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T helper type 2-polarized invariant natural killer T cells reduce disease severity in acute intra-abdominal sepsis

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Summary

Sepsis is characterized by a severe systemic inflammatory response to infection that is associated with high morbidity and mortality despite optimal care. Invariant natural killer T (iNK T) cells are potent regulatory lymphocytes that can produce pro- and/or anti-inflammatory cytokines, thus shaping the course and nature of immune responses; however, little is known about their role in sepsis. We demonstrate here that patients with sepsis/severe sepsis have significantly elevated proportions of iNK T cells in their peripheral blood (as a percentage of their circulating T cells) compared to non-septic patients. We therefore investigated the role of iNK T cells in a mouse model of intra-abdominal sepsis (IAS). Our data show that iNK T cells are pathogenic in IAS, and that T helper type 2 (Th2) polarization of iNK T cells using the synthetic glycolipid OCH significantly reduces mortality from IAS. This reduction in mortality is associated with the systemic elevation of the anti-inflammatory cytokine interleukin (IL)-13 and reduction of several proinflammatory cytokines within the spleen, notably interleukin (IL)-17. Finally, we show that treatment of sepsis with OCH in mice is accompanied by significantly reduced apoptosis of splenic T and B lymphocytes and macrophages, but not natural killer cells. We propose that modulation of iNK T cell responses towards a Th2 phenotype may be an effective therapeutic strategy in early sepsis.

Keywords: acute intra-abdominal sepsis, glycolipids, invariant natural killer T cells, Th2 response

Introduction

Sepsis is an overwhelming systemic inflammatory response to infection [1] that remains the leading cause of death among patients in intensive care units [2,3], with a mortality rate approaching 35% [4]. Additionally, close to one-third of mortalities occurs within the first 72 h of admission [2,5], characterized by an excessive proinflammatory cytokine response that leads to multiple organ failure and death [6–8]. Despite a dramatic increase in the incidence of sepsis over the past 30 years [2], there are no selective therapeutic agents available to reduce the morbidity and mortality of this illness [4,9].

There has been increasing interest in the role of invariant natural killer T (iNK T) cells in regulating host cytokine responses [10–14] and bridging the innate and adaptive immune arms of immunity during sepsis [15–17]. iNK T cells are an evolutionarily conserved subset of T cells that are characterized by the expression of an invariant T cell receptor (TCR)-α chain (Vα24-Jα18 in humans and Vα14-Jα18 in mice), and reactivity to self- and microbial-derived glycolipids presented by the monomorphic major histocompatibility complex (MHC) class I-like molecule CD1d [13,18]. Once activated, iNK T cells rapidly secrete pro- and/or anti-inflammatory cytokines that can subsequently shape the course and nature of immune responses in a variety of disease states [13,14,18].

Although a number of studies have established iNK T cells as potent initiators of an excessive proinflammatory response that promotes lethality in animal models of sepsis [19–21], little is known about the role of these cells in the context of human sepsis. Given their extensive immunoregulatory roles, the manipulation of iNK T cells may provide a potential therapeutic strategy for the treatment of sepsis [22]. In particular, the availability of glycolipids that exhibit distinct immunomodulatory properties allows for a

more comprehensive examination of the function of iNK T cells, and the consequences of their manipulation in disease states [11,23–26]. We therefore sought to characterize the potential of iNK T cell glycolipid agonists to modulate disease severity in an experimental mouse model of acute intra-abdominal sepsis (IAS) [27], and to assess the frequency of iNK T cells in patients with sepsis/severe sepsis.

Materials and methods

Patients and clinical samples

Approval of the study protocol for both the scientific and ethical aspects of this work was obtained from the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects (Approval number: REB103036). This study was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki [28].

Patients aged 18 years and older with a diagnosis of sepsis, severe sepsis or septic shock upon admission to the Medical–Surgical Intensive Care Unit (MS-ICU) at London Health Sciences Centre (LHSC) University Hospital and the Critical Care and Trauma Centre at LHSC Victoria Hospital were recruited prospectively from 1 July 2012 to 31 December 2012. The first day following ICU admission was considered day 1 in the analysis. Sepsis, severe sepsis and septic shock were defined according to established guidelines [29]. Severity of illness was assessed on the Acute Physiology and Chronic Health Evaluation II (APACHE II) score for the first 24 h following diagnosis [30,31]. Exclusion criteria were the presence of immunodeficiency or concomitant immunosuppressive therapy, pregnancy, Do Not Resuscitate (DNR) status and cardiac arrest. Informed consent was obtained directly from each patient or his or her legal representative before enrolment.

Standard cultures in biological samples guided by the presumptive source of the septic insult were performed to assess the presence of bacterial and fungal infection. Species identification was conducted by the LHSC Clinical Microbiology Laboratory. Potentially contaminant microorganisms were not considered. Blood was collected in heparinized vacuum tubes upon admission of the patient to the MS-ICU, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (Invitrogen, Carlsbad, CA, USA) gradient centrifugation, according to published methods [32]. PBMCs were subsequently stained for detection of iNK T cells by flow cytometry.

Animals

All animal experimentation was carried out in strict accordance with the recommendations and guidelines established by the Canadian Council on Animal Care, as well as institutional regulations. Mouse studies were performed according to a protocol approved by the Western University Animal Care and Veterinary Services (Approval number: 2008-034-01).

Female 10–12-week-old mice were used for all experiments. All mice were maintained in a specific pathogen-free facility at Western University. C57BL/6 (B6) mice were obtained from Charles River Canada Inc. (St Constant, Quebec, Canada). Jα18$^{-/-}$ mice, which are on a B6 background and lack iNK T cells [33], were obtained from Dr Luc Van Kaer (Vanderbilt University, Nashville, TN, USA). Green fluorescent protein (GFP)-expressing transgenic mice are B6 background mice with omnipresent enhanced GFP expression under the β-actin promoter, and were kindly provided by Dr Stephen Kerfoot (Western University, London, ON, Canada).

Murine intra-abdominal sepsis (IAS) model

We utilized a faecal-induced peritonitis model of acute IAS in mice, as published previously by our group [27]. Briefly, stool from the caecum of euthanized naive B6 mice was expressed through an enterotomy, homogenized and suspended in 0·9% normal saline (NS) to produce a faecal slurry at a final concentration of 90 mg/ml, for use in experiments on the same day. Mice were injected intraperitoneally with 500 µl faecal slurry or 0·9% NS (sham group). The mice were monitored and scored by two independent investigators – one of whom was blinded to the treatment – every 2–3 h for the first 12 h, and then every 1–2 h thereafter. A murine sepsis score (MSS) was assigned to each mouse based on activity, eye movement, response to exogenous stimuli, breathing rate and pattern and degree of piloerection [27]. As we have demonstrated previously [27], the MSS is a reliable and robust scoring system that has excellent inter-rater reliability (intraclass coefficient = 0·96), high internal consistency (Cronbach's $\alpha = 0{\cdot}92$) and correlates with levels of certain proinflammatory cytokines such as interferon (IFN)-γ and interleukin (IL)-1β during the experimental time-line. Mice were euthanized at 24 h, or earlier if they were deemed to be in significant distress based on our scoring model. Euthanasia was carried out as described above. At the time of euthanasia, intracardiac blood was drawn into 1·5 ml microcentrifuge tubes (BD Biosciences, San Jose, CA, USA) and centrifuged for serum at 1000 ***g*** for 15 min at 4°C.

Glycolipids

Lyophilized OCH was generously provided by the National Institutes of Health (NIH) Tetramer Core Facility (Emory University, Atlanta, GA, USA). Each vial containing 0·2 mg of OCH was solubilized in 1 ml of sterile distilled water, and stored as aliquots at 4°C until use. KRN7000 [α-galactosylceramide (α-GalCer), C26:0/C18:0)] was purchased from Funakoshi Co. Ltd (Tokyo, Japan), solubilized

at 1 mg/ml in dimethylsulphoxide (DMSO) and stored as aliquots at −20°C until use [34]; the control vehicle was 2% DMSO in phosphate-buffered saline (PBS). C20:2 was synthesized and used as published previously [35,36]. For *in vivo* experiments, mice were injected intraperitoneally (i.p.) with a single dose of glycolipid (4 μg/dose) [35] within 20 min after induction of IAS.

Antibodies

For mouse studies, allophycocyanin (APC)-conjugated PBS-57-loaded and -unloaded CD1d tetramers for staining mouse iNK T cells were kindly provided by the NIH Tetramer Core Facility [35]. Fluorescein isothiocyanate (FITC)-conjugated anti-TCR-β, APC-conjugated F4/80, APC-conjugated B220 and phycoerythrin (PE)-conjugated NK1·1 were purchased from eBiosciences (San Diego, CA, USA) or BD Biosciences. For human studies, APC-conjugated PBS-57-loaded and -unloaded CD1d tetramers for staining human iNK T cells were kindly provided by the NIH Tetramer Core Facility, while FITC-conjugated anti-CD3 (SK7), and PE-conjugated anti-CD56 (B159) were purchased from BD Biosciences.

Determination of microbial growth from tissue homogenization and peripheral blood

Whole hearts, lungs (left and right), kidneys (left and right), spleen and liver were removed from euthanized mice and homogenized in 5 ml of phosphate-buffered saline (PBS). Homogenates were serially diluted 1:10 in PBS and plated on brain heart infusion (BHI) agar. Plates were grown aerobically at 37°C overnight to determine tissue colony-forming units (CFU). Intracardiac blood (10 μl) was collected in a heparinized syringe from the right ventricle, serially diluted 1:10 with PBS, and plated on BHI agar to determine blood CFU.

