Selective expression of the activating receptor NKp65 demarcates human ILC3 from mature NK cells

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Abstract

Innate lymphocytes comprise cytotoxic Natural Killer (NK) cells and tissue-resident innate lymphoid cells (ILC) that are subgrouped according to their cytokine profiles into group 1 ILC (ILC1), ILC2, and ILC3. However, cell surface receptors unambiguously defining or specifically activating such ILC subsets are scarcely known. Here, we report on the physiologic expression of the human activating C-type lectin-like receptor (CTLR) NKp65, a high affinity receptor for the keratinocyte-associated CTLR KACL. Tracking rare NKp65 transcripts in human blood, we identify ILC3 to selectively express NKp65. NKp65 expression not only demarcates “bona fide” ILC3 from likewise RORγt-expressing ILC precursors and LTi-cells, but also from mature NK cells which acquire the NKp65-relative NKp80 during a Notch-dependent differentiation from NKp65+ precursor cells. Hence, ILC3 and NK cells mutually exclusively and interdependently express the genetically coupled sibling receptors NKp65 and NKp80. Much alike NKp80, NKp65 promotes cytotoxicity by innate lymphocytes which may become relevant during pathophysiological reprogramming of ILC3. Altogether, we report the selective expression of the activating immunoreceptor NKp65 by ILC3 demarcating ILC3 from mature NK cells and endowing ILC3 with a dedicated immunosensor for the epidermal immune barrier.
Introduction

Cytokine-producing innate lymphoid cells (ILC) mostly reside in epithelial barrier tissues, are involved in tissue homeostasis and importantly contribute to early immune responses to invading pathogens (Artis and Spits, 2015; Colonna, 2018). To cope with a broad range of pathogens, subsets of ILC have evolved with a distinct cytokine signature maintained by the transcription factors T-bet (ILC1), GATA3 (ILC2), and RORγt (ILC3), respectively (Artis and Spits, 2015; Colonna, 2018). ILC1 typically secrete IFN-γ upon stimulation with IL-12 and IL-18, ILC2 produce IL-4, IL-5, and IL-13 when exposed to IL-25 and IL-33, and ILC3 produce IL-17 and/or IL-22 when activated with IL-1β and IL-23 (Artis and Spits, 2015; Colonna, 2018). In mice, the development of ILC depends on the transcriptional regulator Id2 and the common γ-chain of cytokine receptors, reflecting the essential requirement for IL-7 (ILC2, ILC3) or IL-15 signaling (ILC1, NK cells), respectively (Artis and Spits, 2015; Colonna, 2018; Diefenbach et al., 2014). In addition to ‘bona fide’ ILC3, lymphoid tissue-inducer (Lti) cells and more plastic ILC precursor-like innate lymphocytes lacking expression of natural cytotoxicity receptors (NCR) also express RORγt, with their classification being an ongoing matter of debate (Mjosberg and Spits, 2016; Montaldo et al., 2015). NCR originally were identified as activating receptors broadly expressed by human NK cells and comprise NKp30, NKp44, and NKp46 (Koch et al., 2013; Moretta et al., 2001). While Lti cells are essential for the development of secondary lymphoid organs, NCR+ILC3 play a prominent role in the protection and homeostasis of epithelia, as they are a major source of IL-22 (Cella et al., 2009; Gronke et al., 2019; Mjosberg and Spits, 2016; Montaldo et al., 2015). In fact, tonsillar NKp44+ILC3 were originally reported as NK-22 cells due to their capacity to secrete IL-22 (Cella et al., 2009). Though NCR expression by ILCs was noted early on and NCR triggering stimulates some cytokine secretion (Cella
et al., 2009; Glatzer et al., 2013; Salimi et al., 2016), the physiologic relevance of NCR for the immunobiology of ILC remains unknown. This is in contrast to NK cells, where NCR and Natural Killer Gene complex (NKC)-encoded C-type lectin-like receptors (CTLR) NKG2D and NKp80 are known to trigger NK cytotoxicity thereby promoting immunity against tumors and viral infections (Koch et al., 2013; Ullrich et al., 2013; Welte et al., 2006). To further elucidate the biology of human innate lymphocytes (i.e. NK cells and ILC) through characterization of orphan receptors, we previously identified the activating receptor NKp65 (Spreu et al., 2010). NKp65 (encoded by \textit{KLRF2}) and its close relative NKp80 (\textit{KLRF1}) are peculiar human immunoreceptors, because (i) they relay activating signals through a cytoplasmic hemITAM (Bauer and Steinle, 2017; Bauer et al., 2017; Dennehy et al., 2011), (ii) they are NKC-encoded in tight genetic linkage with their ligands (Bartel et al., 2013), (iii) they and their respective ligands are structurally related CTLR interacting in a highly symmetrical manner (Bartel et al., 2013; Li et al., 2013; Spreu et al., 2010). However, while the NKp80 ligand 'activation-induced C-type lectin' (AICL) is surfacing on activated leukocytes such as myeloid cells or NK cells (Klimosch et al., 2013; Welte et al., 2006), expression of the high affinity NKp65 ligand ‘keratinocyte-associated C-type lectin’ (KACL) is mostly restricted to keratinocytes (Bauer et al., 2015; Spreu et al., 2007; Spreu et al., 2010). NKp80 is broadly expressed on NK cells and some effector T cells (Kuttruff et al., 2009; Welte et al., 2006), while the physiologic expression of NKp65 remained elusive. NKp65 was only found on NK-92 cells, a non-Hodgkin’s lymphoma-derived cell line (Gong et al., 1994), and was shown to activate NK-92 effector responses upon KACL engagement (Spreu et al., 2010). Since NKp65 was undetectable on other human NK cell lines, peripheral blood NK cells or other leukocytes, and no \textit{KLRF2} sequences were then deposited in public databases, doubts on a physiologic relevance
of NKp65 emerged. The lack of homologues of NKp65 in rodents further precluded investigations in mice.

