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Neutrophilic Granulocytes Modulate Invariant NKT Cell Function in Mice and Humans

Gerhard Wingender,*† Marcus Hiss,‡ Isaac Engel,* Konrad Peukert,§ Klaus Ley,§ Hermann Haller,‡ Mitchell Kronenberg,* and Sibylle von Vietinghoff‡,§

Invariant NKT (iNKT) cells are a conserved αβTCR+ T cell population that can swiftly produce large amounts of cytokines, thereby activating other leukocytes, including neutrophilic granulocytes (neutrophils). In this study, we investigated the reverse relationship, showing that high neutrophil concentrations suppress the iNKT cell response in mice and humans. Peripheral Vo14 iNKT cells from spontaneously neutrophilic mice produced reduced cytokines in response to the model iNKT cell Ag α-galactosyl ceramide and expressed lower amounts of the T-box transcription factor 21 and GATA3 transcription factor than did wild-type controls. This influence was extrinsic, as iNKT cell transcription factor expression in mixed chimeric mice depended on neutrophil count, not NKT cell genotype. Transcription factor expression was also decreased in primary iNKT cells from the neutrophil-rich bone marrow compared with spleen in wild-type mice. In vitro, the function of both mouse and human iNKT cells was inhibited by coinoculation with neutrophils. This required cell–cell contact with live neutrophils. Neutrophilic inflammation in experimental peritonitis in mice decreased iNKT cell T-box transcription factor 21 and GATA3 expression and α-galactosyl ceramide-induced cytokine production in vivo. This was reverted by blockade of neutrophil mobilization. Similarly, iNKT cells from the human peritoneal cavity expressed lower transcription factor levels during neutrophilic peritonitis. Our data reveal a novel regulatory axis whereby neutrophils reduce iNKT cell responses, which may be important in shaping the extent of inflammation. The Journal of Immunology, 2012, 188: 3000–3008.

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nvariant NKT (iNKT) cells are T lymphocytes characterized by the expression of an invariant Vα14-Jα18 TCR rearrangement (Vo14-Jα18 TCR (Vo24i NKT cells) in humans (1–3). They recognize glycolipids, such as the foreign Ag α-galactosyl ceramide (αGalCer), components of bacterial cell walls, and also endogenous lipids (2) when presented by CD1d, a nonpolymorphic MHC class I Ag-presenting molecule homolog (2, 4). iNKT cell antigenic stimulation rapidly induces effector functions such as cytokine production and cytotoxicity (1–3). iNKT cell cytokines include IFN-γ, generally controlled in T lymphocytes by the T-box transcription factor 21 (T-bet), and IL-4 controlled by GATA3 (5). iNKT cells play a role in host defense, particularly against some Gram-negative bacteria such as Borrelia, Sphingomonas, and Salmonella (6), but also Gram-positive pathogens such as Streptococcus pneumoniae as well as virus (7). They are also involved in a range of chronic and acute inflammatory processes including allergic asthma, ischemia-reperfusion injury, and atherosclerosis (1, 3). Clinical trials are currently exploring the potential of in vitro-expanded iNKT cells for the treatment of metastatic neoplasms (8).

Neutrophilic granulocytes (neutrophils) are the most abundant innate immune cells in blood (9). Although neutrophil counts are remarkably stable under resting conditions (10, 11), many bacterial and fungal infections, and also hormones such as catecholamines and glucocorticoids, rapidly upregulate circulating blood neutrophil counts, making them a dynamic indicator of the extent of inflammation (9). In a number of adhesion molecule-deficient mice, such as β2 integrin gene-deficient (CD18; Itgb2−/−) and E- and P-selectin-deficient (Sele−/−; Selp−/−) mice, circulating neutrophil counts are spontaneously elevated (12).

iNKT cells modulate inflammatory processes, including neutrophilic inflammation. For example, iNKT cells stimulated neutrophil infiltration into the lung (13), ischemic kidney (14), and liver during Listeria infection (15), but inhibited neutrophil invasion in cholestatic liver damage (16). Furthermore, iNKT cells altered cytokine production by neutrophils, inhibiting IL-10 and increasing IL-12 secretion (17). However, data on the reverse relationship (i.e., neutrophils modulating iNKT cells) have not been reported. In this study, we investigated whether neutrophil concentration influences iNKT cell function and demonstrate a marked suppressive effect for both mouse and human iNKT cells.