Preparation of murine hepatic, splenic and omental cell suspensions

To obtain hepatic lymphoid mononuclear cells (MNCs), mice were euthanized and livers were flushed with sterile PBS before they were harvested and pressed through a 40-μm nylon mesh. The resulting homogenate was washed in cold PBS, resuspended in a 33·75% Percoll PLUS solution (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and spun at 700 ***g*** for 12 min at room temperature. The pelleted cells were then treated with ammonium–chloride–potassium (ACK) lysis buffer to remove erythrocytes and washed in cold PBS prior to staining. To obtain omental lymphoid MNCs, the spleen, pancreas and omentum were removed *en-bloc* and suspended in ice-cold PBS. The omenta floated above the spleen–pancreas complex and were removed and processed similar to the liver. Spleens were processed with a tissue homogenizer, and the resulting homogenate was washed in cold PBS. The pelleted cells were treated with ACK lysis buffer for 4 min to remove erythrocytes, and washed in cold PBS prior to staining.

Adoptive transfer of iNK T cells into Jα18$^{-/-}$ mice

Hepatocytes and splenocytes were isolated as described previously from transgenic GFP mice. CD4$^+$ T cell populations were obtained using the EasySep® mouse CD4$^+$ T cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada), as per the manufacturer's instructions. iNK T cells were further enriched by sorting with anti-CD3 and anti-CD1d tetramer on a FACSAriaIII flow cytometric cell sorter (London Regional Flow Cytometry Facility, London, ON, Canada). Cell populations were used only when purity was >95% as determined by flow cytometry. For adoptive transfer experiments, 5×10^5 iNK T cells were transferred intravenously (i.v.) into Jα18$^{-/-}$ mice. Eighteen hours after the transfer, mice were given IAS and monitored as already described.

Flow cytometry

Mouse hepatic, splenic and omental cells (1×10^6) and human PBMCs (1×10^6) were washed with cold fluorescence activated cell sorter (FACS) buffer [PBS + 2% fetal bovine serum (FBS) + 0·1% sodium azide] and incubated with 5 μg/ml anti-mouse CD16/CD32 mAb (clone 2·4G2, Fc-block; eBiosciences) for 20 min on ice before staining with fluorescent monoclonal antibodies (mAbs) diluted in FACS buffer at 4°C for 30–40 min. Cells were then washed and flow cytometry was performed using FACSCanto II (BD) with FlowJo software (Treestar, Ashland, OR, USA). The gating strategy used for the analysis of apoptotic and necrotic cells is shown in Supporting information, Fig. S1.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from hepatic, splenic and omental tissues using the TRIzol reagent (Invitrogen) and resuspended in nuclease-free water (Invitrogen). Quality control of samples was carried out using a Nanodrop ND-1000 spectrophotometer. cDNA was prepared using 500 ng of RNA by Superscript III RNase H$^-$ reverse transcriptase with oligo dT priming (Invitrogen). Quantitative real-time PCR reactions were carried out in triplicate from every transcription reaction using the ABI Prism 7900HT apparatus (Perkin Elmer, Waltham, MA, USA) with Taqman probes (Invitrogen). The sequences of the primers and Taqman probes used in this study were as follows: Vα14: 5′-TGGGAGATACTCAGCAACTCTGG-3′; Jα18: 5′-CAGGTATGACAATCAGCTGAGTCC-3′; and Vα14 probe FAM: 5′-FAM-CACCCTGCTGGATGACACTGC

CAC-TAMRA-3′. Quantitative analysis was performed by the comparative ΔΔCt method by using the Taqman glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression assay (Invitrogen) as an internal control. Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines were followed [37].

Enzyme-linked immunosorbent assays (ELISA)

Cytokine concentrations were determined by commercially available specific ELISA assays for IL-4 and IFN-γ (Ready-Set-Go kits; eBioscience). Optical densities (ODs) were measured on a Benchmark Microplate Reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm for both cytokines.

Multiplex cytokine quantification assay

Serum was analysed by bead-based multiplex assay for 32 different cytokines, chemokines and growth factors (Eve Technologies, Calgary, Alberta, Canada), including granulocyte–colony stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, keratinocyte chemoattractant (KC), leukaemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), monocyte chemotactic protein (MCP)-1, monocyte–colony-stimulating factor (M-CSF), monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, regulated on activation, normal T cell expressed and secreted (RANTES), tumour necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Multiplex data was visualized using a cytokine heat map that was generated using Matrix2png [38].

Histological analysis and TUNEL staining

Liver, spleen and omentum from sham B6 mice, and vehicle-, OCH- and KRN7000-treated septic B6 mice were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) or subjected to terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining using a commercially available kit (Calbiochem, Billerica, MA, USA). At least four stained sections per spleen from four animals per treatment group were examined and scored for apoptosis by a board-certified veterinary pathologist (I. W.) who was blinded to the study design. Apoptosis was defined histologically by the presence of cell clusters with nuclear shrinkage (karyorrhexis), dark eosinophilic cytoplasm, intact plasma membrane and relative paucity of surrounding inflammatory cells within the splenic follicles on H&E staining [39]. Scores assigned to each animal were as follows: 0 for complete absence of apoptosis; 1 for mild presence of apoptosis (0–15% per follicle); 2 for moderate apoptosis (16–30% per follicle); and 3 for severe apoptosis (31–45% per follicle). Photomicrographs were taken using a Nikon DS-Fi1 digital camera using NIS-elements software.

Statistical analysis

For murine experiments, statistical comparisons were performed using analysis of variance (ANOVA) or Mann-Whitney *U*-test (GraphPad Prism). Survival curves were calculated by the Kaplan–Meier method. For cytokine analysis, results from multiple experiments were pooled and analyzed by one-way ANOVA with *post-hoc* comparisons using Tukey's tests.

For human subjects, differences between groups were assessed using the Mann–Whitney *U*-test or χ^2 test for continuous and categorical variables, respectively. Survival curves were calculated by the Kaplan–Meier method and compared by log-rank test. In all analyses, $P < 0{\cdot}05$ was considered statistically significant.

Results

Peripheral blood iNK T cells are elevated in patients with sepsis/severe sepsis

We first sought to determine if patients with sepsis had an altered frequency of iNK T cells in their peripheral blood compared to non-septic patients. We prospectively evaluated 30 patients who were admitted to LHSC Critical Care and Trauma Centre for sepsis or non-sepsis-related critical illness; 23 patients were diagnosed with sepsis/severe sepsis, while seven patients were non-septic trauma patients (Table 1). In the non-septic group, three patients (43%) had sustained traumatic head injuries and four patients (57%) had emergency surgery for trauma (two liver resections; one abdominal aortic surgery; one spine stabilization operation). Groups were similar in age and severity of illness, as calculated by the APACHE II score [30]. However, the gender distribution was significantly different between the two groups, with a preponderance of males in the non-septic group ($P < 0{\cdot}0001$). Most of the patients in the septic group had intra-abdominal sepsis (44%) or lower respiratory tract infections (39%), as confirmed by diagnostic tests. In 30 and 17% of septic patients, respectively, a single Gram-positive or Gram-negative pathogen was identified, while multiple organisms were identified in 30% of the septic group. In 17% of septic patients, the microbial agent was not identified, while one patient (4%) had fungal candidaemia (Table 1).

When lymphocyte subpopulations were assessed by flow cytometry and compared, the septic group had a higher

Table 1. Demographics and clinical characteristics of patients with and without sepsis.

Demographic and clinical characteristics	Non-septic ($n = 7$)	Septic ($n = 23$)	*P*-value
Median age (years)	61	59	0·433
Gender, *n* (%)			<0·0001
Male	6 (85)	13 (57)	
Female	1 (15)	10 (43)	
Mean APACHE II score, *n*	23	16	0·377
Disease severity on admission, *n* (%)			
Sepsis	–	16 (69)	–
Severe sepsis	–	7 (31)	–
Septic shock	–	0 (0)	–
Source of infection, *n* (%)			
Intra-abdominal	–	10 (44)	–
Pneumonia	–	9 (39)	–
Other[†]	–	4 (17)	–
Documented microbial agent, *n* (%)			
Gram-positive	–	7 (30)	–
Gram-negative	–	4 (17)	–
Fungi	–	1 (4)	–
Polymicrobial	–	7 (30)	–
None/unknown	–	4 (17)	–

[†]Includes patients with skin and soft-tissue infections (2) and urosepsis (2). APACHE II = Acute Physiology and Chronic Health Evaluation II.

median percentage of T cells among total lymphocytes (Table 2; Fig. 1a,b). Moreover, the iNK T:T cell ratio was significantly higher in the septic group (Table 2). Patients in the septic group stayed in hospital for a significantly longer time, although in-hospital mortality was similar between the two groups (Fig. 1c).

iNK T cells are pathogenic in intra-abdominal sepsis

Given our finding of elevated iNK T cell proportions in human sepsis/severe sepsis, and the studies that have demonstrated the pathogenicity of iNK T cells in animal models mimicking chronic polymicrobial sepsis [15,22], we studied iNK T cells in a well-controlled mouse model of acute IAS [27]. This particular model was chosen for several reasons. First, it represents a validated model of acute generalized peritonitis [40], a symptom that affects more than 75% of patients who are admitted to the hospital with intra-abdominal sepsis [41]. Secondly, our model results in the rapid and sustained activation of innate immune response pathways [27,40], facilitating further study into iNK T cells and their interaction with the innate and adaptive immune systems. Because iNK T cells can rapidly produce pro- and/or anti-inflammatory cytokines in response to stimuli and shape the subsequent immune responses in various diseases [42,43], we hypothesized that these cells would affect disease severity and survival in IAS. Compared to B6 mice, we observed a significant reduction in sepsis severity (Fig. 2a) and mortality (Fig. 2b) in Jα18$^{-/-}$ mice, which lack iNK T cells [33]. Whereas an intraperitoneal injection of a

Table 2. Comparison of outcomes among patients with and without sepsis.