Here we report on our quest for human cells physiologically expressing NKp65 thereby identifying ILC3 to selectively express NKp65 as opposed to selective NKp80 expression by mature NK cells, and revealing an as of yet unknown molecular link between human ILC3 and the skin immune barrier.
Results

Tracing and identification of NKp65-expressing lymphocytes. At the outset of this study (in 2009), public databases did not contain entries for KLRF2 transcripts indicating that NKp65 may be, if at all, sparsely expressed or expressed by a rare human cell type. In our original study on NKp65, reporting singular expression by the human cell line NK-92, we also detected very low levels of KLRF2 transcripts in NK cell-enriched lymphocytes (Spreu et al., 2010). Hence, we hypothesized that NKp65 may be expressed by rare hematopoietic cells sharing surface markers with NK cells. Therefore, we commenced a systematic search for leukocytes harbouring KLRF2 transcripts. Quantitative PCR-based screens detected very low levels of KLRF2 transcripts in peripheral blood mononuclear cells (PBMC) of most healthy donors. KLRF2 levels varied ~100-fold between donors, and were ~200- to ~20,000-fold lower than levels of NK-92 (Fig. S1). Magnetic bead-based fractionation of PBMC and subsequent qPCR-based analyses of purified lymphocyte fractions detected KLRF2 transcripts almost exclusively in a fraction of cells isolated by an NK cell enrichment procedure (Fig. 1A). Since soluble KACL (sKACL) tetramers were the only available reagents to detect NKp65, we generated an NKp65-specific mAb, designated OMAR1, by immunizing mice with both recombinant sNKp65 and P815-NKp65 transfectants. Specificity of OMAR1 for NKp65 was demonstrated by selective binding of OMAR1 to immobilized sNKp65, to NKp65-transfected 293T cells, and to NK-92MI cells, respectively (Fig. 1B, Fig. S2). Pre-incubation of NK-92MI cells with OMAR1 precluded binding of sKACL tetramers, thus demonstrating that OMAR1 efficiently blocks NKp65-KACL interactions (Fig. 1C). Extensive analyses of freshly isolated blood leukocytes using OMAR1 did not uncover NKp65-expressing cells (data not shown). Hence, we considered that NKp65 expression may require cytokine-mediated induction and incubated NK cell-enriched PBMC with various mixtures of cytokines. In fact, cytokine-activated cells
cultured in presence of IL-2, IL-12, and IL-18, and marked by induced expression of NKp44, comprised a small fraction of cells which were bound by OMAR1 (Fig. 1D and 1E). OMAR1 binding was abrogated upon pre-incubation of OMAR1 with sNKp65 indicating specificity (Fig. 1D). NKp65 specificity of OMAR1 binding to these cells was ultimately demonstrated by qPCR of FACSorted OMAR1+ cells which contained abundant levels of KLRF2 transcripts comparable to levels of NK-92 cells, while FACSorted-OMAR1- cells lacked KLRF2 transcripts (Fig. 1F). Subsequently, FACSorted NKp65+NKp44+ cells and NKp65-NKp44+ cells of several donors were subjected to comparative transcriptional profiling: while transcripts of receptors typically defining mature NK cells, such as KIRs, were markedly underrepresented in NKp65+ cells as compared to NKp65- cells, NKp65+ cells showed an enhanced expression of c-kit (CD117), IL-1 receptor 1 (IL1R1), and the transcription factor AHR (Fig. 1G), an expression pattern which is indicative of ILC3 (Mjosberg and Spits, 2016). In addition, a qPCR-based screen of human tissues for KLRF2 transcripts revealed that most tissues contained very low levels of KLRF2 transcripts well in line with the known sparsity of ILC3, while slightly increased levels of KLRF2 transcripts were detected for tonsils, placenta, and small intestine very well matching the enrichment of ILC3 in these tissues (Doisne et al., 2015; Simoni et al., 2017) (Fig. 1H, Fig. S3).

