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## **Licensed human natural killer cells aid dendritic cell maturation via TNFSF14/LIGHT**

Tim D. Holmes<sup>1,4</sup>, Erica B. Wilson<sup>1</sup>, Emma V.I. Black<sup>1</sup>, Andrew V. Benest<sup>1,5</sup>, Candida Vaz<sup>2</sup>, Betty Tan<sup>2</sup>, Vivek M. Tanavde<sup>2,3</sup> and Graham P. Cook<sup>1,6</sup>.

1. Leeds Institute of Cancer and Pathology, University of Leeds School of Medicine, Wellcome Brenner Building, St. James's University Hospital, Leeds LS9 7TF, UK.

2. Bioinformatics Institute, Agency for Science, Technology and Research (A\*STAR), Biopolis, Singapore.

3. Institute of Medical Biology, A\*STAR, Biopolis, Singapore.

4. Present Address; Karolinska Institute, Karolinska University Hospital Huddinge Stockholm, Sweden.

5. Present Address; Tumour Vascular Biology Group, Cancer Biology and Stem Cells, School of Medicine, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, UK.

6. Corresponding author

**Running Title:** NK cell TNFSF14 aids DC maturation

## **Abstract**

Interactions between natural killer (NK) cells and dendritic cells (DC) aid DC maturation and promote T cell responses. Here, we have analysed the response of human NK cells to tumor cells and we identify a pathway by which NK-DC interactions occur. Gene expression profiling of tumor-responsive NK cells identified the very rapid induction of TNFSF14 (also known as LIGHT), a cytokine implicated in the enhancement of anti-tumor responses. TNFSF14 protein expression was induced by three primary mechanisms of NK cell activation, namely via the engagement of CD16, by the synergistic activity of multiple target cell-sensing NK cell activation receptors and by the cytokines IL-2 and IL-15. For anti-tumor responses, TNFSF14 was preferentially produced by the licensed NK cell population, defined by the expression of inhibitory receptors specific for self-MHC class I molecules. In contrast, IL-2 and IL-15 treatment induced TNFSF14 production by both licensed and unlicensed NK cells, reflecting the ability of pro-inflammatory conditions to override the licensing mechanism. Importantly, both tumor and cytokine activated NK cells induced DC maturation in a TNFSF14-dependent manner. The coupling of TNFSF14 production to tumor-sensing NK cell activation receptors links the tumor immune surveillance function of NK cells to DC maturation and adaptive immunity. Furthermore, regulation by NK cell licensing helps to safeguard against TNFSF14 production in response to healthy tissues.

## **Significance**

As well as having potent cytotoxic activity, natural killer (NK) cells have a regulatory role and interactions between NK cells and dendritic cells (DC) aid DC maturation and adaptive immunity. However, the mechanisms underpinning NK-DC cross talk are poorly defined. We show that tumor cells induce the rapid production of the cytokine TNFSF14 (also known as LIGHT) in human NK cells and that these NK cells induce DC maturation in a TNFSF14-dependent manner. The synergistic activity of NK cell activation receptors in licensed NK cells couples the release of cytotoxic granules to TNFSF14 production. Thus, NK cell activation by tumor cells is linked to the initiation of adaptive immunity via TNFSF14-mediated NK-DC cross talk.

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## **Introduction**

Natural killer (NK) cells play an important role in protecting the host against viral infection and cancer. As well as having potent cytotoxic activity, NK cells are endowed with immunoregulatory activity (1, 2). For example, NK cell activation induces the production of chemokines, such as MIP1- $\alpha$  and IL-8, and pro-inflammatory cytokines such as interferon (IFN)- $\gamma$ , granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor- $\alpha$  (TNF). These molecules regulate the recruitment and activity of numerous immune cell types (1, 2). Importantly, NK cells can promote development of T cell responses via NK-DC interactions that favour both DC maturation and NK cell activation (3-5), with NK cell-derived IFN- $\gamma$  skewing T cell differentiation towards the Th1 phenotype (6, 7).

Cytotoxic activity and cytokine production are coupled to signalling pathways downstream of a repertoire of activating and inhibitory receptors; signals from activating receptors (including NKG2D, DNAM-1, 2B4 and the natural cytotoxicity receptors, NKp30, NKp44 and NKp46) compete with those from inhibitory receptors (such as KIRs and CD94/NKG2A) to regulate activation. In addition, NK cells express CD16, the low affinity receptor for IgG, conferring antibody dependent cellular cytotoxicity (ADCC) (8-10). Activation thus coordinates the killing of target cells, the induction of inflammation and the promotion of adaptive immunity. This potent cytotoxicity and pro-inflammatory activity must be strictly controlled to minimise damage to healthy tissue. Functional competency of unstimulated NK cells is achieved via a process termed licensing or education (11-14). This ensures that only those NK cells expressing inhibitory receptors for self-MHC class I can respond to target cells and NK cells that lack inhibitory receptors for self MHC class I molecules are rendered hyporesponsive, preventing them from attacking healthy cells expressing normal levels of MHC class I molecules.

