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Group 2 innate lymphoid cells express functional NKp30 receptor inducing type 2 cytokine production1

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Abstract

Group 2 innate lymphoid cells (ILC2) are important in effector functions for eliciting allergic inflammation, parasite defence, epithelial repair and lipid homeostasis. ILC2 lack rearranged antigen-specific receptors, and while many soluble factors such as cytokines and lipid mediators can influence ILC2, direct interaction of these cells with microenvironment and other cells has been less explored. Natural cytotoxicity receptors are expressed by subsets of ILC1 and ILC3 and thought to be important for their effector function, but have not been shown to be expressed by ILC2. Therefore, we sought to investigate the expression and functional properties of the natural cytotoxicity receptor NKp30 on human group 2 innate lymphoid cells.

A subset of *ex vivo* and cultured ILC2 express NKp30 that upon interaction with its cognate activatory ligand B7-H6 induces rapid production of type 2 cytokines. This interaction can be blocked by NKp30 blocking antibody and an inhibitory ligand, galectin-3. Higher expression of B7-H6 was observed in lesional skin biopsies of patients with atopic dermatitis; and incubation of keratinocytes with pro-inflammatory and type 2 cytokines upregulated B7-H6 leading to increased ILC2 cytokine production. NKp30-B7-H6 interaction is a novel cell contact mechanism that mediates activation of ILC2 and identifies a potential target for the development of novel therapeutics for atopic dermatitis and other atopic diseases.

Keywords

Group 2 innate lymphoid cells (ILC2); Nkp30; B7-H6; Atopic dermatitis (AD)

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Introduction

Innate lymphoid cells are novel effectors of the immune system that contribute to diverse forms of inflammation and defence (1). They depend on the transcriptional repressor ID2 and cytokines that signal through the common gamma chain of IL-2. The lack of expression of common lineage markers and expression of IL-7Rα are shared features in this family. Based on their phenotype and function they have been divided into three main groups (2). Group 1 ILC include NK cells and ILC1 that produce IFN-γ and TNF-α (3). Group 2 ILC express GATA-3 (4) and ROR-α transcription factors (5), and we and others have shown human skin ILC2 produce type 2 cytokines IL-4, IL-5 and IL-13 in response to IL-33, IL-25, TSLP and PGD2 (6, 7). Group 3 ILC are ROR-γt dependent and comprise lymphoid tissue inducers, natural cytotoxicity receptor $(NCR)^{+}$ and NCR^{-} ILC3 that express IL-22 alone or in combination with IL-17 respectively (8–10). Innate lymphoid cell family members do not possess a rearranged antigen-specific receptor and are thought to rely on a combination of activating and inhibitory signals for their effector functions, but the mechanisms are unclear (11). Several studies have evaluated the expression of the NCRs, NKp44 (NCR2 and CD336), NKp46 (NCR1 and CD335) and NKp30 (NCR3 and CD337) on group 1 and 3 innate lymphoid cells in humans, but the expression of these markers has not yet been demonstrated on group 2 ILCs in humans (12). Here we sought to investigate the expression and function of NKp30 on human group 2 innate lymphoid cells.

NKp30 is an activating type I immunoglobulin-like transmembrane receptor that is not present in mice, but is found mainly on human NK cells (13). It has one Ig-like extracellular domain that connects to the transmembrane region by a short 6-amino acid stem. It is encoded in the extremely polymorphic telomeric end of the class III region of the human MHC locus (3, 14). Alternative splicing of exon 4 gives rise to three main distinct isoforms NKp30a, NKp30b and NKp30c. Upon interaction with its ligands, isoforms a and b are believed to convey cytotoxicity responses, release of IFN-γ and TNF-α by NK cells and trigger dendritic cell maturation (3, 15, 16) whereas splice variant NKp30c reduces IFN-γ production, cytotoxicity and increases the production of IL-10 by NK cells (3). A prevalent expression of isoform c on NK cells has been seen in patients with gastrointestinal stromal tumour (GIST) and associated with reduced survival due to defective IFN-γ, TNF-α and IL-12 production, defective NK-DC dialog and increased immunosuppression and IL-10 production (3). Several physiological, tumour and viral markers have been identified as NKp30 ligands including CMV tegument protein pp65 (17), Duffy binding-like (DBL)—1α domain of Plasmodium falciparum erythrocyte membrane protein—1 (PfEMP–1) (18), nuclear factor HLA-B-associated transcript 3 (BAT3)(19) and tumour associated cell surface protein B7-H6 (20).

Here we investigate the expression of NKp30 on group 2 innate lymphoid cells *ex vivo* and on cultured ILC2. Using quantitative PCR we identify the splice variants of NKp30 and show that incubation of ILC2 with plate bound B7-H6 or cell lines expressing this protein induced production of type 2 cytokines. This interaction can be inhibited by NKp30 blocking antibodies and the soluble blocking ligand, Galectin-3. We further established that activation of NKp30 induces the canonical pathway of NFκB signalling. This report

identifies a functionally important activatory cell contact receptor for ILC2, showing the involvement of NKp30 in ILC2-induced type 2 immune responses.

Materials and Methods

Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from healthy adult donors under local ethics approval (NRES Committee South Central, Oxford C, 09/H0606/71). ILC2 were isolated and cultured as previously described (6). Briefly, lineage (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, CD123 and FcεRI) negative, $CD45^+$, $CD127^+$, $CRTH2^+$ ILC2 population was sorted into 96-well plates at the density of 100 cells per well and re-suspended in mixed lymphocyte reaction (MLR) of gamma-irradiated peripheral blood mononuclear cells (PBMCs) from 3 healthy volunteers (2×10^6 cells/ml) coupled with 100 IU/ml of IL-2. After 4 to 6 weeks, the growing wells were tested by flow cytometry staining and resorted until a pure population of lineage negative $CRTH2^+$ $IL7R\alpha^+$ ILC2 was achieved (Supplemental Fig.1A). Keratinocyte line (HaCaT) was cultured in tissue culture flasks (Corning Incorporated, USA) in DMEM media supplemented with 10% FCS at 37°C with 5% CO2 and split on reaching confluence (approximately every 3–4 days). K562, Jurkat and THP-1 cell lines were cultured in RPMI-1640 supplemented with 10% FCS, Amino acids (MEM Non-Essential Amino Acids Solution 11140-050 Life Technologies) and HEPES (83264 Sigma). Cells were maintained at 0.2×10^6/ml density. For HaCat incubation with cytokines, IFN-γ was used at the concentration of 300 U/mL (21–24). All other cytokines were used at a concentration of 100ng/ml (25).