Materials and Methods

Animals, bone marrow transplantation, and adoptive thymocyte transfer

Animal experiments were approved by the Animal Care Committee at the La Jolla Institute for Allergy and Immunology. Wild-type (wt; CD45.2) C57BL/6 mice and congenic B6.SJL-PtprcPepc/BoyJ (CD45.1) were from The Jackson Laboratory (Bar Harbor, ME). B6.129-Tcra-Jtm1Tgi/BoyJ (Tcrα−/−) mice were kindly provided by Dr. Solomon Reiner (Cleveland Clinic Foundation, Cleveland, OH). BM from wild-type (wt) and thymocyte-deficient (Tcrα−/−) mice were transplanted into lethally irradiated C57BL/6 mice (wt; CD45.2) at 105 BM cells/mouse, followed by 105 total CD3−/TCRβ− cells/mouse. After 12 weeks, recipient mice were killed, and Tcrα TCRhi+ T cells were analyzed by flow cytometry. CD3−/TCRβ− cells were gated as TCRβ−CD3− cells. Mice were maintained according to the recommendations of the European Community Council (1997) and the NIH Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Animal Care Committee at the La Jolla Institute for Allergy and Immunology, La Jolla, CA.
proceed for 6 h before cells were washed, stained, and analyzed by flow cytometry. Stimulation of NKT cells and resuspended in full media. Transwells (0.4-

Near-infrared LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) and BD Fix-Perm for intracellular staining (BD Pharmingen, San Jose, CA) were used according to the manufacturer’s instructions. Purification of mouse CD1d and preparation of αGalCer-loaded CD1d tetramers was as described (26). Mouse iNKT cells were defined as CD8α−CD19−tetramer−TCRβ+, human iNKT cells as CD19−CD3ε−Vα24i− cells. Flow cytometry analysis was performed on a BD FACS Calibur, FACSCanto, or LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Results
Decreased function of Vα14i NKT cells from spontaneously neutrophilic mice
Mice deficient for the β subunit of β2 integrins (CD18, Itgb2−/−) were used as indicated in the figure legends, and p values < 0.05 were considered significant. Data are expressed as mean ± SEM. The p values are indicated with *p < 0.05, **p < 0.01, and ***p < 0.001.

Ex vivo stimulation, human iNKT cell expansion, stimulation, and cytotoxicity assay
For ex vivo stimulation, 10⁶ murine splenocytes or thymocytes were coincubated in 200 μl full RPMI medium (with penicillin/streptomycin and L-glutamine (Gibco, Life Technologies, Carlsbad, CA)) with 2 × 10⁶ bone marrow neutrophils (unless otherwise stated) recovered by flushing the bones with pyrogen-free HBSS without calcium and magnesium and enriched by density gradient centrifugation as described (22). Alternatively, bone marrow neutrophils were purified using a Neutrophil Enrichment Kit (#19762; StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. Cells were counted in a hemocytometer, and viability was assessed by trypan blue exclusion. Human PBMCs and granulocytes were isolated by density gradient centrifugation as described (23). A total of 3 × 10⁶ PBMC and 2.5 × 10⁶ polymorphonuclear leukocytes (PMN) were coincubated in 500 μl full RPMI medium (with penicillin/streptomycin and L-glutamine) and incubated for 7 d as described (24, 25), washed, and stained with CFSE (Invitrogen, Carlsbad, CA) at 1 μM (αGalCer-loaded) and 0.1 μM (control) according to the manufacturer’s instructions (21). Stimulation of iNKT cells with αGalCer with and without neutrophils was done for 4 h unless otherwise indicated; cytotoxicity was allowed to proceed for 6 h before cells were washed, stained, and analyzed by flow cytometry. Human IFN-γ ELISA was from BioLegend (San Diego, CA).

