Chronic stress physically spares but functionally impairs innate-like invariant T cells

Highlights

- iT cells are unusually refractory to stress- and glucocorticoid-induced apoptosis
- Chronic stress curbs both T_{H1}- and T_{H2}-type responses orchestrated by iT cells
- Stress incapacitates iNKT cells via intrinsic glucocorticoid receptor signaling
- Stress-elicited glucocorticoids impair the antitumor activity of iNKT cells

In brief

Invariant T cells are emergency responders to infection and cancer. Rudak et al. report that psychological stress unusually spares these innate-like lymphocytes but alters or impairs their cytokine production and cytotoxic and/or antimetastatic capacities through a cell-autonomous, glucocorticoid receptor-dependent mechanism. This may explain certain aspects of stress-induced immunosuppression.
Chronic stress physically spares but functionally impairs innate-like invariant T cells

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SUMMARY

The deleterious effects of psychological stress on mainstream T lymphocytes are well documented. However, how stress impacts innate-like T cells is unclear. We report that long-term stress surprisingly abrogates both T helper 1 (TH1)- and TH2-type responses orchestrated by invariant natural killer T (iNKT) cells. This is not due to iNKT cell death because these cells are unusually refractory to stress-inflicted apoptosis. Activated iNKT cells in stressed mice exhibit a “split” inflammatory signature and trigger sudden serum interleukin-10 (IL-10), IL-23, and IL-27 spikes. iNKT cell dysregulation is mediated by cell-autonomous glucocorticoid receptor signaling and corrected upon habituation to predictable stressors. Importantly, under stress, iNKT cells fail to potentiate cytotoxicity against lymphoma or to reduce the burden of metastatic melanoma. Finally, stress physically spares mouse mucosa-associated invariant T (MAIT) cells but hinders their TH1-/TH2-type responses. The above findings are corroborated in human peripheral blood and hepatic iNKT/MAIT cell cultures. Our work uncovers a mechanism of stress-induced immunosuppression.

INTRODUCTION

Long-term stress due to persistent negative emotions or repeated exposure to environmental stressors carries adverse immunological consequences (Glaser and Kiecolt-Glaser, 2005). Individuals experiencing greater relative levels of perceived stress mount weaker responses to vaccination and infection (Glaser and Kiecolt-Glaser, 2005). Stress has also been linked to cancer progression in animal models, often implicating diminished or dysregulated antitumor immunity (Kamiya et al., 2019; Kokolus et al., 2013; Partecke et al., 2016; Saul et al., 2005; Yang et al., 2019). According to prospective epidemiological studies, a higher degree of stress is associated with elevated incidences of neoplasia and cancer mortality in previously healthy individuals, and with poorer prognosis in cancer patients (Batty et al., 2017; Chida et al., 2008). Despite such findings, the mechanisms underlying stress-induced immunosuppression are ill defined.

A stress response is typically launched via the coordinated activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis (Glaser and Kiecolt-Glaser, 2005). Dense innervation of various organs by the SNS, including lymphoid tissues, enables targeted release of specific neurotransmitters into the extracellular milieu in which immune cells operate (Glaser and Kiecolt-Glaser, 2005). Stress-elicited defects in antimicrobial and antitumor immunity are often attributable to the operation of the SNS and/or the HPA axis (Pruett, 2003).

Previous studies on stress and immunity have focused heavily on conventional T (T_{conv}) cells (Calcagni and Elenkov, 2006). By inducing apoptosis in CD4+CD8+ thymocytes and mature T_{conv} cells in stressed mice exhibit a “split” inflammatory signature and trigger sudden serum interleukin-10 (IL-10), IL-23, and IL-27 spikes. iNKT cell dysregulation is mediated by cell-autonomous glucocorticoid receptor signaling and corrected upon habituation to predictable stressors. Importantly, under stress, iNKT cells fail to potentiate cytotoxicity against lymphoma or to reduce the burden of metastatic melanoma. Finally, stress physically spares mouse mucosa-associated invariant T (MAIT) cells but hinders their TH1-/TH2-type responses. The above findings are corroborated in human peripheral blood and hepatic iNKT/MAIT cell cultures. Our work uncovers a mechanism of stress-induced immunosuppression.
Figure 1. Prolonged stress impairs iNKT cells’ capacity to trigger IL-4 and IFN-γ production and potentiates an abnormal inflammatory response to glycolipid Ags 

(A) WT B6 mice were restrained for 12 h. Control animals remained undisturbed but were deprived of food and water. Mice subsequently received αGC, αCGC, or vehicle (Veh) i.p., or a combination of IL-12 and IL-18 i.v. 

(B and C) At the indicated time points post-αGC injection, serum IL-4 (B) and IFN-γ (C) were quantified by ELISA (n = 10 per group). 

(D and E) Two hours after αGC injection, IL-4+ and IFN-γ+ cell frequencies among hepatic (D) and splenic (E) TCRb+PBS-57-loaded CD1d tetramer+iNKT cells were determined by flow cytometry. 

(F) Two, 12, and 24 h after αGC (or Veh) administration, serum cytokine levels were measured via multiplex assays, and average values (n = 3 per cohort) were used to generate a heatmap. 

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cells, glucocorticoids shrink the size of T cell pools (Haeryfar and Berczi, 2001; Herold et al., 2006). Moreover, stress creates T helper 2 (Th2) bias (Cascaini and Elenkov, 2006), for instance by favoring interleukin-4 (IL-4) production in the context of ex vivo Tconv cell stimulation (Hu et al., 2014) or within tumor microenvironments (TMES) (Hou et al., 2013). NE (Elenkov et al., 2000), NPY (Battari et al., 2014), and glucocorticoids (Taves and Ashwell, 2020) have each been reported to permit or promote Th2-skewed phenotypes while inhibiting Th1-type responses.

To date, how psychological stress affects innate-like invariant T (iT) cells, including invariant natural killer T (iNKT) and mucosa-associated invariant T (MAIT) cells, has not been explored. This is a critical question given the remarkable immunomodulatory, cytotoxic, antitumor, antibacterial, and antiviral properties of these cells (Haeryfar and Mallevaey, 2015; Haeryfar et al., 2018; Lisbonne et al., 2004; Rudak et al., 2018; Zhang et al., 2019).

iNKT cells bear a CD1d-restricted, glycolipid-reactive, semi-invariant T cell receptor (TCR), which contains a canonically rearranged α chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) paired with one of only few Vβ chain choices (Lantz and Bendelac, 1994). Activated iNKT cells swiftly produce copious quantities of Th1, Th2, and/or Th17 cytokines that shape ensuing immune responses (Matsuda et al., 2008). The prototypic glycolipid ligand of iNKT cells, α-galactosylceramide (αGC) (Kawano et al., 1997), prompts the transactivation of several effector cell types, including NK cells (Smyth et al., 2002), precursors to mature NK (pre-mNK) cells (Choi et al., 2019), and CD8+ Tconv cells (Nakagawa et al., 2004). Therefore, αGC and its analogs are pursued as potential therapeutics (Fuji and Shimizu, 2019; Matsuda et al., 2008).

MAIT cells too harbor iTCR α chains (typically Vα19-Jα33 in mice and Vα27.2-Jα33 in humans) and minimal Vβ chain diversity (Lepore et al., 2014; Rahimpour et al., 2013). They are abundant in human peripheral blood (PB) and strategically positioned in the human liver, lungs, and mucosal layers (Dusseaux et al., 2011; Franciskiewicz et al., 2016; Tang et al., 2013), the ports of entry for many pathogens and common sites of neoplastic transformation or metastatic growth. Upon stimulation with vitamin B intermediates of bacterial and fungal origin (e.g., 5-[2-oxopropylidineamino]-6-邻里tibiaminouracil; 5-OP-RU) (Corbett et al., 2014), certain drugs and drug-like metabolites (Keller et al., 2017; Kjer-Nielsen et al., 2002), and other compounds presented by major histocompatibility complex (MHC)-related protein 1 (MR1), MAIT cells quickly produce a wide array of inflammatory mediators, including Th1, Th2, and Th17-type cytokines (Kelly et al., 2019; Kurioka et al., 2017), and transactivate key downstream effectors (Sallo et al., 2017). Although best known for their antimicrobial activities, MAIT cells may also play significant roles in various TMESs (Haeryfar et al., 2018; Yao et al., 2020). Both iNKT and MAIT cells can also respond to viral infections in a TCR-independent fashion, primarily through cytokines such as IL-12 and IL-18 (Tyznik et al., 2008; van Wilgenburg et al., 2016).

Here, we leveraged multiple mouse models of psychological stress as well as human cell culture systems to investigate the impact of stress on iT cell functions.

**RESULTS**

**Psychological stress impairs the ability of iNKT cells to trigger IL-4 and IFN-γ production and forces them to promote an atypical systemic inflammatory signature**

iNKT cells are unconventional, innate-like T cells with emergency response roles in antitumor and antimicrobial immunity (Fuji and Shimizu, 2019); yet, how stress shapes iNKT cell responses has been largely unknown.

We compared wild-type (WT) C57BL/6 (B6) mice that were left undisturbed or subjected to prolonged physical restraint (Figure 1A) for their in vivo IL-4 and interferon-γ (IFN-γ) responses to αGC (Figures 1A–1C). The primary source of IL-4 in response to this glycolipid are iNKT cells—hence, the rapidity with which they release this cytokine (Crowe et al., 2003). To our surprise, confinement stress resulted in dramatically reduced IL-4 levels (Figure 1B), which goes against the Th2 paradigm of stress and immunity (Cascaini and Elenkov, 2006; Hou et al., 2013; Hu et al., 2014). Peak IFN-γ levels were also similarly decreased in stressed animals (Figure 1C).

iNKT cells are not the only source of serum IFN-γ, which is also secreted by secondary effectors such as NK cells after αGC administration (Hayakawa et al., 2001; Smyth et al., 2002). To assess the impact of stress on IFN-γ production by iNKT cells, hepatic (Figure 1D) and splenic (Figure 1E) iNKT cells were identified via CD1d tetramer staining and examined for their intracellular cytokine content 2 h after αGC treatment. Consistent with serum cytokine results, far fewer iNKT cells from stressed mice generated IL-4 or IFN-γ (Figures 1D, 1E, and S1A). Furthermore, purified hepatic iNKT cells, which were sorted after restraint stress and exposed to αGC in co-cultures with CD11c+ bone-marrow-dervied dendritic cells (BMDCs), were weak cytokine producers (Figure S1B). In contrast, upon ex vivo stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, iNKT cells from stressed animals demonstrated intact IL-4 and IFN-γ production capacities (Figure S1C). PMA and ionomycin work synergistically to activate protein kinase C and Ca2+/calmodulin-dependent kinases, resulting in T cell activation independently of TCR engagement (Chatila et al., 1989).

Therefore, under stress, poor iNKT cell responses to cognate antigens (AgS) are likely due to impaired iTCR-proximal signaling events. We found splenic iNKT cells from stressed animals to express reduced levels of iTCR αβ, CD28, and inducible T cell costimulator (ICOS) (Figure S1D). These changes were accompanied by decreased phospho-SLP76 (pY128) levels and a trend toward diminished phospho-CD3ζ (pY142) and phospho-ZAP70 (pY319)/phospho-Syk (pY525) levels, but not phospho-Lck (pY505), in hepatic iNKT cells (data not shown).
Intrinsic host factors dictate or contribute to skewed cytokine responses in genetically diverse mammals. For instance, B6 and BALB/c mice are considered T\textsubscript{h}1- and T\textsubscript{h}2-dominant animals, respectively (Mills et al., 2000; Watanabe et al., 2004). Previous reports have suggested differences between these strains in terms of susceptibility to stress (Flint and Tinkle, 2001). However, similar to B6 mice, BALB/c mice that had been stressed before receiving \(\alpha\)GC had lower blood IL-4 and IFN-\(\gamma\) levels (Figure S2A).