We have analysed the consequences of human NK cell activation by tumor cells. Our results reveal induction of the TNF superfamily member TNFSF14, also known as Lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed on T lymphocytes (LIGHT)(15). We show that activated NK cells produce TNFSF14 in response to different stimuli, that tumor cells induce TNFSF14 production by licensed NK cells and that TNFSF14 producing NK cells aid DC maturation during NK-DC cross-talk.

## Results

### **Gene expression profiling of tumor-stimulated NK cells identifies induction of a putative immunomodulatory pathway.**

Gene expression profiling has provided great insight into NK cell function (16-18). NK cells respond very rapidly to target cells and we reasoned that analysing the changes in gene expression that occur during the early stages of activation by target cells might reveal new features of these responses.

Activation of NK cells by tumor cells results in the rapid exocytosis of cytotoxic granules and the expression and release of cytokines. Granule exocytosis exposes the granule membrane protein CD107 on the NK cell surface, allowing NK cells that have responded to a tumor target (here termed R-NK) to be separated from non-responding (NR-NK) cells using fluorescence activated cell sorting (FACS). We purified human peripheral blood NK cells, co-cultured them (in the absence of exogenous cytokines) for four hours with K562 tumor cells and sorted the R-NK and NR-NK fractions (Fig. 1A). We performed gene expression profiling on R-NK and NR-NK fractions from two sample sets, one derived from a single healthy donor and the other using fractions pooled from three additional healthy donors. Not surprisingly given donor variability, more genes were differentially regulated in the pooled sample compared to the single donor (1108 genes and 670 genes respectively), but there were 541 genes that were differentially regulated by a factor of 1.5 or more ( $P < 0.05$ ) in both sample sets (Fig. 1B and C and supplementary dataset 1). We focused our analysis on genes whose expression was upregulated in the R-NK cells; induction of genes encoding TNF (TNFSF2), GM-CSF (CSF2), IFN- $\gamma$  (IFNG) and CD69 was detected in R-NK cells as expected (Fig. 1C). Quantitative RT-PCR analysis of mRNA from R-NK and NR-NK cells confirmed induction of a selection of the genes identified by the array analysis. For example, CSF2 and IFNG were induced by some two hundred-fold and fifty-fold respectively in the R-NK cells (Fig. 1B). In addition, several KIR genes and KLRC1 (encoding NKG2A) demonstrated greater expression in the R-NK fraction, consistent with the higher level of MHC class I inhibitory receptor expression in functionally responsive (educated or licensed) human NK cells (12, 19). Manual inspection of genes upregulated in the R-NK fraction revealed many molecules associated with an immune activation phenotype, including cell surface receptors, signalling components and transcription factors, as well as genes associated

with NK cell effector functions (Fig. 1C and supplementary table 1). As well as TNF itself, the R-NK fraction demonstrated increased expression of several TNF superfamily (TNFSF) and TNF receptor superfamily (TNFRSF) members. In particular, we identified four molecules from the same immunoregulatory network; the immunoglobulin superfamily molecule CD160 and the TNF superfamily molecules TNFSF14 (LIGHT), TNFSF15 (also known as TNF-like Ligand 1A; TL1A) and TNFSF6 (Fas Ligand; FasL). These genes encode cell surface molecules involved in a complex regulatory network involving both *cis* and *trans* receptor-ligand interactions that regulate immune activation events in other cell types (20, 21) (Fig. 1D). Both CD160 and TNFSF14 are ligands of TNF receptor superfamily molecule TNFRSF14, also known as Herpesvirus entry mediator (HVEM) (15, 22). Like other TNFSF receptors, HVEM signals via TNF Receptor Associated Factors (TRAFs) to induce NF- $\kappa$ B activity (23, 24). Furthermore, TNFSF14, TNFSF15 and TNFSF6 activity are all antagonised by TNFSFR6B (Decoy Receptor 3; DcR3) (25). The potential that tumor cells might induce NK cell expression of molecules within this network prompted us to explore the regulation and consequences of their expression in more detail.