Cell preparation and staining for flow cytometry
The following Abs were used for flow cytometry: anti-mouse: CD1d (1B1), CD3e (145.2C11, 17A2), CD19 (1D3, 6D5), CD45 (30-F11), CD15-PE (A20), CD45.2 (104), CD69 (H1F6), CD122 (TM-β1), CD154 (CD40L, MR1), 7/4, Ly6G (1A8), Ly6C(H1K4.1), Gr1 (RB6-8C5), T-bet (4B10), GATA3 (5.50-823), TF-α (MP6-XT22), IL-4 (11B1), and IFN-γ (XMG1.2); anti-human: anti-CD1d (1D1a), CD3e (HIT3a), CD19 (HB19), Vγ24/1a14 (6B11), IFN-γ (45.B3), and T-bet (4B10). Abs were purchased from Abcam (Cambridge, MA), BD Biosciences (San Diego, CA), BioLegend, eBioscience (San Diego, CA), or Invitrogen. Near-infrared LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) and BD Fix-Perm for intracellular staining (BD Pharmingen, San Jose, CA) were used according to the manufacturer’s instructions. Purification of mouse CD1d and preparation of αGalCer-loaded CD1d tetramers was as described (26). Mouse iNKT cells were defined as CD8α−CD19−tetramer−TCRβ+, human iNKT cells as CD19−CD3ε−Vα24i− cells. Flow cytometry analysis was performed on a BD FACS Calibur, FACSCanto, or LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Sample preparation
Blood and peritoneal fluid were recovered after local ethics board approval (MH1 2010/807) and written informed consent according to the declaration of Helsinki. In stable peritoneal dialysis (PD) patients (n = 10, 64% male, mean age 55 y [range 20–73 y], mean time on PD 34 mo [5–124 mo], four previous peritonitis episodes in four patients) and patients with acute peritonitis (n = 4, 75% male, mean age 53 y [range 28–69 y], mean time on PD 33 mo [6–84 mo], three previous peritonitis episodes in one patient), cells were recovered from peritoneal outflow of overnight dwells or the first peritonitic outflow before initiation of therapy. Leukocyte counts were assessed in the clinical laboratory at Hanover Medical School.

Ex vivo stimulation, human iNKT cell expansion, stimulation, and cytotoxicity assay
Data were analyzed using FlowJo software (Tree Star, Ashland, OR).
$\text{Igb}2^{-/-}$ than in wt mice (Fig. 1B, Supplemental Fig. 2). Similar decreases in cytokine production were seen in cells from spleen and liver (Fig. 1B). Baseline expression of the activation marker CD122, which constitutes a part of the IL-15R and is important for $\text{iNKT}$ cell survival (28), and the TNF family member CD154 (CD40L) was similar (Fig. 1C and data not shown). However, whereas induction of CD69 after activation was normal, CD154 upregulation on $\text{Igb}2^{-/-}$ $\text{iNKT}$ cells upon $\alpha$GalCer stimulation was significantly reduced (Fig. 1C). Decreased cytokine production in response to $\alpha$GalCer was apparent in both NK1.1 and CD4-negative and -positive subpopulations to a very similar degree (data not shown). NK cells are rapidly activated downstream of $\text{iNKT}$ cell stimulation, a process referred to as trans-activation (29, 30). In line with the reduced $\text{iNKT}$ cell cytokine production and CD154 expression, NK cell trans-activation, measured by IFN-γ production by NK cells, was greatly reduced in neutrophilic $\text{Igb}2^{-/-}$ mice (Fig. 1D). These data demonstrate impaired activation and cytokine production by $\text{iNKT}$ cells from neutrophilic mice in vivo.

**Decreased T-bet and GATA3 transcription factor expression of $\text{V}a14i\text{NK}$ cells from neutrophilic mice**

To gain insight into the mechanism for the decreased cytokine responses by $\text{V}a14i\text{NK}$ cells, we analyzed the expression of the T-bet and GATA3 transcription factors, critical for IFN-γ and IL-4 expression, respectively, in conventional CD4+ T lymphocytes (5). Transcription factors were analyzed by flow cytometry after intracellular staining in thymic and peripheral $\text{iNKT}$ cells from $\text{Igb}2^{-/-}$ and wt mice (Fig. 2A, 2B). $\text{Igb}2^{-/-}$ splenic $\text{iNKT}$ cells contained significantly less of either transcription factor than wt cells (Fig. 2C, 2D). In contrast, the T-bet and GATA3 expression levels in thymic INKT cells were similar in wt and $\text{Igb}2^{-/-}$ mice (Fig. 2), arguing against a developmental cause of this difference. Also, CD1d expression was not different in spleens or thymus of $\text{Igb}2^{-/-}$ compared with wt mice (data not shown).