**NKp65 is selectively expressed by ‘bona fide’ ILC3.** In order to validate that NKp65 is expressed by ILC3, we analyzed tonsillar lymphocytes: levels of KLRF2 transcripts are highest in tonsils (Fig. 1H, Fig. S3) and tonsils are known as a rich source of human ILC3 (Cella et al., 2009; Glatzer et al., 2013). Flow cytometric analyses of tonsillar lymphocytes gated according to the phenotypic definition of ILC3 (lineage-negative (lin-) CD117+CD127+NKp44+ cells) (Mjosberg and Spits, 2016; Montaldo et al., 2015) and
assessed for OMAR1 binding, revealed that ILC3 of most, but not all, donors markedly expressed NKp65 at the cell surface (Fig. 2A and B). NKp65 expression by tonsillar ILC3 was further corroborated by the specific binding of sKACL tetramers to ILC3 which was blocked by pre-incubation of ILC3 with OMAR1 (Fig. 2C). Such NKp65⁺ ILC3 were found to homogeneously express NKp44, IL1R1, and CD161, while expression of CD56 and CCR6 varied (Fig. 2D), well in accord with the reported phenotype of ILC3 (Mjosberg and Spits, 2016; Montaldo et al., 2015). Of note, NKp65⁺ ILC3 were always found to be devoid of NKp80 (Fig. 2D), the only close relative of NKp65 which marks mature NK cells (Freud et al., 2016; Welte et al., 2006). In accord with a selective expression of NKp65 by ILC3, NKp65 was detected on IL-22 secreting tonsillar innate lymphocytes, but not on IFN-γ-secreting cells (Fig. 2E and F). In accord with their ILC3-ness, tonsillar NKp65⁺ cells expressed RORγt, but not EOMES (Fig. 2G). In tonsil sections, NKp65⁺ cells showed a scattered distribution as previously reported for ILC3 (Cella et al., 2009), with many, but not all NKp44⁺ cells co-expressing NKp65, further demonstrating that NKp65⁺ cells are ILC3 (Fig. 2H and I). By contrast, NKp65 was undetectable on other subsets of tonsillar or peripheral blood innate lymphocytes including ILC1, ILC2, and mature NK cells (Fig. S4). While committed ILC3 markedly express NKp65, other RORγt-expressing innate lymphocytes such as LTi cells (NCR⁺CCR6⁺) or ILC3-like precursor cells (NCR⁻CCR6⁺⁻) poorly express NKp65, if at all (Fig. 2J). Collectively, these data demonstrate that NKp65 expression marks 'bona fide’ ILC3 in humans.

**NKp65 expression by ILC3-like NK cell progenitors.** A recent series of studies proposed a multi-stage developmental pathway of human NK cells based on flow cytometric analyses of tonsillar lymphocytes (Freud et al., 2016; Scoville et al., 2017; Scoville et al., 2019). According to this model, NK cells develop from early tonsillar
progenitors 1 (ETP1) (CD34+, CD117-, IL1R1-) and ETP2 (CD34+, CD117+, IL1R1+) via a common innate lymphocyte progenitor (CILP) (CD34+, CD117+, IL1R1-) into RORγt+ expressing ILC3-like progenitor cells (stage 3 cells: CD56+, CD94+, NKp80+, RORγt+) which acquire CD94 (stage 4a cells: CD56+, CD94+, NKp80+, RORγt+), and ultimately differentiate into 'bona fide' mature NK cells (CD56+, CD94+, NKp80+, RORγt+, EOMES+, CD16+ (stage 4b) or CD16- (stage 5)) (Freud et al., 2016; Scoville et al., 2017; Scoville et al., 2019) marked by de novo expression of the NKp65 sibling receptor NKp80 (Freud et al., 2016). Using tonsillar lymphocytes, we assessed NKp65 expression by these phenotypically defined developmental stages of innate lymphocytes and found NKp65 markedly and selectively expressed by stage 3 and stage 4a cells (well in line with their ILC3-ness) (Fig. 3B), whereas early progenitors (ETP/CILP) (Fig. 3A) as well as mature NKp80+ NK cells (stages 4b and 5) (Fig. 3B) were devoid of NKp65. Hence, expression of NKp65 versus NKp80 demarcates RORγt+ ILC3-like progenitor cells from mature RORγt+EOMES+ tonsillar NK cells in a mutually exclusive manner.

NKp65-expressing ILC3-like lymphocytes differentiate into NKp80-expressing NK cells in a Notch-dependent manner. To further elucidate the expression and function of NKp65 during the development of innate lymphocytes, we attempted to generate NKp65-expressing cells by in vitro differentiation from human CD34+ hematopoietic stem cells. To this aim, CD34-enriched cells from healthy donors were co-cultured in vitro with OP9 feeder cells and cytokines as previously reported (Ahn et al., 2013). NKp65+ cells first emerged at ~day 7 of culture (not shown), and after two to four weeks, almost all cells represented CD56+NKp80- innate lymphocytes, here termed in-vitro generated ILC-like cells (ivILC). These ivILC comprise two major subpopulations, CD117hiCD94- (stage 3)
and CD117tagCD94+ (stage 4a) cells that both markedly express NKp65 (Fig. 4A). In contrast to reports of tonsillar stage S4a lymphocytes expressing RORγt (Freud et al., 2016), stage S4a ivILC were RORγt+EOMES+, while stage S3 ivILC were RORγt+EOMES- (Fig. 4B) indicating that NKp65 is not strictly co-expressed with RORγt, at least in vitro. However, mutually exclusive expression of NKp80 and NKp65 was also observed for ivILC, as there were minor populations of mature NKp80+ and/or CD16+ NK cells which lacked surface NKp65 (Fig. 4C). Next, we addressed whether NKp65+ stage S4a cells have the capacity to mature into NKp80-expressing NK cells. To this aim, FACSorted-NKp65+ cells were cultivated on feeder cells in the presence or absence of Notch ligands. Of note, in co-cultures with OP9-DLL1 or −DLL4 feeder cells, but not in the absence of Notch ligands, NKp80 expression was readily induced demonstrating that NKp80 acquisition strictly depends on the Notch signaling pathway (Fig. 4D). Induction of NKp80 expression was especially enhanced in co-cultures with OP9-DLL1 cells and accompanied by a down-modulation of NKp65 (Fig. 4D and E).

