to enhance the relative frequency of trNK cells. B6 (**A** and **B**) and BALB/c (**C** and **D**) mice were treated rabbit IgG or AsGM1 Ab day −1, and organs were harvested 1 d later and analyzed for trNK and cNK cells (defined as NKp46$^+$ cells). (A) Representative dot plots of NK cell subsets in B6 kidney, liver, lung, and spleen after IgG (*top row*) or AsGM1 (*bottom row*) treatment. (B) Frequency distribution of NK subsets in B6 mice across multiple organs based on CD49a and CD49b expression treated with IgG or AsGM1 Ab. (C) Representative dot plots of NK cell subsets in BALB/c kidney, liver, lung, and spleen after IgG (*top row*) or AsGM1 (*bottom row*) treatment. (D) Change in frequency distribution of NK subsets in BALB/c mice from multiple organs based on CD49a and CD49b expression treated with IgG or AsGM1. Each data set has three to six mice per group from independent experiments. *$p < 0.05$ as determined by unpaired *t* test, comparing the frequency of either cNK or trNK in IgG- versus AsGM1 Ab–treated mice.

cells during IRI, we found that trNK cells expressed higher levels of CD160, CD44, and TRAIL than did cNK cells at both 4 and 24 h, in both the naive (sham) and ischemic kidney, with no discernable difference in either NK cell subset following ischemic injury (Fig. 5D, 5F). These distinct phenotypes mirrored differences observed in trNK and cNK cells in the steady-state. Taken