To test whether decreased $\text{iNKT}$ cell transcription factor expression was due to $\beta_2$ integrin deficiency, we employed mice deficient in endothelial and platelet, but not leukocyte selectins (19). These mice were neutrophilic to a similar degree as $\text{Igb}2^{-/-}$ mice (Supplemental Fig. 3A) (12). $\text{iNKT}$ cell characterization of this mouse strain is shown in Supplemental Fig. 1. Also in this strain, T-bet and GATA3 expression in splenic and hepatic $\text{iNKT}$ cells was reduced (Supplemental Fig. 3B, 3C), suggesting neutrophilia as a possible cause of the observed $\text{iNKT}$ cell phenotype.

**Modulation of $\text{V}a14i\text{NK}$ cells by neutrophils is cell extrinsic and reversible**

To test if the phenotype of peripheral $\text{iNKT}$ cells from neutrophilic $\text{Igb}2^{-/-}$ mice was cell intrinsic or environmental, we employed adoptive thymocyte and splenocyte transfers and bone marrow transplantations. We transferred thymocytes from wt or $\text{Igb}2^{-/-}$ mice into normal and neutrophilic hosts. Thymocytes were used as the cell source, as they expressed similar levels of T-bet and GATA3 in both donor strains (Fig. 2). Four to 6 wk later, donor and recipient $\text{iNKT}$ cell T-bet and GATA3 expression levels were analyzed by flow cytometry. The wt and $\text{Igb}2^{-/-}$ thymocytes transferred into $\text{iNKT}$-deficient host mice (Jax18/18), which have normal neutrophil counts (data not shown), expressed similar levels of T-bet and GATA3 in $\text{iNKT}$ cells (Fig. 3A). When thymocytes were transferred to mice with endogenous $\text{iNKT}$ cell populations, host neutrophil counts had a similar influence. For example, $\text{Igb}2^{-/-}$ thymocytes transferred into wt hosts retained relatively higher T-bet and GATA-3 expression, similar to their host counterparts (Fig. 3B). To create a cohort of neutrophilic recipients, we created bone marrow-chimeric recipients with 100% $\text{Igb}2^{-/-}$ bone marrow. Transfer of wt thymocytes into these neutrophilic host mice resulted in lower T-bet and GATA3 transcription factor expression, similar to the host $\text{Igb}2^{-/-}$ INKT cells (Fig. 3B). These data suggest that the decreased expression levels of T-bet and GATA3 observed in $\text{Igb}2^{-/-}$ $\text{iNKT}$ cells was not a cell-intrinsic phenomenon, but rather a consequence of the environment.

To confirm these results in an experimental system in which wt and $\text{Igb}2^{-/-}$ $\text{iNKT}$ cells develop in the same animal, we reconstituted lethally irradiated wt mice with bone marrow from wt and $\text{Igb}2^{-/-}$ mice mixed at an equal ratio. As described (12), transfer of 50% wt/50% $\text{Igb}2^{-/-}$ bone marrow resulted in normal peripheral blood neutrophil counts (in wt bone marrow-transplanted mice, 2.1 ± 0.2 PMN/μl [mean ± SEM]; in 50% wt/50% $\text{Igb}2^{-/-}$, 2.4 ± 0.4; and in $\text{Igb}2^{-/-}$ bone marrow-transplanted mice, 16.9 ± 6.5 PMN/μl). Consistent with the results from transfer of mature cells, in mixed bone marrow chimeras, the expression of T-bet and GATA3 in wt and $\text{Igb}2^{-/-}$ $\text{iNKT}$ cells from the same mouse was similar, irrespective of their genotype (Fig. 3C).

To test if the decrease in T-bet and GATA3 expression in peripheral $\text{iNKT}$ cells in neutrophilic $\text{Igb}2^{-/-}$ mice was reversible, we adoptively transferred splenocytes from wt and $\text{Igb}2^{-/-}$ mice at an equal ratio into $\text{iNKT}$ cell-deficient, normo-neutremic host mice. This completely normalized the transcription factor expression of the $\text{Igb}2^{-/-}$ $\text{iNKT}$ cells by day 3 after transfer (Fig. 3D), indicating that the downregulation was a reversible phenotype. It is of note that both wt and $\text{Igb}2^{-/-}$ bone marrow neutrophils were devoid of CD49d (data not shown), which has recently been proposed as a marker of myeloid-derived suppressor cells (MDSC) (31), a cell type induced in a variety of pathophysiologic conditions, but not present in healthy mice and humans (32–34).