**NKp65+ innate lymphocytes kill KACL+-cells in an NKp65-KACL-dependent manner.**

Next, we addressed functional consequences of NKp65 engagement: cytokine production of tonsillar CD127-enriched lymphocytes or of ivILC was assessed upon various modes of NKp65 engagement through crosslinking with OMAR1 or co-culture with KACL-transfectants. However, NKp65 engagement did not impact on the secretion of cytokines such as TNF, GM-CSF, or IL-22 was observed (Fig. S6, S7). Previously we had reported that NK-92MI cells kill KACL-expressing cells in an NKp65-dependent manner (Spreu et al., 2010). Hence, we assayed ivILC for cellular cytotoxicity using NKR cells, a human T cell line resistant to NK cell lysis (Howell et al., 1985). While control-transduced NKR cells were barely killed, transduction of KACL rendered NKR cells highly susceptible to ivILC-
mediated cytotoxicity demonstrating that NKp65-KACL interaction promotes cellular cytotoxicity (Fig. 5A). NKp65-KACL dependence of NKR-KACL killing by ivILC was further corroborated by the blockade of cytotoxicity in presence of anti-KACL mAb OMA1 which blocks NKp65-KACL interaction (Bauer et al., 2015). U937 cells endogenously express substantial amounts of KACL in addition to other ligands of activating NK receptors (Welte et al., 2006) and, hence, were also assessed for susceptibility to NKp65-mediated killing. Killing assays in the presence and absence of OMA1 demonstrated that killing of U937 by ivILC occurs in a partially KACL-dependent manner. Of note, the extent of KACL-dependent U937 cytolysis correlated with the varying levels of NKp65 expression by ivILC established from different donors (Fig. 5B and C). Altogether, these data show that NKp65 can promote the cytotoxic activity of human innate lymphocytes towards KACL-expressing cells.
Discussion

In the past decade, tissue-resident ILC have entered center stage of immunological research and were shown to act as a first line of innate immune cells in barrier tissues, impacting both on local tissue integrity and on ensuing adaptive immune responses. However, thus far, there is a lack of specific markers to distinguish ILC subsets, in particular also in humans, which impedes research on ILC. Human ILC share expression of the IL-7 receptor γ-chain (CD127) and CD161/NKR-P1A (Diefenbach et al., 2014; Mjosberg and Spits, 2016), the inhibitory counterpart of NKp80 and NKp65 receptors. However, CD127 is also expressed on immature B cells, T cells, and NK cells (Clark et al., 2014; Kang and Coles, 2012), and CD161 is broadly expressed by most NK cells and T cell subsets such as Th17 cells (Fergusson et al., 2014; Lanier et al., 1994). ILC2 are currently defined by expression of CD294 (CRTH2), but CD294 is also expressed by Th2 cells, eosinophils and basophils (Mjosberg et al., 2011). Similarly, known surface markers of ILC3 are shared with NK cells (e.g. NKp44) and RORγt expression is shared with Th17 cells und LTi cells (Artis and Spits, 2015). Here we report selective expression of NKp65 by human ILC3 qualifying NKp65 as a specific marker for this rare cell type which is expected to facilitate not only phenotyping and detection of human ILC3, but also may allow for their selective therapeutic enrichment or depletion. Our findings also advise quantification of KLRF2 transcripts as an easy and direct method to determine ILC3 abundance in specimen of healthy and diseased human tissues which will accelerate studies addressing the contribution of ILC3 to human autoimmune or malignant diseases. Flow cytometric analysis of NKp65 may not only aid in discrimination of ILC3 from mature NK cells, but also from other functionally and developmentally distinct RORγt+ innate lymphocytes such as LTi cells and so-called NCR-ILC3. Consideration of NKp65, its
expression and regulation, will also promote the understanding of the developmental pathways of ILC3-like lymphocytes and NK cells as NKp65 can be used to discern ILC3-like lymphocytes from mature NK cells which are marked by the expression of the NKp65-relative NKp80 (Freud et al., 2016). It will be of immediate interest to determine the underlying mechanistic processes and functional implications of the mutual exclusive expression of the highly related and genetically coupled NKp65 and NKp80 receptors on ILC3-like cells versus NK cells. Thus far, the physiological relevance of NK receptors such as NKp44 expressed on ILC3 is mostly unclear, addressing the unresolved question whether there is immunorecognition and direct activation of ILC3 through receptor-mediated cell-cell interactions. We here report that NKp65+ innate lymphocytes generated in vitro are able to kill KACL-expressing target cells in an NKp65-KACL dependent manner demonstrating that NKp65 can act as an activating receptor of innate lymphocytes. While ILC3 are known as non-cytotoxic cytokine producers, there are recent reports that ILC3 can convert into ILC1-like cytotoxic cells in the presence of inflammatory cytokines (Cella et al., 2019; Freud et al., 2016; Raykova et al., 2017) which is thought to occur in vivo at sites of inflammation. NKp65 binds with exceptionally high affinity to its cognate and genetically linked ligand KACL which in turn is selectively expressed by human keratinocytes (Bauer et al., 2015; Spreu et al., 2010). ILC3 are known to be enriched in diseased skin of psoriasis patients and may initiate or perpetuate disease (Teunissen et al., 2014; Villanova et al., 2014). In mouse models of psoriasis ILC3 were shown to induce disease pathogenesis (Keren et al., 2018; Pantelyushin et al., 2012). Hence, it will be of immediate interest to study potential disease-modifying roles of the high-affinity receptor/ligand pair NKp65-KACL in psoriasis and other inflammatory skin diseases which may require appropriate (transgenic) animal models.
In summary, we here provide the first report on the physiologic expression of the NKC-encoded CTLR NKp65 and demonstrate that NKp65 is selectively expressed by human ‘bona fide’ ILC3. Unlike to previously reported ILC markers, NKp65 is selectively expressed by human ILC3 and therefore appears to represent the most specific marker of this cell type reported to date. NKp65 and its sibling hemITAM-receptor NKp80 are mutually exclusively expressed by developmentally coupled innate lymphocytes shedding a new light on human NK cell development. Further studies will have to define the functional relevance of NKp65 in the intertwined developmental pathways of ILC3 versus NK cells as well as for the immunosurveillance of the KACL-expressing epithelial barrier tissue.
**Materials and Methods**