### **NK cells rapidly express cell surface TNFSF14 in response to target cell stimulation**

We analysed whether tumor-mediated induction of gene expression was mirrored by expression of the corresponding proteins. The K562 tumor cell line induced the expression of CD69, TNFRSF9 (CD137) and TNFSF14 protein at the NK cell surface (Fig 2A). TNFSF14 was induced several fold higher on the R-NK (CD107+) cells compared to their non-responding counterparts (CD107<sup>neg</sup>, NR-NK; Fig. 2A), in agreement with the microarray data. In contrast, little induction of CD160 protein was observed. Increased SELL gene expression (encoding L-selectin, CD62L) was detected in the R-NK population (Fig. 1). However, CD62L was rapidly and completely removed from the cell surface of the R-NK cells (Fig. 2A), consistent with previous observations demonstrating protease mediated CD62L shedding upon NK cell activation (26). The K562 cell line was not unique in its ability to induce TNFSF14 expression in NK cells and several other human cell lines representing both hematopoietic and solid tumors preferentially induced the expression of TNFSF14 on tumor responding (CD107+) NK cells (Fig. S1).

Analysis of the kinetics of TNFSF14 protein expression revealed that like CD69, TNFSF14 was detectable at the cell surface within one and a half hours of tumor cell contact. Furthermore, both CD69 and TNFSF14 remained at the cell surface in response to continual stimulation (Fig. 2B). Although we detected TNFSF14 induction using gene expression profiling, we considered the possibility that the very rapid induction of TNFSF14 at the cell surface might result from the mobilisation of an intracellular pool of pre-existing protein. However, stimulation of NK cells with K562 in the presence of actinomycin D or cycloheximide revealed that the induction of TNFSF14 required *de novo* gene expression and protein synthesis (Fig. 2C).

### **TNFSF14 expression is induced by CD16 and by synergism of multiple NK cell activation receptors**

The rapid induction of TNFSF14 expression in NK cells in response to tumor cell lines suggested that the ligation of tumor-sensing NK cell activation receptors was coupled to TNFSF14 production. We analysed whether cross-linking of individual NK cell activation receptors, or combinations thereof, led to TNFSF14 expression. We loaded mouse P815 cells (bearing Fc receptors) with receptor-specific antibodies, co-cultured these antibody displaying cells with human NK cells for five hours and then analysed NK cell granule exocytosis and TNFSF14 expression (Fig. 3). Modest induction of degranulation and TNFSF14 expression was observed via cross-linking of 2B4 and NKG2D, but this was greatly increased as combinations of activation receptors were ligated. The exception was CD16, where ligation was sufficient to induce TNFSF14 expression on the majority of NK cells. These results indicate that TNFSF14 induction was coupled to the principal activation pathways of NK cells, allowing them to produce this immunomodulatory cytokine when encountering tumor cells or infected cells, either by natural killing mechanisms or via the engagement of CD16.

### **Cytokine-mediated induction of TNFSF14 and its immunomodulatory activity**

Studies on the kinetics of the immune response to infection have shown that NK cell activation is an early event, but is preceded by the synthesis of type I IFN (IFN-I) and monocyte-derived cytokines such as IL-12 and IL-18 (27). Indeed, these cytokines activate NK cells by enhancing cytotoxic activity and IFN- $\gamma$  production (28).

However, neither IFN-I nor a combination of IL-12 and IL-18 was capable of inducing cell surface TNFSF14 expression (Fig. 4A). In contrast, IL-15, a cytokine produced during the early stages of innate immunity (for example, following IFN-I stimulation of DC; (29)) did stimulate TNFSF14 expression, as did IL-2 treatment, consistent with the use of common signalling chains by the IL-2 and IL-15 receptors (Fig. 4A). Both TNF and TNFSF14 can be released from the cell surface via proteolytic cleavage (30, 31) and stimulation of NK cells with PMA and ionomycin induced shedding of both cytokines, whereas stimulation with IL-2 or IL-15 induced TNFSF14 release, but little TNF (Fig. 4B).

Co-culture of TNFSF14 transfected cells with iDC promotes DC maturation in a TNFSF14 dependent manner (32). The expression of TNFSF14 by NK cells and the importance of NK-DC interactions in the shaping of adaptive immunity (5, 33) suggested that NK cell derived TNFSF14 might participate in this cross-talk. Stimulation of immature DC (iDC; generated *in vitro* from CD14<sup>+</sup> monocytes) with purified TNF or TNFSF14 resulted in the increased expression of CD40, CD86 and HLA-DR (Fig. 4C), consistent with pro-inflammatory cytokine mediated differentiation of iDC to a more mature, antigen-presenting phenotype (32, 34). Co-culture of NK cells and DC induces CD86 expression on the DC (3, 4). Co-culture of IL-2 stimulated NK cells with iDC induced the expression of CD86 on the iDC and the use of blocking antibodies demonstrated that CD86 induction was dependent upon both TNFSF14 and TNF (Fig. 4D).

