Paradoxical inhibition of Human Natural Interferon-Producing Cells by the activating receptor NKp44

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Abstract

NK cell-mediated cytoxicity is triggered by multiple activating receptors associated with the signaling adaptor protein DAP12/KARAP. Here we show that one of these receptors, NKp44, is present on a subset of natural interferon-producing cells (IPC) in tonsils. NKp44 expression can also be induced on blood IPC after \textit{in vitro} culture with IL-3. Cross-linking of NKp44 does not trigger IPC-mediated cytotoxicity, but, paradoxically, inhibits IFN-\(\alpha\) production by IPC in response to CpG oligonucleotides. We find that IPC in tonsils are in close contact with CD8\(^+\) T cells and demonstrate that a subset of memory CD8\(^+\) T cells produces IL-3. Therefore, IL-3-mediated induction of NKp44 on IPC may be an important component of the ongoing cross talk between the innate and adaptive immune response that allows memory CD8\(^+\) T cells to control the IPC response to virus.
Introduction

Natural interferon-producing cells (IPC), also termed plasmacytoid dendritic cells (pDC), play an important role in the first line defence against viral infections\textsuperscript{1-3}. IPC recognize viral components, such as CpG-rich DNA and single stranded (ss) RNA, through the Toll-like receptors TLR9 and TLR7, respectively\textsuperscript{4}. Detection of viral infection triggers IPC secretion of type I interferons (IFNs), i.e. IFN-\(\alpha\) and IFN-\(\beta\), which inhibit viral replication in infected cells and activate host immune responses\textsuperscript{5-7}. In particular, type I IFNs enhance the cytotoxicity of NK cells and CD8 T cells\textsuperscript{5,8}, induce survival of CD8 T cells\textsuperscript{9}, activate dendritic cells (DC)\textsuperscript{10}, promote differentiation of helper CD4 T cells into Th1 cells\textsuperscript{5} and the differentiation of memory B cells into antibody-secreting plasma cells\textsuperscript{11,12}. IPC further activate adaptive immune responses by secreting IL-12\textsuperscript{13} and proinflammatory chemokines\textsuperscript{14}, and by presenting antigens to T cells\textsuperscript{15-17}.

IPC are generated in the bone marrow\textsuperscript{2} and circulate in peripheral blood in very low numbers. Upon viral infection, IPC presumably leave the blood stream and migrate into the lymph nodes draining the site of infection through the high endothelial venules (HEV)\textsuperscript{18-20}. IPC localize predominantly in the T cell zone of the lymph node, where they secrete type I IFNs and activate innate and adaptive immune responses. IPC also migrate into inflamed peripheral tissues, particularly skin of patients affected by autoimmune disorders like systemic lupus erythematosus\textsuperscript{21,22}. Moreover, IPC are recruited into tumors, such as ovarian carcinomas and melanomas, but, in these sites, they appear to be functionally
paralysed by tumor microenvironment and incapable of secreting type I IFNs and activating immune responses\textsuperscript{23,24}.

Human IPC are identified by the exclusive expression of a cell surface molecule termed BDCA-2\textsuperscript{25}. BDCA-2 is a C-type lectin receptor that acts as an antigen-capturing molecule. Moreover, engagement of BDCA-2 by a specific antibody inhibits IFN-\(\alpha\) production by IPC in response to CpG. Activation of IPC \textit{in vitro} with TLR ligands causes a rapid downregulation of BDCA-2 from the cell surface, together with increased expression of MHC and costimulatory molecules, such as CD80, CD86 and CD40. IPC also express the alpha chain of IL-3 receptor (CD123) and depend on IL-3 for their survival \textit{in vitro}\textsuperscript{26,27}. Resting and activated IPC express additional cell surface markers of the myeloid lineage (CD31, CD36, CD68) and the lymphoid lineage (CD45RA)\textsuperscript{28}, as well as T cell- and B cell-specific transcripts, like pre-TCR alpha and Spi-B\textsuperscript{2}. Although a rare leukemia with the surface characteristics of IPC expresses the NK cell marker CD56\textsuperscript{29}, no other NK cell surface molecules have been identified on normal IPC to date.