Antibodies

For FACS surface staining the cells were labelled by the following anti human antibodies purchased from Biolegend unless stated otherwise: CD3 (SK7; BD Biosciences), CD19 (SJ25C1; BD Biosciences), CD123 (FAB301C; R&D systems), CD11b (DCIS1/18), CD11c (BU15; Abcam), CD8 (RPA-T8), FcεRI (AER-37 (CRA-1)), CD14 (MφP9; BD biosciences), CD4 (MEM-241), CD45 (H130), ICOS (C398.4A), CD56 (B159), CRTH2 (BM16; Miltenyibiotec), IL-7Rα (A019D5), live/dead violet (L34955; Invitrogen), NKp30 (clone: AF29-4D12), NKp30 blocking antibody (Clone 210845 R&D systems, AF29-4D12 Miltenyi Biotec), Phospho-IκBα (Ser32/36 Cell Signalling 9246), Anti-B7-H6 antibody (ab121794), B7-H6 blocking antibody (17BL.3), CD68 (Y1/82A), Siglec-8 (7C9) and CD16 (3G8).

Quantitative RT-PCR

RNA extraction was performed using RNeasy plus Mini Kit (Qiagen 74134) and TurboCapture 96 mRNA kit (Qiagen 72251). cDNA was prepared using Omniscript RT kit. The following gene expression assays were purchased from Applied Biosystems: GATA3 (Hs00231122_m1), IL-5 (Hs01548712_g1), IL-13 (Hs00174379_m1), GAPDH (Hs99999905_m1), IL-4 (Hs00174122_m1), RORα (HS00536545_m1), NKp30a (Hs01553310-g1), NKp30b (Hs01561746-g1) and NKp30c (Hs01553311-g).

B7-H6 plate bound assay

Coat Corning Costar 9018 (Nunc Maxisorp®) were coated with indicated concentration of recombinant human B7-H6 Fc chimera protein (R&D systems 7144-B7-050) or control protein overnight at 4°C. 5×104 ILC2 were cultured on B7-H6 or isotype control coated plates. After 24 hours the supernatants were collected for cytokine analysis using ELISA or cytokine bead array. Where indicated the cells were pre-incubated with (10μg/ml) Galectin-1 (CF 1152-GA-050/CF, Bio-Techne), Galectin-2 (1153-GA-050, Bio-Techne), Galectin-3 (10289-HNAE-E-SIB, Stratech) for 1 hour before culture with plate bound rhB7-H6 or cytokine treated HaCaTs.

ELISA and ELISpot

Human IL-13 ELISA Ready-SET-Go (88-7439-86), Human IL-13 ELISA Duoset (DY213-05) and Human IL-13 ELISpot^{BASIC} (3470-2A) kit were purchased from eBiosciences, R&D systems and Mabtech, respectively and carried out according to manufacturers' instructions.

Immunohistochemistry

Anti-B7-H6 antibody (Abcam; ab121794), Isotype control (Abcam; ab37416), Ms anti-Rab HRP (344002, MaxDiscovery) and anti-Galectin-3 antibody (AF1154, Bio-Techne) were used to stain formalin-fixed paraffin-embedded skin tissue sections from healthy donors and adult atopic dermatitis patients with moderate-severe disease. DAB signal was quantified using 'Fiji' version of ImageJ.

Isolation of epidermal cells

skin biopsies from healthy donors were cut into wide strips and incubated overnight in 2u/ml dispase at 4°C. Epidermal sheet was peeled from the dermis by forceps and incubated for 15 minutes in 0.5% trypsin+0.02% EDTA at 37°C. The mixture was then pipetted repeatedly and passed through a 40μm strainer.

Suction blister technique

Suction blister cups were applied to the lesional and non-lesional skin of adult atopic dermatitis patients with moderate-severe disease, and not on systemic therapy, at a vacuum pressure of 250–450 mmHg. Blisters were formed within 30–90 min. 24 hours later fluid was aspirated using a 30-gauge needle. Fluids were then centrifuged at 1,500 rpm for 5 min at 4°C and the concentration of cytokines was measured by multi-plex array (Luminex).

Statistical analysis

t tests were performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, USA).

Results

A large subset of group 2 innate lymphoid cells express the natural cytotoxicity receptor NKp30

To investigate recognition strategies that ILC2 employ to sense the microenvironment, the expression of NKp30 on ILC2 was examined. Cultured ILC2 lines were stained for NKp30 (NCR3) and high levels of NKp30 expression were detected (94.6%, Fig. 1A). However, IL-2 is known to induce up-regulation of NKp30 on NK cells and its presence in the culture media could potentially induce up-regulation of NKp30 by ILC2 (26, 27). Therefore to evaluate the expression of this receptor *ex-vivo*, lineage negative (CD3, CD4, CD8, CD14, CD19, CD56, IL3R, FcεRI, CD11b, CD11c) cells that express CD45, CD127 (IL-7Rα), and CRTH2 from peripheral blood of healthy donors were analysed and 52.4 ±11.5% of human ILC2 express NKp30 on their surface *ex-vivo* (n=7, Fig. 1B, with gating in Supplemental Fig. 1A, the expression of NKp30 on NK cells is shown as a reference). Supplemental figure 1B shows the expression of NCRs on group 1, 2 and 3 ILCs *ex-vivo*.

ILC2 predominantly express isoform c of NKp30

Alternative splicing of exon 4 of the NCR3 gene gives rise to different isoforms. In NK cells, crosslinking of each isoform induces distinct signalling pathway and a different pattern of cytokine production. NKp30a and NKp30b isoforms are classified as immune-stimulatory whereas NKp30c is thought to be immune-regulatory (3). To determine the expression of NKp30 isoforms on ILC2, the mRNA levels of NKp30a, b and c isoforms in cultured ILC2 were measured with q-PCR. The data were normalized to the ubiquitously expressed *GAPDH* housekeeping gene and ILC2 were found to predominantly express *NKp30c* (Fig. 1C). Taken together these data raised the possibility that NKp30 may play a role in ILC2 function.