There is sexual dimorphism in sensitivity to various stressors (Buynitsky and Mostofsky, 2009). In addition, iNK T cell frequencies and cytokine profiles, including IL-4 and IFN-\(\gamma\) responses to \(\alpha\)GC, may differ between males and females (Bernin et al., 2016; Gourdy et al., 2005). We found restraint stress to similarly reduce serum IL-4 and IFN-\(\gamma\) concentrations in both sexes (Figure S2B).

IL-4 and IFN-\(\gamma\) are classic T\textsubscript{h}2 and T\textsubscript{h}1 cytokines, respectively. However, numerous other cytokines and chemokines also participate in inflammatory processes. To more widely capture the iNK T cell response landscape amid prolonged stress, we performed multiplex analysis on serum samples collected 2, 12, and 24 h after treatment with \(\alpha\)GC or vehicle. A multitude of cytokines, other than IL-4 and IFN-\(\gamma\), were significantly reduced at one or several time points in the serum of stressed mice. These included IL-2, IL-5, IL-13, eotaxin, granulocytemacrophage colony-stimulating factor (GM-CSF), IFN-\(\gamma\)-inducible protein 10 (IP-10)/CXCL10, monocyte chemoattractant protein-1 (MCP-1)/CCL2, RANTES/CCL5, and tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)) (Figures 1F and S3). In our cytokfluorimetric analyses, we confirmed diminished intracellular levels of IL-2, IL-5, and IL-13 in \(\alpha\)GC-stimulated iNK T cells from stressed animals (Figures S1E–S1Q). In contrast with the above mediators, IL-10, IL-23, and IL-27 became notably detectable at strikingly high quantities in animals that had been restrained (Figures 1F and S3). Furthermore, stress augmented the production of IL-1\(\alpha\), IL-1\(\beta\), macrophage inflammatory protein-1\(\alpha\) (MIP-1\(\alpha\)/CCL3, and MIP-3\(\alpha\)/CCL20 in \(\alpha\)GC-treated mice (Figures 1F and S3) and also resulted in a trend toward increased IL-17A levels at the 12-h time point (\(p = 0.075\)).

There were a number of cytokines whose serum levels were not different between stressed and control cohorts, including granulocyte colony-stimulating factor (G-CSF), IL-6, IL-7, IL-9, IL-15, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-28B/IFNL3, IL-31, IL-33, keratinocyte-derived chemokine (KOCXCL1, leukemia inhibitory factor (LIF), lipopolysaccharide-inducible CXC chemokine (LIX)/CXCL5, macrophage colony-stimulating factor (M-CSF), monokine induced by IFN-\(\gamma\) (MIG)/CXCL9, MIP-1\(\beta\)/CCL4, MIP-2/CXCL2, transforming growth factor \(\beta\)1 (TGF-\(\beta\)-1), TGF-\(\beta\)-2, TGF-\(\beta\)-3, and vascular endothelial growth factor (VEGF) (Figure 1F and data not shown).

Taken together, our cytokine analyses indicate that iNK T cells defy the popular belief that stress indiscriminately steers all T cell responses toward a purely or heavily T\textsubscript{h}2-biased phenotype. Instead, iNK T cells display a mixed signature dominated by “select” pro- and anti-inflammatory cytokines. Even within the same general category, atypical patterns emerged. For example, whereas stress raised the levels of IL-1\(\alpha\) and IL-1\(\beta\) after \(\alpha\)GC injection, TNF-\(\alpha\) was diminished and IL-6 remained unaltered.

Our multiplexing analyses did not include a 6-h time point post-\(\alpha\)GC administration, at which IL-12 reaches its peak levels (Fuji et al., 2002). This was remedied in separate ELISAs in which a nearly 10-fold reduction in IL-12 was evident. To be exact, serum IL-12 levels were 4,355 ± 422 pg/mL and 403 ± 278 pg/mL in control and stressed animals, respectively. Given the prominent role of DCs in IL-12 production following in vivo iNK T cell activation (Blumenfeld et al., 2011; Fuji et al., 2006), we examined the frequency of IL-12\(^{+}\) DCs, which was significantly reduced in stressed mice that had received \(\alpha\)GC 6 h earlier (Figure S1H). The above results are consistent with a “split mini-signature” even among classic pro-inflammatory cytokines.

Next, we determined whether stress alters iNK T cell responses to CD1d-binding glycolipids other than \(\alpha\)GC. We found prolonged restraint (Figure 1A) to suppress cytokine responses triggered by \(\alpha\)-C-galactosylceramide (\(\alpha\)GCC), a T\textsubscript{h}1-polarizing analog of \(\alpha\)GC that carries a single glycosidic O-to-CH\(_2\) substitution (Figure 1G) (Schmieg et al., 2003). To ascertain whether stress compromises CD1d/TCR-independent, cytokine-driven iNK T cell responses, we used a combination of IL-12 and IL-18, which enables NK cells and innate-like T cells to produce IFN-\(\gamma\) (Velázquez et al., 2008). Therefore, we subjected mice to restraint before injecting them with IL-12 and IL-18 (Figure 1A). Both hepatic and splenic iNK T cells from stressed mice failed to produce IFN-\(\gamma\) in this setting (Figure 1H). Similarly, NK cells from a stressed cohort were unable to mount an optimal IFN-\(\gamma\) response to IL-12 and IL-18 (Figure S1J).

Collectively, prolonged psychological stress abrogates IL-4 and IFN-\(\gamma\) responses that are either elicited or potentiated by \(\alpha\)GC-exposed iNK T cells. This finding appears reproducible across different genetic backgrounds in both sexes and irrespective of iNK T cells’ tissue location and activation mode. The mixed inflammatory signature observed in stressed animals represents a stark contrast with T\textsubscript{h}2-polarized T\textsubscript{conv} responses.

Unlike other lymphocyte subsets, iNK T cells are refractory to stress- and glucocorticoid-induced apoptosis

Mediators of stress disrupt certain defense mechanisms by inducing apoptosis in lymphocytes (Herold et al., 2006; Tseng et al., 2005; Yin et al., 2000). As expected, non-parenchymal hepatic mononuclear cell (HMNC) and splenocyte counts were significantly lower in stressed mice than in controls immediately after restraint (2.2 × 10\(^{6}\) ± 0.2 × 10\(^{6}\) versus 3.5 × 10\(^{5}\) ± 0.3 × 10\(^{5}\) for HMNCs; 54.7 × 10\(^{6}\) ± 7.8 × 10\(^{6}\) versus 77.6 × 10\(^{6}\) ± 8.3 × 10\(^{6}\) for splenocytes; \(n = 6\) per group) and also 2 h after \(\alpha\)GC administration (1.2 × 10\(^{6}\) ± 0.1 × 10\(^{6}\) versus 2.3 × 10\(^{5}\) ± 0.1 × 10\(^{5}\) for HMNCs; 33.2 × 10\(^{6}\) ± 7.0 × 10\(^{6}\) versus 62.0 × 10\(^{6}\) ± 8.3 × 10\(^{6}\) for splenocytes; \(n = 5\) per group). Therefore, because physical restraint impeded IL-4 and IFN-\(\gamma\) responses to \(\alpha\)GC (Figure 1), we asked whether cytokine-producing cells had simply died. We found increases, rather than decreases, in hepatic and splenic iNK T cell frequencies in stressed mice (Figure 2A). This was curious because K\(\text{i67}^{+}\) iNK T cell frequencies were similar between stressed and control animals (30.3% ± 6.7% versus 27.0% ± 3.0% for hepatic iNK T cells; 17.6% ± 3.3% versus 21.7% ± 5.3% for splenic iNK T cells; \(n = 4\) per group), dismissing the possibility that iNK T cells had undergone expeditious proliferation during stress. Our finding...
Figure 2. Unlike T<sub>conv</sub> cells, iNKT cells are resistant to glucocorticoid-induced apoptosis

(A) WT B6 mice were left undisturbed or restrained for 12 h, after which HMNCs and splenocytes were harvested and stained with a monoclonal antibody (mAb) to TCR<sub>b</sub> along with empty (control) or PBS-57-loaded CD1d tetramers. Representative dot plots and summary data depict hepatic and splenic iNKT cell frequencies in stressed and control mice.

(B) The absolute numbers of iNKT and TCR<sub>b</sub><sup>+</sup>PBS-57-loaded CD1d tetramer<sup>-</sup>T<sub>conv</sub> cells were also calculated.

(C) In addition, the percentages of iNKT and T<sub>conv</sub> cells containing active caspases were determined by flow cytometry.

(D) Hepatic iNKT and T<sub>conv</sub> cells were purified from ≥5 mice that had been either subjected to 2, 6, or 12 h of restraint stress or left undisturbed. After obtaining cDNA, the indicated gene products were amplified by quantitative PCR. Gene expression fold changes in iNKT and T<sub>conv</sub> cells isolated from stressed mice relative to corresponding cell populations from control animals were calculated using the 2<sup>−ΔΔCt</sup> method and used to generate a heatmap.

(E) Hepatic iNKT and T<sub>conv</sub> cells were analyzed for their intracellular Bcl-2 content.

(F) Hepatic T<sub>conv</sub> cells were enumerated in Nr3c1<sup>−/−</sup> and Nr3c1<sup>−/−</sup>Lck<sup>Cre</sup> mice that had been either restrained or left undisturbed.

(G) Cohorts of WT B6 mice were given corticosterone (CS) or Veh in drinking water for 21 days before they were sacrificed for their livers and spleens, in which iNKT and T<sub>conv</sub> cells were enumerated.

Each symbol in (A)–(C) and (E)–(G) represents an individual mouse, and error bars represent SEM. *p < 0.05, **p < 0.01, ****p < 0.0001 by unpaired Student’s t tests. NS, not significant.
that iNKT cells’ absolute numbers remained stable in contrast with marked drops in Tconv cell numbers (Figure 2B) suggested that iNKT cells were insensitive to stress-provoked apoptosis. This was validated by measuring the intracellular active caspase content of these cells (Figure 2C).

iNKT cells are “pre-activated, memory-like” T cells (D’Andrea et al., 2000; Park et al., 2000). Therefore, we asked whether their resistance to apoptosis was a mere reflection of this feature and thus mimicable by the memory subset of Tconv cells. Naive and memory Tconv pools were distinguished based on CD44 expression (Figure S4A). Similar to unfractonated and naive Tconv cells, and unlike iNKT cells, CD44+ memory Tconv cells were less frequent and contained more active caspases in restrained animals (Figure S4B). Therefore, iNKT cells’ resistance to stress is not linked to their memory-like property.

To shed light on the molecular mediators of cell survival and death in our system, we analyzed the transcript levels of relevant genes in sorted cells 2, 6, and 12 h into restraint stress. Compared with baseline levels, several pro-apoptotic genes were upregulated in Tconv but not in iNKT cells. These included the apoptosis component Apat1, the Bcl-2 family members Bad, Bbc3, Bik, and Pmaip1, and the executioner caspases-3 and -6 (Figure 2D). In fact, a number of these genes were downregulated in iNKT cells. At the protein level, the pro-survival molecule Bcl-2 was elevated fact, a number of these genes were downregulated in iNKT cells.