NKp44 is an activating receptor expressed on human NK cells that belongs to the immunoglobulin (Ig) superfamily\textsuperscript{30,31}. It consists of a single extracellular V-type Ig domain, a transmembrane region and a short cytoplasmic domain. Through its transmembrane region, NKp44 associates with the adaptor molecule DAP12 (KARAP), which contains a tyrosine-based activation motif (ITAM). DAP12 is required for NKp44 cell surface expression and intracellular signalling. The
cytoplasmic domain of NKp44 contains a tyrosine-based motif with unknown function\textsuperscript{32}. NKp44 mediates cytotoxicity against various tumor cells, such as neuroblastoma and choriocarcinoma\textsuperscript{30,31,33,34}. The NKp44 ligand on these tumors has not yet been identified. There may also be a role for NKp44 recognition of virally infected cells\textsuperscript{35}. NKp44 is not expressed on the surface of resting NK cells. Expression is induced upon \textit{in vitro} activation of NK cells with IL-2\textsuperscript{30}. Recently, NKp44 was also found on a subset of NK cells in tonsils, possibly representing \textit{in vivo} activated NK cells\textsuperscript{36,37}. Here we report that tonsil IPC and blood IPC cultured \textit{in vitro} with IL-3 express NKp44. We demonstrate that cross-linking of NKp44 leads to inhibition of IFN-\textgreek{a} production by IPC in response to CpG. Moreover, we show that IPC in tonsils are in close contact with CD8 T cells and that a subset of memory CD8 T cells produces IL-3, indicating a potential cross-talk between IPC and CD8 T cells through IL-3-mediated induction of NKp44.
Materials and Methods

Cell preparations and cultures

Buffy coats from normal healthy volunteers were obtained from the American Red Cross (St. Louis, MO). Peripheral blood from patients with Nasu-Hakola disease was obtained from the Nagasaki Medical Center of Neurology, Nagasaki, Japan\textsuperscript{38}. Peripheral blood mononuclear cells (PBMC) were prepared from buffy coats by standard Ficoll density gradient centrifugation (Ficoll-Paque, Amersham Biosciences, Piscataway, NJ). IPC were isolated from PBMC using the BDCA-4 isolation kit (Miltenyi Biotec, Auburn, CA). Tonsils were obtained from pediatric patients undergoing elective tonsillectomy (Children’s Hospital, Washington University School of Medicine, St. Louis, USA). Approval was obtained from the Washington University School of Medicine and the Nagasaki Medical Center of Neurology institutional review boards for these studies. Informed consent was provided according to the Declaration of Helsinki. Lymphocyte suspensions were prepared from tonsils by dissociation of the tissue, followed by Ficoll density gradient centrifugation. NK92 cells were kindly provided by M.L. Botet (University Pompeu-Fabre, Barcelona, Spain). Peripheral blood NK cells were obtained from CD56\textsuperscript{+}CD3\textsuperscript{−}PBMC as described\textsuperscript{39}. Peripheral blood CD8 T cells were isolated using CD8 MicroBeads (Miltenyi Biotec) and then sorted into CD45RA\textsuperscript{+}2B4\textsuperscript{−} naïve cells, CD45RA\textsuperscript{−}2B4\textsuperscript{−} central memory cells, CD45RA\textsuperscript{+}2B4\textsuperscript{+} effector cells and CD45RA\textsuperscript{−}2B4\textsuperscript{+} effector memory cells on a MoFlo cytometer (Cytomation, Fort Collins, CO). Sorted cells were cultured for 48h in IL-2-containing medium before stimulation with 10^{-7}M PMA and 0.5µg/ml ionomycin (both from Sigma Aldrich,
St. Louis, MO). Following 2h of stimulation, 2µM monensin was added to the cultures and incubation was continued for 4h. Cells were then fixed for detection of intracellular cytokines.

PBMC and purified IPC were cultured in RPMI 1640 (Gibco BRL, Rockville, MD), supplemented with 10% fetal calf serum (HyClone, Logan, UT), GlutaMAX, kanamycin and Na-pyruvate (all from Gibco BRL). IL-3 (R&D Systems, Minneapolis, MN) was added to a final concentration of 20ng/ml, where indicated. For IPC stimulation, 5 µg/ml CpG 2216 (Invitrogen, Carlsbad, CA) or influenza virus WSN strain (a kind gift from Andrew Pekosz, Department of Molecular Microbiology, Washington University, St. Louis, MO) was added to cultures. For receptor cross-linking experiments, cells were stimulated in ELISA plate wells coated with F(ab')_2 goat anti-mouse IgG antibody (10 µg/ml, Southern Biotechnology Associates, Birmingham, AL) and hybridoma supernatants containing the indicated mouse mAb. Alternatively, wells were coated with purified mouse mAbs (10 µg/ml) or their F(ab')_2 fragments (50 µg/ml). F(ab')_2 fragments were prepared from the anti-NKp44 mAb (clone 3.43) and anti-CD155 mAb (clone SKII.4, mouse IgG1, as control antibody) using the ImmunoPure F(ab')_2 Preparation Kit from Pierce (Rockford, IL).