Variable	Non-septic	Septic	*P*-value
Median white blood cell count ($\times 10^9$/l)	10·6	11·5	0·182
Lymphocytes[†], %	16·2	17·6	0·252
Lymphocyte subsets[‡], %			
T cells	36·7	57·8	0·039
NK cells	5·19	12·25	0·274
iNK T cells	0·0041	0·0057	0·138
iNK T:T cell ratio	0·009	0·020	0·047
Mean hospital stay (range), days	12·8 (0–38)	25·2 (4–55)	0·045
In-hospital mortality, *n* (%)	3 (43)	5 (28)	0·955
Cause of death, *n* (%)			0·293
Multi-organ failure	1 (14)	4 (17)	
Cardiac arrest	1 (14)	0 (0)	
Withdrawal of care	1 (14)	1 (4)	

[†]Expressed as a percentage of the total sample analysed on flow cytometry. [‡]Expressed as a percentage of lymphocytes. Median populations are presented. iNK T = invariant natural killer T cells.

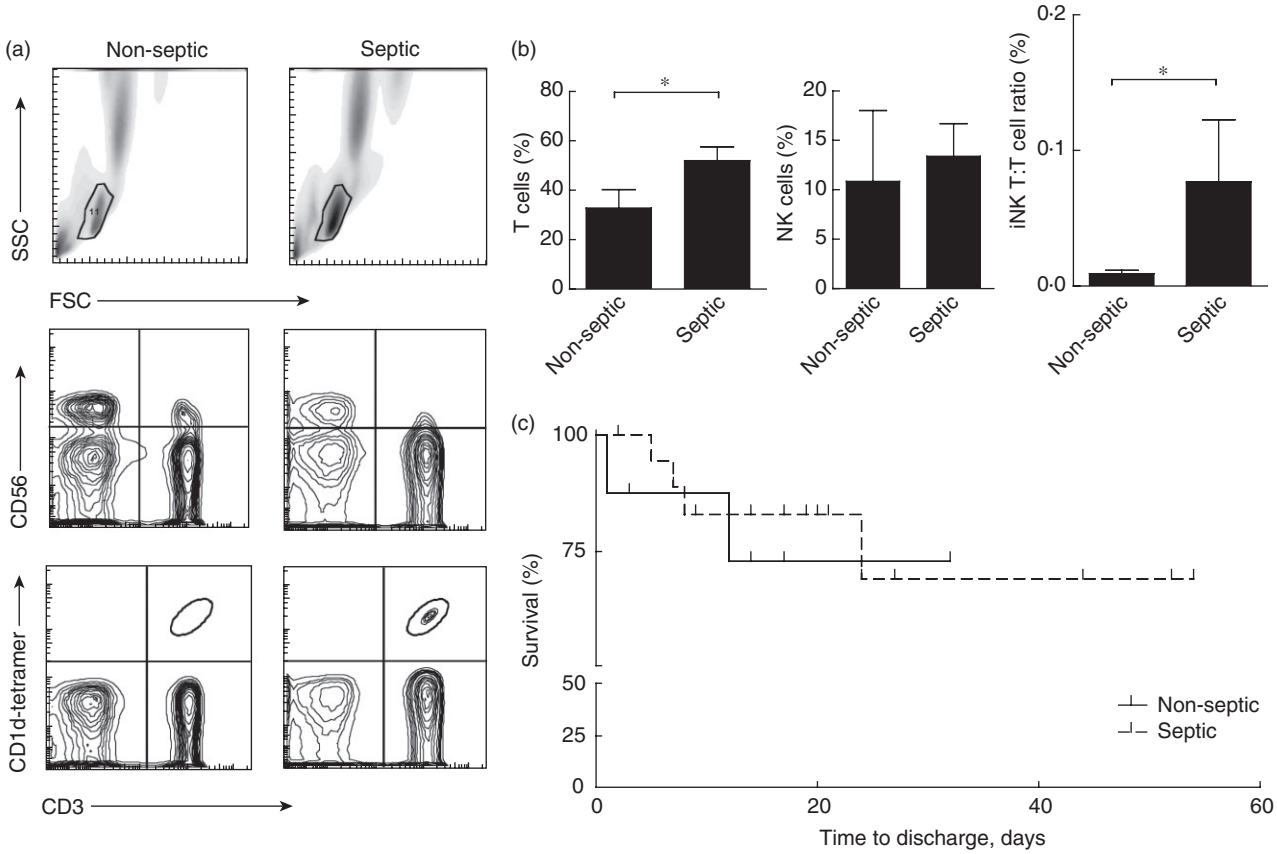

Fig. 1. Characterization of invariant natural killer T (iNK T) cell populations in the blood samples of critically ill patients. (a) Representative flow cytometry plots of peripheral blood sampled from a septic and non-septic patient. (b) Histograms (median ± standard error of the mean) comparing frequency of T cells, NK cells and iNK T:T cell ratios in septic and non-septic patients in the intensive care unit. $^{*}P < 0{\cdot}05$ by Mann–Whitney *U*-test. (c) Kaplan–Meier survival curves from time of blood collection to time of discharge.

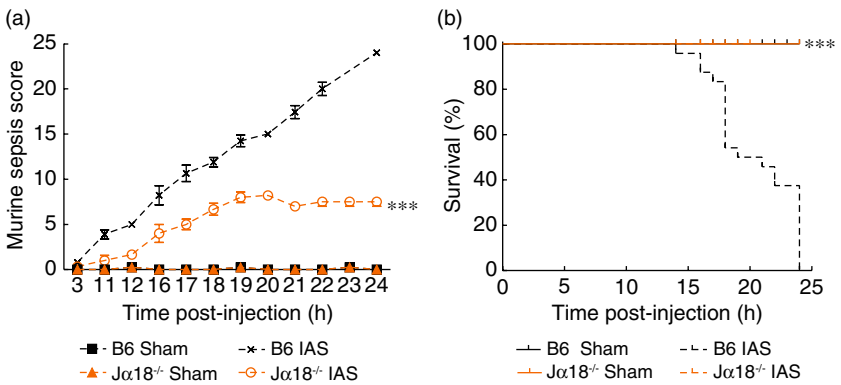

Fig. 2. Invariant natural killer T (iNK T) cells are pathogenic in intra-abdominal sepsis (IAS). (a) B6 and iNK T cell-deficient J$\alpha18^{-/-}$ mice were injected with faecal slurry (90 mg/ml) to induce IAS and monitored during the experimental time-line. Murine sepsis scores were significantly higher compared to sham-treated B6 and J$\alpha18^{-/-}$ mice [injected intraperitoneally with normal saline (NS)] and J$\alpha18^{-/-}$ mice with IAS ($n = 6$ for sham B6 mice, $n = 6$ for J$\alpha18^{-/-}$ mice, $n = 10$ for septic B6 mice, $n = 6$ for septic J$\alpha18^{-/-}$ mice). $^{***}P < 0{\cdot}001$ by two-way analysis of variance test. (b) Mortality for B6 mice with IAS were significantly higher than sham B6 and J$\alpha18^{-/-}$ mice, as well as septic J$\alpha18^{-/-}$ mice ($n = 6$ for sham B6 mice, $n = 6$ for J$\alpha18^{-/-}$ mice, $n = 10$ for septic B6 mice, $n = 6$ for septic J$\alpha18^{-/-}$ mice). $^{***}P < 0{\cdot}001$ by log-rank test.