Stress suppresses γGC-elicited cytokine responses through an iNKT cell-intrinsic, GR signaling-dependent mechanism independently of neurotransmission from post-ganglionic sympathetic neurons

We next attempted to elucidate the upstream neurological pathway(s) governing iNKT cell hypersponsiveness in the context of prolonged restraint stress. Given the paramount role of the SNS in the fight-or-flight response (Elenkov et al., 2000), we first looked into the expression of SNS neurotransmitter receptors in iNKT cells purified from the liver of stress-naive B6 mice. We did not detect Npy1r, Npy2r, Npy4r, Npy5r, or Npy6r transcripts (data not shown), and synthetic NPY failed to modulate cytokine production by the iNKT cell hybridoma DN32.D3 in response to γGC (data not shown). Hepatic iNKT cells had detectable mRNAs encoding the adrenergic receptors Adra2a, Adra2b, Adrb1, and Adrb2 at levels comparable to or lower than those found in matched hepatic Tconv cells (Figure S5A).

In addition, NE inhibited IL-2 production by γGC-stimulated DN32.D3 cells (Figure S5B), which was preventable by β-adrenergic receptor antagonism with propranolol (Figure S5C). In these experiments, the brain served as a negative control because OHDA does not cross the blood-brain barrier (Schober, 2004).

We then shifted our focus back onto the HPA axis and glucocorticoids. Elevated serum CS in stressed mice reassured us that prolonged restraint in our hands could induce robust activation of the HPA axis (Figure 3C). We found pretreatment with the glucocorticoid synthesis inhibitor metyrapone to rescue IL-4 and IFN-γ production in stressed mice that subsequently received γGC (Figure 3A). Sympathectomy was confirmed by reduced tyrosine hydroxylase (TH) staining in the spleens of OHDA-treated animals (Figure 3B). In these experiments, the brain served as a negative control because OHDA does not cross the blood-brain barrier (Schober, 2004).

Although the systemic ablation of GR signaling could relieve iNKT cell hypersponsiveness during stress, it was unclear whether glucocorticoids were acting on iNKT cells directly or on other cell types such as glycolipid Ag-presenting cells. We first demonstrated that iNKT cells from naive mice express the GR, at a greater level than that in matched Tconv cells. This was manifest at both mRNA and protein levels (Figures 4A and 4B). Second, we observed that stress instigates GR signaling in hepatic and splenic NK and B cells during prolonged stress, which was reversible by treatment with the GR antagonist RU486 (Figure S4C).

Exogenous glucocorticoids are prescribed for many diseases and conditions. Therefore, we tested the effect of long-term, oral administration of corticosterone (CS), the main glucocorticoid in rodents (Pruett, 2003), on T cell apoptosis. Similar to endogenous glucocorticoids, CS reduced Tconv cell numbers in B6.Nr3c1fl/flLckcre/WT (Nr3c1flLckcre) mice (Figure 2F). We also observed a sharp numerical decline in hepatic and splenic NK and B cells during prolonged stress, which was reversible by treatment with the GR antagonist RU486 (Figure S4C).

To summarize, iNKT cells’ unusual resilience in the face of a stress response is mediated by glucocorticoid-GR interactions. Importantly, the above results also rule out cell death as the reason behind the dwarfed IL-4 and IFN-γ responses of iNKT cells to glycolipid Ag or cytokines (Figure 1) in stressed animals.

Stress suppresses γGC-elicited cytokine responses through an iNKT cell-intrinsic, GR signaling-dependent mechanism independently of neurotransmission from post-ganglionic sympathetic neurons

We next attempted to elucidate the upstream neurological pathway(s) governing iNKT cell hypersponsiveness in the context of...
Next, we asked whether physical restraint per se alters the activation status of iNKT cells. Consistent with their "pre-activated" phenotype (D’Andrea et al., 2000; Park et al., 2000), hepatic and splenic iNKT cells expressed high surface levels of CD25, CD44, and CD69 and minimal CD62L in their steady state (Figure 4F). Moreover, confinement stress failed to appreciably change these baseline levels (Figure 4F). Therefore, we took a more comprehensive approach by comparing the transcriptional profiles of purified iNKT cells from stressed and control mice. Although stress upregulated Bcl2 and CD127 (Il7ra), the mRNA levels of a number of genes that support the effector functions of iNKT cells were reduced. These include Cd40l, Il18rap, Egr2, Ifr4, Nfatc3, Tbx21, Ifng, Il4, Gzma, Tnf, Tnfsf9, and Tnfsf10 (Figure 4G). Therefore, wide-ranging iNKT cell dysfunctions, beyond select cytokine production, can be expected in the aftermath of restraint-induced GR signaling.

Long-term exposure to multiple unpredictable stressors, but not to the same stressor, hinders iNKT cell functions

Chronic stress can be due to long-term exposure to different stressful events or elements, resulting in sustained glucocorticoid release in the absence of organismal “habituation.” This can be simulated by the chronic variable stress (CVS) model whereby mice are subjected to heterotypic psychological and/or physical stressors, once daily and once nightly, for 21 days (Figure 5A). Given the importance of GR signaling in restraint-induced iNKT cell impairments, we hypothesized that CVS should impede in vivo cytokine production in response to αGC. We first confirmed elevated blood CS levels at the conclusion of the CVS experiment (Figure 5B), consistent with previous reports that CVS continuously activates the HPA axis (Franco et al., 2016). In addition, hepatic and splenic iNKT cells maintained their absolute numbers in mice that had been subjected to CVS (Figure 5C). Finally, and as hypothesized, animals that were injected with αGC after CVS had lower levels of IFN-γ, IL-2 (Figure 5D). We also exposed parallel cohorts of mice repeatedly to the same stressor, physical restraint, for 21 consecutive days before αGC treatment. In this repeated restraint stress (RRS) model, animals predict the stressor and habituate and adapt to it by gradually de-escalating glucocorticoid release (Girotti et al., 2006). Indeed, αGC-induced cytokine production remained impeccable in mice that had undergone RRS (Figure 5D), a sharp contrast with the CVS model.
Figure 4. Stress impedes iNKT cell responses to αGC in a cell-autonomous, GR-dependent manner and creates a transcriptomic signature consistent with extensive iNKT cell dysfunctions

(A) Hepatic iNKT and Tconv cells from naïve B6 mice (n = 10 per experiment) were fluorescence-activated cell sorting (FACS) purified and the Nr3c1 mRNA content of iNKT cells relative to that of Tconv cells was PCR quantitated. Fold-change values were determined in 3 independent experiments.

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The above results further enforce our conclusion that the activation of the HPA axis mediates stress-induced iNKT cell dysfunctions.

Glucocorticoid secretion due to prolonged stress interferes with iNKT cells’ antimetastatic function

iNKT cells participate in anticancer immune surveillance, and their glycolipid agonists have been used in clinical trials for multiple malignancies (Fuji and Shimizu, 2019; van den Heuvel et al., 2011). However, whether stress alters the antitumor property of iNKT cells has not been addressed.

We first assessed the oncolytic capacity of splenocytes from mice that had been subjected to prolonged restraint before they received an αGC injection. Effector cells isolated from stressed mice were less competent in killing YAC-1 lymphoma cells in vitro (Figure 6A). We then used an in vivo killing assay with which to test the ability of αGC-activated NK cells and pre-mNK cells to destroy B6, β2M<sup>-/-</sup> (β2M<sup>-/-</sup>) splenocytes (Choi et al., 2019). Consistent with our in vitro results, β2M<sup>-/-</sup> target cells were only poorly cleared in previously restrained animals (Figure 6B).

Next, we examined whether stress interferes with the ability of αGC-primed iNKT cells to prevent metastatic cancer in vivo. Mice were restrained, or not, before receiving a single intraperitoneal (i.p.) injection of αGC or vehicle, followed shortly by an intravenous (i.v.) inoculum of B16-F10 melanoma cells. Visual enumeration of pulmonary metastases revealed a complete loss of αGC-mediated protection in stressed mice (Figure 6C). This finding was validated by whole-body bioluminescence imaging of tumor-bearing B6 albino mice that showed far greater B16-F10-Red-FLuc (B16-FLuc) tumor burden in their lungs if they were previously restrained (Figure 6D). Furthermore, RU486 treatment before stress reinstated the efficacy of αGC therapy against B16-F10 metastasis (Figure 6E). Therefore, through a GR-dependent pathway, prolonged psychological...
stress abolishes the capacity of iNKT cells to orchestrate antime-tastatic responses.

MAIT cells are resistant to glucocorticoid-induced apoptosis but fail to mount optimal cognate responses under stress

Dubbed “the human cousins” of mouse iNKT cells, MAIT cells are MR1-restricted T cells with key roles in host defense [Legoux et al., 2017; Salou et al., 2019]. They are scarce in conventional laboratory mice (Cui et al., 2015) but frequent in the human PB, liver, and lungs (Dusseaux et al., 2011; Franciszkiewicz et al., 2016; Tang et al., 2013).

We asked whether similar to mouse iNKT cells, human iNKT cells survive exposure to glucocorticoids. We incubated human PB mononuclear cells (PBMCs) (Figure 7A) or HMNCs (Figure 7B) for 24 h with hydrocortisone (HC), the main stress-induced glucocorticoid produced in humans, or with dexamethasone (DEX), a commonly prescribed synthetic glucocorticoid. Exposure to either HC or DEX elevated the intracellular active caspase levels of PB T_conv cells (Figure 7A). By contrast, matched blood iNKT and MAIT cells contained very low and stable levels of active caspases. A similar pattern was evident among hepatic MAIT cells (Figure 7B). Moreover, in two liver samples in which iNKT cells were detectable, there was no evidence of increased caspase activity after incubation with HC or DEX (data not shown).

Similar to mouse iNKT and T_conv cells from stressed animals, which upregulate CD127 in a cell-autonomous, GR-dependent manner to respond to the pro-survival cytokine IL-7 (Figures S6A–S6E), human MAIT, iNKT, and T_conv cells increased their...
Figure 7. Stress and glucocorticoids fail to program MAIT cells for apoptosis but render them hyporesponsive to 5-OP-RU
(A and B) Human PBMCs (A) and HMNCs (B) were exposed to hydrocortisone (HC), dexamethasone (DEX), or Veh for 24 h before they were analyzed by flow cytometry to detect intracellular active caspasases in the indicated T cell subsets. gMFI values are summarized in bar graphs.
(C) Immediately after prolonged restraint stress (or not), HMNCs from B6-MAITCAST mice were stained with 6-FP- (control) or 5-OP-RU-loaded MR1 tetramers along with anti-TCRβ and -B220 mAbs. Representative zebra plots illustrate MAIT cell populations after gating on TCRβ+B220/C0 events, and scatterplots summarize MAIT cell frequencies among total γδ T cells.
(D) The absolute numbers of hepatic MAIT cells were also calculated.
(E) B6-MAITCAST and MR1−/− mice (n ≥ 3) were injected i.p. with 5-OP-RU or Veh, and serum IL-4 and IFN-γ levels were quantified at the indicated time points.
(F) B6-MAITCAST mice were restrained (or left undisturbed), injected with 5-OP-RU (n = 8) or Veh (n = 3 or 4), and bled for serum IL-4 and IFN-γ measurements.