**Cytotoxicity assays**

The target cell lines P815 (murine mastocytoma) and K562 (human erythroleukemia) were labelled with 0.1 mCi chromium-51 (Amersham) for 1h,
washed three times and seeded at 5000 cells/well in a 96 well plate. The indicated effector cells (NK92, activated NK cells or IPC cultured overnight in IL-3) were added in serial dilutions, with a maximum effector/target ratio of 10:1. Cytotoxicity was measured as chromium-51 release following 4h or 18h of culture at 37°C. Specific lysis was calculated from the formula % lysis = 100 x (test cpm – spontaneous cpm)/(total cpm – spontaneous cpm).

**Flow cytometric analysis and immunofluorescence**

Anti-NKp44 mAbs (clones 2.29, and 3.43, both mouse IgG1) were generated in our laboratory by immunizing mice with a NKp44-human Fc fusion protein. Anti-NKp44 (clone 2.29) was conjugated to allo-phycocyanin (Cyanotech, Kailua-Kona, HI) according to standard protocols. Biotinylated anti-TREM-1 antibody was generated in our laboratory and used in combination with Streptavidin-APC (Molecular Probes, Eugene, OR). Unconjugated anti-BDCA-2 antibody (clones 13A11 and AC144) and FITC-conjugated anti-BDCA-2 (AC144) were a kind gift of Miltenyi Biotec (Bergisch Gladbach, Germany). Fluorochrome-labelled antibodies against CD45RA, CD123, Granzyme B, IL-2, IL-3, TNF-α and IFN-γ were purchased from BD Biosciences (San Jose, CA). Fluorochrome-labelled antibodies against CD8, CD19, CD20, CD56, CD14, CD16, CD83, CD86, CD40 and CD244 (2B4) were from Beckman Coulter (Miami, FL). Samples were analysed on a FACSCalibur (BD Biosciences) using the CellQuest software.
Cryosections of tonsil tissues were stained with anti-BDCA-2 (AC144, mouse IgG1) and anti-CD8 (OKT8, mouse IgG2a, from ATCC), followed by biotin-labelled anti-mouse IgG2a (Southern Biotechnology Associates). Antibody binding was detected using a Texas Red-conjugated streptavidin and Alexa 488-conjugated anti-mouse IgG1 antibody (Molecular Probes). Slides were mounted with VectaShield (Vector Laboratories, Burlingame, CA) containing DAPI stain and analyzed on an Olympus BX51 (Olympus, Melville, NY) using the Spot Advanced software.

**Analysis of cytokine and chemokine secretion**

IFN-α levels in culture supernatants were measured by evaluating the inhibition of Daudi cell proliferation with reference to a standard IFN-α curve\cite{41}. Concentrations of the chemokines MCP-1, MIP-1α, MIP-1β, RANTES and IL-8 were assessed using the Chemokine II Cytometric Bead Array (BD Biosciences).
Results

A subset of tonsil IPC expresses NKp44

NKp44 is induced by in vitro activation of NK cells and TCR \( \gamma \delta \) cell clones, while no cell types express NKp44 within PBMC\(^{30,31} \). In addition, NKp44 is present on a subset of tonsil NK cells, presumably corresponding to NK cells activated in vivo\(^{36,37} \). We studied in depth the phenotype of NKp44\(^+ \) cells in human tonsils and observed that NKp44 is expressed not only on NK cells but also on a small cell population that lacks NK cell markers. This cell subset expressed the IL-3 receptor alpha chain (CD123) (not shown) and BDCA-2, a specific marker of IPC\(^{25} \) (Fig 1). NKp44\(^+ \) IPC represented approximately 10-50% of tonsil IPC and expressed NKp44 at lower levels than tonsil NK cells (Fig. 1). In contrast, peripheral blood IPC did not express NKp44 (Fig 1). We conclude that primary IPC express NKp44 in tonsils but not in blood.