Together, these results indicate that lower $\text{iNKT}$ cell T-bet and GATA3 expression in neutrophilic mice is not cell intrinsic, but determined by the environment, and they suggest neutrophil counts as the likely responsible factor.

**Neutrophils modulate $\text{V}a14i\text{NK}$ cell T-bet and GATA3 from wt mice**

The murine bone marrow harbors large numbers of mature neutrophils (11). Therefore, if exposure to increased numbers of
neutrophils decreased T-bet and GATA3 expression, wt bone marrow Vca14i NKT cells might display lower transcription factor expression than cells from other organs. Flow cytometric analyses indeed showed decreased expression of T-bet and GATA3 in bone marrow NKT cells compared with cells from spleen (Fig. 4A) and thymus (data not shown) of the same wt animal. To test whether such downregulation could also be induced in vitro, primary mouse splenocytes and thymocytes were coincubated with mouse bone marrow neutrophils in vitro. This decreased T-bet and GATA3 expression in both splenic and thymic Vca14i NKT cells (Fig. 4B), demonstrating that high local concentrations of resting neutrophils can induce NKT cell downregulation of these transcription factors also in unmanipulated wt mice and that it can be replicated in vitro in short-term cultures. Most causes of neutrophilia in vivo also involve activation of neutrophils by inflammatory mediators leading to activation, degranulation, and distinct forms of cell death (35). Stimulation of neutrophils by TNF-α, N-formylmethionyl-leucyl-phenylalanine, or PMA did not alter the neutrophil-mediated decrease in T-bet expression (data not shown). However, spontaneously apoptotic or heat-killed neutrophils lost their ability to affect Vca14i NKT cell T-bet and GATA3 expression in our in vitro coculture system (data not shown), indicating that live neutrophils were required. Even after density gradient purification, bone marrow contains other cell types. We therefore employed negative selection (BM-PMN neg.sel). NKT cell T-bet and GATA3 expression in BM-PMN was performed with or without physical contact (tw, transwell with 0.4-μm pore size; one of two independent experiments shown).

FIGURE 3. T-bet and GATA3 expression in Vca14i NKT cells is modulated by neutrophil counts. (A) wt and Itgb2−/− thymocytes were transferred to iNKT cell-deficient Jα18−/− mice with normal neutrophil counts and splenic iNKT cells analyzed after 6 wk (an example of n = 4 from two experiments) for T-bet (left panel) and GATA3 (right panel). (B) To test for wt iNKT cell transcription factor expression in neutrophilia, wt (CD45.1) thymocytes were transferred into normal wt (CD45.2) hosts (top panel) or neutrophilic hosts previously reconstituted with Itgb2−/− (CD45.2) bone marrow (bottom panel). Four weeks later, the expression levels of T-bet and GATA3 were analyzed. They were lower in splenic iNKT cells from neutrophilic mice than normal controls, but identical for wt and Itgb2−/− iNKT cells in the same environment (examples from n = 4 transplanted mice per group). (C) wt (CD45.1) and Itgb2−/− iNKT cell T-bet and GATA3 from mixed 50% wt/50% Itgb2−/− bone marrow chimeras were indistinguishable (examples from n = 9). (D) wt (CD45.1) and Itgb2−/− (CD45.2) splenocytes were transferred into Jα18−/− mice to assess the effect of normal neutrophil counts on peripheral Itgb2−/− iNKT cells. T-bet and GATA3 transcription factor expression within iNKT cells on days 0 and 3 after transfer is given (example of n = 3 from two experiments).

FIGURE 4. Neutrophils modulate Vca14i NKT cell transcription factor expression in vivo and in vitro. (A) T-bet and GATA3 expression in wt spleen and bone marrow (BM)-derived iNKT cells. (B) Mouse splenocytes and thymocytes were cultured in the presence or absence of neutrophils (107/ml) for 6 h and T-bet and GATA3 expression determined (examples from two independent experiments). (C) wt splenocytes were cultured with increasing concentrations of wt BM neutrophils purified by density gradient (BM-PMN) and negative Ab selection (BM-PMN neg.sel). iNKT cell T-bet and GATA3 expression is shown in relation to actual neutrophil concentration calculated from flow cytometry (Ly6C+Ly6G+7/4+ cells) (example from n = 3). (D) Coculture with neutrophils (5 × 105/ml) for 4 h was performed with or without physical contact (tw, transwell with 0.4-μm pore size; one of two independent experiments shown).
not shown). To further investigate whether iNKT cell inhibition was due to a soluble factor or cell–cell contact dependent, neutrophils were separated from Vox14i NKT cells using a transwell (Fig. 4D). This completely abolished the neutrophil inhibitory effect on Vox14i NKT cell transcription factor expression.