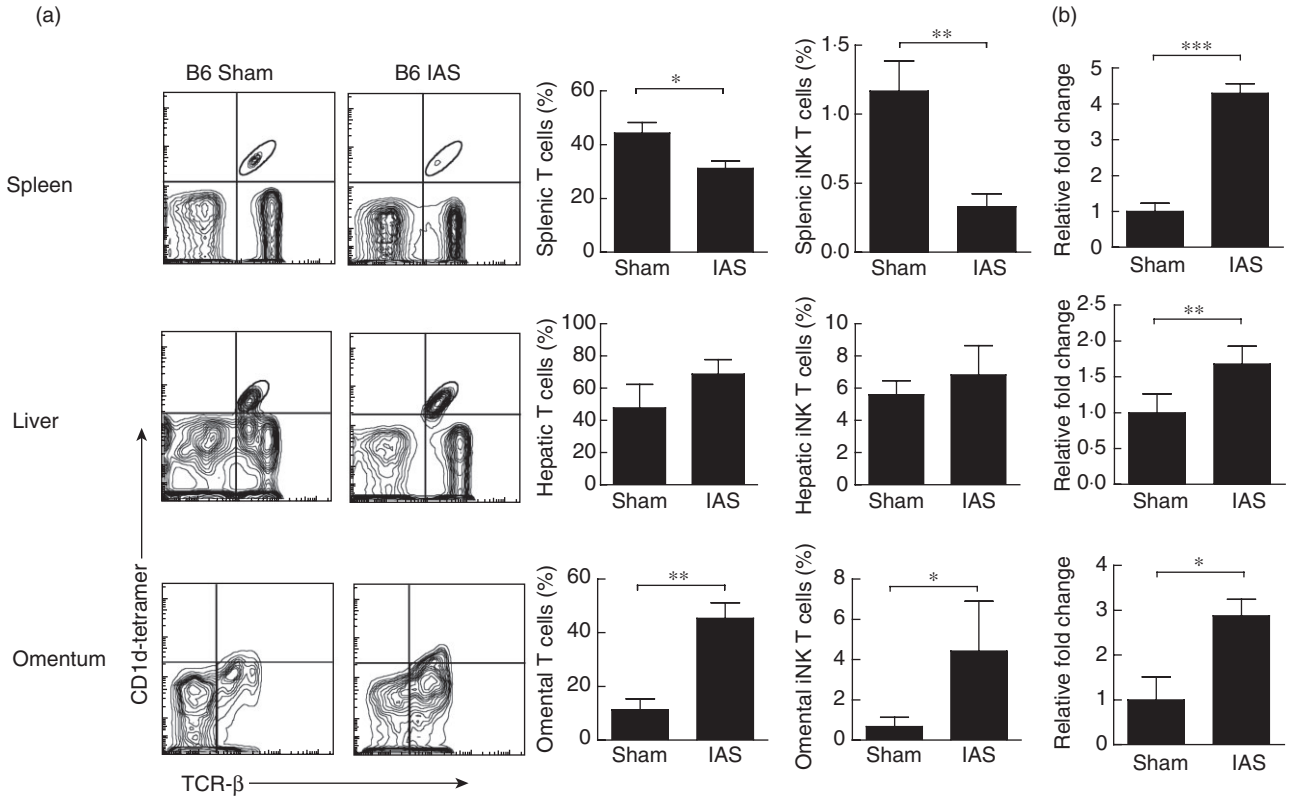

Fig. 3. Tissue-specific distribution of invariant natural killer T (iNK T) cells is altered during intra-abdominal sepsis (IAS). (a) The distribution of $CD3^+$ T and $CD3^+CD1d$ $tetramer^+$ iNK T cells in the spleen and omentum is altered significantly in IAS, but remains unchanged in the liver ($n = 7$, $n = 10$ in sham and IAS groups, respectively). Percentages of cell populations are represented as means ± standard errors of the mean. $^{**}P < 0{\cdot}001$; $^{*}P < 0{\cdot}05$ by Mann–Whitney *U*-test (b) Quantitative reverse transcription–polymerase chain reaction (RT–PCR) using custom-designed probes designed for detecting the invariant T cell receptor (TCR) demonstrates a significantly elevated expression of TCR transcripts within the spleen, liver and omentum following IAS. Relative fold changes of the expression of the invariant TCR were calculated based on the ΔΔCt method after normalizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control ($n = 3$, $n = 5$ in sham and IAS groups, respectively). $^{***}P < 0{\cdot}0001$; $^{**}P < 0{\cdot}001$; $^{*}P < 0{\cdot}05$ by Mann–Whitney *U*-test.

faecal slurry solution (90 mg/ml) in B6 mice resulted in 100% mortality at 24 h (Fig. 2b), the sham B6 and $J\alpha18^{-/-}$ groups, which were injected with NS, as well as the septic $J\alpha18^{-/-}$ group, remained alive. On necropsy, we observed discrete abscess collections overlying the intestines and liver in septic $J\alpha18^{-/-}$ mice, whereas septic B6 mice developed intestinal distension and oedema without abscess formation (unpublished observations). In addition, the adoptive transfer of iNK T cells from GFP transgenic mice into $J\alpha18^{-/-}$ mice and subsequent induction of IAS increased disease severity, in comparison to $J\alpha18^{-/-}$ mice that did not receive iNK T cells (Supporting information, Fig. S2a). Together, these results confirm the pathogenic nature of iNK T cells in IAS.

Tissue-specific distribution of iNK T cells is altered in IAS

Previous animal studies using a model of chronic polymicrobial sepsis found that the frequency of hepatic iNK T cells declined significantly, whereas splenic iNK T cells remained unchanged [15]. We sought to determine whether a similar occurrence would be observed in IAS. Furthermore, we hypothesized that the omentum, which has been described as the 'policeman of the abdomen' for its ability to migrate to and mitigate inflammatory reactions [44], may accommodate increased numbers of iNK T cells post-sepsis.

Using flow cytometry, we determined the frequencies of $TCR\beta^+CD1d$ $tetramer^-$ conventional T cells and $TCR\beta^+CD1d$ $tetramer^+$ iNK T cells in the spleen, liver and omentum. In the spleen, the percentages of conventional T cells and iNK T cells declined significantly post-sepsis (Fig. 3a), while there was no difference in the distribution of iNK T or T cells in the liver (Fig. 3a). In the omentum, the percentages of T cells and iNK T cells increased significantly post-sepsis (Fig. 3a). In $J\alpha18^{-/-}$ mice that were adoptively transferred with iNK T cells from GFP transgenic mice, we detected the presence of these cells within the omentum post-sepsis (Supporting information, Fig. S2b).

We also sought to quantify the transcriptional expression of the invariant TCR following IAS, because the surface receptors of iNK T cells (including TCR and NK1·1) can be down-regulated upon activation [45,46] and become undetectable by flow cytometry using standard reagents [45]. Using the Taqman assay with custom-designed primers that overlap the invariant TCR Vα14-Jα18 splice site and amplify a portion of the TCR [47,48], we observed significant increases in the transcriptional expression of the invariant TCR within the spleen, liver and omentum post-sepsis (Fig. 3b). Together, these results demonstrate that the tissue-specific distribution of iNK T cells is altered significantly during IAS, and that the transcription of the invariant TCR may be increased post-sepsis.

Th2-polarized iNK T cells reduce disease severity in IAS

Multiple groups, including ours, have examined the use of glycolipids to modulate cytokine responses in iNK T cells, and ameliorate disease severity in autoimmune diseases such as type 1 diabetes [35,49] and rheumatoid arthritis [10,12,50]. As the acute phase of intra-abdominal sepsis is characterized primarily by a marked proinflammatory or Th1-type response that contributes to mortality [4,51–54], we hypothesized that administration of a Th2-polarizing glycolipid would reduce disease severity in sepsis. OCH is an iNK T cell agonist which results in a Th2-biased cytokine profile when administered *in vivo* [43,55]. Similar to previous studies by our group and others [32,35,43], we demonstrated that the intraperitoneal injection of OCH into naive B6 mice results in a rapid peak of serum IL-4 at 2 h, and is then reduced significantly at 12–24 h (Supporting information, Fig. S3a); in contrast, serum levels of the Th1-type cytokine IFN-γ peaked at 12 h, but were almost undetectable at 24 h (Supporting information, Fig. S3a). Administration of the prototypical iNK T cell agonist KRN7000 [56] resulted in elevated serum levels of IFN-γ between 12 and 24 h (Supporting information, Fig. S3a). The IL-4:IFN-γ ratio, calculated based on the peak values of these cytokines, was higher for OCH compared to KRN7000, indicating that OCH promotes a Th2-dominant cytokine response *in vivo*.

Treatment with OCH prolonged survival in septic mice compared to both vehicle and KRN7000 treatments (Fig. 4a). Median survival for OCH-treated mice was 28 h compared to 24 and 22 h for vehicle- and KRN7000-treated mice, respectively ($P < 0{\cdot}0001$ by log-rank test). Mice in the OCH group survived beyond 24 h, whereas mortality for vehicle- and KRN7000-treated mice was 100% by 24 h. OCH-treated mice also had a significantly lower MSS after 24 h compared to vehicle- and KRN7000-treated mice with IAS (Fig. 4b). However, there were no statistical differences in MSS between the vehicle and KRN7000 treatments. The reduced MSS for OCH-treated mice derived from significant improvements in respiratory status, an important clinical predictor of mortality in sepsis [57–60]. Most vehicle- and KRN7000-treated mice developed respiratory distress (laboured breathing and reduced respiratory rates) by 15 h post-sepsis, unlike OCH-treated mice that continued to have relatively normal respiratory rates even at 24 h. OCH-treated mice were also more responsive to auditory and touch stimuli, whereas vehicle- and KRN7000- treated mice remained non-responsive and slow-moving or stationary. In addition, we did not observe any differences in disease severity between vehicle- and OCH-treated $J\alpha18^{-/-}$ mice with IAS (Fig. 4c), consistent with the mechanism for the beneficial effects of OCH on sepsis severity and mortality in B6 mice being linked to the specific modulation of iNK T cells.

Next, we analysed the spleens and livers of septic mice treated with the glycolipid agonists, but did not detect differences in splenic or hepatic T cell distributions (Fig. 4d,e, respectively). However, we had significantly reduced detection of iNK T cells in the spleen and liver following glycolipid treatment (Fig. 4d,e). This reflects the down-regulation of the surface TCR that occurs with administration of glycolipid agonists, as shown previously by several groups, including ours [12,32,43,61] (Fig. 4e and Supporting information, Fig. S3b). In particular, we observed a significantly lower detection of iNK T cells following KRN7000 treatment compared to treatment with OCH (Fig. 4d,4e). The differential degree to which the glycolipids down-regulate the surface TCR is a reflection of their differential binding kinetics to iNK T cells. While OCH and KRN7000 down-regulate the surface TCR within 4–12 h post-administration, KRN7000 is approximately 10-fold more potent at down-regulating the TCR after 24 h [61], leading to the results we observed in Fig. 4d,e.