IL-3 induces NKp44 on blood IPC

We investigated whether blood IPC can be induced to express NKp44. Indeed, overnight incubation of IPC purified from peripheral blood in medium containing IL-3 induced NKp44 on the surface of IPC (Fig. 2). Similarly, when whole PBMC were cultured in IL-3, NKp44 became detectable on a small cell subset corresponding to IPC but not on other cell types (Fig. 2). These findings indicate that IL-3 acts specifically on IPC to induce NKp44 expression. NKp44 was already detectable on IPC in low levels after 10 hours in IL-3, it reached maximum levels by about 30 hours of culture and remained stable throughout the
72h period of culture (Fig 3a). We also cultured freshly isolated blood IPC in the presence of IL-3 together with TLR9 and TLR7 agonists, such as CpG and influenza virus, which are known to induce IPC activation and IFN-α secretion. Remarkably, these stimuli inhibited the induction of NKp44 when added at the same time as IL-3 (Fig. 3b). In contrast, when IPC were first cultured with IL-3 to induce expression of NKp44 and then stimulated with CpG or influenza virus, expression of NKp44 did not significantly decrease (data not shown), indicating that TLR ligands can inhibit the induction of NKp44, but do not affect pre-existing NKp44 expression.

**IPC from a DAP12-deficient patient do not express NKp44**

In NK cells, NKp44 associates with DAP12 (KARAP)\(^{32}\). This ITAM-bearing adaptor protein mediates downstream signalling events upon receptor engagement\(^{42,43}\). We sought to determine whether NKp44 associates with DAP12 in IPC. Human IPC are rare blood cells and cannot be grown *in vitro* in sufficient numbers for biochemical demonstration of NKp44/DAP12 association. However, DAP12 is required for cell surface expression of NKp44\(^{32}\). Therefore, we analysed cell surface expression of NKp44 in IL-3 cultured PBMC from a DAP12-deficient patient affected by the Nasu-Hakola disease\(^{38,44}\). We stained granulocytes of this patient with a mAb against TREM1, another known DAP12-associated receptor\(^{40}\), and found no TREM-1 expression, confirming the absence of functional DAP12 (Fig. 4a). Staining of the DAP12-deficient PBMCs with mAbs against with BDCA-2 and CD123 revealed the presence of blood IPC as well as
their expression of the IL-3 receptor alpha chain (Fig. 4b). Following overnight culture of PBMC with IL-3, however, DAP12-deficient IPC failed to express NKp44 (Fig. 4c). We conclude that NKp44 associates with DAP12 in IPC as it does in NK cells.

**NKp44 triggering does not activate IPC-mediated killing of target cells**

Cross-linking of the NKp44 on NK cells triggers cytotoxicity. IPC have not been reported to function as cytotoxic cells, despite their abundant expression of granzyme B. We tested the possibility of IPC-mediated target cell lysis upon NKp44 crosslinking. NKp44+ IPC were cultured with the Fc Receptor (FcR) bearing cell line P815 in the presence of NKp44 antibody to induce receptor cross-linking. Activated NK cells were used as controls. Cytotoxicity was measured in a 4h chromium release assay. No target cell lysis was observed when IPC were used as effector cells, whereas activated NK cells efficiently killed P815 in the presence of NKp44 antibody (Fig. 5a). We also examined the ability of IPC to directly kill the target cell line K562, which is highly susceptible for NK cell-mediated killing. K562 was lysed efficiently by NK cells but not by IPC (Fig. 5b). To investigate whether IPC utilize other mechanisms of cytotoxicity, such as those mediated by members of the TNF receptor family (TRAIL, CD95L), which require longer time spans to induce target cell death, we co-cultured IPC with P815 in the presence of crosslinking NKp44 antibody for 18h. However, no significant target cell lysis was detected in these settings (Fig. 5c). In conclusion, we did not observe any significant IPC-mediated cytotoxicity, neither by triggering
the NKp44 receptor, nor by co-culturing IPC with the typical NK cell target K562, although IPC did contain intracellular granzyme B (Fig. 5d).