Pro-inflammatory cytokines exert their effects on multiple cell types and like TNF, TNFSF14 has previously been shown to induce the expression of ICAM-1 on endothelial cells (35). Accordingly, IL-2 stimulated NK cells induced ICAM-1 expression on human umbilical vein-derived endothelial cells in a TNFSF14-dependent manner (Fig. S2)

### **Licensed NK cells preferentially produce TNFSF14 and activate DC in response to tumor stimulation.**

The induction of TNFSF14 expression was confined to the tumor responsive NK cell population (CD107<sup>+</sup>, R-NK; Fig. 1 and 2). This suggested that TNFSF14 expression might be restricted to NK cells that were functionally licensed by virtue of expressing inhibitory receptors reactive with self-MHC class I molecules. We determined the KIR ligand haplotype of donor blood samples and then analysed K562-induced

TNFSF14 expression on NK cells from these donors according to whether the cells expressed KIR molecules specific for self-MHC class Ia molecules and the inhibitory receptor NKG2A (a receptor for the MHC class Ib molecule, HLA-E). Tumor-induced TNFSF14 expression was approximately ten fold-higher in the cells expressing NKG2A and two self reactive KIRS compared to NK cells lacking expression of these receptors (Fig. 5A and S3). Indeed, NK cells expressing either NKG2A or a single self-reactive KIR expressed significantly more TNFSF14 in response to K562 stimulation than NK cells expressing neither NKG2A nor self-reactive KIRs. Furthermore, the magnitude of TNFSF14 induction was proportional to the number of self-reactive KIRS and greater in the NKG2A<sup>+</sup> compared to the NKG2A<sup>neg</sup> population (Fig. 5B). These results demonstrate that TNFSF14 expression in response to tumor target cells preferentially occurs in the licensed NK cell population and that both NKG2A and KIRs can license TNFSF14 production. The activation of NK cells with IL-2 converts functionally hyporesponsive cells lacking inhibitory receptors for self-MHC class I molecules (i.e. unlicensed cells) into a responsive subset (11, 14). Accordingly, TNFSF14 was produced by both the licensed and unlicensed NK cell subsets when stimulated with either IL-2 or IL-15 (Fig. S4).

We then analysed the ability of the tumor-stimulated NK cells to mediate CD86 expression by iDC. We co-cultured NK cells with K562 tumor cells for four hours and sorted the R-NK and NR-NK cell fractions based on display of CD107 at the cell surface (as performed for the gene expression profiling). These sorted fractions were then analysed for TNFSF14 expression; the bulk of R-NK cells were TNFSF14<sup>+</sup>CD107<sup>+</sup>. Importantly, although some TNFSF14 expression was detected in the NR-NK fraction, the level of expression of TNFSF14 at the cell surface was much higher in the R-NK cell population (Fig. 5C). The sorted R-NK and NR-NK fractions were then co-cultured with autologous iDC for forty-eight hours in the presence of anti-TNFSF14 or a control antibody. The R-NK fraction expressing TNFSF14 induced the expression of CD86 on almost half of the iDC in a TNFSF14-dependent manner. In contrast, the NR-NK fraction produced little TNFSF14 and these NK cells did not induce CD86 expression on the co-cultured iDC (Fig. 5C and D). Thus, licensed NK cells, endowed with the ability to respond to target cell stimulation, induce the expression of TNFSF14 in response to tumor cells and these stimulated licensed cells induce DC expression of CD86 expression in a TNFSF14 dependent manner following NK-DC cross-talk.

## **Discussion.**

These results reveal that NK cell activation, either by tumor cells, IL-2 or IL-15 results in the rapid production of biologically active TNFSF14, a cytokine endowed with potent and diverse immunomodulatory activity, including the ability to enhance anti-tumor immunity (36, 37). Human NK cells expressing TNFSF14 have previously been observed in the gut, where its expression is constitutive (38). Our results demonstrate its very rapid induction on peripheral blood derived NK cells in response to a variety of stimuli. TNFSF14 was originally described as a molecule produced by activated T cells that binds to HVEM (TNFRSF14), the receptor for Herpes Simplex Virus (HSV); TNFSF14 competes with the HSV glycoprotein D molecule for HVEM binding, thereby blocking HSV entry (15). This study also identified lymphotoxin- $\alpha$  as an HVEM ligand and set the stage for subsequent studies from which a complex web of interactions between TNFSF members and both functional and decoy receptors has emerged (20, 21, 25). Several functions have since been attributed to TNFSF14, but the precise outcome of TNFSF14/HVEM interactions are dependent upon the availability of other ligands and receptors in either *cis* or *trans* conformations that compete for interaction with one another (20, 21, 25).