Anti-inflammatory processes are concomitantly initiated to mitigate proinflammatory states in sepsis, both systemically [62–65] and in individual organs [66]. These immunosuppressive mechanisms decrease the responsiveness of cells of the innate and adaptive immune systems, thereby increasing susceptibility to opportunistic and additional infections [67–70]. Importantly, we observed that the use of OCH, which significantly reduced the production of the proinflammatory cytokine IFN-γ [12,35,43], did not worsen the microbial load of septic mice, compared to vehicle and KRN7000 treatments (Fig. 4f). Therefore, administration of the Th2-polarizing glycolipid OCH did not result in overt susceptibility to microbial infection. Additionally, OCH-treated mice that survived to 48 h demonstrated a significantly lower bacterial count in all tested organs, compared to OCH-treated mice that died at 24 h (data not shown). Sham mice, as expected, did not demonstrate bacterial organ counts (data not shown).

Lastly, we tested the effect of a second Th2-polarizing glycolipid, C20:2, on disease severity in IAS, to confirm whether the Th2-biased modulation of iNK T cells was responsible for ameliorating disease severity. C20:2 is a

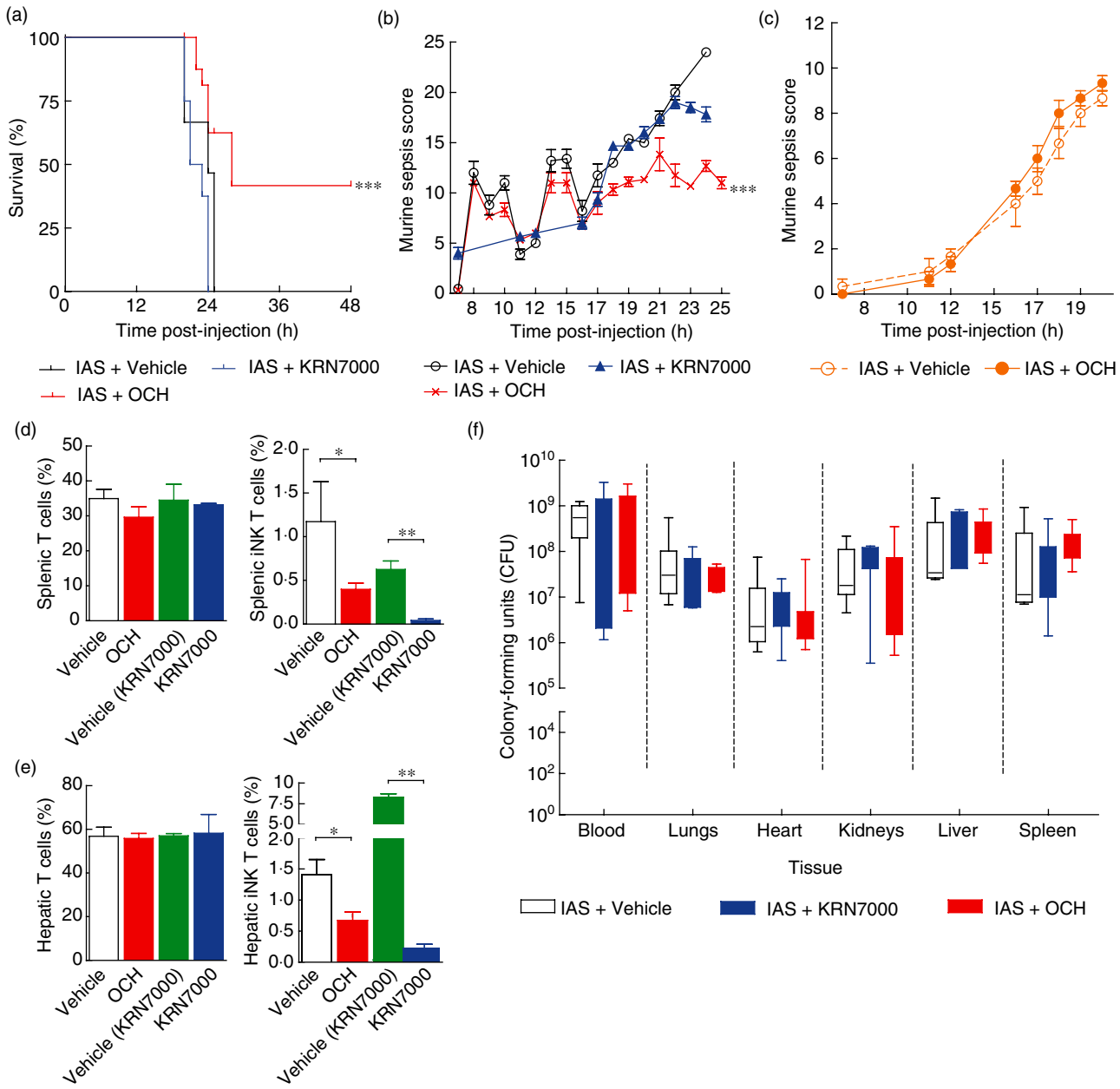

Fig. 4. T helper type 2 (Th2)-polarizing glycolipid OCH reduces disease severity in intra-abdominal sepsis (IAS). C57BL/6 (B6) mice were injected concomitantly intraperitoneally with faecal slurry (FS; 500 μl of 90 mg/ml solution) to induce intra-abdominal sepsis (IAS), and vehicle, OCH or KRN7000. (a) OCH-treated mice had prolonged survival significantly compared to vehicle- and KRN7000-treated mice ($n = 19$, $n = 15$, $n = 8$ for OCH, vehicle and KRN7000 groups, respectively). ***$P < 0{\cdot}001$ by log-rank test. (b) OCH-treated mice demonstrated significantly reduced disease severity compared to vehicle-treated and KRN7000-treated mice ($n = 19$, $n = 15$ and $n = 8$ mice, respectively, for OCH, KRN7000, and vehicle groups). ***$P < 0{\cdot}001$ by two-way analysis of variance (ANOVA) with Bonferroni post-test. (c) iNK T-deficient J$\alpha 18^{-/-}$ mice were given FS (500 μl of a 90 mg/ml solution) to induce IAS and treated concomitantly with OCH or vehicle. Murine sepsis scores were similar between vehicle and OCH-treated mice ($n = 3$ per group). (d,e) Administration of OCH and KRN7000 resulted in significantly reduced detection of $CD3^+CD1d$ tetramer$^+$ invariant natural killer T (iNK T) cells among septic B6 mice compared to vehicle treatments. The percentages of $CD3^+$ T cells remained unchanged with administration of iNK T-specific glycolipid agonists ($n = 6$, $n = 4$, $n = 6$, and $n = 3$ for vehicle, OCH, vehicle (KRN7000) and KRN7000 groups, respectively). *$P < 0{\cdot}05$; **$P < 0{\cdot}01$ by Mann–Whitney *U*-test. (f) Bacterial counts in blood and multiple organs were similar between vehicle-, OCH- and KRN7000-treated mice with sepsis ($n = 7$–9 per group). Data are representative of at least three independent experiments.

potent agonist with a capacity to bind and activate iNK T cells that is significantly stronger than OCH [35,43]; administration of C20:2 in naive B6 mice also resulted in a more pronounced Th2 response at 24 h than OCH [35,43] (Supporting information, Fig. S3a). When septic B6 mice were treated with C20:2, we observed a significant reduction in MSS between 20 and 24 h compared to vehicle-treated mice (Supporting information, Fig. S4a), with improved respiratory status at the observed time-points. These results confirm the novelty of manipulating iNK T cells into a Th2-biased state for the mitigation of disease severity in IAS. However, the MSS continued to rise in C20:2-treated mice, in contrast to OCH, where the MSS reached a plateau (Fig. 4b). Based on these results, we elected to focus on OCH and the means by which it improves mortality in IAS.

The proinflammatory cytokine profile in IAS is ameliorated by administration of OCH

In order to further understand the impact of the glycolipid agonists on the septic response, we assessed the concentrations of 32 cytokines and chemokines from the sera and spleens of vehicle-, OCH- and KRN7000-treated septic mice, as well as sham-treated mice, at 24 h (Fig. 5a–c). We focused on this time-point because we have shown previously that the systemic levels of proinflammatory cytokines demonstrate the strongest correlation with the MSS at 24 h [27], whereupon we also observed the largest differences between glycolipid treatments. In the serum, mean concentrations of IL-17 were significantly lower in the OCH-treated mice compared to KRN7000-treated mice. The concentration of IL-13 was higher in the sera of OCH-treated mice compared to KRN7000- and vehicle-treated mice. In the spleen, IFN-γ, IL-3, IL-4, IL-17 and TNF-α were elevated significantly in the KRN7000-treated group compared to the OCH-treated group. Therefore, the administration of OCH significantly reduced the levels of proinflammatory cytokines in IAS.

Treatment with OCH reduces splenocyte apoptosis significantly in IAS

We next sought to elucidate the reason for the improved survival among septic mice that were treated with OCH. When we performed histopathological analysis on the spleen, liver and omentum of septic B6 mice treated with KRN7000 or OCH (Fig. 6a), we found a significant reduction of apoptotic cells within the spleens of OCH-treated mice compared to vehicle- and KRN7000-treated mice. The presence of karyorrhexic nuclei within clusters of cells with eosinophilic cytoplasm was observed in the white pulp of the spleen by H&E staining, and subsequently confirmed as apoptotic cells by TUNEL staining, particularly in vehicle- and KRN7000-treated mice. Based on histopathological scoring, OCH-treated mice had mild apoptosis, whereas vehicle-treated and KRN7000-treated mice had moderate and severe apoptosis, respectively (Fig. 6b).