(legend continued on next page)
surface CD127 levels upon exposure to glucocorticoids (Figure S6F). This phenomenon may serve as a defense mechanism at the cellular level.

In vivo studies on MAIT cells can be challenging due to the low frequency of these cells in standard mouse strains. Therefore, we used B6-MAITCAST mice that contain ~20 times more MAIT cells than do WT B6 mice (Cui et al., 2015). Subjecting these mice to prolonged restraint stress, rather than decreased, hepatic MAIT cell frequencies among αβ T cells (Figure 7C), accompanied by enhanced CD127 expression (Figure S6G). Furthermore, stress failed to reduce absolute MAIT cell numbers (Figure 7D).

To assess the impact of stress on MAIT cell functions, we first demonstrated that a single i.p. injection of 5-OP-RU gives rise to early serum IL-4 and IFN-γ spikes in B6-MAITCAST mice but not in MR1−/− B6-MAITCAST (MR1−/−) MAIT-deficient animals (Figure 7E). This indicated a requirement for MAIT cells in these responses and provided us with readily available controls to work with. B6-MAITCAST mice that had undergone restraint stress before they received 5-OP-RU had significantly lower blood levels of IL-4 and IFN-γ compared with control animals (Figure 7F). Likewise, as quickly as 30 min post-5-OP-RU administration, IL-4 and IFN-γ became undetectable inside MAIT cells from control mice but not in stressed animals (Figure 7G). Therefore, stress lessens MAIT cell capacities to potentiate Th1- and Th2-type responses in vivo.

**DISCUSSION**

iT cells link the innate and adaptive arms of immunity (Matsuda et al., 2008; Rudak et al., 2018). Therefore, revealing how stress affects iT cell responses is important both from a basic biological standpoint and in light of their therapeutic potentials.

We demonstrate that stress compromises the ability of iNKT cells to trigger Th1- and Th2-type responses and to promote antimitastatic immune surveillance. Mechanistically, this hyporesponsive state is dependent upon direct GR signaling in iT cells, which remain uniquely and remarkably recalcitrant to glucocorticoid-inflicted apoptosis.

Many studies to date have suggested that mediators of stress promote Th2-type immunity (Calcagni and Elenkov, 2006; Hou et al., 2013; Hu et al., 2014). Tamada et al. argued that glucocorticoids selectively retain IL-4-producing NKT cells in the T cell repertoire (Tamada et al., 1998). Our findings indicate that psychological stress limits NKT cell-mediated Th2-type responses, albeit not exclusively. In fact, stress generates a mixed inflammatory signature and also skews NKT cell responses in favor of select anti-inflammatory cytokines, namely IL-10 and IL-27, but not TGF-β. To what extent these immunosuppressive cytokines inhibit Th1 and/or Th2 immunity following NKT cell stimulation remains to be determined. We also found that stressed mice exposed to zGC generate greater levels of Th17-associ-
we have found that a brief period (15 min) of physical restraint, which results in acute stress (Dhabhar et al., 2012), fails to alter zGC-elicited cytokine production (data not shown). Consistent with the above theory, plasma NE and epinephrine levels reach their peak as early as 5–20 min after restraint in rats whereas CS levels peak later, around 60 min following physical restraint (Kvetnansky et al., 1978, 1993).

In a mouse model of cerebral stroke, Wong et al. demonstrated that NE released by post-ganglionic sympathetic neurons induces IL-10 production by hepatic iNKT cells, which leads to immunosuppression and secondary bacterial infection (Wong et al., 2011). However, whether or not SNS mediators, including NE, interfere with TCR-mediated iNKT cell activation was not assessed. Nielsen and coworkers reported that sustained adrenergic receptor stimulation influences the efficacy of zGC-based cancer immunotherapy only minimally (Nielsen et al., 2018), and our current work indicates that SNS mediators do not appreciably impact iNKT cell responses to glycolipid Ags. Therefore, in the absence or presence of concomitant TCR stimulation, adrenergic receptor signaling may have different outcomes.

iNKT cell impairments in stressed animals were accompanied by increased levels of GILZ, a transcriptional target of the GR (Moncorz et al., 2019). GILZ exerts broad anti-inflammatory activities mediated, partially, by protein-protein interactions that inhibit NF-κB and AP-1 (Ronchetti et al., 2015). It has been speculated that GILZ binds directly to DNA to repress the transcription of several genes that control T cell functions (Yosef et al., 2015). GILZ was recently shown to abolish the efficacy of immunostimulatory therapies against murine tumors, and greater GILZ expression within TMEs correlated with poorer prognosis in human cancers (Yang et al., 2019). It is conceivable that GILZ contributes to suppressed iNKT cell functions in stressed individuals. As such, selective inhibitors of GILZ may be beneficial.

We recently reported that long-term stress upregulates the immune checkpoint molecule T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) on iNKT cells in a cell-autonomous, GR-dependent fashion (Rudak et al., 2019). Our preliminary results indicate that systemic blockade of TIGIT expression within TMEs correlated with poorer prognosis in human cancers (Yang et al., 2019). It is conceivable that TIGIT contributes to suppressed iNKT cell functions in stressed subjects. As such, selective inhibitors of TIGIT may be beneficial.

Unlike Tconv cells that are restricted by polymorphic MHC molecules, iNKT and MAIT cells recognize cognate Ags complexed with monomorphic molecules, namely CD1d and MR1, respectively (Kawano et al., 1997; Treiner et al., 2003). Therefore, CD1d and MR1 ligands should work across genetically distinct individuals, which makes iT cell attractive targets in immunotherapeutic interventions for microbial infections and/or malignancies (Haeryfar and Mallevaey, 2015; Rudak et al., 2018). A growing body of evidence implicates psychological stress as an obstacle to cancer immunotherapy (Lei et al., 2011; Sommershoj et al., 2017; Yang et al., 2019), and our findings provide a mechanism of stress-induced immunosuppression with wide-ranging repercussions for antitumor immunity. In addition, stress curbed or drastically altered systemic inflammatory responses to zGC and 5-OP-RU, both of which are derived from microbes. It is thus likely that stress also impedes iT cell responses to pathogens in natural or therapeutic settings, which will be a subject of future investigations.

STAR METHODS

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**QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at [https://doi.org/10.1016/j.celrep.2021.108979](https://doi.org/10.1016/j.celrep.2021.108979).

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**AUTHOR CONTRIBUTIONS**

P.T.R. designed and performed experiments, analyzed and interpreted data, and wrote the initial manuscript. J.C. and K.M.P. performed experiments and analyzed data. K.L.S., D.N.J., and P.J.F. interpreted data. S.M.M.H. conceived the idea, obtained funding, administered the project, designed experiments, analyzed and interpreted data, and edited the final manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Alexa 700-conjugated anti-mouse/human B220 (Clone RA3-6B2) | Thermo Fisher Scientific | Cat # 56-0452-82; RRID: AB_891458 |
| Alexa 700-conjugated anti-human CD3 (Clone UCHT1) | Thermo Fisher Scientific | Cat # 56-0038-42; RRID: AB_10597906 |
| FITC-conjugated anti-mouse CD4 (Clone GK1.5) | Thermo Fisher Scientific | Cat # 11-0041-81; RRID: AB_464891 |
| PE-eFluor610-conjugated anti-mouse CD11c (Clone N418) | Thermo Fisher Scientific | Cat # 61-0114-80; RRID: AB_2574529 |
| PerCP-Cy5.5-conjugated anti-mouse CD28 (Clone 37.51) | Thermo Fisher Scientific | Cat # 45-0281-80; RRID: AB_925744 |
| FITC-conjugated anti-mouse/human CD44 (Clone IM7) | Thermo Fisher Scientific | Cat # 15-0441-81; RRID: AB_468748 |
| PE-conjugated anti-mouse/human GILZ (Clone CFMKG15) | Thermo Fisher Scientific | Cat # 12-4033-82; RRID: AB_1659717 |
| PE-conjugated anti-mouse/human IL-5 (Clone TRFK5) | Thermo Fisher Scientific | Cat # 47-7021-82; RRID: AB_1235004 |
| eFluor660-conjugated anti-mouse IL-12p35 (Clone 4D10p35) | Thermo Fisher Scientific | Cat # 46-5698-82; RRID: AB_11040981 |
| APC-eFluor780-conjugated anti-mouse/rat IgG1 (Clone OX-19) | Thermo Fisher Scientific | Cat # 45-4902-82; RRID: AB_1272001 |
| APC-eFluor780-conjugated anti-mouse/rat IgG2a (Clone eBRG1) | Thermo Fisher Scientific | Cat # 45-4888-80; RRID: AB_906260 |
| APC-eFluor780-conjugated anti-mouse/rat IgG2x (Clone eBR2x) | Thermo Fisher Scientific | Cat # 45-4714-82; RRID: AB_906257 |
| APC-eFluor780-conjugated anti-mouse/rat IgG2a: IgG1 (Clone P3.6.2.8.1) | Thermo Fisher Scientific | Cat # 25-4714-42; RRID: AB_1548705 |
| APC-eFluor780-conjugated anti-mouse/rat IgG2x: IgG1 (Clone P3.6.2.8.1) | Thermo Fisher Scientific | Cat # 12-4301-82; RRID: AB_470047 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| PE-Cy7-conjugated rat IgG2b isotype control (Clone eB149/10H5) | Thermo Fisher Scientific | Cat # 25-4031-82; RRID: AB_891624 |
| eFluor660-conjugated rat IgG2a isotype control (Clone eBR2a) | Thermo Fisher Scientific | Cat # 50-4321-80; RRID: AB_10598640 |
| APC-eFluor780-conjugated rat IgG1x isotype control (Clone eBRG1) | Thermo Fisher Scientific | Cat # 47-4301-80; RRID: AB_1271986 |
| PE-Cy7-conjugated rat IgG1x isotype control (Clone R3-34) | BD Biosciences | Cat # 557645; RRID: AB_306762 |
| PE-Cy7-conjugated rat IgG1x isotype control (Clone eBRG1) | Thermo Fisher Scientific | Cat # 25-4301-82; RRID: AB_470198 |
| PerCP-eFluor 710-conjugated rat IgG2ax isotype control (Clone eBR2a) | Thermo Fisher Scientific | Cat # 46-4321-82; RRID: AB_1834455 |
| PE-conjugated mouse IgG2a isotype control (Clone G155-178) | BD Biosciences | Cat # 556653; RRID: AB_396517 |
| PE-Cy7-conjugated Armenian hamster IgG isotype control (Clone eBio299Arm) | Thermo Fisher Scientific | Cat # 25-4888-82; RRID: AB_470204 |
| FITC-conjugated Armenian hamster IgG isotype control (Clone eBio299Arm) | Thermo Fisher Scientific | Cat # 11-4888-85; RRID: AB_470038 |
| PE-conjugated rat IgG2bx isotype control (Clone eB149/10H5) | Thermo Fisher Scientific | Cat # 12-4031-82; RRID: AB_470042 |
| Anti-mouse/rat tyrosine hydroxylase (polyclonal) | Abcam | Lot # 117112 |
| Anti-mouse/rat/human/cow/fruit fly/sheep/plant/Xenopus/yeast/zebrafish β-actin (Clone mAbGEa) | Thermo Fisher Scientific | Cat # MA1-744; RRID: AB_2223496 |
| HRP-conjugated goat anti-Rabbit IgG (H+L) (Polyclonal) | Thermo Fisher Scientific | Cat # 31460; RRID: AB_228341 |
| HRP-conjugated goat anti-mouse IgG (H+L) (Polyclonal) | Thermo Fisher Scientific | Cat # 31430; RRID: AB_228307 |
| Anti-mouse TIGIT (Clone 1B4) | Cell Essentials, Inc. (Boston, MA): http://www.cell-essentials.com | Lot # 111704 |
| Mouse IgG1x isotype control (Clone MOPC-21) | BioXCell | Cat # BE0083; RRID: AB_1107784 |