**Cells.** Peripheral blood mononuclear cells (PBMC) of healthy human donors were isolated from buffy coats obtained from the German Red Cross Blood Service by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare). NK cells were isolated from PBMC using MACS technology and the human NK cell isolation kit (Miltenyi) according to the manufacturer’s protocol. The purity of isolated CD3^−^CD56^+^ cells always was > 95% as analyzed by flow cytometry. Purified NK cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM, Sigma-Aldrich) supplemented with 10% FCS (Biochrom). 100 U/ml IL-2 (Roche), 5 ng/ml IL-12, and/or 5 ng/ml IL-18 (Miltenyi or R&D Systems) were added as indicated. Magnetic bead based fractionation of PBMC was done by either positive selection via PE conjugated mAbs (CD4, CD8 and CD19) and anti-PE microbeads (Miltenyi) or negative selection using TCRγ/δ^+^ T cell or NK cell isolation kit (both Miltenyi) according to the manufacturer’s protocol. Surgical samples of human tonsils were obtained from the Department of Otorhinolaryngology, University Hospital Frankfurt am Main, with approval of the local Ethics Committee. Tonsils were cut in small pieces, enzymatically digested with 0.5 mg/ml Collagenase D (Roche) and 3000 U/ml DNAse I (Roche) at 37°C, and mechanically disrupted using a gentleMACS Dissociator (Miltenyi). Cell suspension was passed over a 100 µm cell strainer and mononuclear cells were isolated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare). Cells stained with anti-NKp44-PE (Beckmann Coulter) were enriched using anti-PE microbeads and MACS technology (Miltenyi) and subsequently cultured in RPMI medium supplemented with 10% FCS, 1% non-essential amino acids (Sigma-Aldrich), and 50 ng/ml IL-7, IL-23, and/or IL-1β (Miltenyi) as indicated. For intracellular cytokine detection, tonsillar ILCs were enriched with anti-APC microbeads (Miltenyi) after incubation with anti-
CD127-APC. NK-92 cells were cultured in IMDM with 10% FCS, 10% horse serum (Sigma-Aldrich) and 100 U/ml IL-2, U937 in RPMI-1640 medium (Sigma-Aldrich) with 10% FCS, and 293T cells in DMEM medium (Sigma-Aldrich) supplemented with 10% FCS. 293T cells co-expressing NKp65 and eGFP were obtained by transient transfection with the plasmid pIRES2-eGFP containing the full length NKp65 cDNA (Spreu et al., 2010) in front of the eGFP reading frame. 293 cells ectopically expressing KACL and the NKp65 non-binding mutant R158E/I161K were generated as previously described (Bauer et al., 2015). NKR transductants were previously described (Neuss et al., 2018) and cultivated in RPMI medium supplemented with 10% FCS and 0.6 µg/ml puromycin (AppliChem). OP9 cells were cultured in MEMα GlutaMAX (Thermo Fisher) medium supplemented with 20% FCS.

**In vitro-differentiation of innate lymphocytes.** For *in vitro*-differentiation of innate lymphocytes (ivILC), human CD34⁺ hematopoietic stem cells (HSCs) were enriched from G-CSF-mobilized healthy donor apheresis samples using MACS CD34 microBeads (Miltenyi) after Ficoll density gradient centrifugation. Purified CD34⁺ HSCs (>90% purity) were suspended in HSC medium (DMEM and Ham’s F-12 medium (2:1 mixture) (Thermo Fisher) supplemented with 10% heat-inactivated human AB sera (Sigma-Aldrich), 1% penicillin/streptomycin, 1% L-Glutamine, 25 μM of β-mercaptoethanol, 20 μg/ml of ascorbic acid, and 0.05 μg/ml of sodium selenite (Sigma-Aldrich)) and seeded on a monolayer of nearly confluent OP9-mock cells. Human recombinant IL-7 (20 ng/ml), IL-15 (10 ng/ml), SCF (20 ng/ml), and Flt3-L (10 ng/ml) (all from Miltenyi) were added as indicated and cells cultivated for up to nine weeks with a weekly transfer to a fresh monolayer of feeder cells in fresh cytokine-containing medium. For cell sorting ivILCs were generated on OP9-mock feeder cells and harvested after 28 days in culture. Cells were
sorted on a FACS Aria cell sorter (BD Biosciences). NKp65\textsuperscript{+} cells were subsequently cultured on OP9-mock, OP9-DLL1, or OP9-DLL4 cells for five days.

**Cytotoxicity assays.** *In vitro*-generated ILC (ivILC) (day 30 to 50 of culture) were co-cultured with U937 or NKR-KACL cells in presence of 10 µg/ml mAb OMA1 or an isotype control at ratios ranging from 0.5:1 to 2:1. Prior to co-culture, target cells were labeled with FarRed (Thermo Fisher Scientific) and ivILC with CFSE (Thermo Fisher Scientific), respectively, according to the manufacturer’s protocol. After 15 h co-culture at 37°C, FarRed\textsuperscript{+} cells were counted using a FACS Aria (BD Biosciences). To determine % cytotoxicity, counts of all remaining FarRed\textsuperscript{+} target cells in co-cultures were divided by counts of all FarRed\textsuperscript{+} target cells in parallel cultures without ivILC.