In the omentum of vehicle- and KRN7000-treated mice we noted a significant increase in lymphocytes, whereas fewer lymphocytes were observed in the omentum of OCH-treated mice (Fig. 6a). We did not observe overt differences in liver histopathology among vehicle-, OCH- and KRN-treated mice. When we examined the histology of C20:2-treated septic mice, we observed a decrease in apoptosis compared to KRN7000-treated mice. However, the degree of apoptosis in C20:2-treated mice were higher than OCH-treated mice with IAS (Supporting information, Fig. S4b).

We then performed flow cytometry on spleens harvested from vehicle-, OCH- and KRN7000-treated mice with IAS to determine the immune cell populations that had undergone apoptosis (Fig. 7). Treatment with OCH reduced the apoptosis of T and B cells significantly compared to vehicle- and KRN7000-treated mice. However, there were no differences in the frequency of apoptotic macrophages between the KRN7000 and OCH groups, although both treatments reduced the frequency of apoptosis significantly compared to vehicle-treated mice. With respect to NK cell apoptosis, we observed a trend towards reduced apoptosis in KRN7000-treated mice. Together, these results demonstrate that different glycolipid agonists of iNK T cells differentially mitigate the apoptosis of splenic lymphocytes, but not NK cells and macrophages. Moreover, Th2-polarizing glycolipids reduce lymphocyte apoptosis significantly within the spleen, a critical predictor of mortality in severe sepsis and septic shock [71–73].

Discussion

iNK T cells exert profound and diverse regulatory functions in health and disease, bridging the innate and adaptive defence mechanisms in a variety of immune responses [14,18,74]. Here, we demonstrate that patients with sepsis/severe sepsis have significantly elevated proportions of iNK T cells and that OCH, a Th2-polarizing glycolipid agonist of iNK T cells, profoundly reduces disease severity in acute IAS, with significantly reduced lymphocyte apoptosis within the spleen. These findings introduce iNK T cells as potential therapeutic targets for the treatment of sepsis.

Glycolipid ligands of iNK T cells have been used successfully in experimental models of autoimmune diseases [12,35,49,50], transplantation [23,25] and malignancy [33,75]. KRN7000 [56] reduced morbidity and mortality associated with murine graft-*versus*-host disease [25,76], while OCH mitigated disease severity in non-obese diabetic mice [49], experimental autoimmune encephalomyelitis [55] and collagen-induced arthritis [10,50]. OCH also prevented disease symptoms in a humanized mouse model of

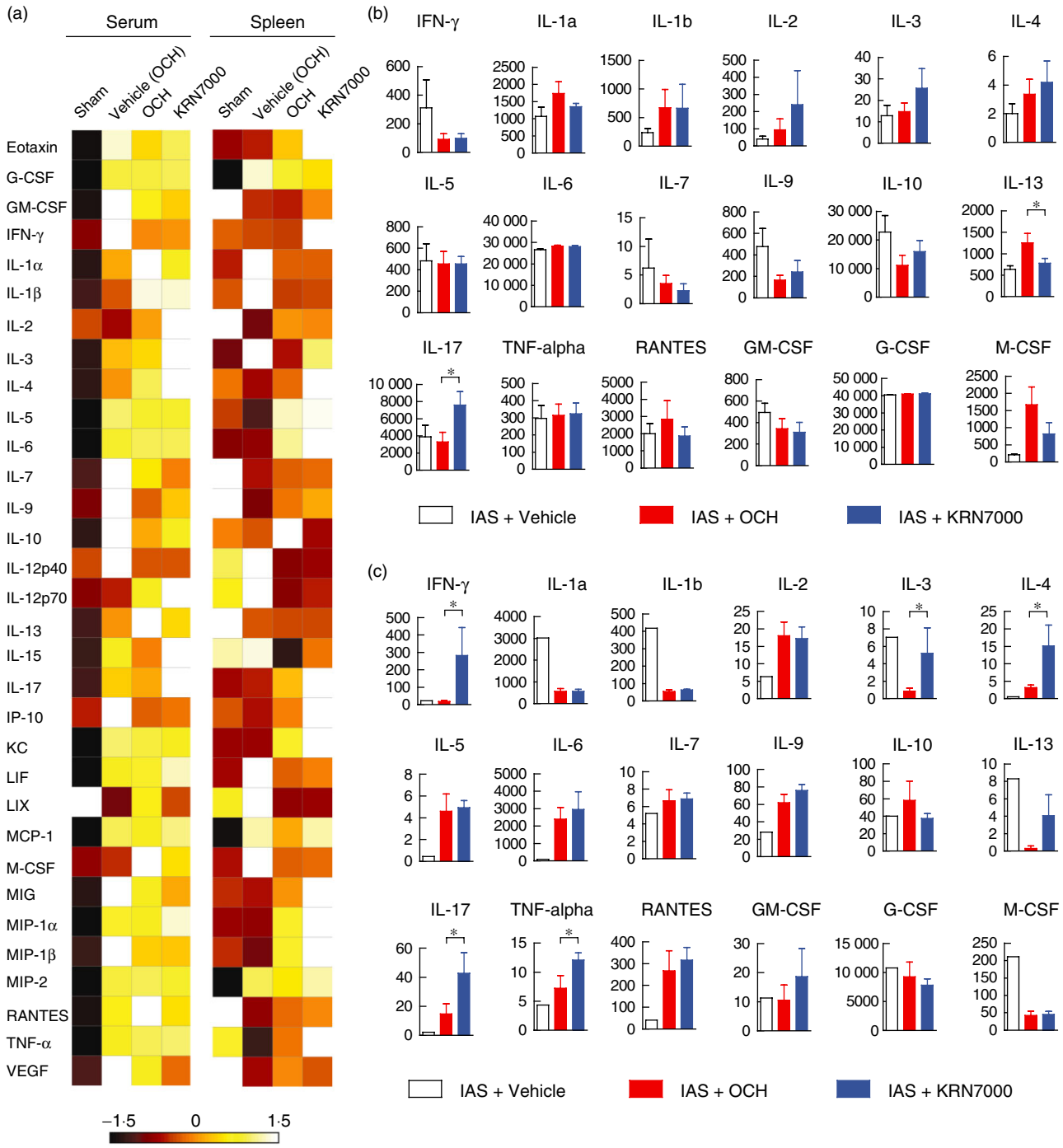

Fig. 5. Cytokine levels at 24 h in the sera and spleens of septic B6 mice. (a) Sera and spleen homogenates from vehicle-, OCH- and KRN7000-treated B6 mice with intra-abdominal sepsis (IAS) were analysed for 32 inflammatory cytokines by multiplex array, and displayed as a heat map ($n = 4$ mice per group). Concentrations of invariant natural killer T (iNK T) cell-specific cytokines are shown from sera (b) and spleen homogenates (c) of septic mice treated with vehicle, OCH or KRN7000 ($n = 4$–8 per group). Concentrations of cytokines are shown in pg/ml. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ by one-way analysis of variance with *post-hoc* Tukey's multiple comparison test. Data are representative of at least three independent experiments.