**Neutrophils modulate Vox24i NKT cell function in vitro**

To test if neutrophils would similarly impact primary human Vox24i NKT cells, PBMCs were cocultured with elevated human neutrophil concentrations for 4 h. This significantly decreased iNKT cell T-bet and GATA3 expression (Fig. 5A). Similar to the murine system, neutrophil stimulation did not alter the neutrophil-mediated decrease in Vox24i NKT cell T-bet expression and IFN-γ production (data not shown).

Vox24i NKT cells are infrequent in human peripheral blood, but can be expanded in vitro (24, 25). In our hands, this expansion resulted mainly in IFN-γ–producing iNKT cells with IL-4 barely above detection limit. When in vitro-expanded human iNKT cells were exposed to neutrophils, T-bet expression decreased significantly (Fig. 5B). Restimulation with αGalCer in the presence of neutrophils resulted in significantly less IFN-γ secretion to the supernatant than control cells (Fig. 5C). Individual cell IFN-γ production was assessed by flow cytometry after intracellular staining. Coincubation with neutrophils resulted in a significantly smaller proportion of IFN-γ+ iNKT cells (Fig. 5D). Vox24i NKT cell cytotoxicity was assessed after a 6-h coculture with αGalCer-loaded, CFSE-labeled PBMCs. Addition of freshly isolated neutrophils significantly decreased αGalCer-mediated cytotoxicity (Fig. 5E, 5F). However, the use of neutrophil-derived supernatants or the separation of the neutrophils in culture using a transwell abolished their effect on Vox24i NKT cells (Fig. 5G and data not shown). These data show that inhibition of iNKT cells by high neutrophil concentrations applies similarly to mouse and human cells and demonstrate that cell–cell contact is required for neutrophils to impair iNKT cell function.

**Neutrophilic inflammation decreases Vox14i NKT cell cytokine production in vivo**

To investigate the effect of inflammatory neutrophilia on iNKT cell function in vivo, peritonitis was induced in wt mice by injection of thioglycollate (Fig. 6). After 3 d, iNKT cells were stimulated in vivo by injection of αGalCer and analyzed after 90 min. αGalCer did not alter the inflammatory peritoneal cavity leukocyte count (Fig. 6A). However, iNKT cell cytokine production was significantly lower in cells recovered from a neutrophilic compared with a normal environment (Fig. 6B). iNKT cell T-bet and GATA3 expression levels were also decreased in mice with peritonitis and correlated well with iNKT cell cytokine production (Fig. 6C). Accumulation of peritoneal leukocytes, mostly neutrophils, was significant at 6 h after thioglycollate injection (Fig. 6D) (20). When we assessed the time course of T-bet and GATA3 expression levels in peritoneal iNKT cells, we found them to be already decreased at this time (Fig. 6E). Neutrophil recruitment in peritonitis is to a large degree CXCL1 chemokine dependent and can be prevented by CXCR2 chemokine receptor blockade (Fig. 6D) (20). CXCR2 blockade also normalized the expression of the transcription factors in peritoneal iNKT cells (Fig. 6E), demonstrating that the reduction was not due to a direct effect of thioglycollate on iNKT cells. Altogether, these data indicate that acute, inflammatory neutrophilia induces downregulation of T-bet and GATA3 and impaired cytokine production following iNKT cell Ag stimulation in vivo.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Neutrophils modulate Vox24i NKT cell function in vitro. (A) Human PBMCs isolated by density gradient centrifugation were cultured in the presence or absence of neutrophils (5 × 10⁶/ml) for 4 h. Neutrophils significantly decreased T-bet and GATA3 expression in Vox24i NKT cells (n = 4). (B) T-bet expression of in vitro-expanded Vox24i NKT cells was assessed after 4 h coincubation with neutrophils (PMN; 10⁶/ml). (C) In vitro-expanded Vox24i NKT cells were exposed to αGalCer (100 ng/ml) in the presence and absence of neutrophils (PMN; 10⁶/ml). IFN-γ concentration in the supernatant after 4 h was determined by ELISA (n = 3). (D) Individual iNKT cell (CD3⁺Vox24⁺CD19⁻) IFN-γ was determined by flow cytometry (n = 5). (E and F) iNKT cell cytotoxicity against fresh PBMC loaded with 100 ng/ml αGalCer. PBMCs were differentially stained with CFSE (1 μM for αGalCer exposed, 0.1 μM for control cells), mixed, and incubated in RPMI for 6 h with and without iNKT cells and freshly isolated neutrophils (PMN; 10⁶/ml). The proportion of αGalCer-labeled (CFSE₈) and CFSElow (control PBMC) was determined by flow cytometry and is expressed as αGalCer labeled relative to control PBMCs in (E) (n = 3 independent experiments). (G) αGalCer stimulation of in vitro-expanded human iNKT cells was conducted in the presence or absence of 10⁶/ml neutrophils with or without physical contact (transwell with 0.4-μm pore size) for 10 h (n = 4, Bonferroni after one-way ANOVA). *p < 0.05, **p < 0.01.
Vα24i NKT cell T-bet expression is decreased in neutrophilic peritonitis