**Biological samples**

- Human tumor-free liver samples: This paper/University Hospital at London Health Sciences Centre
- Healthy human blood samples: This paper

**Chemicals, peptides, and recombinant proteins**

- APC- or PE-conjugated PBS-57-loaded mouse or human CD1d tetramer: NIH Tetramer Core Facility
- APC- or PE-conjugated unloaded mouse or human CD1d tetramer: NIH Tetramer Core Facility
- APC- or PE-conjugated 5-OP-RU-loaded mouse or human MR1 tetramer (Corbett et al., 2014): NIH Tetramer Core Facility
- APC- or PE-conjugated 6-FP-loaded mouse or human MR1 tetramer (Corbett et al., 2014): NIH Tetramer Core Facility
- αGC: Funakoshi
- αCGC: NIH Tetramer Core Facility
- 5-amino-6-D-ribitylaminouracil (5-ARU): Dr. Olivier Lantz
- Methylglyoxal solution: Sigma-Aldrich
- Phorbol 12-myristate 13-acetate (PMA): Sigma-Aldrich
- Ionomycin: Sigma-Aldrich
- Brefeldin A: Sigma-Aldrich
- Recombinant mouse GM-CSF: Peprotech
- Recombinant mouse IL-4: Peprotech
- Recombinant mouse IL-12p70: Peprotech
- Recombinant mouse IL-18: R&D Systems
- 6-hydroxydopamine hydrobromide: Sigma-Aldrich
- Corticosterone: Sigma-Aldrich

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Hydrocortisone      | Sigma-Aldrich | Cat # H0888 |
| Dexamethasone       | Sigma-Aldrich | Cat # D4902 |
| Metyrapone          | Sigma-Aldrich | Cat # M2696 |
| RU486               | Sigma-Aldrich | Cat # M8046 |
| Norepinephrine      | Sigma-Aldrich | Cat # A7257 |
| Neuropeptide Y      | Sigma-Aldrich | Cat # N5017 |
| Propranolol hydrochloride | Sigma-Aldrich | Cat # P0884 |
| Percoll PLUS        | GE Healthcare | Cat # 17-5445-01 |
| Ficoll-Paque PLUS   | GE Healthcare | Cat # 17-1440-02 |
| Na$_2^{51}$CrO$_4$  | PerkinElmer | Cat # NEZ030S001MC |
| Triton X-100        | Sigma-Aldrich | Cat # T8787 |
| XenoLight D-Luciferin | PerkinElmer | Cat # 127799 |
| 7-aminoactinomycin D (7-AAD) viability dye | Thermo Fisher Scientific | Cat # 00-6993-50 |

**Critical commercial assays**

| Amersham ECL Prime Western Blotting Detection Reagent | GE Healthcare | Cat # RPN2232 |
| eBioscience Ready-SET-Go! Mouse IFN-γ ELISA Kit | Thermo Fisher Scientific | Cat # 88-7314-88 |
| eBioscience Ready-SET-Go! Mouse IL-2 ELISA Kit | Thermo Fisher Scientific | Cat # 88-7024-88 |
| eBioscience Ready-SET-Go! Mouse IL-4 ELISA Kit | Thermo Fisher Scientific | Cat # 88-7044-88 |
| DetectX Corticosterone Enzyme Immunoassay Kit | Arbor Assays | Cat # K014-H1 |
| Intracellular Fixation & Permeabilization Buffer Set | Thermo Fisher Scientific | Cat # 88-8824-00 |
| Foxp3/Transcription Factor Staining Buffer Set | Thermo Fisher Scientific | Cat # 00-5523-00 |
| FITC CaspaTag Pan-Caspase In Situ Assay Kit | EMD Millipore | Cat # APT420 |
| EasySep Mouse CD11c Positive Selection Kit II | STEMCELL Technologies | Cat # 18780A |
| PureLink RNA Mini Kit | Thermo Fisher Scientific | Cat # 12183018A |
| SuperScript VILO cDNA Synthesis Kit | Thermo Fisher Scientific | Cat # 11755-050 |
| Taqman Fast Advanced Master Mix | Thermo Fisher Scientific | Cat # 4444557 |
| CellTrace CFSE Cell Proliferation Kit | Thermo Fisher Scientific | Cat # C34554 |

**Experimental models: Cell lines**

| Mouse: YAC-1 lymphoma cells | ATCC | Cat # TIB-160; RRID: CVCL_2244 |
| Mouse: B16-F10 melanoma cells | Dr. Ann Chambers, Western University | Available from ATCC (Cat # CRL-6475; RRID: CVCL_0159) |
| Mouse: B16-F10-Red-FLuc (B16-FLuc) melanoma cells | PerkinElmer | Cat # BW124734 |
| Mouse: DN32.D3 hybridoma cells | Dr. Albert Bendelac, University of Chicago | N/A |

**Experimental models: Organisms/strains**

| Mouse: C57BL/6 | Charles River Canada | Cat # 027; RRID: IMSR_CRL:027 |
| Mouse: BALB/c | Charles River Canada | Cat # 028; RRID: IMSR_CRL:028 |
| Mouse: i2M$^{−/−}$: B6.129P2-B2m$^{tm1Unc/Jc}$DcrJ | Dr. Anthony Jevnikar, Western University | Available from The Jackson Laboratory (Cat # 002087; RRID: IMSR_JAX:002087) |
| Mouse: B6 albino: B6(Cg)-Tyr$^{+/-}$/J | The Jackson Laboratory | Cat # 000058; RRID: IMSR_JAX:000058 |
| Mouse: B6.Nr3c1$^{f/f}$: B6.Cg-Nr3c1$^{tn1.1Jda/J}$ | The Jackson Laboratory | Cat # 021021; RRID: IMSR_JAX:021021 |
| Mouse: B6.Lck$^{cre/cre}$: B6.Cg-Tg(Lck-cre)548Jxm/J | The Jackson Laboratory | Cat # 003802; RRID: IMSR_JAX:003802 |
| Mouse: Nr3c1$^{f/f}$Lck$^{cre/cre}$: B6.Nr3c1$^{f/f}$Lck$^{cre/cre}$/J | This paper | N/A |
| Mouse: Lck$^{cre/cre}$: B6.Lck$^{cre/cre}$/J | This paper | N/A |
| Mouse: B6-MAIT$^{CAST}$ (Cui et al., 2015) | Dr. Olivier Lantz | N/A |
| Mouse: MR1$^{-/-}$: B6-MAIT$^{CAST}$ (Cui et al., 2015) | Dr. Olivier Lantz | N/A |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Mansour Haeryfar (Mansour.Haeryfar@schulich.uwo.ca).

Materials availability
The B6.Nr3c1fl/flLckcre/wt and B6.Lckcre/wt mouse lines generated in this study can be bred using commercially available parental strains as described under Experimental model and subject details. This study did not generate any other new unique reagents.

Data and code availability
This study did not generate/analyze datasets or codes.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Adult WT B6 and BALB/c mice, between 8 and 16 weeks of age, were purchased from Charles River Canada (Saint-Constant, QC). B6(Cg)-Tyr2/J (B6 albino) mice (#000058) were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-MAITCAST mice, a congenic strain harboring a larger MAIT cell compartment relative to WT B6 mice, and MAIT cell-deficient MR1/C0/B6-MAITCAST mice have been previously described (Cui et al., 2015). B2M−/− mice were provided by Dr. Anthony Jevnikar (Western University, London, ON). Nr3c1Lckcre mice, whose T cells lack the GR, were generated by crossing B6.Nr3c1fl/fl (Jackson #021021) with B6.Lckcre/cre mice (Jackson #003802), followed by backcrossing the offspring with Nr3c1fl mice. Lckcre mice were generated by crossing offspring with WT B6 mice. PCR-based genotyping was conducted throughout breeding. Animals were housed in an institutional barrier facility with constant light/dark cycles. Prior to any experiment, mice were randomly assigned to treatment groups. Both male and female mice were included in our initial serum cytokine analyses (Figures 1B, 1C, 7E, 7F, and S2) and immunophenotyping studies (Figures 2A, 2B, 7C, and 7D). Male mice were used in all other in vivo experiments. Cohorts were always age- and sex-matched. Mouse experiments were conducted following Animal Use Protocols 2010-241, 2018-093 and 2018-130, which were approved by the Animal Care Committee of Animal Care and Veterinary Services at Western University.

Human specimens
HMNCs were obtained from tumor-free liver samples from patients undergoing surgical resection, without prior neoadjuvant therapy, at University Hospital (London Health Sciences Centre, London, ON). Three patients had undergone surgery for colorectal liver metastasis, one for ampullary cancer, one for ampillary adenoma, and one for pancreatic cancer. Patients had a mean age of 64 (range: 37-80) and were all male.

PBMCs were isolated from 4 healthy blood donors, two males and two females, with a mean age of 37 (range: 29-51). Human specimens were collected after obtaining written, informed consent from participants as per study protocols 5545, 2597 and 113362 approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects.

Cell lines
The mouse lymphoma cell line YAC-1 (ATCC TIB-160) was grown at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMAX-I, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL
streptomycin, and 10 mM HEPES, which has been referred to as complete medium. The mouse melanoma line B16-F10 was provided by Dr. Ann Chambers (Western University, London, ON) and maintained in MEM Alpha medium supplemented with 10% FBS. Luciferase-expressing B16-FLuc cells (PerkinElmer #BW124734) were grown in RPMI 1640 containing 10% FBS. DN32.D3, a CD4 CD8 NKT hybridoma cell line from Dr. Albert Bendelac (University of Chicago, Chicago, IL), was cultured in RPMI 1640 containing 10% FBS, 2 mM GlutaMAX-I, and 0.1 mM MEM nonessential amino acids.

METHOD DETAILS

Models of psychological stress
To induce prolonged confinement stress, mice were held horizontally for 12 h inside well-ventilated 50-mL conical tubes. This immobilization procedure prompts psychological stress in rodents without causing pain or physical compression and activates the SNS and the HPA axis (Buynitsky and Mostofsky, 2009). Cage-mate controls remained undisturbed but were deprived of access to food and water for 12 h. To model acute stress, mice were restrained for 15 minutes (Dhabhar et al., 2012) while control animals were left in home cages without food and water.

Repeated restraint stress (RRS) was inflicted by subjecting mice to 21 days of physical immobilization for 1 h daily. The experimenter and the time at which each stressor was applied remained constant throughout the entire procedure.