**Engagement of NKp44 inhibits IFN-α secretion in response to CpG**

To further evaluate the function of NKp44 in IPC, we tested the effects of NKp44 engagement on cytokine production. We incubated IPC with IL-3 to induce NKp44 and then cultured NKp44+ IPC in plates coated with anti-NKp44 antibody in the presence of CpG, which induces IFN-α and chemokine secretion. Remarkably, NKp44 cross-linking consistently inhibited CpG-induced IFN-α secretion (Fig. 6a); the degree of inhibition varied between IPC preparation by 2-16 fold. This ability of NKp44 to inhibit the IFN-α response of IPC to CpG was reminiscent of the inhibitory function previously reported for the C-type lectin BDCA-225. However, comparison of NKp44 and BDCA-2 effects on IFN-α secretion revealed that BDCA-2 mediates stronger inhibition than NKp44 (Fig. 6b). IPC express the Fc gamma receptor FcγRII47,48, and IgG-Fc can inhibit IFN-α the response of IPC to viruses49. Additionally, engagement of FcγRs can drastically modulate the cytokine profile of other hematopoietic cells such as monocytes/macrophages50,51. To address whether the anti-NKp44 mAb inhibits IFN-α response of IPC by engaging an FcγR, we performed experiments with anti-NKp44 F(ab’)2 fragments, which do not engage Fc receptors. Plate-bound anti-NKp44 F(ab’)2 fragments induced an inhibition of IFN-α response to CpG similar to that seen with complete antibody (Figure 6b), demonstrating that inhibition is not mediated by interaction of the antibody with the FcγRII. In
contrast to CpG-induced IFN-α, NKp44 cross-linking did not significantly inhibit CpG-induced secretion of proinflammatory chemokines such as MIP-1α and MIP-1β (Fig. 6c and d). We conclude that ligation of NKp44 selectively inhibits IFN-α response to CpG.

**CD8 T cells with memory phenotype produce IL-3**

Since IL-3 is required for NKp44 expression on IPC, we asked which cells may provide IL-3 to IPC in vivo. Mast cells and eosinophils are a well-established source of IL-3. However, these cell types are unlikely to be present in the T cell area and around HEV of secondary lymphoid organs, where IPC are mainly located. In fact, analysis of tonsil sections by immunofluorescence with antibodies specific for IPC and other cell types revealed that IPC are often positioned in close contact to CD8 T cells (Fig. 7a and b). However, whether CD8 T cells produce IL-3 is not known. We previously demonstrated that CD8 T cells can be divided into four distinct subsets, based on the expression of CD45RA and the cell surface receptor 2B4: a) CD45RA+/2B4- cells correspond to naïve T cells; b) CD45RA+/2B4high cells are effector T cells; c) CD45RA-/2B4- cells represent memory T cells; d) CD45RA-/2B4+ cells are effector-memory T cells. Since most of the CD45RA-/2B4- cells express CCR7 (not shown), they largely overlap with the well-described subset of central memory cells. We sorted these four subsets of CD8 T cells from peripheral blood and assessed their capacity to produce IL-3 by intracellular staining. Remarkably, we found that only memory CD8 T cell produce IL-3 (approximately 20-70% of the cells in different
individuals) (Fig. 7c). IL-3 production correlated with IL-2 production and lack of intracellular perforin. In contrast, effector or effector-memory subsets produced high levels of IFN-γ and perforin but not IL-3 (Fig. 7c). The naïve subset solely produced IL-2 (not shown). This result suggests that memory CD8 T cells activated by or in close proximity to IPC may provide the IL-3 required for IPC survival and NKp44 upregulation.
Discussion

In this report, we demonstrate that a subset of tonsil IPC and IL-3-cultured blood IPC express the NK cell receptor NKp44 and that engagement of NKp44 inhibits IFN-α responses of IPC to CpG. NKp44 was originally considered a specific marker for activated NK cells and some TCR-γδ T cell clones\textsuperscript{30,31}. NKp44 was then detected on some tonsil NK cells\textsuperscript{36,37}. This is the first report to describe the presence of an activating NK cell receptor on human IPC. Because IPC in a DAP12-deficient patient lacked NKp44, it is likely that in normal IPC NKp44 reaches the cell surface and signals through DAP12, as in the case of NK cells. Blood IPC expressed NKp44 upon \textit{in vitro} culture with IL-3. None of the other cell types that express the receptor for IL-3, such as basophils, monocytes and monocyte-derived DC, expressed NKp44 upon culture with IL-3. Moreover, IL-3 did not induce NKp44 expression on NK cells, consistent with their lack of IL-3R (data not shown). Although IL-2 can induce NKp44 on NK cells\textsuperscript{30}, it was unable to do so on IPC, which also express the IL-2R alpha chain (CD25) (data not shown).