To test whether inflammatory neutrophilia in vivo also decreased human iNKT cell function, we investigated peripheral blood and peritoneal cavity Vα24i NKT cells at baseline and in peritonitis. Peritoneal iNKT cells were recovered from the outflow fluid of patients treated with chronic PD for renal replacement therapy. Total peritoneal fluid leukocyte concentrations were very low under resting conditions (Fig. 7A, 7B). Most leukocytes from peritoneal cavity of stable PD patients were lymphocytes (data not shown), most likely a resident population (36, 37). Vα24i NKT cells in the human peritoneal cavity have not been described, but were readily detected among CD3ε+ T cells (Fig. 7C). Conventional T cells in human peritoneum predominantly produce IFN-γ (37). Indeed, expression of the Th2 transcription factor GATA3 was at the detection limit in peritoneal Vα24i NKT cells (data not shown). However, the Th1 transcription factor T-bet was expressed and was significantly higher in Vα24i NKT cells from the peritoneal cavity than from peripheral blood (Fig. 7D). Acute peritonitis results in a massive neutrophil influx into the peritoneum, accounting for >90% of the leukocytes (Fig. 7B and data not shown), resulting in a concentration (cells per microliter) similar to peripheral blood. In peritonitis, T-bet expression in peritoneal Vα24i NKT cells was similar to or lower than in blood iNKT cells from the same patient (Fig. 7D). These data show that T-bet expression of primary Vα24i NKT cells from the neutrophil-poor peritoneal cavity is higher than in peripheral blood and decreases in response to neutrophilic inflammation in humans in vivo.

Discussion

Our data show for the first time, to our knowledge, that neutrophilic granulocytes inhibit iNKT lymphocyte function in mice and humans, both under resting conditions and during inflammation in vivo. We observed downregulation of iNKT cell baseline T-bet and GATA3 expression and decreased responses to the iNKT cell Ag αGalCer, regarding both cytokine production and CD154 (CD40L) upregulation. NK cell trans-activation to produce IFN-γ, an important pathway for amplification of immune responses downstream of iNKT cell activation, was also impaired. These effects were reversible.

Mouse and human iNKT cells and neutrophils differ in numbers and tissue distribution. Although neutrophils are more frequent in human than mouse blood and constitute the most common leu-
neutrophil population there, the mouse bone marrow contains a large pool of mature neutrophils (11). However, similar effects of neutrophil concentration were observed in mice and humans. The expression by iNKT cells of T-bet and GATA3 was decreased in the neutrophil-rich bone marrow environment in mice below the amount in splenic iNKT cells under resting conditions. Similarly, human blood iNKT cells expressed lower T-bet than iNKT cells from the relatively neutrophil-poor peritoneal cavity. The downregulation of these transcription factors and iNKT cell functions could readily be reproduced in vitro by exposing human or mouse iNKT cells to neutrophils. Taken together, our data indicate similar iNKT cell responses to neutrophil concentrations in both species.

