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Neutrophil 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 iNKT 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. Neutrophil 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: 000–000.

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-129Pepc+/+ (CD45.1) were from the Jackson Laboratory (Bar Harbor, ME). B6.129-Tcra-Jtm1Tgi

Abbreviations used in this article: αGalCer, α-galactosyl ceramide; iNKT, invariant NKT cell; Igβ2, β2 integrin gene; MDSC, myeloid-derived suppressor cell; PD, peritoneal dialysis; PMN, polymorphonuclear leukocyte; T-bet, T-box transcription factor 21; wt, wild-type.

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The online version of this article contains supplemental material.
and cytotoxicity assay

Medium with 100 ng/ml m
m5m

were treated with trimethoprim-
sulfomethoxazole in drinking water for 2 wk after transplantation. Ex-
periments were performed 3 to 4 mo after bone marrow transplantation. Ad-
optive transfer of CDS-enriched (Milenyi Biotec, Aubeck, CA) thyr-
mocytes and splenocytes was done after a single irradiation (400 rad) and
cells analyzed at the indicated time points. Blood for leucocyte counts was
taken via tail bleeding into EDTA-coated capillary tubes and analyzed with
an automatic analyzer (Hemavet 950FS; DREW Scientific, Oxford, CT).

αGalCer application and peritoni
tis model

αGalCer (2,53,5,4R)-1-O-(α-galactopyranosyl)-N-hexacosanoyl-2-amino-
1,3,4-octadecanetriol (KRN7000; Kirin Pharma, Gunma, Japan) was given
by i.v. injection 90 min before analysis (1 μg/mouse). For induction of
peritoni, 1 ml BBL fluid thioglycollate medium (BD Biosciences,
Sparks, MD) was injected i.p., and cells were recovered by washing twice
with 5 ml PBS at the indicated time points as described (20). Anti-CD122
(R&D Systems, Minneapolis, MN) was injected i.v. (30 μg/mouse). Cell
preparation from liver, spleen, and thymus was essentially as described
(21).

Human samples

Blood and peritoneal fluid were recovered after local ethics board approval
(MHH 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], nine
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 peritonitis outflow before initiation of therapy. Leucocyte counts were
assessed in the clinical laboratory at Hannover Medical School.

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
10% FCS) 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 × 107 polymorphonuclear leucocytes (PMN) were coincubated in 500
μl full media.

For human iNKT cell expansion, total PBMCs were cultured in full
medium with 100 ng/ml αGalCer for 7 d as described (24, 25), washed,
and resuspended in full media. Transwells (0.4-μm pore size) were from
Corning (Corning, NY). For the cytotoxicity assay, fresh PBMC were
incubated with 100 ng/ml αGalCer in full RPMI for 1 h, washed, and
labeled 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 and with 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 cytom
tery

The following Abs were used for flow cytometry: anti-mouse: CD1d (1B1),
CD3ε (145.2C11, 17A2), CD19 (1D3, 6D5), CD45 (30-F11), CD45.1-P
(A20), CD45.2 (104), CD69 (H1.2F3), CD122 (TM-β1), CD154 (CD40L,
MR1), 7/4, Ly6G (1A8), Ly6C(HK.1.4), Gr1 (RB6-8C5), T-bet (4B10),
GATA3 (LS5-823), TNF-α (MP6-XT22), IL-4 (11B11), and IFN-γ
(X51-812); anti-human: CD1d (51.1), CD3e (BHI3a), CD19 (BHI19),
β2m24A18 (6B11), IFN-γ (45.B3), and T-bet (4B10). Abs were purchased
from Abcam (Cambridge, MA), BD Biosciences (San Jose, CA), Bio-
Legend, eBioscience (San Diego, CA), or Invitrogen. Near-infrared LIF/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 prep-
paration of αGalCer-loaded CD1d tetramers was as described (26). Mouse
iNKT cells were defined as CD8ααCD19 tetramer-TCRβα, human
iNKT cells as CD19CD3ε+Vα24+ cells. Flow cytometry analysis was
performed on a BD FACS Calibur, FACS Canto, or LSR II (BD Biosciences).
Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Two-tailed Student t test or ANOVA with appropriate post hoc test was
used as indicated in the figure legends, and p values < 0.05 were con-
sidered significant. Data are expressed as mean ± SEM. The p values are
indicated with *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