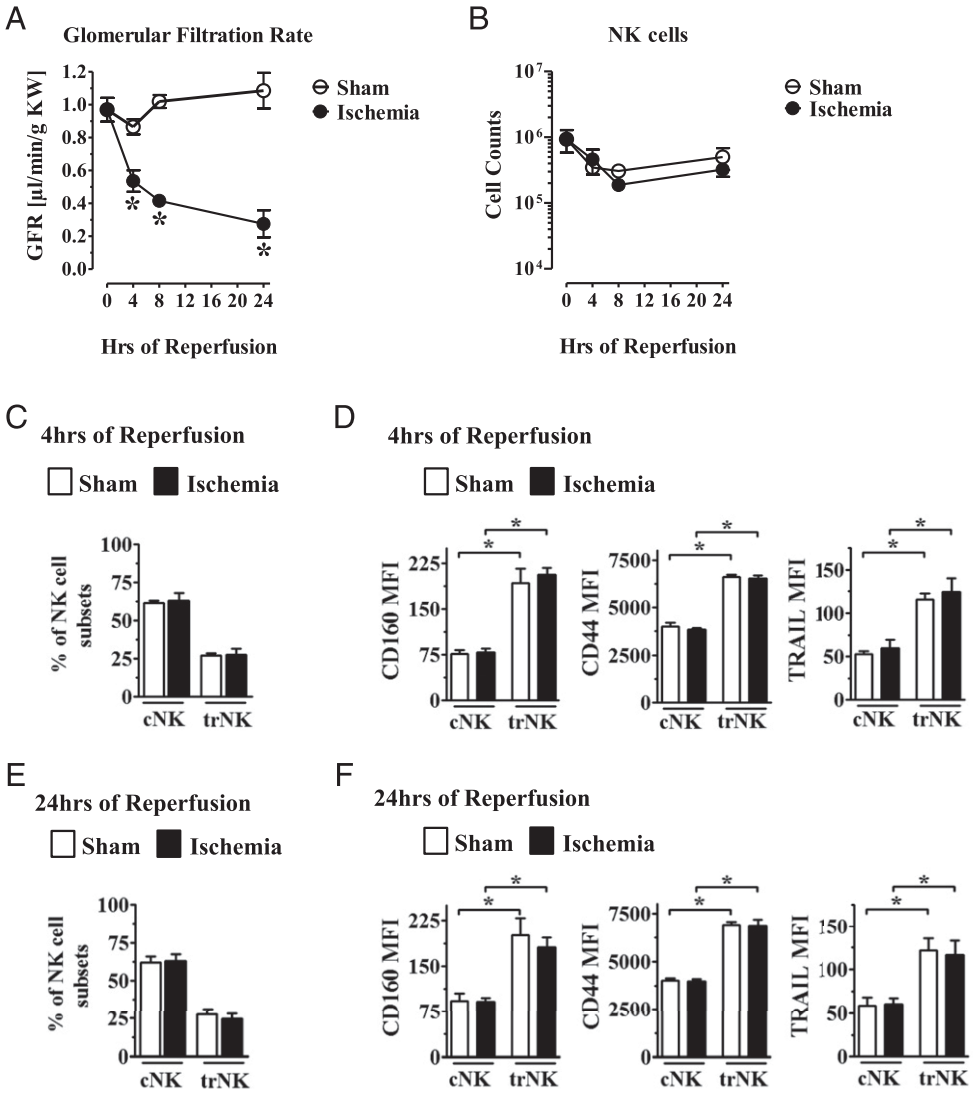

FIGURE 5. IRI in the kidney does not profoundly alter phenotype of trNK and cNK cells. B6 mice were subjected to either sham surgery (open bars) or 30 min of ischemia (filled bars) and analyzed as described. (**A**) After 4, 8, and 24 h of reperfusion, kidney function was measured by GFR. (**B**) B6 mice were subjected to IRI and after 4, 8, and 24 h of reperfusion, NK cells were quantified using Percoll purification and analyzed by flow cytometric analysis by staining for $CD45^+CD3^-NK1.1^+$ expression. (**C**) B6 mice were subjected to sham or 30 min of ischemia and analyzed for the frequency of trNK and cNK cells at 4 h after IRI. (**D**) Flow cytometric analysis of mean expression of activation markers on trNK versus cNK cells at 4 h after IRI in the kidney. Each data set has four to six mice per group. (**E**) B6 mice were subjected to sham or 30 min of IRI surgeries and analyzed for the frequency of trNK and cNK cells at 24 h after IRI. (**F**) FACS analysis of mean expression of activation markers on trNK versus cNK at 24 h after IRI in the kidney. Each data set has four to six mice per group from independent experiments. $*p < 0.05$ as determined by unpaired *t* test, comparing either values from the same time point between sham and ischemia (A), or by one-way ANOVA with significance indicated between cNK and trNK (D and F).

together, these data suggest that trNK and cNK cells have a relatively stable number and cell surface phenotype during acute ischemic kidney injury, with trNK cells showing evidence for a heightened basal activation state.

NK1.1-depleting Ab protects mice from kidney dysfunction during IRI

The use of anti–NK1.1 Ab as a depleting strategy for NK and NKT cells is well established in the field. Whereas anti-NK1.1–depleting studies have been shown to be protective in some studies of ischemic kidney injury (47), the role of NK cells in the hanging weight model of kidney injury remains to be elucidated. To test this, B6 mice were treated with either isotype or anti-NK1.1–depleting Ab (administered on days −3 and −1), subjected to IRI on day 0, and allowed to recover for 1 d. Depletion of NK cells ($CD45^+CD3^-NKp46^+$) was confirmed in the kidney by flow cytometry (Fig. 6A), with NK1.1 Ab treatment resulting in an equivalent depletion of both cNK and trNK cells (Fig. 6B, 6C). Whereas isotype-treated animals subjected to IRI had a pronounced impairment in kidney function as measured by GFR, anti-NK1.1–treated mice showed a clear improvement in kidney function, with GFR values nearly restored to values measured from sham, nonischemic mice (Fig. 6D).

The NK1.1 Ag is expressed by both NK and NKT cells, both of which have been implicated in AKI (45, 47, 57, 58). To test the role of NKT cells in this model, we quantified the number of NKT cells ($CD45^+CD3^+NK1.1^+$) at 4, 8, and 24 h after IRI and found no significant changes in total numbers (Supplemental Fig. 4A, 4B). Importantly, when comparing the number of NK and NKT cells in the ischemic kidney, NK cells were much more abundant than NKT cells (Supplemental Fig. 4C).