B7-H6 induces production of type 2 cytokines by ILC2

B7-H6 is a self-ligand that is expressed on the surface of some tumour cell lines (14). It belongs to the B7 family of receptors that encode ligands for CD28 and CTLA-4 (B7-1 and B7-2), but unlike other members of the B7 family it is thought to be selective for NKp30 and does not bind CD28 or other NCRs. It is composed of 2 immunoglobulin extracellular domains (a distal V-like and a proximal C-like domain) with an adjacent phase 1 intron. Both the front and back β sheets of NKp30 bind to the V-like domain of B7-H6 via hydrophobic interactions (14, 28). To investigate whether NKp30 expressed on ILC2 mediates effector functions, ILC2s were treated with a recombinant human B7-H6 Fc chimeric protein, a selective NKp30 ligand. Cross-linking NKp30 receptor on ILC2 by plate bound B7-H6 protein induced production of increased amounts of IL-13 (Fig. 2A). Galectin-3 is a β-galactosidase binding protein expressed on the surface of tumour cells and promotes proliferation and metastasis. Galectin-3 interacts with *N*-glycosylated sites (Asn-42, Asn-121) of NKp30 ectodomain and interferes with ligand binding (29). Incubation of ILC2 for 1 hour with soluble NKp30 blocking ligand, Galectin-3, before performing the B7-H6 plate bound assay reduced the expression of IL-13 (Fig. 2B) whereas it did not affect PGD2 stimulation which is mediated through CRTH2 (data not shown). The inhibitory effect of Galectin-3 was not observed with Galectin-1 and Galectin-2 (Fig. 2B).

Next we screened a panel of tumour cell lines for their ability to activate ILC2, including cells with differential expression of HLA class I molecules in order to investigate whether HLA may influence ILC2 function through interaction with non-polymorphic HLA-binding receptors. The human B lymphoblastoid cell line .221 lacks expression of surface class I. Native .221 cells or .221 cells transfected with specific HLA variants, did not induce production of IL-13 by ILC2 (Fig. 2C). In contrast, K562 cells (HLA^{low} myelogenous leukemia line), THP-1 cells (monocytic leukemia line) and Jurkat cells (T cell leukemia line) induced IL-13 expression by ILC2 (Fig. 2C). Further analysis showed that these cell lines all express B7-H6 on their cell surface, whereas the .221 cell line was negative. There was no expression of BAT3, another NKp30 ligand, on the surface of the tumour cell lines (Fig. 2D).

Similar to its effect on ILC2 interaction with plate-bound rhB7H6, Galectin-3 had an inhibitory effect on NKp30-mediated interaction of ILC2 and K562 (Supplemental Fig 1C). Comparing K562-mediated activation of ILC2 with PGD_2 and IL-33 activation, showed that K562 cells are as potent inducers of IL-13 expression by ILC2 as PGD_2 and IL-33 (Fig. 2E). To investigate whether IL-33 or PGD2 can modify NKp30, we stimulated ILC2 with increasing concentrations of IL-33 and PGD2. No significant change was observed in the expression of NKp30 isoforms on ILC2 following stimulation with IL-33 and PGD2 (Supplemental Fig. 2A). To examine the effect of NKp30 mediated activation of ILC2 on the expression of cytokine receptors and CRTH2, we incubated ILC2 with plate bound B7-H6 and isolated mRNA at various time points. 3 hours after incubation with B7H6 the mRNA levels encoding the IL-33 receptor (ST-2), and PGD2 receptor (CRTH2) were significantly reduced (Fig. 2F). IL-25 receptor (IL-17RB) expression was moderately reduced. Following initial downregulation, the ST2 mRNA level increased above the basal level. To test whether K562 also induced expression of other cytokines we used multiplex cytokine analysis. Activation of ILC2 by K562 cells induced production of IL-2, IL-3, IL-4, IL-5, IL-13, IL-8 and GM-CSF (Fig. 3A) but did not increase production of IFN-γ and IL-10 (data not shown). Analysis of the level of these cytokines in the blister fluid of skin of patients with atopic dermatitis confirmed the biological significance of the levels of produced cytokines by activated ILC2 (Supplemental Fig. 2B).

To confirm that the ILC2 activation is mediated by NKp30, we incubated the cells with increasing concentrations of NKp30 specific antibody for 1 hour before culture with the K562 cell line. Blocking NKp30 reduced the K562-induced cytokine production in a dose dependent manner (Fig. 3A and Supplemental 2C). Incubating K562 cells with B7H6 blocking antibody (30) for one hour before culturing with ILC2 significantly inhibited production of IL-13 (Fig. 3B).

NKp30 mediated activation of ILC2 is contact dependent as supernatant from cultured tumour cell lines could not activate ILC2 (Fig. 3C) and indeed 24-plex and 7-plex analysis of K562 supernatant did not reveal any known ILC2 stimulating cytokine or chemokines (ILC2 stimulating cytokines are shown in Supplemental Fig. 2D). In fact, ILC2 produced a similar pattern of cytokines either by K562-mediated cross-linking of NKp30 receptor or plate-bound rhB7H6 activation but rhB7H6 provided a stronger signal (Supplemental Fig. 2E).

To confirm the NKp30 mediated rapid effector functions of ILC2 *ex-vivo*, we incubated freshly isolated ILC2 from the blood of healthy donors with the K562 tumour cell line and showed that K562 alone do not produce IL-13, but it can indeed activate ILC2 and induce their production of IL-13 (Fig. 3D).

As discussed above NK cells express different isoforms of NKp30, whereas ILC2 mainly express NKp30c isoform. To compare the cytokine production profile of activated NK cells and ILC2, we incubated both cell populations with rhB7H6. Upon interaction with B7H6, NK cells produced significant amounts of IFN-γ, TNF-α and IL-10 whereas ILC2 did not. ILC2 mainly produced type 2 cytokines, IL-13 and IL-5. Although NK cells also expressed these cytokines but the ability of ILC2 in producing type 2 cytokines was significantly higher (Fig. 3E).

Therefore NKp30 expressed on ILC2 is functionally active and mediating interaction between ILC2 and immortal laboratory tumour cell lines suggesting they may have a role in the interaction with malignant or stressed host cells.

B7-H6 is expressed on basal epidermis in normal tissue and suprabasal epidermis in lesional skin biopsies of atopic dermatitis patients

Several studies have reported the expression of B7-H6 on tumour cell lines. To investigate whether B7-H6 is also expressed in non-malignant tissue, sections of healthy skin and lesional skin biopsies of patients with atopic dermatitis were evaluated. Interestingly, we found expression of B7-H6 protein in healthy skin tissue but this was confined to the basal layer of epidermis. In contrast lesional skin biopsies from adult patients with atopic dermatitis showed B7-H6 expression throughout the suprabasal layers of the epidermis and myeloid derived populations in the dermis (Fig. 4A and supplemental Fig. 3A). Objective quantification of immunohistochemistry staining of B7H6 expression in skin biopsies of healthy controls and atopic dermatitis patients is shown in Fig 4B.