Depression of iNKT cell function in neutrophilic mice could have been developmentally induced phenotype. However, the expression levels of T-bet and GATA3 were similar in the thymus of wt and neutrophilic mice. Furthermore, when we tested for the effect of neutrophilia on mature cells, iNKT cell depression was readily observed in mature splenic and thymic Vo14i NKT cells in vivo and in vitro and in human peripheral blood Vo14i NKT cells. Also, Vo14i NKT cells from neutrophilic mice readily recovered T-bet and GATA3 expression after adoptive transfer to a wt environment. These data suggest that neutrophils provide a short-term and reversible modulatory effect on iNKT cell activation. Acute neutrophilia is an early and often short-lived response to infection, stress, and trauma (9). In such conditions, neutrophils are often activated, degranulate, and produce reactive oxygen species. Activated neutrophils retained their inhibitory function for iNKT cells, although it was not increased, indicating that iNKT cell inhibition by neutrophils is not restricted to resting conditions and therefore could be of general importance during inflammations in vivo.

T-bet and GATA3 transcription factors are critical for the expression of IFN-γ and IL-4/IL-13, respectively, in peptide-reactive or conventional CD4+ T lymphocytes (5). T-bet and GATA3 deficiency severely affect Vo14i NKT cell differentiation (38, 39), and therefore, data on their role in mature iNKT cells are limited. However, retroviral-mediated expression of T-bet (40) or GATA3 (41, 42) in Vo14i NKT cells increased cytokine production in response to αGalCer in vitro, suggesting a functional role in cytokine production by activated iNKT cells. Our data (Fig. 6C) demonstrate a correlation of T-bet and GATA3 expression with iNKT cell cytokine content assessed by intracellular cytokine staining. However, given the broad suppressive effect of neutrophils on the production of other cytokines by iNKT cells, including TNF-α, IL-13, and GM-CSF, it is likely that interaction with neutrophils leads to downregulation of additional transcription factors.

It is of note that CD49d-negative granulocytes isolated from normal mice and a large number of healthy donors suppressed iNKT cell function in our study. This suggests the suppressive cells are not exclusively MDSC, which although heterogeneous, are at least in part a CD49d+ (31) population with both granulocytic and monocytic phenotypes. Furthermore, MDSC typically are found in disease (e.g., infection and different forms of malignancies), and they are not usually present in healthy organisms, which provided the sources of the neutrophils in our study (32–34). Regulation of conventional T lymphocytes by neutrophils has been suggested by enhanced T cell activation in neutropenic animals (43, 44). Data on a mechanism for this, however, are controversial. Some reports described roles for soluble molecules such as NO induced by IFN-γ (45), IL-10 (46), or arginase liberated from dying cells (47). However, in other settings, direct interaction of neutrophils and APC appeared to be required (17, 44). In our experiments, iNKT cell inhibition by neutrophils depended on the presence of live cells and required cell–cell contact. Currently, no cell–cell contact dependent mechanism for T cell suppression by neutrophils has been described, and therefore, the inhibition of iNKT cells by neutrophils we observed in this study likely represents a novel mechanism. Potential candidates for cell-surface molecules that could be important in the iNKT cell–neutrophil interaction are inhibitory molecules that have been reported to be involved in T cell–APC interactions (48–52). Although we did not observe changes in PD-1, BTLA, GITR, or CD152 iNKT cell-surface expression, whether they have a role in iNKT cell–neutrophil interaction remains to be established.

Inhibition of iNKT cells by neutrophilic granulocytes could play an important role in a number of pathophysiologic conditions that activate iNKT cells. Inhibition of iNKT cells by neutrophilia may be beneficial in settings of otherwise overwhelming iNKT cell activation (e.g., during sepsis and bronchial asthma) (1, 3), and it may contribute to reduced iNKT cell function in chronic inflammatory conditions such as atherosclerosis (53). However, an increased concentration of neutrophils also may inhibit beneficial iNKT cell responses, such as cytotoxicity or cytokine secretion required for the elimination of malignancies (1, 3) or pathogenic bacteria (7). Interestingly, several, although not all (54), pathogens in which host protection requires an iNKT cell response, such as Borrelia burgdorferi and Rickettsiae, and several viral infections do not usually elicit a strong neutrophilic response (7).

In summary, our report describes inhibition of iNKT cell activation by neutrophils, both in vitro and in vivo, and in mice as...
well as humans in both steady-state conditions and during inflammatory conditions. Deliberate modulation of this interaction may be potentially beneficial for induction of stronger iNKT cell responses to neoplasms and pathogens and for limiting allergic or autoimmune activity.

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Disclosures

The authors have no financial conflicts of interest.

References