Decreased function of Vα14i NKT cells from spontaneously
neutrophilic mice

Mice deficient for the β subunit of β2 integrins (CD18, Itgb2-/-) are
spontaneously neutrophilic (Fig. 1A) (12, 18). We did not ob-
serve a significant difference in the number of splenic NKT cells,
but the number of NKT cells in the liver, where in mice a major
iNKT cell population is located (1–3), was reduced in agreement
with previous reports (27) (Supplemental Fig. 1). The wt and
neutrophilic Itgb2-/- mice were injected with the potent iNKT cell
Ag αGalCer (2) and cytokine production was determined via in-
tracelluar staining. The proportion of NKT cells that produced
IFN-γ, TNF-α, or IL-4 was significantly smaller in neutrophilic

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Itgb2<sup>−/−</sup> 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 nK<sup>i</sup>T 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 Itgb2<sup>−/−</sup> nK<sup>i</sup>T cells upon αGalCer stimulation was significantly reduced (Fig. 1C). Decreased cytokine production in response to αGalCer was apparent in both NK1.1 and CD4<sup>+</sup>-negative and -positive subpopulations to a very similar degree (data not shown). NK cells are rapidly activated downstream of nK<sup>i</sup>T cell stimulation, a process referred to as trans-activation (29, 30). In line with the reduced nK<sup>i</sup>T cell cytokine production and CD154 expression, NK cell trans-activation, measured by IFN-γ production by NK cells, was greatly reduced in neutrophilic Itgb2<sup>−/−</sup> mice (Fig. 1D). These data demonstrate impaired activation and cytokine production by nK<sup>i</sup>T cells from neutrophilic mice in vivo.

Decreased T-bet and GATA3 transcription factor expression of V<sup>α</sup>14i NKT cells from neutrophilic mice

To gain insight into the mechanism for the decreased cytokine responses by V<sup>α</sup>14i NKT 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<sup>+</sup> T lymphocytes (5). Transcription factors were analyzed by flow cytometry after intracellular staining in thymic and peripheral nK<sup>i</sup>T cells from Itgb2<sup>−/−</sup> and wt mice (Fig. 2A, 2B). Itgb2<sup>−/−</sup> splenic nK<sup>i</sup>T 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 nK<sup>i</sup>T cells were similar in wt and Itgb2<sup>−/−</sup> mice (Fig. 2), arguing against a developmental cause of this difference. Also, CD1<sup>d</sup> expression was not different in spleens or thymus of Itgb2<sup>−/−</sup> compared with wt mice (data not shown).

To test whether decreased nK<sup>i</sup>T cell transcription factor expression was due to β<sub>2</sub> integrin deficiency, we employed mice deficient in endothelial and platelet, but not leukocyte selectins (19). These mice were neutrophilic to a similar degree as Itgb2<sup>−/−</sup> mice (Supplemental Fig. 3A) (12). nK<sup>i</sup>T 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 nK<sup>i</sup>T cells was reduced (Supplemental Fig. 3B, 3C), suggesting neutrophilia as a possible cause of the observed nK<sup>i</sup>T cell phenotype.

Modulation of V<sup>α</sup>14i NKT cells by neutrophils is cell extrinsic and reversible