It is therefore plausible to speculate that widespread expression of B7-H6 in the skin of patients with atopic dermatitis can lead to NKp30-mediated activation of ILC2. This finding is compatible with our earlier observation that ILC2 resident in the skin of patients with atopic dermatitis show an activated phenotype (6). The presence of ILC2 in the epidermis ((31) and supplemental fig. 3B) favours their migration into or retention in the epidermis and supports their possible interaction with keratinocytes.

To further understand whether the widespread expression of B7-H6 in patients' skin contributes to the NKp30-mediated activation of ILC2, the effect of the immortalized human keratinocyte cell line (HaCaT) on ILC2 activation was tested. A relatively low expression of B7-H6 (Fig. 4C) was observed in HaCaTs. However B7-H6 is known to be up-regulated under inflammatory conditions (32). Therefore we incubated the HaCaT cell line with IFN-γ, TNF-α, epithelial cytokines, mast cell products and type 2 cytokines.

Consistent with earlier reports, the pro-inflammatory cytokines IFN-γ and TNF-α induced significant up-regulation of B7-H6. Interestingly, type 2 cytokines IL-13 and IL-4 also significantly upregulated B7-H6, whereas epithelial cytokines (IL-25, IL-33 and TSLP) and

the mast cell derivative PGD_2, did not significantly up-regulate cell surface expression of B7-H6 on HaCaT cells (Fig. 4C). We next treated HaCaTs with increasing concentration of IFN-γ or IL-13, then washed, trypsinised, counted and subsequently cultured the cells with ILC2 for 24 hours. The ability of ILC2 to produce IL-13 was correlated to the dose of IFN-γ or IL-13 used to treat HaCaT cells (Fig. 4D). Interestingly, pre-incubation with soluble Galectin-3 for 1 hour before culturing with IL-13 and IFN-γ treated HaCaTs reduced the expression of IL-13 (Fig. 4E). Galectin-3 is expressed by myeloid derived cells ((33), Supplemental Fig. 3A) at similar level in healthy skin and lesional biopsies of atopic dermatitis patients as shown by objective analysis of Galectin-3 staining of 5 donors (Supplemental Fig 3C).

Rapid NKp30 mediated activation of ILC2 triggers the canonical pathway of NFkB activation

All NKp30 isoform monomers have arginine charged residues in their transmembrane regions that couple with aspartate amino acids in CD3ζ homodimers or CD3ζ/FcRγ heterodimers by a salt bridge (34). CD3ζ is an ITAM bearing adaptor molecule essential for transmitting downstream signalling (14). Isoform b is constantly associated with CD3ζ while isoform c shows a weaker interaction. NKp30 cross-linking triggers the canonical pathway of nuclear factor κB (NFκB) activation. NFκB dimers are bound to the inhibitory protein IκB. IκB kinase complex (IKK) consists of IKKα, IKKB and regulatory protein NEMO and phosphorylates IκB. Phosphorylated IκB undergoes proteasomal degradation that releases NFκB to translocate to the nucleus (35). To study the signalling pathway involved in NKp30-mediated ILC2 activation, intracellular FACS analysis of these cells confirmed the increase in phosphorylation of IκB (Fig. 5A). To evaluate whether the increase in production of cytokines was due to the release of preformed cytokines or increase in gene expression, we incubated ILC2 with plate bound B7-H6 protein for up to 24 hours which increased production of IL-13 protein (Fig. 5B) and increased mRNA expression of *IL-13*, *IL-4*, *IL-5*, *AREG* (amphiregulin) and enhanced expression of *GATA-3* (Fig. 5C) but did not affect *ROR-α* expression (data not shown).

Discussion

In 1999 Moretta *et al.* identified natural cytotoxicity receptors with activating properties that can trigger an immune response on recognition of cognate cellular and viral ligands and therefore play an important role in NK cell anti-tumour and anti-viral cytotoxicity (36). Although NCRs have been detected on type 1 and type 3 innate lymphoid cells, their specific functions have not yet been fully elucidated (37, 38). Here we identified and characterized NKp30 expression on group 2 innate lymphoid cells, showing an important role in induction of type 2 cytokines.

NKp30 protein is encoded by the NCR3 gene located in the highly polymorphic telomeric end of the class III region. (39). NK cell sub-populations can express all three of the commonest isoforms (a, b, c) and the relative contribution of each receptor depends on the expression levels on the cell surface. Interestingly, ILC2 showed predominant expression of the putative immune-modulatory splice variant, NKp30c and lower expression of NKp30a

and b isoforms. We show that NKp30 engagement on ILC2 augments the production of type 2 cytokines IL-4, IL-5, IL-13 and GM-CSF as well as other inflammatory cytokines such as IL-2, IL-3 and IL-8. These cytokines have a crucial role in allergic and inflammatory conditions and genes encoding many of these cytokines are located at the same chromosome locus 5q31-33 (40). Proliferation and differentiation of $CD34^+$ progenitor cells into basophils and mast cells are highly dependent on IL-3. IL-3 also regulates dendritic cell differentiation from monocytes (41, 42). IL-8 is a potent neutrophil chemo-attractant (43, 44) and contributes to allergy and severe asthma (45, 46). GM-CSF has been increasingly appreciated as a type 2 cytokine (47, 48) and a neutrophil and eosinophil survival factor (49, 50).

Another difficulty that limits our understanding of the role of NKp30 is poor characterisation of its ligands and their distribution on normal tissues under homeostatic and inflammatory conditions. Two self-ligands have been identified to bind to NKp30; a novel member of B7 family receptors, B7-H6, and a largely intracellular protein BAT3, which can be expressed on the cell surface under certain conditions (14, 20, 28, 51). Consistent with activation of NKp30 expressing cells through B7-H6, our data demonstrated that plate bound recombinant human B7-H6 can induce production of IL-13 by ILC2 cells in a dose dependent manner. Interestingly incubation of multiple tumour cell lines with ILC2 showed that cell lines which activated ILC2s and induced production of IL-13 such as THP1, K562 and Jurkat, lacked cell surface staining of BAT3 while maintained B7-H6 expression; suggesting that B7-H6 is the main NKp30 ligand being identified on the laboratory tumour cell lines. We do not have availability of a B7-H6 blocking antibody to rule in these findings, but the correlative data are supported by the recombinant B7-H6 data. Indeed IL-13 production was diminished upon prior incubation of ILC2s with increasing concentration of NKp30 blocking antibody or soluble NKp30 blocking ligand, Galectin-3. We showed that the IL-13 production occurs rapidly, consistent with an early innate immune function to respond during the initial phases of an immune response. Although stimulation with IL-33 and PGD2 did not alter the expression of NKp30 isoforms, NKp30 mediated activation of ILC2 down-regulated expression of cytokine receptors IL-33, IL-17RB and PGD2 suggesting a negative feedback mechanism making ILC2 less responsive to further activation at early stages. The expression level of cytokine receptors were increased after 8 hours, in case of ST2 this increase was above basal levels. Stimulation with epidermal cytokines may follow the initial NKp30 mediated activation of ILC2.