The chronic variable stress (CVS) model entailed daily exposures to heterotypic psychological or physical stressors for 21 days. One brief stressor during the light cycle and one overnight (O/N) stressor during the dark cycle were introduced each day. In no particular order, brief stressors included placement in a 4 °C environment for 1 h, physical restraint for 1 h, horizontal cage shaking at 80 rpm for 1 h, and placement in 30 °C water for 15 minutes. O/N stressors included water deprivation, cage tilting at a 45-degree angle, constant exposure to light, wet bedding (through pouring ~200 mL of water onto cage bedding), and food deprivation. Unlike RRS, mice fail to habituate to unpredictable stressors in the CVS model and display continuously elevated stress responses as a consequence (Franco et al., 2016). Parallel cohorts of non-stressed control mice were left undisturbed with food and water ad libitum for 21 days.

Chemical sympathectomy
Six days before mice were subjected to prolonged restraint stress, 6-hydroxydopamine (OHDA) (Sigma-Aldrich) was administered i.p. at 200 mg/kg in a vehicle containing 0.9% NaCl and 10−7 M ascorbic acid in phosphate-buffered saline (PBS). Successful sympathectomy was confirmed by immunoblotting for TH in splenic and brain tissues of OHDA-treated mice. We used a rabbit polyclonal Ab (ab117112 from abcam) to capture mouse TH, and an anti-mouse TH (Abcam, #ab125828) mAb served as a loading control. Blots were digitally imaged using a C-DiGit Blot Scanner (LI-COR Biosciences).

Administration and pharmacological inhibition of glucocorticoids
To simulate stress-elicited rise in glucocorticoids, corticosterone (CS) (Sigma-Aldrich) was administered orally. CS was dissolved in absolute ethanol and then diluted in standard drinking water to yield a final concentration of 25 μg/mL of water with 1% ethanol. CS-containing water was provided for 21 days with weekly replenishments. This regimen gives rise to CS serum levels that are comparable to those found in chronically stressed mice as we previously reported (Rudak et al., 2019).

In several experiments, mice were injected i.p. with 200 mg/kg of the glucocorticoid synthesis inhibitor metyrapone (Sigma-Aldrich) or with 25 mg/kg of the GR antagonist RU486 (Sigma-Aldrich) 1 h prior to prolonged restraint stress.

In vivo administration of NKT cell ligands/stimuli
Glycolipid stimulation of NKT cells was achieved via i.p. administration of 100 μg/kg of KR7000/αGC (Funakoshi, Tokyo, Japan) or 200 μg/kg of αCGC in a vehicle containing 5.6% sucrose, 0.75% L-histidine and 0.5% Tween-20, which was further diluted in PBS. αGC was supplied by the NIH Tetramer Core Facility (Atlanta, GA). To stimulate NKT cells in a cytokine-dependent manner, each animal was injected i.v. with 2 ng recombinant mouse IL-12 (Peprotech, Rocky Hill, NJ) plus 200 ng recombinant mouse IL-18 (R&D Systems) in PBS.

To activate MAIT cells, mice were injected i.p. with 200 μL of PBS containing 20 μL of a 5-OP-RU stock solution. The stock solution was prepared by mixing equal volumes of 2 mM 5-amino-6-D-ribitylaminouracil (5-ARU) and 2 mM methylglyoxal in DMSO for 24 h at room temperature. Aliquots were stored at −80 °C until use. Control mice received vehicle (2 mL methylglyoxal in DMSO) diluted in PBS.

Where indicated, mice were given 200 μg of a TIGIT-blocking mAb (clone 1B4) or a mouse IgG1 isotype control (clone MOPC-21 from BioXCell) 1 h prior to αGC administration.

Quantification of serum cytokines and CS
Mice were bled immediately after stress or at 2, 12 and 24 h post-treatment with αGC, αCGC, 5-OP-RU, or an appropriate vehicle. Sera were isolated, aliquoted and stored at −20 °C. Mouse IFN-γ, IL-2 and IL-4 concentrations were measured using eBioscience CatKey.
mixed with purified CD11c+ BMDCs at an (STEMCELL Technologies) containing Ficoll-Paque PLUS (GE Healthcare).  

**Hepatic** Quantitative PCR analyses were interrogated using a BD FACSCanto II flow cytometer equipped with BD FACSDiva version 6.1.2 software. (Applied Biosystems). Normalized Ct (Table S1. Cycle threshold (Ct) values from amplified transcripts were generated using a StepOne Plus Real-Time PCR instrument and the resulting mixture was plated in Custom Taqman Array Fast Plates (Thermo Scientific). Taqman Fast Advanced Master Mix (Applied Biosystems) was added to each cDNA sample, Total RNA was isolated using a PureLink RNA Mini Kit (Thermo Scientific), and cDNA was synthesized using a SuperScript VILO g mAb (clone 2.4G2) to prevent non-specific binding to Fc3 is isolate human PBMCs, uncoagulated blood from healthy donors was spun at 1,200 g 700 (Ammonium-Chlorine-Potassium) lysis buffer for 3 minutes at room temperature. HMNCs were isolated from mouse livers or human 37 °C for subsequent cytokine measurements. Upon completion of the culture, cells were harvested using a cell scraper and CD11c+ BMDCs were magnetically enriched using an EasySep Mouse CD11c Positive Selection Kit II (STEMCELL Technologies).

**Quantitative PCR analyses**  
Hepatic iNKT and/or Tconv cells from ≥ 5 stressed or control mice were sorted to 100% purity using a BD FACSaria III Cell Sorter. Total RNA was isolated using a PureLink RNA Mini Kit (Thermo Scientific), and cDNA was synthesized using a SuperScript VILO cDNA Synthesis Kit (Thermo Scientific). Taqman Fast Advanced Master Mix (Applied Biosystems) was added to each cDNA sample, and the resulting mixture was plated in Custom Taqman Array Fast Plates (Thermo Scientific) containing probe/primer sets listed in Table S1. Cycle threshold (Ct) values from amplified transcripts were generated using a StepOne Plus Real-Time PCR instrument (Applied Biosystems). Normalized Ct (ΔCt) values were calculated by subtracting each Ct value by that of Actb and/or Tbp. The following formula was used to determine the relative mRNA content of the cells: Fold Change = 2-ΔΔCt.

**Generation of bone marrow-derived dendritic cells (BMDCs)**  
Marrow cells were flushed out of femurs and tibias of B6 mice and depleted of erythrocytes. Cells were then washed, filtered and placed inside a T75 polystyrene flask at a density of 1 × 10^6 cells/mL of complete medium supplemented with 10 ng/mL each of recombinant mouse GM-CSF and IL-4 (Peprotech, Rocky Hill, NJ). Cultures were maintained for 6 days at 37 °C in a humidified atmosphere containing 6% CO2. Every other day, non-adherent cells were discarded, and cultures were replenished with fresh medium, GM-CSF and IL-4. Upon completion of the culture, cells were harvested using a cell scraper and CD11c+ BMDCs were magnetically enriched using an EasySep Mouse CD11c Ex vivo and in vitro treatments and stimulations DN32.D3 cells were seeded at 1 × 10^5 cells/well of a U-bottom microplate and treated for 20 minutes with NE (0.1-10 μM) or NPY (10^-6-1 μM), both from Sigma-Aldrich, before they were stimulated with 100 ng/mL of αGC. Where indicated, cells were pretreated with 10 μM propranolol hydrochloride (Sigma-Aldrich) for 20 minutes before they were exposed to NE. After 24 h at 37 °C, cell viability was assessed by 7-AAD staining, and the IL-2 content of supernatants was measured by ELISA. Hepatic mouse iNKT cells were FACSorted from ≥ 5 stressed animals or controls. In a U-bottom microplate, iNKT cells were mixed with purified CD11c+ BMDCs at an iNKT:DC ratio of 2:1. Co-cultures were stimulated with 100 ng/mL of αGC for 24 h at 37 °C, after which cell-free supernatants were stored at −20 °C for subsequent cytokine measurements.

In indicated experiments, HMNCs and splenocytes were seeded at 5 × 10^5 cells/well in a microplate and stimulated with 15 ng/mL of PMA plus 500 ng/mL of ionomycin in the presence of 10 μg/mL brefeldin A, all of which were purchased from Sigma-Aldrich. After 2 h at 37 °C, cells were washed and stained for intracellular cytokines.

Hydrocortisone (HC) and dexamethasone (DEX) were purchased from Sigma-Aldrich, dissolved in absolute ethanol and diluted in RPMI 1640. In a U-bottom microplate, 1 × 10^6 human HMNCs/well or 5 × 10^5 human PBMCs/well were incubated for 24 h at 37 °C in complete medium containing 0.01-10 μM HC or DEX.

**Cytofluorometric analyses**  
After cervical dislocation, mouse spleens were mechanically homogenized and depleted of erythrocytes through exposure to ACK (Ammonium-Chlorine-Potassium) lysis buffer for 3 minutes at room temperature. HMNCs were isolated from mouse livers or human tumor-free liver samples. Specimens were homogenized, and parenchymal cells were removed by density gradient centrifugation at 700 × g in 33.75% Percoll PLUS (GE Healthcare). This was followed by treatment with ACK lysis buffer to eliminate erythrocytes. To isolate human PBMCs, uncoagulated blood from healthy donors was spun at 1,200 × g in 50 mL SepMate PBMC Isolation Tubes (STEMCELL Technologies) containing Ficoll-Paque PLUS (GE Healthcare).  

Before surface staining, mouse cell suspensions were incubated for 10 minutes on ice with 5 μg/mL of an anti-mouse CD16/CD32 mAb (clone 2.4G2) to prevent non-specific binding to Fcγ receptors. Cell surface staining was conducted for 30 minutes at 4 °C in PBS containing 2% FBS. Intracellular detection of cytoplasmic proteins was performed using the Intracellular Fixation & Permeabilization Buffer Set (Thermo Scientific). To stain nuclear proteins, we used the Foxp3/Transcription Factor Staining Buffer Set (Thermo Scientific). A FITC Caspase Pan-Caspase In Situ Assay Kit (EMD Millipore) was used to detect intracellular active caspases.  

Mouse iNKT cells were defined as TCRβ+PBS-57-loaded mCD1d tetramer+ cells, and human iNKT cells as CD3+PBS-57-loaded hCD1d tetramer+ cells, while empty CD1d tetramers served as staining controls. Mouse MAIT cells were defined as B220 TCRβ+5-OP-RU-loaded mMR1 tetramer+ cells, and human MAIT cells as CD3+5-OP-RU-loaded hMR1+ cells. 6-formylpterin (6-FP)-loaded MR1 tetramer reagents, which do not react with the TCR of MAIT cells, were utilized in parallel as staining controls. We identified mouse Tconv cells as TCRβ+PBS-57-loaded mCD1d tetramer+ cells, and human Tconv cells as CD3+PBS-57-loaded hCD1d tetramer+ 5-OP-RU-loaded hMR1 tetramer+ cells. Mouse NK cells, B cells and DCs were immunophenotyped as TCRβ- NK1.1+, TCRβ-B220+ and TCRβ-CD11c+ cells, respectively. Staining with isotype controls was used to draw gates as appropriate. The fluorochrome-conjugated mAbs and tetramer reagents employed in this study are listed in the Key Resources Table. Cells were interrogated using a BD FACS compensated by 7-AAD staining, and the IL-2 content of supernatants was measured by ELISA.