**Recombinant proteins and antibodies.** Soluble recombinant ectodomains of KACL (sKACL: Ile 46 through Leu 185) and NKp65 (sNKp65: Ser 63 through Val 207) were purified from supernatants of 293 stably transfected with the respective cDNA containing BirA-, c-myc- and six-histidine-tags as previously reported (Spreu et al., 2010). Ectodomains were biotinylated using BirA ligase (Avidity) and, immediately before use, either immobilized on streptavidin-coated microspheres (Bangs Laboratories) or tetramerized using APC-conjugated streptavidin (Jackson ImmunoResearch). NKp65-specific mAb OMAR1 was generated by standard hybridoma technology. In brief, BALB/c mice were immunized with both P815-NKp65 and sNKp65, and resulting hybridoma screened for specific reactivity against 293 cells expressing a NKp65-pIRES2-eGFP construct. Immunization of mice was approved by the local regulatory authorities. Purified OMAR1 was biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Scientific). NKp80-
specific mAb 5D12 (Welte et al., 2006) and KACL-specific mAbs OMA1 (Spreu et al., 2010) and OMA6 (Bauer et al., 2015) were described previously.

**Intracellular cytokine detection assays.** CD127⁺-enriched cells from human tonsils were co-cultured with anti-biotin MACSiBead particles (Miltenyi) at a bead to cell ratio of 5:1 or with P815 cells at a 2:1 ratio, pre-loaded with biotinylated (MACSiBead) or unconjugated antibodies (P815) (anti-NKp65 mAb OMAR1, anti-NKp44 mAb or IgG1 isotype control) in the presence or absence of cytokines IL-1β and IL-23 (both 5 pg/ml) as indicated. For positive control, cells were stimulated with PMA (25 ng/mL, Sigma-Aldrich) and ionomycin (1 µM, Sigma-Aldrich). After 2 h of co-culture, GolgiStop (BD Bioscience) was added and incubation continued for another 18 h. After 20 h cells were permeabilized, stained with various antibodies and assessed by flow cytometry. For analyses, gates were set on lin⁻(CD3, CD14, CD19, CD34) CD127⁺ cells. *In vitro*-generated ILC (day 43 in culture) were stimulated with plate bound mAbs or co-cultured with 293-KACL cells expressing either KACL protein or KACL mutant R158E/I161K (Bauer et al., 2015). Cytokines IL-1β and IL-23 (both 10 ng/ml) or PMA/ionomycin was added as indicated. Cells were cultured for 1 h prior to addition of GolgiStop. After 6 h incubation, cells gated as lin⁻CD45⁺CD56⁺CD11a⁻ were analyzed for intracellular cytokines by flow cytometry.

**Immunohistochemistry and immunofluorescence.** Frozen sections of human tonsils were incubated overnight at 4°C with mAb OMAR1, anti-NKp44 (BioLegend) or isotype control after blocking with Bloxall blocking solution (Vector Laboratories). Subsequently, sections were stained with the Super Sensitive Multilink-Label-AP kit (Biogenix) and developed with permanent red (Dako). Slides were counterstained with Hämalaun, evaluated by microscopy and scanned using an Aperio ScanScope slide scanner (Leica).
For immunofluorescence, tissue samples were embedded and frozen in Tissue-Tek. Sections were fixed in cold acetone for 10 min and subsequently dried at room temperature for 10 min. After rehydration in TBS specimen were blocked in 10% goat serum (Sigma-Aldrich) in 3% BSA/TBS and subsequently probed with anti-NKp65 mAb OMAR1 and rabbit polyclonal anti-NKp44 (Biorbyt) for 1 h at room temperature. Secondary antibody staining was performed using an Alexa Fluor Plus 647-conjugated goat anti-mouse IgG Ab (Thermo Scientific) and an Alexa Fluor 488-conjugated goat anti-rabbit IgG Ab (Thermo Scientific). Sections were embedded and counterstained with ProLong Gold antifade mountant with DAPI (Thermo Scientific). Stained sections were visualized using a DMI6000B microscope connected to a DFC3000G camera (Leica Microsystems). 2x2 bin mode was used.

**Flow cytometry.** Cells were incubated with the indicated mAb (10 µg/ml), or fluorochrome-conjugated tetramers (10 µg/ml) for 30 min at 4°C. After washing, cell bound antibodies were stained with either fluorochrome-conjugated goat anti-mouse IgG antibodies or APC-conjugated streptavidin (Jackson ImmunoResearch). In some experiments, cells were subsequently stained with fluorochrome-conjugated antibodies specific for human antigens. Fluorescence stainings were analyzed on a FACS Canto II and data analyzed using FlowJo (Tree Star). Dead cells were excluded from the analysis by staining with DAPI (AppliChem) or fixable viability dye eFluor™ 450 (eBioscience). For sorting of NKp65^+ versus NKp65^- lymphocytes, cytokine-treated cells were stained with anti-NKp65 mAb OMAR1 and anti-NKp44, and living NKp44^+ cells sorted using a FACS Aria (BD Biosciences). To assess expression of intracellular cytokines, cells were fixed with Cytofix/Cytoperm (BD) according to the manufacturer’s instructions and subsequently stained with cytokine-specific antibodies using saponin buffer. Transcription
factor stainings with fluorochrome-conjugated antibodies against RORγt, EOMES, and GATA3 were done with the FoxP3 staining kit (eBioscience) according to the manufacturer`s protocol. Antibodies are detailed in Table S1.