Until recently B7-H6 was believed to be absent on normal tissues and restricted to tumour and transformed cells (51). However in 2013 Matta and colleagues showed that B7-H6 could be induced on non-transformed cells in various conditions of cell stress such as infections and inflammation (30). B7-H6 was selectively upregulated on $CD14^+$ $CD16^+$ pro-inflammatory monocytes and neutrophils when stimulated with TNF-α and IL-1β *in vitro* and in septic conditions *in vivo* (30). These data have guided us to propose some molecular bases for the direct interaction and cross-talk between NKp30 positive ILC2 and ligand expressing tissues. Staining of formalin fixed, paraffin-embedded sections of normal human skin showed low level of B7-H6 expression in the basal layer of epidermis whereas B7-H6 expressing cells were detected throughout the epidermis in lesional tissue sections of patients with atopic dermatitis. Dermal cell populations expressing B7-H6 were also

observed which mainly consisted of $CD11b^+$ and $CD11c^-$ $CD68^+$ cells (Supplemental Fig3A).

The unstimulated keratinocyte cell line, HaCaT, showed low levels of B7-H6 expression but incubation with pro-inflammatory cytokines TNF-α and IFN-γ and type 2 cytokines IL-13 and IL-4 increased expression of B7-H6. It has been well established that type 2 cytokines are found in high concentrations in lesions of atopic dermatitis patients (AD) (52–55) but type 1 cytokines IFN-γ and TNF-α have also been found, particularly during the chronic stage of AD (56).

The expression of B7-H6 by keratinocytes provides a novel perspective on the link between inflammation and NKp30 mediated activation of ILC2s. Keratinocytes in AD lesions may not only activate ILC2 by producing epithelial cytokines, but also direct interaction via B7-H6 can trigger production of type 2 cytokines. Therapeutic strategies to modulate ILC2 activity may provide novel treatment approaches for individuals with AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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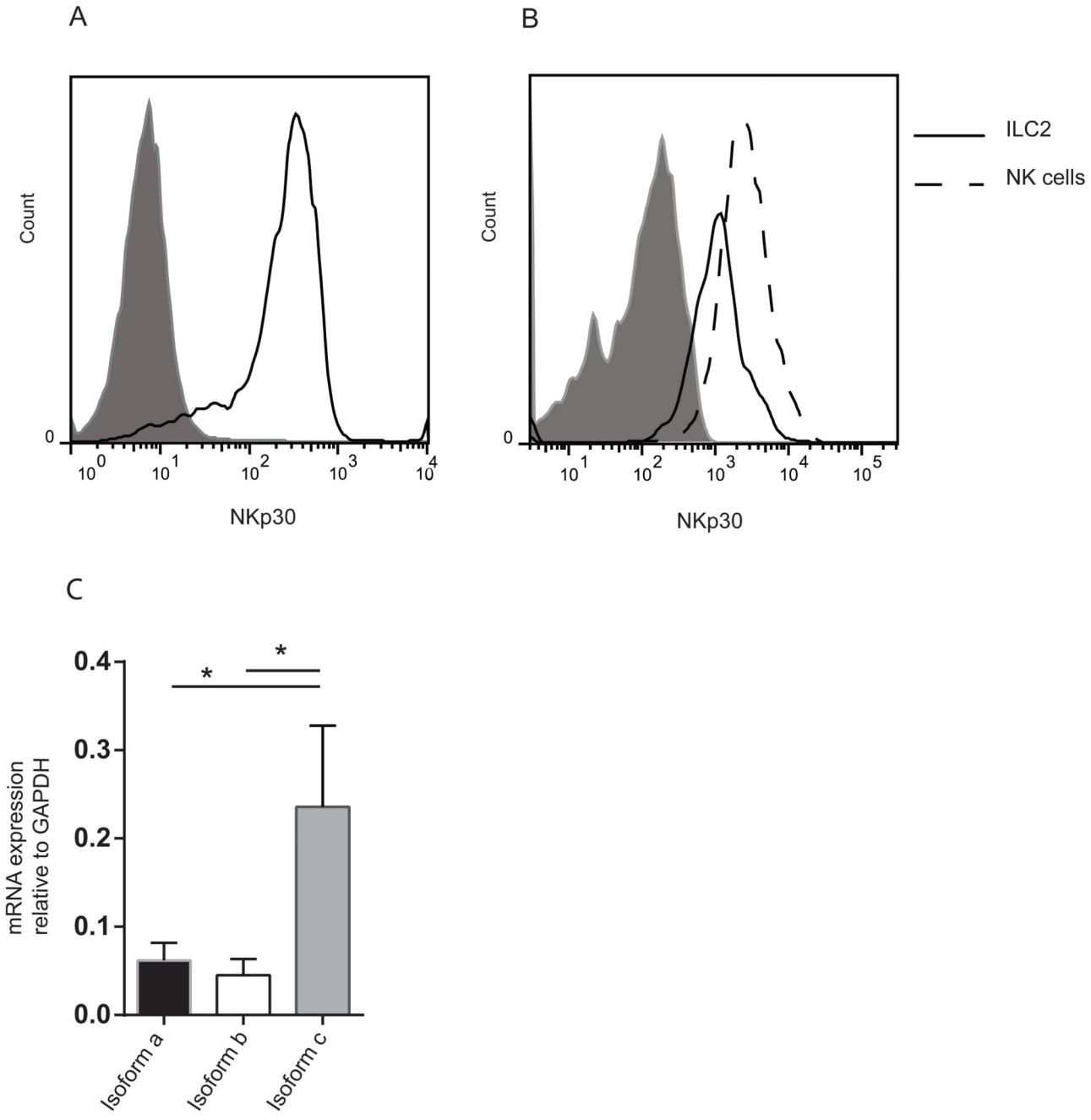

Figure 1. ILC2 express natural cytotoxicity receptor NKp30.

Cultured ILC2 (A) or *ex vivo* NK cells and ILC2 (B) were examined for NKp30 expression by flow cytometry. Black and dotted lines are NKp30 staining of ILC2 and NK cells respectively. Filled histogram is isotype control staining. ILC2 were gated as lineage negative (CD3, CD4, CD8, CD14, CD19, CD56, CD11b, CD11c, IL-3R, FcεRI), $CD45^+$, IL-7Rα$^+$, $CRTH2^+$. NK cells were gated as $CD3^-$ $CD45^+$ $CD56^+$. Representative example stains of seven donors are shown. (C) Expression of different isoforms of NKp30 on ILC2

was measured by quantitative RT-PCR. mRNA levels were normalized to the house keeping gene *GAPDH* (n=9). * P<0.05.