Hepatic iNKT and/or Tconv cells from ≥ 5 stressed or control mice were sorted to 100% purity using a BD FACSaria III Cell Sorter. Total RNA was isolated using a PureLink RNA Mini Kit (Thermo Scientific), and cDNA was synthesized using a SuperScript VILO cDNA Synthesis Kit (Thermo Scientific). Taqman Fast Advanced Master Mix (Applied Biosystems) was added to each cDNA sample, and the resulting mixture was plated in Custom Taqman Array Fast Plates (Thermo Scientific) containing probe/primer sets listed in Table S1. Cycle threshold (Ct) values from amplified transcripts were generated using a StepOne Plus Real-Time PCR instrument (Applied Biosystems). Normalized Ct (ΔCt) values were calculated by subtracting each Ct value by that of Actb and/or Tbp. The following formula was used to determine the relative mRNA content of the cells: Fold Change = 2-ΔΔCt.

**Generation of bone marrow-derived dendritic cells (BMDCs)**  
Marrow cells were flushed out of femurs and tibias of B6 mice and depleted of erythrocytes. Cells were then washed, filtered and placed inside a T75 polystyrene flask at a density of 1 × 10^6 cells/mL of complete medium supplemented with 10 ng/mL each of recombinant mouse GM-CSF and IL-4 (Peprotech, Rocky Hill, NJ). Cultures were maintained for 6 days at 37 °C in a humidified atmosphere containing 6% CO2. Every other day, non-adherent cells were discarded, and cultures were replenished with fresh medium, GM-CSF and IL-4. Upon completion of the culture, cells were harvested using a cell scraper and CD11c+ BMDCs were magnetically enriched using an EasySep Mouse CD11c Positive Selection Kit II (STEMCELL Technologies).

**Ex vivo and in vitro treatments and stimulations**  
DN32.D3 cells were seeded at 1 × 10^5 cells/well of a U-bottom microplate and treated for 20 minutes with NE (0.1-10 μM) or NPY (10^-6-1 μM), both from Sigma-Aldrich, before they were stimulated with 100 ng/mL of αGC. Where indicated, cells were pretreated with 10 μM propranolol hydrochloride (Sigma-Aldrich) for 20 minutes before they were exposed to NE. After 24 h at 37 °C, cell viability was assessed by 7-AAD staining, and the IL-2 content of supernatants was measured by ELISA.

Hepatic mouse iNKT cells were FACSorted from ≥ 5 stressed animals or controls. In a U-bottom microplate, iNKT cells were mixed with purified CD11c+ BMDCs at an iNKT:DC ratio of 2:1. Co-cultures were stimulated with 100 ng/mL of αGC for 24 h at 37 °C, after which cell-free supernatants were stored at −20 °C for subsequent cytokine measurements.

In indicated experiments, HMNCs and splenocytes were seeded at 5 × 10^5 cells/well in a microplate and stimulated with 15 ng/mL of PMA plus 500 ng/mL of ionomycin in the presence of 10 μg/mL brefeldin A, all of which were purchased from Sigma-Aldrich. After 2 h at 37 °C, cells were washed and stained for intracellular cytokines.

Hydrocortisone (HC) and dexamethasone (DEX) were purchased from Sigma-Aldrich, dissolved in absolute ethanol and diluted in RPMI 1640. In a U-bottom microplate, 1 × 10^6 human HMNCs/well or 5 × 10^5 human PBMCs/well were incubated for 24 h at 37 °C in complete medium containing 0.01-10 μM HC or DEX.
51-chromium (⁵¹Cr) release assay
YAC-1 target cells were incubated for 90 minutes at 37°C with 100 μCi Na₂⁵¹CrO₄ (PerkinElmer). Labeled target cells were washed and then co-cultured at indicated effector:target ratios with splenocytes from stressed or control B6 mice that had received αGC or vehicle 24 hours before cytotoxicity assays. Cell-free supernatants were collected 4 h later, in which the ⁵¹Cr activity was quantified using a PerkinElmer Wizard 1470 Automatic Gamma Counter. Experimental release (ER) values were obtained from wells in which effector and target cells were both present. Spontaneous release (SR) and total release (TR) were measured from wells containing medium alone or 1% Triton X-100, respectively. Cytotoxicity was calculated using the following formula: % specific lysis = [(ER-SR) / (TR-SR)] × 100.

In vivo killing assay
Donor splenocytes from WT B6 and β2M−/− mice were labeled with 0.2 μM and 2 μM 5-(and-6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE), respectively, and used as target cells. Cells were washed, mixed at a 1:1 ratio, and analyzed by flow cytometry before injection. Approximately 1 × 10⁷ cells from the resulting suspension were injected i.v. into stressed recipients that were treated with αGC or vehicle. Two h later, animals were sacrificed for their spleen in which the remaining CFSEhi and CFSElo target cells were traced by flow cytometry. In vivo killing was calculated using the following formula: % specific killing = {1 - [(CFSEhi events in recipient / CFSElo events in recipient) / (CFSEhi events pre-injection / CFSElo events pre-injection)]} × 100 as we previously described (Choi et al., 2019).

Metastatic melanoma model
Six h after administration of αGC or vehicle, stressed and control B6 mice received between 2.5 × 10⁵ – 1 × 10⁶ B16-F10 cells in 200 μL PBS i.v. Fourteen days later, lungs were harvested for digital imaging, and distinct tumor nodules on each lung were visually counted. Lungs harboring > 400 nodules were deemed to carry too many nodules for accurate enumeration. Therefore, they were conservatively represented as containing at least 400 nodules.

As an alternative method of measuring the metastatic tumor burden, B6 albino mice were injected with 5 × 10⁵ B16-FLuc cells. After 21 days, they were anaesthetized with isoflurane and injected i.p. with 3 mg XenoLight D-Luciferin (PerkinElmer) in PBS. For up to 35 minutes thereafter, whole body bioluminescence imaging was conducted in an IVIS Lumina XRMS In Vivo Imaging System (PerkinElmer).

QUANTIFICATION AND STATISTICAL ANALYSIS
Flow cytometry results were analyzed using FlowJo version 10.0.7 software (Tree Star, Ashland, OR). The relative pixel intensities of bands in western blot images were quantified using Image Studio version 3.1.4 software. For bioluminescence imaging, total signal (photons/second/cm²/steradian) was quantified by region-of-interest analysis using LivingImage software (PerkinElmer).

Throughout this investigation, objective quantification methods, as opposed to subjective scoring, were used. Therefore, blinding was not necessary. Sufficient sample sizes were not statistically predetermined but were consistent with those from comparable studies and based on our prior experience.

Student’s t tests or one- or two-way ANOVA were employed, as appropriate, using GraphPad Prism version 6.0 software (La Jolla, CA). *, **, *** and **** denote statistically significant differences with p < 0.05, p < 0.01, p < 0.001 and p < 0.0001, respectively. Details related to sample sizes, measures of dispersion and the specific statistical tests used can be found in figure legends.
Supplemental information

Chronic stress physically spares
but functionally impairs
innate-like invariant T cells