To test if the phenotype of peripheral nK<sup>i</sup>T cells from neutrophilic Itgb2<sup>−/−</sup> mice was cell intrinsic or environmental, we employed adoptive thymocyte and splenocyte transfers and bone marrow transplantations. We transferred thymocytes from wt or Itgb2<sup>−/−</sup> 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 nK<sup>i</sup>T cell T-bet and GATA3 expression levels were analyzed by flow cytometry. The wt and Itgb2<sup>−/−</sup> thymocytes transferred into nK<sup>i</sup>T-deficient host mice (Jα18<sup>−/−</sup>), which have normal neutrophil counts (data not shown), expressed similar levels of T-bet and GATA3 in nK<sup>i</sup>T cells (Fig. 3A). When thymocytes were transferred to mice with endogenous nK<sup>i</sup>T cell populations, host neutrophil counts had a similar influence. For example, Itgb2<sup>−/−</sup> thymocytes transferred into wt hosts retained relatively higher T-bet and GATA3 expression, similar to their host counterparts (Fig. 3B). To create a cohort of neutrophilic recipients, we created bone marrow-chimeric recipients with 100% wt 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 Itgb2<sup>−/−</sup> nK<sup>i</sup>T cells (Fig. 3B). These data suggest that the decreased expression levels of T-bet and GATA3 observed in Itgb2<sup>−/−</sup> nK<sup>i</sup>T 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 Itgb2<sup>−/−</sup> nK<sup>i</sup>T cells develop in the same animal, we reconstituted lethally irradiated wt mice with bone marrow from wt and Itgb2<sup>−/−</sup> mice mixed at an equal ratio. As described (12), transfer of 50% wt/50% Itgb2<sup>−/−</sup> 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% Itgb2<sup>−/−</sup>, 2.4 ± 0.4; and in Itgb2<sup>−/−</sup> 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 Itgb2<sup>−/−</sup> nK<sup>i</sup>T 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 nK<sup>i</sup>T cells in neutrophilic Itgb2<sup>−/−</sup> mice was reversible, we adoptively transferred splenocytes from wt and Itgb2<sup>−/−</sup> mice at an equal ratio into nK<sup>i</sup>T cell-deficient, normo-neutemic host mice. This completely normalized the transcription factor expression of the Itgb2<sup>−/−</sup> nK<sup>i</sup>T cells by day 3 after transfer (Fig. 3D), indicating that the downregulation was a reversible phenotype. It is of note that both wt and Itgb2<sup>−/−</sup> 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 nK<sup>i</sup>T 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 V<sup>α</sup>14i NKT 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

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**FIGURE 2.** Decreased transcription factor expression in V<sup>α</sup>14i NKT cells from neutrophilic mice. (A and B) T-bet and GATA3 expression in nK<sup>i</sup>T cells recovered from thymus and spleen was analyzed by flow cytometry after intracellular staining. (C and D) Both were significantly reduced in peripheral nK<sup>i</sup>T cells recovered from spleen of Itgb2<sup>−/−</sup> compared with wt mice (expressed as mean fluorescence intensity relative to wt cells) (n = 8–10 from three to four independent experiments). ***p < 0.001.
neutrophils can induce i

(Fig. 4B), demonstrating that high local concentrations of resting GATA3 expression in both splenic and thymic Vα bone marrow neutrophils in vitro. This decreased T-bet and GATA3 expression is shown in relation to actual neutrophil concentration calculated from flow cytometry (Ly6C1Ly6G7/4+ cells) (example from n = 3). (D) Coculture with neutrophils (5 × 10⁷/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).

FIGURE 4. Neutrophils moderate Vα14+ iNKT 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 neutrophils 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 modulated by neutrophil counts. (107/ml) for 6 h and T-bet and GATA3 expression determined (examples from n = 3). (D) Coculture with neutrophils (5 × 10⁷/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).

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 and heat-killed neutrophils lost their ability to affect Vα14+ iNKT cell T-bet and GATA3 expression in our in vitro coculture systems (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 to obtain highly purified neutrophils. Comparing the increase in T-bet and GATA3 transcription factor expression relative to absolute neutrophil numbers, measured by flow cytometry, revealed highly similar dose responses (Fig. 4C), indicating that indeed the neutrophils in the mixture were responsible for the iNKT cell inhibitory effect.

Coculture with neutrophils did not alter PD-1, BLTA GITR, or CD152 on the iNKT cell surface (data not shown). The wt and CD14−/− bone marrow neutrophils did not differ in their ability to induce downregulation of T-bet and GATA3 in iNKT cells (data

neutrophils decreased T-bet and GATA3 expression, wt bone marrow Vα14+ iNKT 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 iNKT 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 cocultured with mouse bone marrow neutrophils in vitro. This decreased T-bet and GATA3 expression in both splenic and thymic Vα14+ iNKT cells (Fig. 4B), demonstrating that high local concentrations of resting neutrophils can induce iNKT cell downregulation of these tran-
not shown). To further investigate whether iNKT cell inhibition was due to a soluble factor or cell–cell contact dependent, neutrophils were separated from V\textsubscript{α}14i NKT cells using a transwell (Fig. 4D). This completely abolished the neutrophil inhibitory effect on V\textsubscript{α}14i NKT cell transcription factor expression.

**Neutrophils modulate V\textsubscript{α}24i NKT cell function in vitro**

To test if neutrophils would similarly impact primary human V\textsubscript{α}24i 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 V\textsubscript{α}24i NKT cell T-bet expression and IFN-γ production (data not shown).