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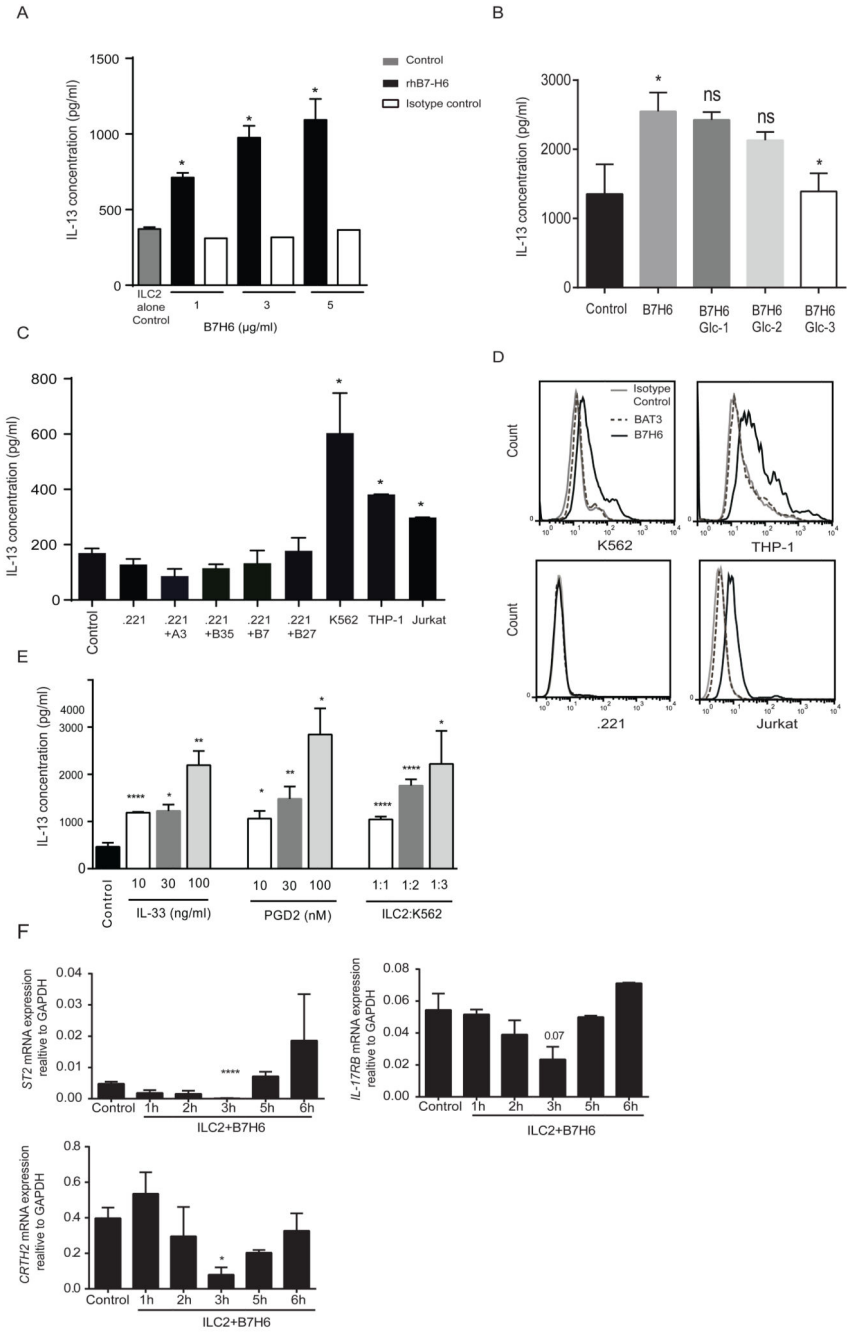

Figure 2. NKp30 ligand, B7-H6, activates ILC2.

(A) IL-13 production by ILC2 was measured by ELISA after 24 hours of culture with increasing concentration of rhB7H6 or protein control (n=5). Statistical comparisons were made compared to ILC2 alone. (B) IL-13 production by ILC2 was measured by ELISA 4 hours after culture with 5μg/ml plate bound B7H6, with and without 1 hour pre-incubation with soluble Galectin-1 (10μg/ml), Galectin-2 (10μg/ml) and Galectin-3 (10μg/ml) (n=4) . (C) ILC2s were cultured with tumour cell lines for 24 hours and production of IL-13 cytokine was measured by ELISA (n=5). Statistical comparisons were made compared to

ILC2 alone control. (D) Flow cytometry was used to examine expression of B7-H6 and BAT3 protein on the surface of K562, THP-1, .221 and Jurkat tumour cell lines. (E) IL-13 production by ILC2 was measured by ELISA after 4 hours stimulation with 10 ng/ml, 30ng/ml and 100ng/ml IL-33, 10nM, 30nM and 100nM PGD_2 and 1:1, 2:1 and 3:1 ILC2:K562. (F) mRNA expression of *ST2* (IL-33R), *IL-17RB* (IL-25R) and *CRTH2* following culture with rhB7-H6 for indicated times. Statistical comparisons were made compared to ILC2 alone control. * P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