Given that the NK1.1 Ab can deplete both NK and NKT cells, we sought to test the impact of NK1.1 Ab treatment on mice genetically

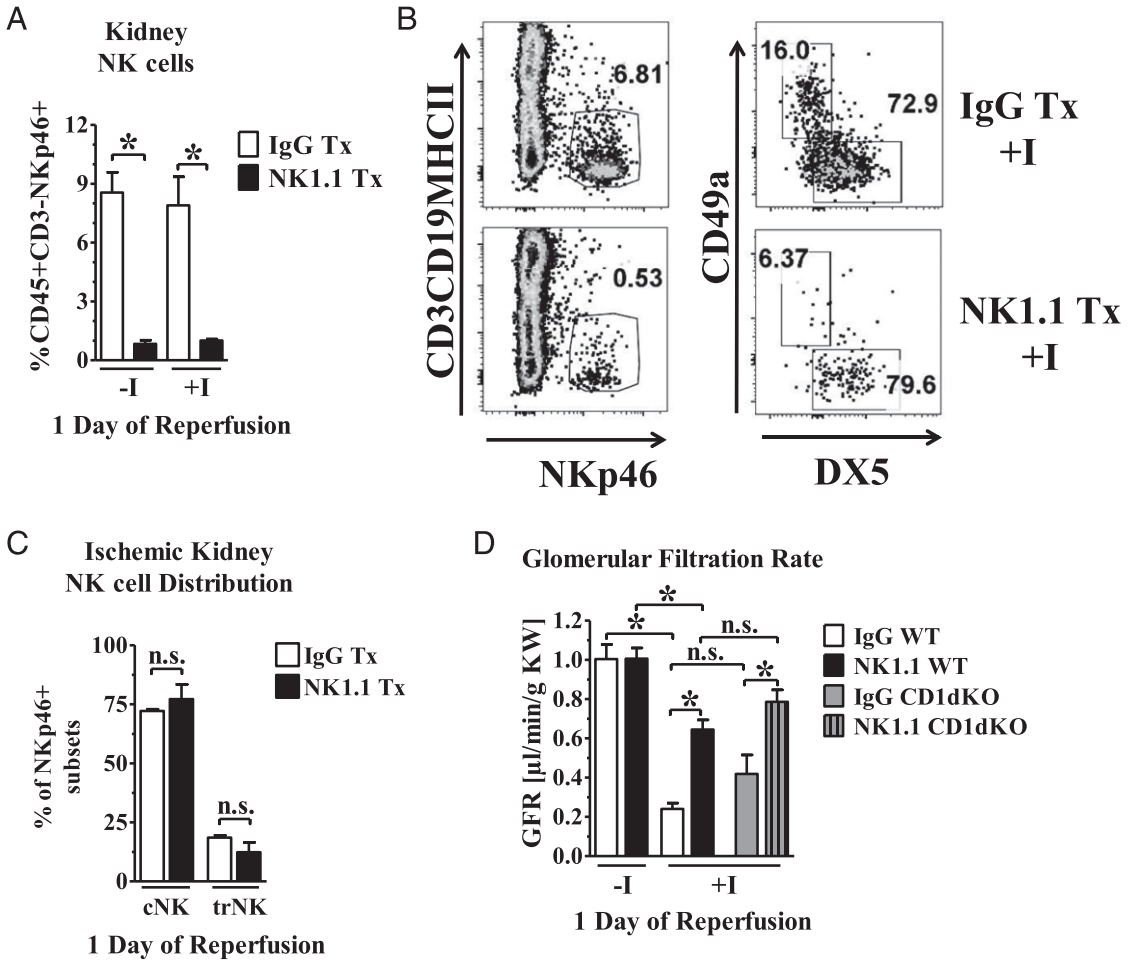

FIGURE 6. NK1.1 Ab treatment effectively depletes trNK and cNK cells in the kidney and attenuates kidney damage. B6 mice were treated with IgG or NK1.1 Ab on days −3 and −1 and subjected to IRI on day 0 and analyzed at day +1. (**A**) The frequency of NK cells in the kidneys of B6 mice that were treated with IgG or NK1.1 Ab and subjected to sham (−I) or IRI (+I) surgeries at 1 d after reperfusion. (**B** and **C**) B6 ischemic kidneys treated with either IgG or NK1.1 Ab were analyzed by flow cytometric analysis for distribution of trNK versus cNK cells, with frequencies plotted in (C). (**D**) Quantification of GFR in B6 and CD1d KO mice that received either IgG or NK1.1 Ab treatment in sham (−I) or ischemic (+I) conditions. Each data set has three to six mice per group from independent experiments. *$p < 0.05$ as determined by unpaired t test (C), comparing IgG- and NK1.1-treated mice or by one-way ANOVA with a Bonferroni posttest correction for multiple comparisons (A and D).