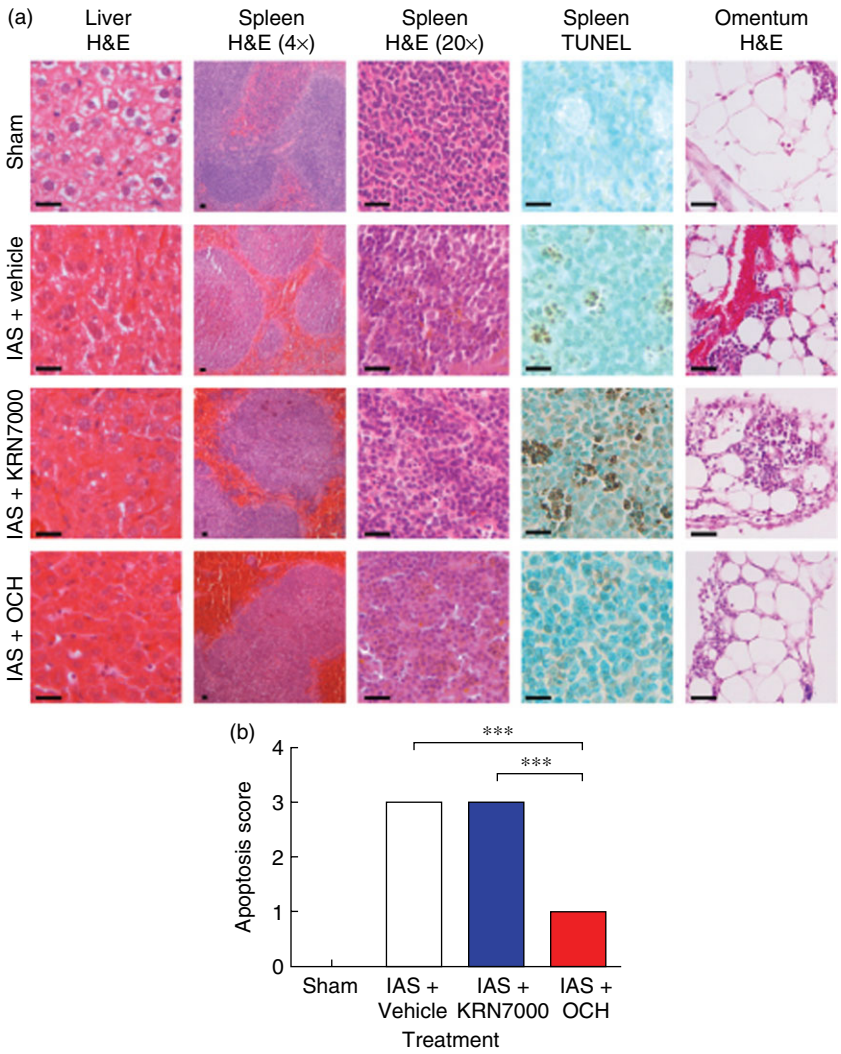

Fig. 6. Histopathology of septic B6 mice treated with glycolipid agonists of invariant natural killer T (iNK T) cells. (a) Treatment with OCH significantly reduced apoptosis within the spleen compared to vehicle- and KRN7000-treated mice with intra-abdominal sepsis (IAS), both by haematoxylin and eosin (H&E) staining as well as terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining. Lymphocyte migration to the omentum is also ameliorated in OCH-treated mice compared to vehicle- and KRN7000-treated mice. There were no histopathological differences in the liver. Images are representative of four animals per treatment group (size bar, 50 μm). (b) Histopathological scoring of the degree of apoptosis observed within the spleens of sham and septic B6 mice treated with vehicle, KRN7000 or OCH ($n = 4$ animals per treatment group). Apoptosis was defined histologically by the presence of cell clusters with nuclear shrinkage (karyorrhexis), dark eosinophilic cytoplasm, intact plasma membrane and relative paucity of surrounding inflammatory cells within the splenic follicles on H&E staining. Scores assigned to each animal by a blinded independent pathologist were as follows: 0 for complete absence of apoptosis; 1 for mild presence of apoptosis (0–15% per follicle); 2 for moderate apoptosis (16–30% per follicle); and 3 for severe apoptosis (31–45% per follicle). $^{***}P < 0{\cdot}0001$ by two-tailed Mann–Whitney *U*-test.

citrullinated fibrinogen-induced inflammatory arthritis [12], and delayed Th1-mediated cardiac allograft rejection in mice [23].

In our study, we show that the administration of OCH ameliorated the severe proinflammatory Th1-type response associated with IAS and reduced mortality. Although proinflammatory cytokines such as IFN-γ and TNF-α contribute to immune responses against bacterial infections [77], elevated levels of these cytokines are also associated with poor outcomes and decreased survival in sepsis [8,27,78]. Pinsky *et al.* [8] demonstrated that a significant proportion of patients who die from sepsis have markedly elevated levels of proinflammatory cytokines compared to survivors of sepsis, while Bozza and colleagues [6] correlated elevated levels of proinflammatory cytokines with early mortality (within 24–48 h of admission). We therefore elected to utilize an acute, non-resuscitative mouse model of septic shock characterized by a markedly elevated proinflammatory cytokine profile [27], in order to mimic septic patients at significant risk for early mortality. As confirmed in this study, the treatment of septic mice with KRN7000 resulted in a Th1-type response at 24 h [14,43,79] and did not affect disease severity. In addition, elevated levels of the Th2 cytokine, IL-13, may be contributing to the significant improvements in respiratory status and disease severity that we observed in OCH-treated mice. A potent anti-inflammatory cytokine [80,81], IL-13, is produced in large quantities by alveolar macrophages in the lung during polymicrobial sepsis [80], and has been shown to protect mice from endotoxic shock when administered *in vivo* [82]. Because a compromised respiratory status significantly increases morbidity and mortality in sepsis [57–59], the selective Th2-biased modulation of iNK T cells may tip the overall balance of the cytokine response in favour of a Th2 response and provide a novel strategy to prevent this complication.

The relative deficiency of proinflammatory cytokines has also been associated with increased susceptibility to additional infections. However, we did not observe an increase in microbial load within the blood and organs of

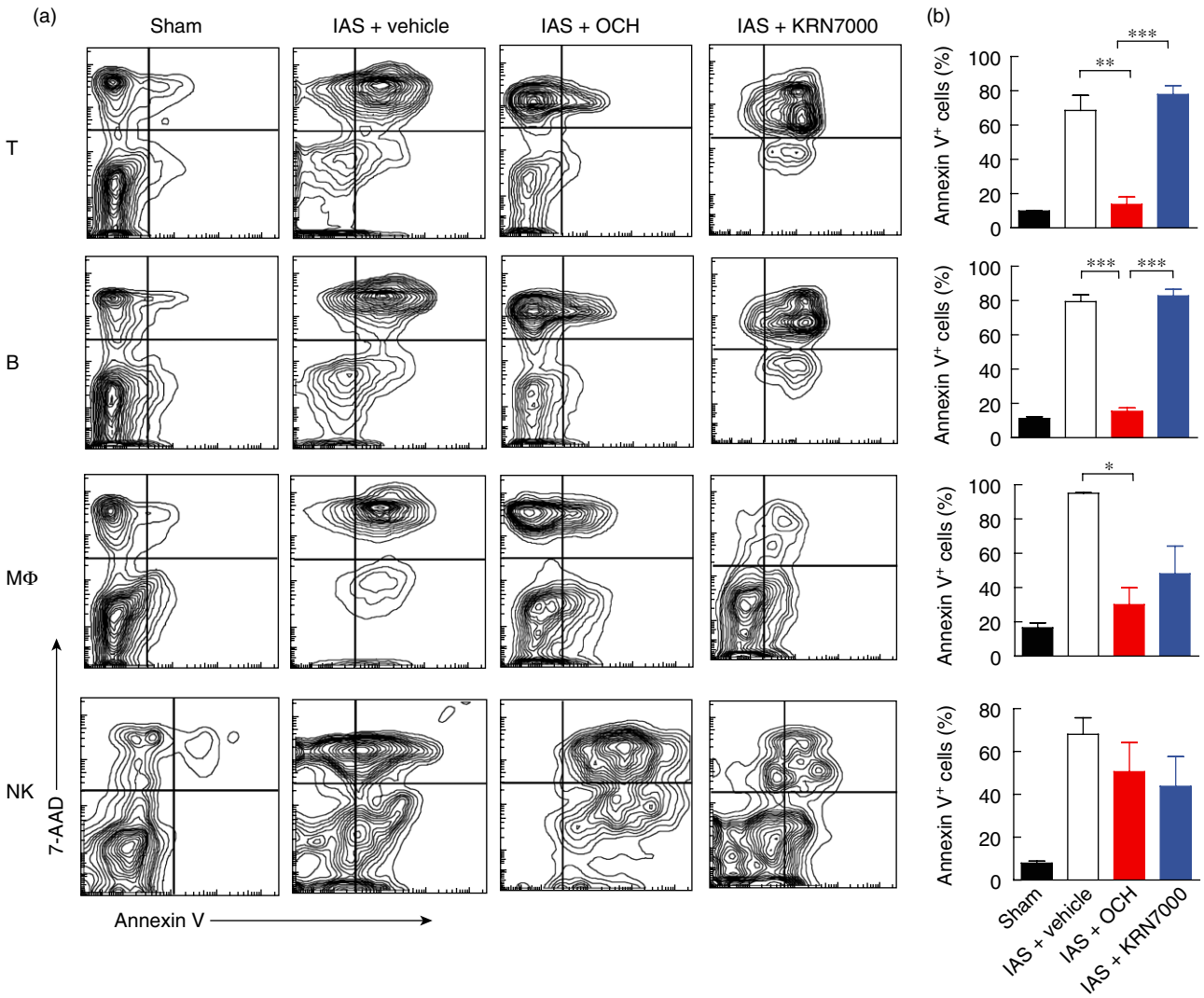

Fig. 7. Analysis of apoptotic cell populations in the spleens of septic B6 mice. Splenocytes from sham and septic B6 mice treated with OCH, KRN7000 or vehicle were stained for T, B and natural killer (NK) cells and macrophages, and stained further for annexin V (a marker for early apoptosis) and 7-aminoactinomycin D (7-AAD) viability dye. Early and late apoptotic cells (annexin V^+ $7AAD^-$ and annexin V^+ $7AAD^+$ cells, respectively) were quantified and compared between treatments. OCH treatment significantly reduced apoptosis among T and B cells, as well as macrophages, but not NK cells ($n = 3$–6 mice per group). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ by one-way analysis of variance with Tukey's *post-hoc* multiple comparison test. Data are representative of three independent experiments.

OCH-treated mice compared to vehicle-treated mice with IAS. As OCH is a less potent agonist than KRN7000, with lower binding affinity for the invariant TCR compared to the latter [43,61], the administration of a single dose of OCH may have affected only a portion of iNK T cells, thereby abrogating rather than eliminating the proinflammatory response. In addition, other immune cells which are not directly affected by glycolipid administration may continue to participate in bacterial clearance, including NK cells, which also produce significant amounts of IFN-γ [77]. Any differences in microbial counts between KRN7000- and vehicle-treated mice may have been masked by the excessive proinflammatory response that is inherent in our sepsis model [27]. Lastly, our study also confirms that the manipulation of iNK T cells alone can dramatically alter outcomes in sepsis, given that iNK T-deficient mice are resistant to mortality from sepsis, and disease severity was unaffected by glycolipid treatment in these animals.

Interestingly, the use of C20:2, another Th2-polarizing glycolipid that is significantly more potent at inducing a Th2 bias compared to OCH [35,36,83] and suppresses downstream NK cell function [43], also mitigated sepsis severity significantly. Unlike OCH, however, C20:2-treated mice continued to worsen, while the histopathology of the spleen demonstrated a higher degree of apoptosis following treatment with C20:2 compared to OCH; these findings may be explained by the relatively short half-life of C20:2 compared to OCH [35,43]. Nevertheless, the results of our

study suggest that the Th2-biased manipulation of iNK T cells may be a viable therapeutic strategy in sepsis, although optimization of the timing and frequency of glycolipid usage may be needed to provide the most effective results.