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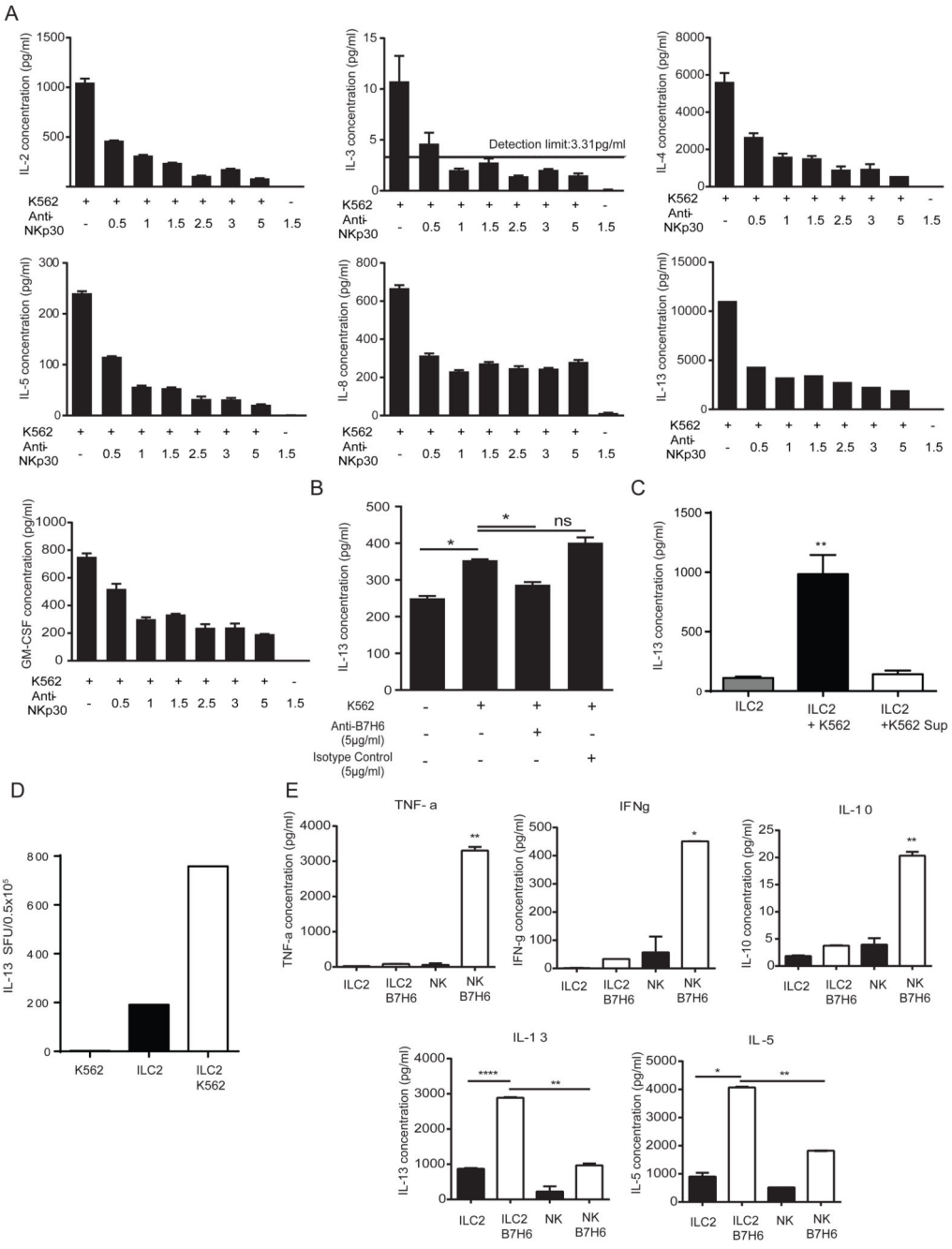

Figure 3. Crosslinking of NKp30 receptor on ILC2 increases production of type 2 cytokines.

(A) Cytokine production was measured by cytokine multiplex analysis of ILC2 supernatant following 4 hour incubation with K562 tumour cell in the absence or presence of anti-NKp30 antibody. (B) Production of IL-13 by ILC2 after incubation with K562 for 4 hours with and without pre-incubation with B7-H6 blocking or isotype control antibody (5µg/ml) (C) IL-13 production by ILC2 was measured using ELISA following 24 hours incubation with K562 cells or supernatant from K562 cells (n=3). (D) Freshly isolated ILC2 were co-cultured with K562 tumour cells for 24 hours and IL-13 cytokine production was measured

by ELISpot (SFU, spot forming units; figure representative of 3 experiments). (E) Cytokine analysis of supernatant of NK cells and ILC2 following 4 hours of incubation with B7-H6. * P<0.05, **P<0.01, ***P<0.001, ****p<0.0001.

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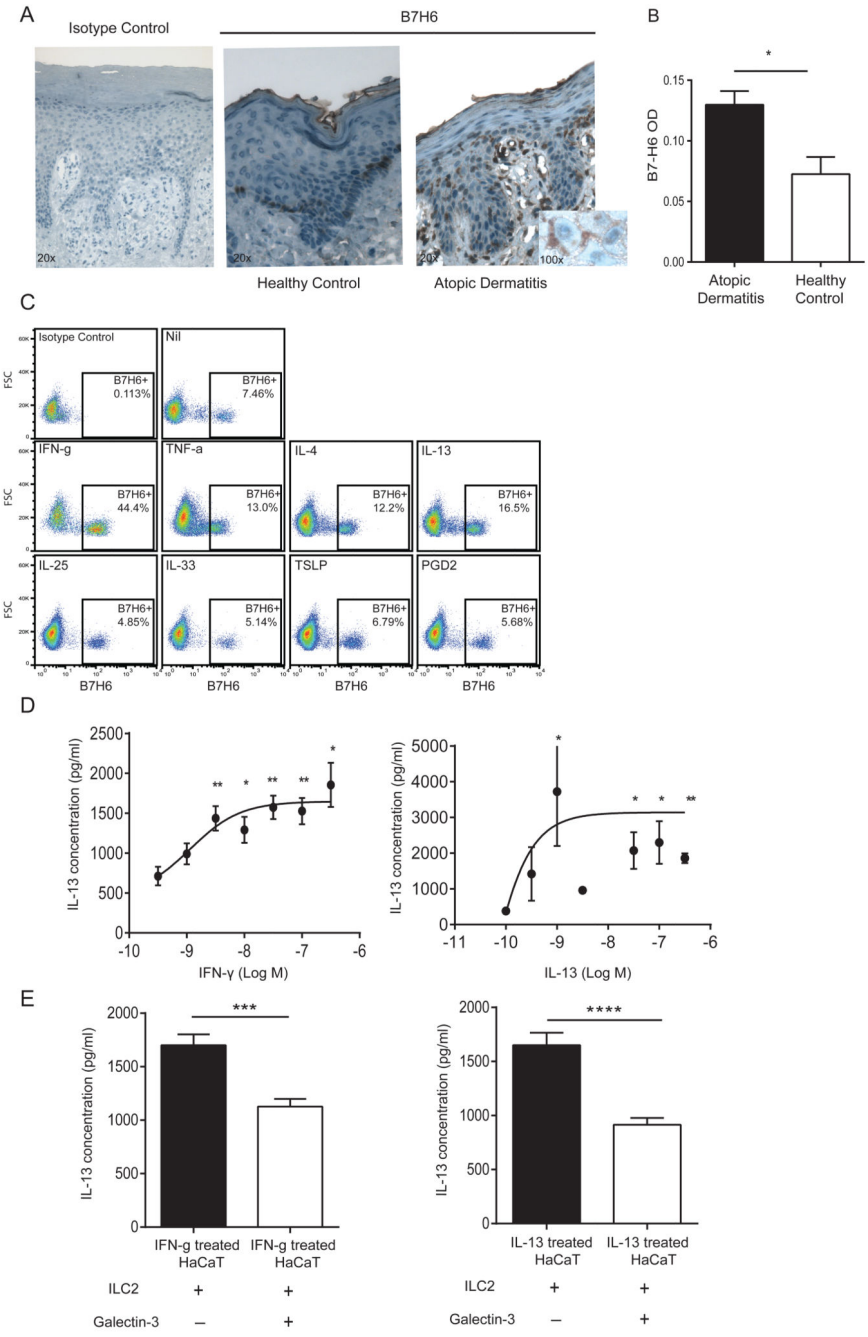

Figure 4. Higher expression of B7-H6 is found in lesional skin biopsies of AD patients.