In NK cells, NKp44 ligation triggers exocytosis of lytic granules and secretion of IFN-γ against tumor cells expressing yet unknown NKp44 ligands\textsuperscript{30,31,33,34}. Since IPC express high levels of granzyme B\textsuperscript{45,46}, we evaluated whether IPC triggering through NKp44 induces cytotoxicity. However, our experiments showed no evidence for NKp44-mediated killing of FcR+ cells coated with an anti-NKp44 antibody. In addition, IPC were unable to directly kill classical NK target cells,
such as K562. These results are consistent with the original characterization of IPC as a cell type unambiguously distinct from NK cells\(^7\). In spite of its ability to deliver activating signals, NKp44 paradoxically reduced IFN-\(\alpha\) responses of IPC to CpG. This inhibition was consistent throughout many experiments, although the degree of inhibition varied depending on the donor, the expression level of NKp44, and the activation state of IPC after isolation from PBMC. The signalling mechanism of this NKp44-mediated inhibition of CpG-induced secretion of IFN-\(\alpha\) is yet unknown. It has been shown that the C-type lectin BDCA-2, which also inhibits IFN-\(\alpha\) responses to CpG, induces a strong \(\text{Ca}^{2+}\) mobilization\(^{25}\). Thus, NKp44 may inhibit IPC function through DAP12 by triggering a cascade of protein tyrosine phosphorylation, which leads to activation of PLC\(\gamma\) and \(\text{Ca}^{2+}\) mobilization\(^{42,43}\). This activating pathway may trigger the secretion of yet unidentified cytokines that inhibit IFN-\(\alpha\) secretion. This possibility is corroborated by previous studies showing that cross-linking of certain activating receptors in monocytes/macrophages, such as the FcRs and ILT1, promote secretion of IL-10, which inhibits release of IL-12\(^{50,51,54}\). NKp44 and/or DAP12 may also inhibit IPC by recruiting cytoplasmic protein tyrosine phosphatases or other inhibitory mediators. Tyrosine phosphatases are typically recruited through inhibitory tyrosine-based inhibitory motifs (ITIM)\(^{55}\). Campbell et al. have recently reported the presence of an ITIM-like sequence in the cytoplasmic region of NKp44, although they were unable to demonstrate an inhibitory function of NKp44 in NK cells\(^{32}\). DAP12 does not contain ITIMs, but a tyrosine-based activation motifs (ITAM) that recruits protein tyrosine kinases\(^{42,43}\). However, recent evidence
indicates that in certain conditions, ITAMs can recruit tyrosine phosphatases instead of tyrosine kinases and mediate inhibition\textsuperscript{56}.

In our study, we demonstrated that tonsil IPC frequently co-localize with CD8 T cells and that, within CD8 T cells, a subset with a memory phenotype produces IL-3, which is required to induce NKp44 expression in IPC. Thus, CD8 T cells may provide an important source of IL-3 for induction of NKp44 in IPC \textit{in vivo}. On the other hand, TLR ligands inhibited IL-3-mediated induction of NKp44. Based on these observations, we can propose the following model. Upon viral infection, NKp44\textsuperscript{-} blood IPC enter secondary lymphoid organs presumably through high endothelial venules\textsuperscript{18,19}. Here, activation of IPC through TLRs prevents expression of NKp44 and induces secretion of type I IFNs, activating innate responses. The development of adaptive immunity reduces the viral load and, consequently, IPC stimulation through TLRs. Moreover, activated memory CD8 T cells secrete IL-3 and induce NKp44 on IPC, thus effectively downmodulating the innate immune response. As NKp44 can bind viral components, such as hemagglutinins\textsuperscript{35}, ligation of NKp44 may further downregulate innate responses by IPC. Moreover, tumor microenvironment was shown to induce functional paralysis of tumor associated-IPC\textsuperscript{23,24}. Thus, expression of yet unknown NKp44 ligands by tumor cells may provide a mechanism for inhibition of tumor-associated IPC.
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Figure 1. IPC express NKp44 in tonsil but not in peripheral blood.

Mononuclear cells were prepared from tonsils and peripheral blood and stained with mAbs against BDCA-2 and NKp44.

Figure 2. IL-3 induces expression of NKp44 in peripheral blood IPC.