V\textsubscript{α}24i 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\textsuperscript{+} iNKT cells (Fig. 5D). V\textsubscript{α}24i NKT cell cytoxicity 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 V\textsubscript{α}24i 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 V\textsubscript{α}14i 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. 6F), 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.** Neutrophils modulate V\textsubscript{α}24i NKT cell function in vitro. (A) Human PBMCs isolated by density gradient centrifugation were cultured in the presence or absence of neutrophils (5 × 10\textsuperscript{5} ml) for 4 h. Neutrophils significantly decreased T-bet and GATA3 expression in V\textsubscript{α}24i NKT cells (n = 4). (B) T-bet expression of in vitro-expanded V\textsubscript{α}24i NKT cells was assessed after 4 h coculture with neutrophils (PMN; 10\textsuperscript{7} ml). (C) In vitro-expanded V\textsubscript{α}24i NKT cells were exposed to αGalCer (100 ng/ml) in the presence and absence of neutrophils (PMN; 10\textsuperscript{7} ml). IFN-γ concentration in the supernatant after 4 h was determined by ELISA (n = 3). (D) Individual iNKT cell (CD\textsuperscript{3+}V\textsubscript{α}24iCD\textsuperscript{19+}) 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 full RPMI for 6 h with and without iNKT cells and freshly isolated neutrophils (PMN; 10\textsuperscript{7} ml). The proportion of αGalCer-labeled (CFSE\textsuperscript{hi}) and CFSE\textsuperscript{low} (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 10\textsuperscript{7} 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.
VA24i 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 VA24i 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). VA24i 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 VA24i NKT cells (data not shown). However, the Th1 transcription factor T-bet was expressed and was significantly higher in VA24i 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 VA24i 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 VA24i 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-
kocyte 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 a 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 Vα14i iNKT cells in vivo and in vitro and in human peripheral blood Vα24i iNKT cells. Also, Vα14i iNKT 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 Vα14i iNKT 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 Vα14i iNKT 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 not generally found in healthy organs (52). Furthermore, MDSC typically suppressed the downstream of the neutrophil, which is likely to represent a novel mechanism. Potential candidates for cell–cell contact dependent mechanism for iNKT cell inhibition by neutrophils have been described for soluble molecules such as NO induced by IFN-γ (24), IL-10 (46), and 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 neutrophil 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


Supplemental figure 1: iNKT cell distribution and profile in wt, Itgb2−/− and Sele−/− Selp−/− mice

(A) iNKT cells were enumerated among TCRβ+ cells in spleen, thymus, bone marrow (BM), liver and peripheral lymphnodes (Ln) of wt (n=4-7), Itgb2−/− (n=4-7) and Sele−/− Selp−/− (n=2) mice, (n.d. = not done). (B) Phenotyping of splenic iNKT cells revealed an increased proportion of CD4+ cells among both, NK1.1+ and NK1.1− iNKT cells in Itgb2−/− mice (n as part A).
Supplemental figure 2: Gating strategy for iNKT cell assessment and Intracellular cytokine production in response to αGalCer in peripheral Vα14iNKT cells from spontaneously neutrophilic mice.

(A) iNKT cells were defined as αβTCR⁺, CD19⁻, CD8α⁻, CD44⁺, αGalCer-loaded-CD1d-tetramer⁺ (tet⁺) cells.

(B,C) Mice were injected with αGalCer 90 min before sacrifice and iNKT cell cytokine production in spleen (B) and liver (C) was assessed (representative examples of intracellular cytokine staining).
Supplemental figure 3: Reduced T-bet and GATA3 expression in splenic and hepatic iNKT cells from neutrophilic Sele^{-/-}Selp^{-/-} mice

(A) Peripheral blood neutrophil counts were significantly elevated in P and L selectin deficient (Sele^{-/-}Selp^{-/-}) compared to wt mice (n=18 (wt) and 3 (Sele^{-/-}Selp^{-/-})). (B,C) T-bet (B) and GATA3 (C) expression in splenic, thymic and hepatic iNKT cells was analyzed by flow-cytometry after intracellular staining (expressed relative to wt cells) (n = 2 per group). Both were reduced in iNKT cells from spleen and liver of Sele^{-/-}Selp^{-/-} compared to wt mice.