We also show that the tissue-specific distribution of iNK T cells is altered during IAS, with significant reductions in the spleen and a concomitant rise in the omentum. The human omentum has been described as the 'policeman of the abdomen' for its ability to adhere to sites of intra-abdominal pathology and prevent widespread pathogen contamination [84,85]. Similarly, the murine omentum has been shown to facilitate the regeneration of damaged tissues [44]. These results, as well as the findings of Lynch *et al.* who demonstrated that the human omentum contained a rich reservoir of iNK T cells [86], prompted us to examine the murine omentum, wherein we observed a significant increase in iNK T cells post-sepsis. Our observation that the omentum became enlarged during IAS correlates with findings by Shah *et al.* [44], and represents a unique feature of this organ that has not been noted in other secondary lymphoid structures such as lymph nodes or spleens. T cells were also noted to be increased significantly in the omentum during IAS, corroborating observations made by Carlow *et al.* [87] in a caecal ligation and puncture (CLP) model of polymicrobial sepsis. Our results with respect to the tissue-specific distribution of iNK T cells post-sepsis contrast with the findings of Hu *et al.* [15], who demonstrated a significant reduction in hepatic iNK T cells but no changes in the frequency of splenic iNK T cells in the CLP model. We propose that splenic iNK T cells mobilize more readily during acute sepsis compared to hepatic iNK T cells, as a recent study by Barral *et al.* [17] showed that splenic iNK T cells patrol the red pulp and marginal zones of the spleen, rapidly sample blood-borne antigens and display migratory capabilities. This may explain our observed changes in splenic iNK T cell frequency post-sepsis, and additionally suggests that the iNK T cells we detected in the omentum post-sepsis may have originated from the spleen, given that the two organs are physically attached to each other [44]. Interestingly, we observed a contrast between the surface detection of the invariant TCR and detection of the invariant TCR transcripts within splenic iNK T cells post-sepsis. Given that different subsets of iNK T cells ($CD4^+$, $CD4^-CD8^-$, $NK1{\cdot}1^+$ and $NK1{\cdot}1^-$ populations) differ in their gene expression [88] and cytokine profiles [89], and may also differ in their organ distribution, splenic iNK T cells may be delayed in their surface re-expression of the invariant TCR compared to hepatic and omental iNK T cells, even though they may be transcriptionally active.

In this study, we also demonstrate that Th2-polarized iNK T cells reduce apoptosis significantly within the spleen, particularly among T and B cells as well as macrophages. iNK T cells can rapidly sense, and are activated by, apoptotic cell death [90,91]. Wermeling *et al.* [91] showed that in B6 mice injected with apoptotic cells, activated iNK T cells reduce B cell survival. Moreover, the cytokine profile of these iNK T cells was altered towards a Th2-type response, albeit in *ex-vivo* splenocyte cultures rather than *in vivo* [91]. Our group has demonstrated previously that Th2-polarized iNK T cells undergo less apoptosis in a model of autoimmune diabetes compared to Th1-biased iNK T cells [36]. No studies to date, however, have examined the impact of modulating iNK T cell phenotype on sepsis-induced apoptosis. The latter is an especially important phenomenon with significant immunological and clinical implications. The apoptosis of T and B cells significantly impairs the adaptive immune response and, by disabling cross-talk between the adaptive and innate immune systems, also impairs the latter [9,66,92]. These mechanisms lead to an immunosuppressive phase in septic patients, which may result in additional secondary infections that substantially increase mortality [9,92]. Hotchkiss *et al.* observed a striking apoptosis-induced loss of cells of the innate and adaptive immune systems in the spleen during sepsis, including $CD4^+$ and $CD8^+$ T cells, B cells and dendritic cells [51,73]. Additionally, T cells that come into contact with macrophages and dendritic cells that have ingested apoptotic cells either become anergic or undergo apoptosis themselves [93]. Therefore, the significant reduction in splenic lymphocyte apoptosis following treatment with OCH may preserve the function and efficacy of immune cells, prevent anergy and mitigate the immunosuppressive phase during sepsis. Interestingly, apoptosis of NK cells appeared to be reduced by treatment with OCH and KRN7000, although the trend is more pronounced for the latter. Because NK cells also produce significant amounts of IFN-γ [77], their apoptosis in the spleens of vehicle- and OCH-treated mice may explain the reduced levels of splenic IFN-γ in these two groups.

We have also demonstrated that the proportion of circulating iNK T cells (as a percentage of circulating T cells) is elevated early in the septic process for critically ill patients, corroborating a recently published study by Heffernan *et al.* [94]. We elected to present and compare the iNK T:T cell ratio in our study for two reasons: because of their relative paucity, small differences in the number of iNK T cells between populations may not be observed if only their absolute numbers are considered. In addition, we observed large variations in the numbers of lymphocytes, T cells and NK cells among septic patients due to the heterogeneity of their infections. Accounting for this variation necessitated the calculation of iNK T cells as a fraction of the overall circulating T cell repertoire in each patient. Given their propensity to rapidly produce significant quantities of pro- and/or anti-inflammatory cytokines, the increased proportion of iNK T cells suggests that these cells may play a prominent role in promulgating the immune response in septic patients. Furthermore, the proportion of iNK T cells is not increased in patients who have sustained significant inflammatory responses due to trauma, suggesting that

these cells may be responding specifically to microbial pathogens in humans. Consequently, the detection of increased proportions of iNK T cells may also serve as an important biomarker to differentiate septic from non-septic patients early in the disease process, thereby facilitating rapid and targeted interventions for the disease.

Given the failure of many immunotherapeutic drugs in the treatment of sepsis [95,96], alternative agents have been sought to combat this disease with some success [97–103]. The results of our study demonstrate that iNK T cells should be further considered as potential targets for immunotherapy in sepsis, and that modulation of the iNK T cell phenotype towards a Th2 response has a protective effect during acute infection.

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Disclosure

S. A. P. is a consultant for Vaccinex, Inc. (Rochester, NY), which has commercial interests related to the development of therapies involving iNK T cell activating glycolipids. R.V. A., S. M. M. H. and J. K. M. are named as co-inventors on a patent application relating to the use of iNK T cell-activating glycolipids for the treatment of sepsis. The other authors do not have any actual or potential conflicts of interest to disclose.

Author contributions

R. V. A., S. M. M. H. and J. K. M. conceived and designed the study. R. V. A. performed the experiments, conducted the analysis and interpretation of the data, and drafted the manuscript. S. A. P. provided C20:2 and instructions for its preparation and administration. D. M. and S. X. X. assisted with experiments, while I. W. performed histological analysis of the tissue samples. T. M., D. F. and C. M. supervised the collection of blood samples from patients. All authors reviewed and revised the manuscript for important intellectual content, and approved the final version of the article.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Gating strategy to identify the percentage of apoptotic and necrotic immune cell populations. CD3-allophycocyanin (APC) is shown as an example, but the same strategy was used for macrophages (F480-APC), B cells (B220-APC) and natural killer (NK) cells [NK1·1-phycoerythrin (PE)].

Fig. S2. Adoptive transfer of invariant natural killer T (iNK T) cells into iNK T-deficient Jα18$^{-/-}$ mice. iNK T cells were isolated and sorted from green fluorescent protein (GFP)-expressing transgenic mice, and injected intravenously into Jα18$^{-/-}$ mice. After 18 h, mice were administered a faecal slurry (500 µl of a 90 mg/ml solution) intraperitoneally to induce intra-abdominal sepsis (IAS) and monitored for 24 h. (a) Adoptive transfer of iNK T cells significantly increased the severity of sepsis compared to Jα18$^{-/-}$ mice that did not receive iNK T cells. (b) Adoptively-transferred iNK T cells moved into the omentum of Jα18$^{-/-}$ mice following IAS, as detected by flow cytometry, compared to adoptively transferred iNK T cells in sham Jα18$^{-/-}$ mice.

Fig. S3. Effect of glycolipid agonists on cytokine expression in naive B6 mice, and on invariant natural killer T (iNK T) cells in septic B6 mice. (a) Naive B6 mice were injected intraperitoneally with 4 µg OCH or KRN7000 or C20:2, and bled at 2, 12 and 24 h post-injection. Serum samples were assayed for interleukin (IL)-4 and interferon (IFN)-γ by enzyme-linked immunosorbent assay (ELISA). Each data point shows mean (± standard error of the mean) of two or three mice from one representative experiment. Vehicle-treated mice had cytokine levels below limits of detection. (b) B6 mice were given an intraperitoneal injection of faecal slurry (500 µl of a 90 mg/ml solution) to induce intra-abdominal sepsis (IAS) and treated concomitantly with 4 µg of vehicle, OCH or KRN7000. After 24 h, mice were killed and cell suspensions from the liver and spleen were stained for the flow cytometric detection of CD1dtetramer + T cell receptor (TCR)β^+ iNK T cells.

Fig. S4. C57BL/6J (B6) mice were injected intraperitoneally with 500 µl of faecal slurry (FS) (90 mg/ml) to induce intra-abdominal sepsis (IAS), and injected concomitantly with 4 µg of the glycolipid C20:2 or vehicle solution. (a) Murine sepsis scores for septic mice treated with C20:2 or vehicle ($n = 5$, $n = 10$ mice for C20:2 and vehicle groups, respectively). $^{***}P < 0{\cdot}001$ by two-way analysis of variance test. (b) After 24 h, septic B6 mice treated with C20:2 were killed, and the liver, spleen and omentum were removed and processed for histopathological analysis. These images are representative of five septic B6 mice that were treated with C20:2 (size bar, 25 µm).