deficient in NKT cells (e.g., Jα18- or CD1d-deficient mice). Whereas mice deficient in the TCR gene segment Jα18 have a deficit in NKT cells, recent studies have shown that these mice also have major alterations in TCR repertoire (59). Based on this, we tested CD1d1 × CD1d2–deficient mice, which are defective in NKT cell development, without other known defects in lymphocyte development (60, 61). As expected, anti–NK1.1 Ab treatment profoundly depleted NK cells in CD1dKO mice (Supplemental Fig. 4D). Whereas isotype-treated CD1dKO mice subjected to ischemia showed decreased GFR, similar to wild-type mice, anti–NK1.1 Ab treatment significantly improved GFR in both CD1dKO and wild-type ischemic mice (Fig. 6D). Based on these data, we conclude that the primary protective effect of anti–NK1.1 Ab treatment results from depletion of NK cells, not NKT cells.

AsGM1 preferentially ablates cNK cells, showing that trNK cells promote AKI

Whereas the above data identify NK cells as mediators of ischemic injury, they do not discriminate between the relative contributions of trNK and cNK cells, because NK1.1 Ab treatment equivalently depletes both NK cell subsets. Given the differential expression of AsGM1 on trNK and cNK cells in the kidney, and their differential susceptibility to AsGM1 Ab depletion, we hypothesized that AsGM1 Ab treatment may allow us to dissect the relative contributions of trNK and cNK cells to ischemic kidney injury. To test this, we treated B6 mice with anti–AsGM1 Ab depletion and subjected these mice to either sham (nonischemia) or ischemic injury. Anti–AsGM1 Ab treatment resulted in a profound reduction of kidney NK cells in these mice, comparable to the magnitude of depletion observed following anti–NK1.1 Ab treatment (compare Figs. 6B and 7A). Consistent with our previous data, anti–AsGM1 Ab resulted in a prominent increase in the relative frequency of trNK cells in the ischemic kidney (Fig. 7A, 7B).

Next, we tested the consequence of Ab treatment of kidney injury and function. First, we examined the consequence of anti–NK1.1 and anti–AsGM1 Ab treatment on the early induction of apoptosis after ischemic injury, measured by active caspase-3 staining at 4 h after reperfusion. These studies showed that anti–NK1.1 Ab-injected mice had a significantly decreased number of apoptotic cells relative to anti–AsGM1 Ab-injected mice (Supplemental Fig. 4E–I). To investigate the consequence of these Abs on kidney function at 24 h reperfusion, we measured kidney function by GFR, a gold standard for kidney function, comparing