Upper panel: IPC isolated from peripheral blood were stained with mAbs against BDCA-2 and NKp44 before (left) and after (right) overnight culture in IL-3. Lower panel: Total PBMC were cultured overnight in the absence (left) or presence (right) of IL-3, and analyzed for expression of BDCA-2 and NKp44.
**Figure 3.** IL-3-induced NKp44 expression persists during IPC culture, while CpG and virus block NKp44 induction.

(a) Peripheral blood IPC were cultured in the presence of IL-3. The mean fluorescence intensity (MFI) of anti-NKp44 staining was measured at 10h, 21h, 32h, 48h and 72h of culture.

(b) Freshly isolated peripheral blood IPC were cultured in IL-3-containing medium in the presence of CpG, influenza virus (WSN), or Poly (I:C). NKp44...
expression was evaluated after 16h of culture. CpG and WSN blocked NKp44 induction, while the TLR3 agonist poly (I:C) did not influence NKp44 expression, consistent with the lack of TLR3 in IPC.

**Figure 4. DAP12-deficient IPC do not express NKp44.**

(a) To confirm DAP12-deficiency, we evaluated granulocytes for expression of TREM1, a DAP12-associated receptor (right). TREM1 expression was determined by gating on CD16-positive granulocytes (left).
(b) The presence of IPC in DAP12-deficient PBMC was confirmed by analyzing PBMC for expression of BDCA-2 and CD123. Lineage-positive cells (CD3⁺, CD14⁺, CD16⁺, CD20⁺, CD56⁺) were excluded from the analysis.

(c) Total PBMC from a normal donor and the DAP12-deficient patient were cultured overnight in the presence (right) or absence (left) of IL-3. Selective expression of NKp44 on IPC was evaluated by counterstaining with BDCA-2. CD19⁺, CD14⁺ and CD56⁺ cells were excluded from the analysis.

4h cytotoxicity assay

Figure 5. NKp44 does not elicit IPC-mediated cytotoxicity.

(a, c) Redirected lysis of the FcR-bearing cell line P815 in the presence of an anti-NKp44 antibody was determined by a 4h (a) or 18h (c) chromium release
assay. Activated NK cells (a) or the NK cell line NK92 (c) were included as controls.

(b) Direct killing of K562 cells by IPC or NK92 was tested in a 4-hour chromium release assay.

(d) Intracellular granzyme B expression in IPC. Bold profile shows anti-granzyme B staining; thin profile represents an isotype-matched control antibody.

Figure 6. Crosslinking of NKp44 on IL-3-cultured IPC reduces IFN-α responses to CpG.
Peripheral blood IPC were cultured overnight in IL-3 and then transferred to wells containing plate-bound anti-NKp44 antibody, in the presence or absence of CpG.
as IFN-α-inducing stimulant. After 20h, culture supernatants were tested for the presence of IFN-α (a, b), MIP-1α (c) and MIP-1β (d). For IFN-α, two representative experiments are shown, which were carried out with whole antibody (a), or with F(ab’)2 fragments (b) to cross-link NKp44. In some experiments, IPC were also stimulated on anti-BDCA-2-mAb-coated plates for comparison. Black bars: control antibody; grey bars: anti-NKp44 mAb; white bars: anti-BDCA-2 mAb.

Figure 7. CD8 T cells are in close proximity of IPC in tonsil and provide a source of IL-3.
(a, b) Co-localization of CD8 T cells and IPC in the T cell areas of tonsil. Cryosections from tonsils were stained with anti-BDCA-2 and anti-CD8, followed by Alexa488-conjugated and Texas Red-conjugated secondary reagents. BDCA-
2 is shown in green, CD8 in red and DAPI nuclear stain in blue. Some of the HEVs are marked by white profiles. GC, germinal center.

(c) CD8 T cells were magnetically enriched from peripheral blood and sorted into CD45RA⁻/2B4⁻ central memory cells (left column), CD45RA⁻/2B4⁺ effector-memory cells (middle column), CD45RA⁺/2B4⁺ effector cells (right column) and CD45RA⁺/2B4⁻ naïve cells (not shown). Cytokine production of the sorted cell subsets was assessed by intracellular staining with anti-IL-2, IL-3, IFN-γ and TNF-α following 6h of stimulation with PMA and ionomycin. Inserts in upper panel represents FACS profiles of individual CD8 T cell subsets after intracellular staining with an anti-perforin antibody.
Paradoxical inhibition of human natural interferon-producing cells by the activating receptor NKp44

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