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Supplemental Figure 1. Prolonged physical restraint impairs the ability of iNKT cells to elicit T\textsubscript{H1} and/or T\textsubscript{H2}-type cytokine responses to αGC or to a combination of IL-12 and IL-18, but not to a combination of PMA.
and ionomycin (related to Figure 1). (A) B6 mice were physically restrained (or not) for 12 h before they were given αGC. Two hours later, HMNCs and splenocytes were stained with mAbs against IL-4 and IFN-γ and analyzed by flow cytometry. Representative dot plots illustrate the frequencies of IL-4+ and IFN-γ+ iNKT cells after gates were set based on isotype control staining. (B) Sorted hepatic iNKT cells pooled from ≥5 stressed or control B6 mice were stimulated ex vivo with 100 ng/mL of αGC in the presence of CD11c+ BMDCs. After 24 h, IL-4 and IFN-γ levels in culture supernatants were measured. (C) HMNCs and splenocytes from stressed and control B6 mice were stimulated for 2 h with 15 ng/mL of PMA and 500 ng/mL of ionomycin before intracellular levels of IL-4 and IFN-γ in iNKT cells were determined by flow cytometry. (D) HMNCs and splenocytes from restrained or control animals were stained with loaded CD1d tetramer or mAbs against indicated molecules. After gating on iNKT cells, the gMFI of staining for each molecule is depicted. (E-G) As in (A), but summary data indicates the frequencies of iNKT cells staining positively for mAbs against IL-2 (E), IL-5 (F) and IL-13 (G). (H) B6 mice were restrained (or not) for 12 h before they were given αGC. Six h later, HMNCs were stained with an anti-IL-12p35 mAb or a rat IgG2aκ isotype control. The frequency of IL-12+ DCs was determined after gating on TCRβ-CD11c+ events. (I) Mice that had been restrained or left undisturbed were injected with IL-12 and IL-18 one h before the percentages of IFN-γ+ events among TCRβ-NK1.1+ NK cells were determined. Representative flow plots and summary data are shown. Each symbol represents an individual mouse, and error bars represent SEM. *, ** and *** denote significant differences with p<0.05, p<0.01 and p<0.001, respectively, by unpaired Student’s t-tests. NS = not significant
Supplemental Figure 2. Prolonged stress impairs cognate iNKT cell responses in male and female B6 and BALB/c mice (related to Figure 1). (A) WT BALB/c mice were subjected to physical restraint or left undisturbed for 12 h before they were injected i.p. with αGC or Veh. At indicated time points, IL-4 and IFN-γ in serum samples were quantified by ELISA (n=8-9/group). (B) Data presented in Figure 1B-C and Figure S2A were segregated by sex. The kinetics of serum cytokine levels following αGC (or Veh) administration in male (B6: n=5; BALB/c: n=5) and female (B6: n=5; BALB/c: n=3-4) mice are depicted. Error bars represent SEM. **, *** and **** denote differences with p<0.01, p<0.001 and p<0.0001, respectively, using two-way ANOVA with Dunnett’s post-hoc analysis.
Supplemental Figure 3. Prolonged physical restraint before αGC administration alters the serum concentrations of a wide range of inflammatory mediators (related to Figure 1). WT B6 mice were restrained or left undisturbed for 12 h before they were injected with αGC or Veh (n=3/cohort). Two, 12 and 24 h later, mice were bled and serum levels of indicated mediators were quantified by cytokine/chemokine multiplexing. *, **, *** and **** denote significant differences between stressed and control animals receiving αGC with $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively, using two-way ANOVA with Tukey’s post-hoc analysis.
Supplemental Figure 4. Unlike iNKT cells, naïve and memory T_{conv} cells, NK cells and B cells are prone to stress-induced apoptosis (related to Figure 2). (A-B) Splenocytes and HMNCs from restrained and control B6 mice were stained for surface TCRβ, surface CD44 and intracellular active caspases. A rat IgG2bκ isotype control was used to set the gate for CD44 staining. (A) Representative FACS plots illustrate our gating strategy to distinguish between splenic CD44^{+} and CD44^{-} populations among TCRβ^{+}CD1d tetramer^{-} events, which correspond to memory and naïve T_{conv} cells, respectively. Gates containing T_{conv} cells with intracellular active caspases are also shown. (B) The absolute numbers of hepatic and splenic memory and naïve T_{conv} cells and the percentages of T_{conv} cells staining positively for active caspases are depicted. (C) Separate cohorts of B6 mice were treated i.p. with RU486 or Veh.
One hour later, animals were subjected to prolonged restraint stress or were left undisturbed for 12 h. HMNCs and splenocytes were prepared shortly afterwards and stained with a panel of mAbs against TCRβ, NK1.1 and B220. TCRβ-NK1.1+ NK cells and TCRβ-B220+ B cells were identified by flow cytometry, and their absolute numbers were calculated. Each symbol represents an individual mouse, and error bars represent SEM. *, ** and *** denote differences with \( p < 0.05 \), \( p < 0.01 \) and \( p < 0.001 \), respectively, using unpaired Student’s \( t \)-tests (B) or one-way ANOVA with Dunnett’s post-hoc analysis (C). NS = not significant
Supplemental Figure 5. iNKT cells express adrenergic receptors and respond weakly to αGC in the presence of NE in an in vitro setting (related to Figure 3). (A) HMNCs from 10 naïve B6 mice were pooled and co-stained with an anti-TCRβ mAb and PBS-57-loaded CD1d tetramers. iNKT and T_{conv} cells were FACS-purified for cDNA synthesis and gene expression analysis by quantitative PCR. The expression of indicated genes by iNKT cells relative to T_{conv} cells was calculated using the $2^{-\Delta\Delta C_{T}}$ method. Data from 3 independent experiments were used to determine fold change values. (B) DN32.D3 cells were exposed for 20 minutes to indicated concentrations of norepinephrine (NE) before they were stimulated with 100 ng/mL of αGC. The IL-2 content of culture supernatants was quantified after 24 h by ELISA (n=3-5). (C) DN32.D3 cells were pretreated for 20 minutes with propranolol before they were exposed to NE and subsequently stimulated with αGC (n=4). After 24 h, IL-2 was measured in supernatants (n=4) and cellular viability was assessed by 7-AAD staining (n=3). Error bars represent SEM. *, ** and *** denote differences with $p<0.05$, $p<0.01$ and $p<0.001$, respectively, by one-way ANOVA with Dunnett’s post-hoc analysis (B) or by unpaired Student’s t-tests (C).
Supplemental Figure 6. Mouse and human iNKT, MAIT and T$_{conv}$ cells upregulate CD127 in response to GR signaling (related to Figure 7). B6 mice were subjected to 12 h of restraint stress (or not). Shortly afterwards, HMNCs and splenocytes were prepared and stained to detect CD127 expression on the surface of iNKT and T$_{conv}$.
cells. Open and filled histograms correspond to staining with an anti-CD127 mAb and a rat IgG2ακ isotype control, respectively, after gating on hepatic iNKT cells (A). Cumulative data depicting the gMFI of CD127 staining in indicated populations are also shown (B). Nr3c1Δ and Nr3c1ΔLckcre mice were physically restrained (or not) and assessed for CD127 expression in their iNKT and Tconv cell compartments (C-D). Blue and red histograms correspond to CD127 expression on hepatic iNKT cells from control and stressed mice, respectively (C), and cumulative data for indicated cell populations are summarized using bar graphs (D). Separate cohorts of WT B6 mice received RU486 (or Veh) i.p. 1 h before they were physically restrained (or not). The gMFI of CD127 staining is shown (E). Human PBMCs were cultured for 24 h in the presence of hydrocortisone (HC), dexamethasone (DEX) or vehicle before they were stained with either an anti-CD127 mAb or a mouse IgG1κ isotype control. The gMFI of CD127 staining in iNKT, MAIT and Tconv cells was assessed by flow cytometry (n=5) (F). B6-MAITCAST mice were physically restrained or left undisturbed for 12 h before they were sacrificed for their liver. HMNCs were stained with a mAb to CD127 or a rat IgG2ακ isotype control. After gating on TCRβ+B220−MR1 tetramer+ MAIT cells, the percentages of CD127+ cells and the gMFI of CD127 staining were determined (G). Each symbol in (B, D-E and G) represents an individual mouse. Error bars represent SEM. *, **, *** and **** denote differences with $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively, using unpaired Student’s $t$-tests (B, D and G) or one-way ANOVA (E-F).
Supplemental Figure 7. Stress-induced iNKT cell impairments are partially mediated by TIGIT and do not last long after the stressor is removed (related to Figure 4). (A) B6 mice were restrained or left undisturbed
before they received a 200-μg i.p. dose of an anti-mouse TIGIT mAb (or a mouse IgG1κ isotype control) followed by αGC administration as schematically illustrated. Mice were bled at indicated time points, and serum IFN-γ levels were quantified (n=8-9/group). (B-C) Separate cohorts of B6 mice were physically restrained or left undisturbed for 12 h. Mice were then returned to standard housing conditions for one day (B) or seven days (C) before they were injected i.p. with αGC. Serum IFN-γ and IL-4 levels were measured at indicated time points (n=4/group). Error bars represent SEM. * and ** denotes differences with p<0.05 and p<0.01, respectively, using two-way ANOVA with Sidak’s post-hoc analysis.
**Supplemental Table 1: Taqman-based qPCR primer/probe sets used in this study (related to STAR Methods).**

| Target | Assay Identification |
|--------|----------------------|
| Abl1   | Mm00802029_m1        |
| Actb   | Mm00607939_s1        |
| Adra1a | Mm00442668_m1        |
| Adra1b | Mm00431685_m1        |
| Adra1d | Mm01328600_m1        |
| Adra2a | Mm00845383_s1        |
| Adra2b | Mm00477390_s1        |
| Adra2c | Mm00431686_s1        |
| Adrb1  | Mm00431701_s1        |
| Adrb2  | Mm02524224_s1        |
| Adrb3  | Mm02601819_g1        |
| Aifm1  | Mm00442540_m1        |
| Anxa5  | Mm01293059_m1        |
| Apaf1  | Mm01223702_m1        |
| Api5   | Mm00500189_m1        |
| Atf5   | Mm004179654_m1       |
| Bad    | Mm00432042_m1        |
| Bag1   | Mm01208593_m1        |
| Bag3   | Mm00443474_m1        |
| Bak1   | Mm00432045_m1        |
| Bax    | Mm00432051_m1        |
| Bbc3   | Mm00519268_m1        |
| Bcl2   | Mm00477631_m1        |
| Bcl2a1a| Mm03646861_mH        |
| Bcl2l1 | Mm00437783_m1        |
| Bcl2l11| Mm00437796_m1        |
| Bid    | Mm00432073_m1        |
| Bik    | Mm00476123_m1        |
| Birc2  | Mm00431811_m1        |
| Birc3  | Mm01168413_m1        |
| Birc5  | Mm00599749_m1        |
| Bmf    | Mm00506773_m1        |
| Bnip2  | Mm00443990_m1        |
| Card10 | Mm00459941_m1        |
| Card6  | Mm01297056_m1        |
| Casp1  | Mm00438023_m1        |
| Casp2  | Mm00432314_m1        |
| Casp3  | Mm01195085_m1        |
| Casp4  | Mm00432307_m1        |
| Casp6  | Mm00438053_m1        |
| Casp7  | Mm00432324_m1        |
| Casp8  | Mm00802247_m1        |
| Casp9  | Mm00516563_m1        |
| Chlb   | Mm01343092_m1        |
| Gene   | Accession     |
|--------|---------------|
| Cd27   | Mm01185212_g1 |
| Cd28   | Mm00483137_m1 |
| Cd40lg | Mm00441911_m1 |
| Cd44   | Mm01277161_m1 |
| Cd69   | Mm01183378_m1 |
| Cd274  | Mm03048248_m1 |
| Cdk2   | Mm00443947_m1 |
| Cdk4   | Mm00726334_s1 |
| Cflar  | Mm01255578_m1 |
| Cradd  | Mm01226172_m1 |
| Csf1   | Mm00432686_m1 |
| Dad1   | Mm01319221_m1 |
| Dff4a  | Mm00438410_m1 |
| Dffb   | Mm00432822_m1 |
| Diablo | Mm01194441_m1 |
| Egr2   | Mm00456650_m1 |
| Egr3   | Mm00516979_m1 |
| Fadd   | Mm00438861_m1 |
| Fas    | Mm01204974_m1 |
| Fasl   | Mm00438864_m1 |
| Fos    | Mm00487425_m1 |
| Foxo3  | Mm01185722_m1 |
| Foxp1  | Mm00474848_m1 |
| Gata3  | Mm00484683_m1 |
| Gzma   | Mm01304452_m1 |
| Gzmb   | Mm00442834_m1 |
| Hells  | Mm00468580_m1 |
| Icam1  | Mm00516023_m1 |
| Icos   | Mm00497600_m1 |
| Ifng   | Mm01168134_m1 |
| Ifnar1 | Mm00439544_m1 |
| Ifnar2 | Mm00494916_m1 |
| Igf1r  | Mm00802831_m1 |
| Il10ra | Mm00434151_m1 |
| Il12rb1| Mm00434189_m1 |
| Il18rap| Mm00516053_m1 |
| Il2    | Mm00434256_m1 |
| Il2ra  | Mm01340213_m1 |
| Il2rb  | Mm00434268_m1 |
| Il4    | Mm00445259_m1 |
| Il7ra/Cd127 | Mm00434295_m1 |
| Irf4   | Mm00516431_m1 |
| Itch   | Mm01246513_m1 |
| Jak1   | Mm00600614_m1 |
| Jak3   | Mm00439962_m1 |
| Jun    | Mm00495062_s1 |
| Gene   | Accession          |
|--------|--------------------|
| Lat    | Mm00456761_m1     |
| Lta    | Mm00440228_gH     |
| Myb    | Mm00501741_m1     |
| Naip2  | Mm00440446_m1     |
| Nfatc1 | Mm00479445_m1     |
| Nfatc2 | Mm00477776_m1     |
| Nfatc3 | Mm01249200_m1     |
| Nfkb1  | Mm00476361_m1     |
| Notch1 | Mm00435249_m1     |
| Npy1r  | Mm04208490_m1     |
| Npy2r  | Mm01218209_m1     |
| Npy4r  | Mm01220859_m1     |
| Npy5r  | Mm00443855_m1     |
| Npy6r  | Mm00627550_m1     |
| Nr3c1  | Mm00433832_m1     |
| Nr4a1  | Mm01300401_m1     |
| Pim2   | Mm00454579_m1     |
| Pmaip1 | Mm00451763_m1     |
| Polb   | Mm00448234_m1     |
| Prf1   | Mm00812512_m1     |
| Ptger2 | Mm00436051_m1     |
| Ripk1  | Mm00436354_m1     |
| Rnf128 | Mm00480990_m1     |
| Sell   | Mm00441291_m1     |
| Sphk2  | Mm00445021_m1     |
| Stat3  | Mm01219775_m1     |
| Stat6  | Mm01160477_m1     |
| Tbp    | Mm00446973_m1     |
| Tbx21  | Mm00450960_m1     |
| Tgfb1  | Mm01178820_m1     |
| Tnfa   | Mm00443258_m1     |
| Tnfrsf10b | Mm00457866_m1   |
| Tnfrsf14 | Mm00619239_m1   |
| Tnfrsf4 | Mm00442039_m1     |
| Tnfrsf9 | Mm00441899_m1     |
| Tnfsf10 | Mm01283606_m1     |
| Tnfsf14 | Mm00444567_m1     |
| Tnfsf8  | Mm00437153_m1     |
| Traf1  | Mm00493827_m1     |
| Traf2  | Mm00801978_m1     |
| Traf3  | Mm00495752_m1     |
| Trp53bp2 | Mm00557629_m1   |
| Xiap   | Mm01311594_mH     |
| Zbtb16 | Mm01176868_m1     |
| Zc3hc1 | Mm01168068_m1     |