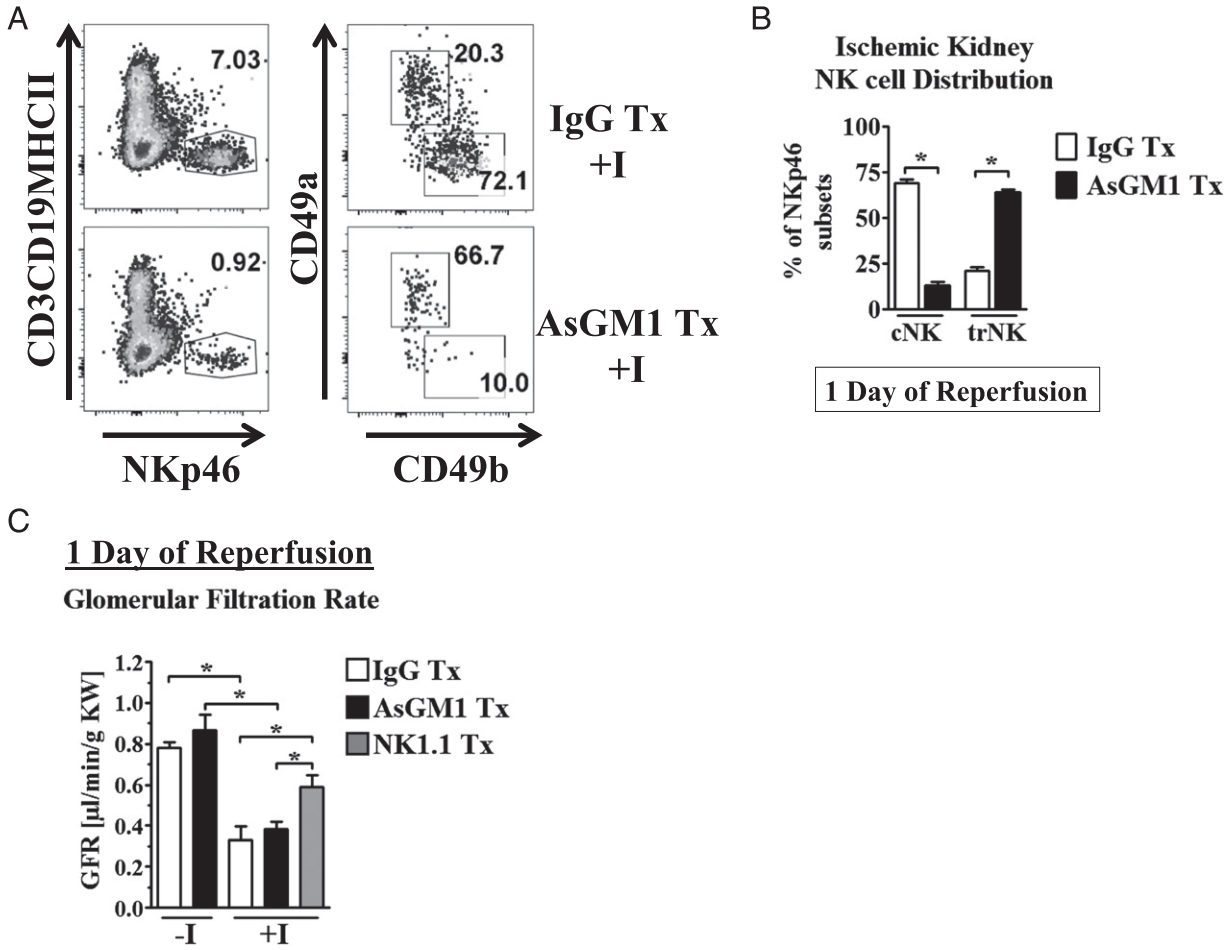

FIGURE 7. AsGM1 Ab preferentially depletes cNK cells, showing that trNK cells promote AKI. B6 mice were treated with IgG or AsGM1 Ab on day −1 or treated with NK1.1 Ab on days −3 and −1 and then subjected to sham (−I) or IRI (+I) surgeries on day 0 and analyzed at day +1. (**A**) Representative dot plots of kidneys 24 h after IRI from B6 mice that were treated with IgG or AsGM1 Ab and analyzed for the frequency of NKp46$^+$ NK cells (*left*) and the frequency of trNK and cNK cells in either IgG-treated (*top*) or AsGM1-treated (*bottom*) mice. (**B**) Quantification of the frequency of trNK and cNK cells after IgG or AsGM1 Ab treatment in ischemic kidneys. (**C**) B6 mice treated with IgG, AsGM1, or NK1.1 Ab were given sham (−I) or IRI (+I) surgeries and GFR was measured 1 d after surgery. Each data set has five to eight mice per group from independent experiments. *$p < 0.05$ as determined by unpaired *t* test (B), comparing IgG- and AsGM1-treated mice or by one-way ANOVA with a Bonferroni posttest correction for multiple comparisons (C).

isotype-, anti–AsGM1 Ab-, and anti–NK1.1 Ab-injected mice. Strikingly, whereas anti–NK1.1 Ab treatment conferred protection, anti–AsGM1 Ab treatment failed to protect mice from IRI despite a similar reduction in overall NK cell percentages (Fig. 7C). Given that anti-AsGM1 poorly depletes trNK cells, in contrast to anti–NK1.1 Ab treatment, these data identify that trNK cells are sufficient to promote IRI in the kidney.