(A) B7-H6, expression in healthy control (n=11) and atopic dermatitis lesions (n=6) was examined by immunohistochemistry. (B) Quantification of B7-H6 immunohistochemistry signal using 'Fiji' version of 'ImageJ' in healthy control (n=11) and atopic patients skin lesions (n=6). (C) The expression of B7-H6 on the keratinocyte cell line (HaCaT) was evaluated after 72 hours of incubation with IFN-γ (300U/ml), TNF-α (100ng/ml), IL-4 (100ng/ml), IL-13 (100ng/ml), IL-25 (100ng/ml), IL-33 (100ng/ml), TSLP (100ng/ml), PGD_2 (100nM) by flow cytometry and compared with isotype control staining. (D)

Following 72 hours of incubation with increasing concentration of IFN-γ or IL-13, HaCaTs were washed, trypsinised and equal numbers were cultured with ILC2 for 24 hours. The production of IL-13 was measured in the supernatant by ELISA (n=3). (E) IL-13 production by ILC2 was measured by ELISA after incubation with 10μg/ml Galectin-3 for 1 hour prior to culture with IFN-γ (300U/ml) or IL-13 (100ng/ml) treated HaCaTs. * P<0.05, **P<0.01, *** P<0.001, **** P<0.0001.

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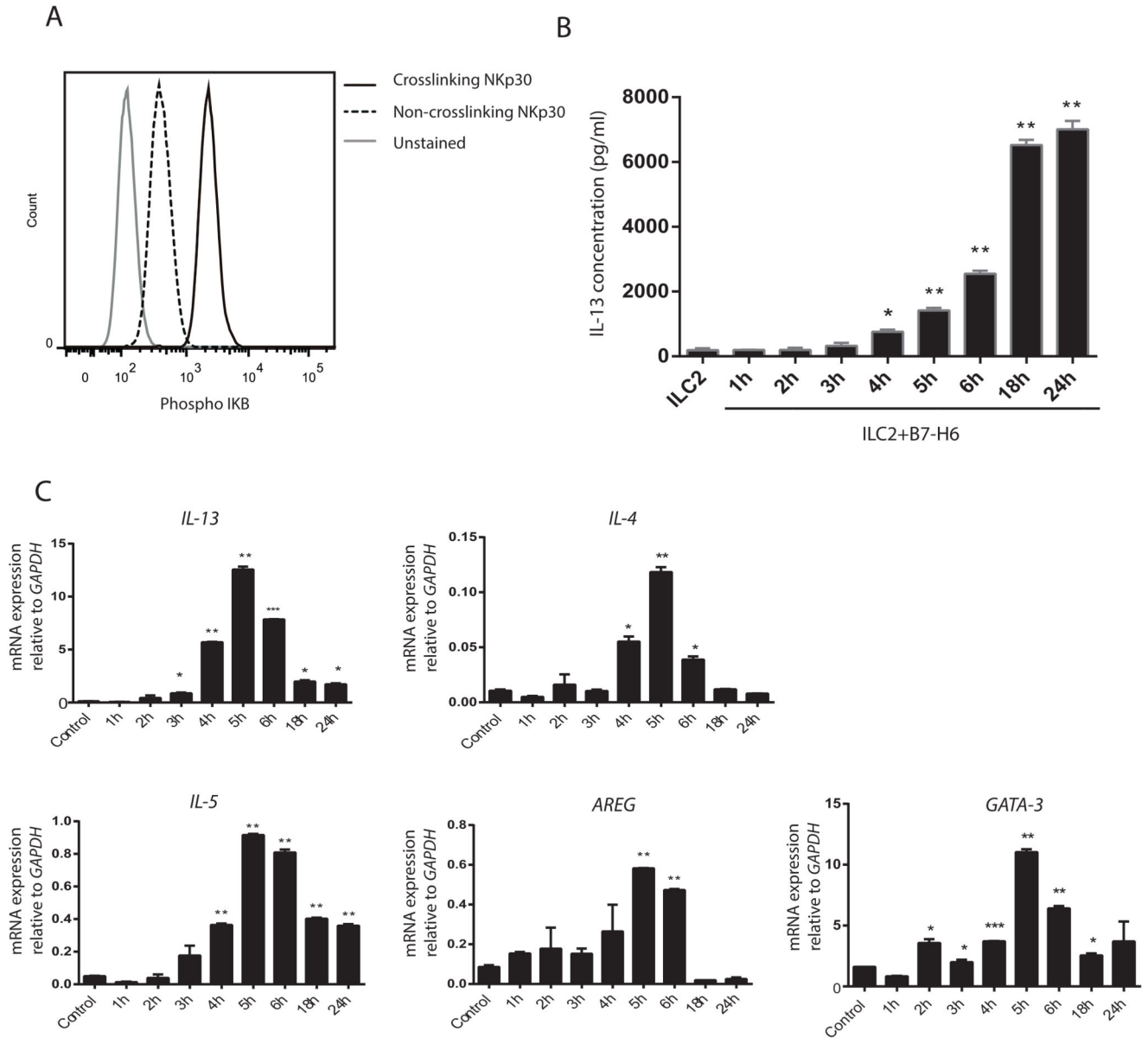

Figure 5. NKp30 mediated activation of ILC2 induces the NFκB signalling pathway.

(A) The expression of phosphorylated IκB in ILC2s was evaluated by flow cytometry after 4 hours of culture with K562 cells (data representative of 4 experiments). (B) IL-13 production by ILC2 in the absence or presence of plate bound B7-H6 was measured by ELISA, and (C) mRNA expression was quantified using RT-PCR following incubation with plate bound B7-H6 (n=2). Statistical comparisons were made with ILC2 alone control. P<0.05, **P<0.01.