**Microarray analysis.** Freshly isolated NK cells from four healthy human donors (age range: 25 to 30 years; one male, three females) were stimulated with indicated concentrations of IL-2, IL-12 and IL-18 for four days. NKp65+ and NKp65- subpopulations of NKp44+ cells, respectively, were sorted on a FACS Aria cell sorter (BD Biosciences). Cells were processed for RNA isolation using a RNeasy Micro Kit (Qiagen) and cDNA generated using standardized protocols (Ovation Pico WTA System V2 and Encore Biotin Module, NuGEN). Microarray hybridization to GeneChip Human Gene 1.0 ST arrays (Affymetrix) was performed according to the manufacturer's protocol. Bioinformatic analysis was done with the statistical computing environment R version 2.12 (URL http://www.R-project.org/). Probe level normalization was conducted using the variance stabilization method (Huber et al., 2002) and probeset summarization was calculated using the median polish method on the normalized data (Tukey, 1970). For each probeset a robust additive model was fitted across the arrays, considering the different sensitivity of the probesets via the probe effect. To reduce the dimension of the microarray, data was filtered with an intensity filter (the intensity of a gene should be above 100 in at least 0.25 percent of the samples, if the group size is equal) and a variance filter (the interquartile range of log2 intensities should be at least 0.5, if the group size is equal). After expression intensity filtering fold changes (FC) and p-values were calculated with the two sample t-test (variance=equal) to identify genes that are differentially expressed between two groups. For the multiple testing problems a False Discovery Rate (FDR) was used
(Hochberg and Benjamini, 1990). The data are accessible through GEO Series accession number GSE133772.

**Quantitative RT-PCR.** RNA was isolated from cells using peqGold Trifast Reagent (Peqlab) followed by DNAse I digestion and reverse transcription using M-MLV reverse transcriptase (Promega). For tissue screens of KLRF2 transcripts, RNA was purchased from the FirstChoice human total RNA survey panel (Ambion). For sorted NKp65+ cells, a cells-to-cDNA II kit was used (Thermo Scientific). Transcript levels were analyzed with primer pairs specific for NKp65 (5’-tgacctgaagttctggcataaa-3’/5’-tgtcctgatagactgctgacg-3’) in combination with probe # 14 (Universal Probe Library, Roche). Input was normalized to human TATA box-binding protein (hTBP) or 18S rRNA TaqMan assay reagents (VIC labelled; Applied Biosystems) as indicated. Amplifications were performed on a StepOne Plus machine (Applied Biosystems) using a FastStart Universal Probe Master mix (Roche). Relative gene expression was calculated with the \( \Delta \Delta C_T \) method.

**Statistical analyses.** Data are routinely shown as mean ± SD. Unless stated otherwise, statistical significance was determined by one-way Analysis of Variance (ANOVA) with Tukey`s post test using GraphPad Prism 7.0. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

**Data and materials availability**

The KACL- and NKp65-specific monoclonal antibodies will be commercially available within the next years, thereby ensuring highest quality standards and fair access to the research community. Microarray data sets are available at:

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References


Figure 1. Tracing NKp65-expressing lymphocytes. (A) Abundance of KLRF2 transcripts in PBMC and MACS-fractionated subpopulations thereof from a healthy human donor. (B and C) NKp65-specificity of mAb OMAR1. Flow cytometric analyses of (B) 293T cells transfected with a bicistronic KLRF2 cDNA-eGFP construct and stained with OMAR1 or an isotype control (IgG1) plus fluorochrome-conjugated goat anti-mouse Ig antibodies (GaM), and of (C) NK-92MI cells pre-incubated with OMAR1 (black) or an isotype control (gray) and subsequently stained with both APC-labelled sKACL tetramers and PE-conjugated GaM. (D and E) NKp65 is expressed on a small population of cytokine-
activated blood lymphocytes. PBMC enriched for NK cells by MACS as in (A) and cultured for four days with IL-2, IL-12, and IL-18, were subsequently analyzed by flow cytometry for surface expression of NKp65 using OMAR1 on cytokine-activated (i.e. NKp44⁺) cells (D, middle panel). For control, cells were stained with an isotype control (D, left) or with OMAR1 pre-incubated with sNKp65 (D, right). Gates were set for DAPI-CD3⁻ cells. (E) Frequencies of NKp65⁺ cells among activated NKp44⁺ lymphocytes generated as in (D) from PBMC of n = 6 donors represented by different symbols. (F) Levels of KLRF2 transcripts among FACS-purified NKp65⁺NKp44⁺ and NKp65⁻NKp44⁺ lymphocytes from (D) normalized to levels of MACS-purified NK cells of the same donor. Representative of two independent experiments. (G) Differential gene expression by FACS-purified NKp65⁺NKp44⁺ versus NKp65⁻NKp44⁺ blood lymphocytes activated as in (D). Differential expression of indicated genes was averaged from microarray data obtained from analyses of two individual preparations of cells from a total of n = 4 healthy donors. (H) Abundance of KLRF2 transcripts in various human tissues. (A, F, H) Copy numbers of KLRF2 transcripts were determined by qPCR, normalized, and set relative to samples marked with an asterisk. nd, not detectable. For comparison, KLRF2 transcript levels of NK-92 cells are included. Data are shown as means of triplicates ± SD.
Figure 2. Selective NKp65 expression by human ILC3. (A) Lin^−CD117^+CD127^+NKp44^+ lymphocytes from human tonsils of $n = 2$ donors were analyzed for NKp65 surface expression using OMAR1-bio plus SA-APC (black line). (B) Levels of NKp65 expression (SFI with mean ± SD) on Lin^−NKp44^+ cells from NKp44-enriched tonsillar cells of $n = 20$ donors analyzed in 18 independent experiments. (C) Binding of sKACL tetramers to Lin^−
CD161\(^{+}\)NKp44\(^{+}\) tonsillar lymphocytes (black line). Specificity of sKACL staining is demonstrated by blockade through pre-incubation of cells with OMAR1 (dashed line). (D) Lin\(^{-}\)CD117\(^{-}\)CD127\(^{-}\)NKp65\(^{+}\) tonsillar lymphocytes of donor 1 (A) were assessed for expression of CD56, CD161, IL1R1, CCR6, and NKp80. (C and D) Comparable results were obtained for a total of \(n = 3\) donors in two independent experiments. (E and F) Tonsillar cells were stimulated with (E) IL-1\(\beta\) plus IL-23, or (F) IL-12 plus IL-18 for four hours, and subsequently, NKp65 expression of resulting (E) lin\(^{-}\)IL-22\(^{+}\) cells or (F) lin\(^{-}\)IFN-\(\gamma\)\(^{+}\) cells assessed using OMAR1-bio plus SA-APC (black line). Representative of two independent experiments. (G) Permeabilized lin\(^{-}\)CD117\(^{-}\)CD127\(^{-}\)NKp65\(^{+}\) tonsillar lymphocytes were stained for ROR\(\gamma\)t and EOMES. Representative of two independent experiments. (H) Immunohistochemical stainings of tonsil sections with OMAR1 (middle), anti-NKp44 (right) or an isotype control (left), counterstained with Mayer’s hematoxylin. Representative of two independent experiments. (I) Immunofluorescence analysis of tonsil sections co-stained with OMAR1, anti-NKp44, and DAPI. Representative of three independent experiments. (J) Flow cytometric analyses of human tonsillar ILC3 for NKp65 expression. NCR\(^{+}\)ILC3 (NKp44\(^{+}\)), LTi (NKp44\(^{-}\)CCR6\(^{+}\)), and NCR\(^{-}\)ILC3 (NKp44\(^{-}\)CCR6\(^{-}\)) were stained with OMAR1-bio plus SA-PE (solid line). Representative of two independent experiments. (A, C-G, J) Shaded histograms represent control stainings.
Figure 3. ILC3-like NK cell progenitors express NKp65. (A) Human tonsillar cells gated for Lin^CD34^−CD45RA^+^ cells were analyzed for NKp65 expression on early progenitor stages ETP1 (CD117^−^IL1R1^−^), ETP2 (CD117^+^IL1R1^−^), and CILP (CD117^+^IL1R1^+) using OMAR1-bio plus SA-PE (black lines). (B) Lin^CD56^ human tonsillar lymphocytes were gated for stage S3, S4a, S4b, and S5 progenitor and mature NK cells based on the expression of NKp80, CD16, CD117, and CD94 as defined in (Freud et al., 2016), and assessed for NKp65 expression with OMAR1-bio plus SA-PE (black line). (A and B) Representative data for four donors analyzed in two independent experiments. Shaded histograms are control stainings.
Figure 4. NKp65-expressing lymphocytes differentiate into NKp80-expressing NK cells in a Notch-dependent manner. (A) NKp65 expression (OMAR1-bio plus SA-PE, solid line) on stage S3-like and S4a-like cells among in vitro-differentiated innate lymphocytes (ivILC) is shown. Representative for 8 individual samples analyzed in six
independent experiments (control stainings: filled histograms). (B) Cytometric analysis of ivILC generated from CD34+HSC of a representative donor. Permeabilized cells gated as lin−CD56−NKp65+ and either CD117hiCD94− or CD117intCD94+ cells were assessed for expression of transcription factors EOMES and RORγt, respectively. (C) Mutual exclusive surface expression of NKp65 and either NKp80 (middle) or CD16 (right) by ivILC (gate: lin−CD56+) (left). Representative result of six independent experiments. (D and E) Flow cytometric analyses of sorted NKp65+ precursor cells (Fig. S5) after five days in culture on OP9-GFP, -DLL1 or -DLL4 feeder cells, respectively. (D) NKp80 expression on lin−CD56−CD94+ and lin−CD56−CD16+ cells, respectively. (E) NKp65 expression was assessed on lin−CD56−CD94+ cells using OMAR1-bio plus SA-PE (solid line; SFI indicated). Isotype control stainings are filled.
Figure 5. NKp65 triggers cytotoxicity of human innate lymphocytes. (A) KACL-dependent killing of NKR-KACL transductants by ivILC. Cytotoxicity of ivILC towards NKR-KACL or NKR-mock cells was assessed in presence of OMA1 or an isotype control (IgG2a). (B and C) KACL-dependent lysis of U937 cells by ivILC. (B) Cytotoxicity in presence of OMA1 or an isotype control (IgG2a) at different E/T ratios. (C) Cytotoxicity of ivILC derived from three different donors against U937 (E/T = 1:1) in presence of OMA1 or an isotype control with respective NKp65 expression levels (SFI) of ivILC indicated below. (A-C) Depicted are means of triplicates ± SD. of one representative of three independent experiments with ivILCs generated from three unrelated donors. Statistical significance was calculated using two-way ANOVA with Tukey’s post hoc test. ns, not significant. \( * P < 0.05; ** P < 0.01; **** P < 0.0001. \)