Discussion

NK cells are sentinels in the immune response that rapidly detect changes in the cellular environment to produce cytokines and to induce target cell apoptosis. Whereas NK cells have been implicated in diverse responses (e.g., virus infection, DNA damage, cellular transformation, and IRI), a common trigger of NK cells is the detection of cellular stress, which either increases NK cell activating ligands or decreases NK cell inhibitory ligands (62). Recent data indicate that NK cells not only circulate, but that a subset of trNK cells are retained in situ (42, 43). In the present study, we hypothesized that trNK cells function as important mediators in IRI by being poised for immediate response to tissue injury. To test this, we studied this process in the kidney and found that 1) trNK cells are present in the kidney; 2) trNK cells in the kidney, and other tissues, have reduced expression of AsGM1, a common target in NK cell depletion studies; and 3) anti–AsGM1 Ab depletion preferentially spares kidney trNK cells. Based on this knowledge, we directly compared how two different methods of NK cell depletion that differed in their ability to deplete trNK cells impacted IRI. Our studies show that although trNK cells are numerically minor, these cells are potent mediators of ischemic tissue injury.

NK cells are regulated by the relative engagement of activating and inhibitory NK cell receptors by their respective ligands on neighboring cells (62). A common theme of these responses is that cellular stress in target cells can either increase activating ligands (e.g., MICA in humans and Rae-1 in mice) or decrease inhibitory receptor ligands (e.g., MHC class I). In this context, IRI, the process of transient oxygen insufficiency (i.e., hypoxia) to a tissue, is associated with the induction of cellular stress markers such as MICA (in humans) and Rae-1 (in mice), which are potent activating ligands for activating NK cells (45, 63–65), placing NK cells as central mediators of ischemic tissue injury.

In the present study, we examined the role of NK cells in IRI by using a mouse model of ischemic AKI, a common human pathology characterized by ischemia that can result from various etiologies, including diabetes, major surgery, sepsis, and drug toxicity (44). Whereas previous studies highlighted the importance of NK cells in

ischemic AKI using patch-clamping models of AKI, which are associated with intrarenal coagulation (45–47), the role of cNK and trNK cells in ischemic injury had not yet been studied.

Our identification of trNK cells as an important cell type in mediating IRI damage emphasizes how local NK cell responses can trigger tissue injury in the absence of NK cell recruitment. It is also notable that trNK cells express low levels of two inhibitory receptors, KLRG1 and CD244, suggesting that trNK cells may have an increased propensity toward cytotoxicity resulting from decreased inhibitory signaling. Importantly, KLRG1$^-$ NK cells have been reported in other studies that were not cNK cells (CD3$^-$ NK1.1$^+$DX5$^-$), suggesting that in some contexts, absence of KLRG1 may be another marker for trNK cells (66–68). Additionally, CCR2 was exclusively on a subset of CD49a$^+$ trNK cells in unmanipulated and ischemic kidney (data not shown), consistent with reports of CCR2$^+$ NK cells during lung inflammation (39); these data suggest that trNK cells may play a role in the CCL2/CCR2 chemokine axis important for monocyte recruitment. Note that whereas our studies highlight a role for trNK cells in the early stages of acute ischemic injury, at this time we have not focused on later stages of ischemic injury when recruitment of cNK cells may further enhance tissue damage (29).

AsGM1 was identified as a NK cell marker >30 y ago (69, 70), and depleting regimens using anti-AsGM1 Ab have been critical in showing the requirement of NK cells in diverse experimental settings (71–76). Indeed, whereas AsGM1 Ab depletion is not uniquely restricted to NK cells (e.g., see Ref. 22), this method remains in use today, both to deplete NK cells in mouse strains that do not express the PK136-reactive NK1.1 allele (e.g., BALB/c), and as a complementary approach to anti–NK1.1 Ab studies in NK1.1-expressing mice (e.g., B6).

Our data indicating differential expression of AsGM1 on subsets of trNK cells provide a new level of consideration in the use of AsGM1 Ab depletion. First, by comparing and contrasting the impact of AsGM1- and NK1.1-based depletion methods on trNK and cNK cells and IRI, our studies illustrate a new approach to distinguish trNK and cNK cell functions in vivo. Second, our data identify an unanticipated heterogeneity of AsGM1 expression among trNK cells. Whereas AsGM1 Ab significantly depletes cNK cells, this Ab regimen is unable to deplete at least a subset of trNK cells. As such, AsGM1 Ab results in incomplete depletion of trNK cells, an especially important consideration when studying tissues enriched in trNK cells (e.g., kidney, liver, pancreas, and uterus). Notably, a simple approach to understanding the utility and limitations of AsGM1 Ab depletion studies can be obtained by analyzing AsGM1 expression on cNK and trNK cells in the organ of interest. Our studies also emphasize the importance of analyzing NK cell depletion studies not only in the blood (which lacks trNK cells) but also in situ. Notably, analysis of NK cell depletion in the spleen and/or peripheral blood (which is devoid of trNK cells) or use of DX5 as an NK marker (which is not expressed on trNK cells) may significantly overestimate the efficiency of Ab-based depletion in various tissues by failing to consider trNK cell reservoirs. Given these caveats, we consider it likely that the functional contribution of trNK cells remains underappreciated, especially in studies based solely on AsGM1-depleting Abs.

Depending on the organ and strain of mouse, trNK cells represent a sizable fraction of the NK cell pool with the long-term capacity to monitor and influence tissue function. Although the function of trNK cells remains an active area of investigation, trNK cells appear to have unique capabilities, demonstrated both by their ability to directly facilitate delayed type hypersensitivity reactions (42) and by their unique cytokine and cytolytic profile (43). Given that trNK cells are retained in tissue and poised for activation, our data identify trNK cells as an early player in the IRI response in the kidney. Although future studies are required to determine the functional mechanisms by which trNK cells promote IRI, it is clear that trNK cells are sufficient to induce tissue damage even after AsGM1 Ab depletion reduced the overall number of trNK cells.

Based on these data, we hypothesize that trNK cells may be major mediators of ischemic injury in multiple organs. Curiously, trNK cells have tissue-specific differences in terms of their development, with T-bet–dependent liver and skin trNK cells, in contrast to T-bet– and NFIL3-independent kidney and uterine trNK cells (43). Although developmental and functional commonalities between kidney and uterine trNK cells remain to be further explored, it is notable that uterine trNK cells appear to regulate spiral artery arborization in the uterus (77). It is tempting to speculate that kidney trNK cells may also be involved in vascular changes, whether during ischemic injury or during development.

An important future goal in understanding trNK cells will be to determine the functional heterogeneity of trNK cells, both within an individual tissue and between different tissue types. Based on our studies, we propose that AsGM1 expression can serve as one additional parameter that may provide insights into trNK cell subsets. Examples of this include the heterogeneity of AsGM1 expression within trNK cells in the kidney, with some cells expressing little to no AsGM1 in contrast to other AsGM1^{high} trNK cells. One notable exception to the heterogeneous, reduced expression of AsGM1 on trNK cells is in uterine trNK cells in B6 mice, consistent with an increasing literature suggesting unique developmental and functional properties of uterine NK cells (43, 77, 78). Whereas the biologic consequence of differential expression of AsGM1 remains unknown, in the future it will be important to determine whether AsGM1 serves as an indicator of trNK maturation, localization, and/or directly shapes the functionality of trNK cells.

trNK cells are a recently described subset of NK cells, whose functions remain an active area of investigation. By studying trNK cells in the kidney and other organs, our data identify an unanticipated differential expression of AsGM1 on trNK cells in multiple organs and strains of mice. These data emphasize that AsGM1 expression may provide a powerful new approach by which to gain fundamental new insights into NK cell development and function. Furthermore, to our knowledge, our studies provide the first evidence that trNK cells play a central role in ischemic tissue injury.

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Disclosures

The authors have no financial conflicts of interest.

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