The ectopic expression of TNFSF14 at tumor sites enhances CTL responses (36, 37). Our results reveal that NK cells can provide the TNFSF14 upon interaction with tumor cells. Ligation of tumor sensing NK cell activation receptors thus coordinates cytotoxic granule exocytosis (leading to tumor cell destruction) with the production of chemokines, TNF, IFN- $\gamma$  and, as shown here, the rapid expression of TNFSF14. Collectively, these molecules aid local responses, such as the recruitment of inflammatory and immune cells to the lesion, and promote the maturation of DCs and skewing towards Th1 responses. A role for TNF in NK cell induced DC maturation has been reported previously (3, 5). Our results show that TNFSF14 also participates in this process. Whilst TNF and TNFSF14 share certain pro-inflammatory activities, TNFSF14 has non-redundant functions; TNFSF14 deficient mice exhibit reduced migration of cells to lymph nodes during immune responses and TNFSF14 (from a source other than T cells) is required in these animals to initiate T cell responses (39). TNFSF14 alone is not as potent as TNF in promoting DC maturation and other factors are likely required (32). The requirement for TNFSF14 in promoting adaptive responses varies according to the antigenic dose, with lower doses showing

an increased dependency on this cytokine (39). Our data, together with that previously reported (4), shows that IL-2 stimulated NK cells make little TNF. However, IL-2 activated NK cells produce TNF following NKp30 ligation and promote DC maturation in a TNF-dependent (and NKp30 dependent) manner (5). Hence, IL-2 induces TNFSF14 directly and NKp30 ligation (by the DC) induces TNF. Both cytokines then induce DC maturation. Resting NK cells have been previously shown to induce DC maturation in a TNF dependent manner (3). However, these assays were performed in the absence of tumor cells and were optimal in the presence of LPS, suggesting that in this case, DC-derived TNF may be driving the maturation process.

In our experiments, NK cells stimulated with tumor cells alone (without exogenous cytokines) produced TNFSF14, and NK-DC co-culture induced DC maturation in a TNFSF14 dependent manner, establishing a role for TNFSF14 and the HVEM axis in NK-DC cross-talk. We co-cultured both sorted R-NK and NR-NK cells with iDC and only the TNFSF14 producing R-NK cells promoted DC maturation. Importantly, immature DC express both HVEM and TNFSF14 (32, 40) and we cannot formally exclude a role for DC-derived TNFSF14 in the DC maturation process. However, our results with NK cells support data from Morel *et al*, showing that the TNFSF14 transfected cells delivered TNFSF14-dependent (and HVEM-dependent) maturation signals to iDC (32), and experiments in which TNFSF14 supplied in the *trans* configuration signals through HVEM to activate NF- $\kappa$ B (41). NK cells are also activated as a result of NK-DC cross-talk (4, 42, 43) and additional interactions between members of the HVEM regulatory network have been shown to activate NK cells (44). In addition, DC-derived IL-15 is of major importance in NK cell activation (29, 45) and the ability of IL-15 to induce TNFSF14 production by NK cells provides a basis for the activation of both NK cells and DC during their interaction. Interleukin-2 is mainly produced by CD4<sup>+</sup> cells during adaptive immune responses (46). The ability of IL-2 to induce TNFSF14 production by NK cells may simply reflect the sharing of signaling chains by the IL-2 and IL-15 receptors. However, it is now evident that NK cell responses are not confined to the early stages of an immune response but are more durable, persisting into the adaptive phases when IL-2 is more abundant (27, 47-51).

Our gene expression profiling identified the rapid induction of a number of transcripts encoding cytokines and chemokines, including GM-CSF, IFN- $\gamma$ , TNF, IL-3, IL-8 and CCL3/MIP1 $\alpha$ . The rapid induction of these genes (and others) can be

attributed in part to the presence of AU-rich elements (AREs) in the 3' untranslated regions that stabilise pre-existing mRNA upon cellular activation. Indeed, AREs were first identified in the CSF2 gene (52) and have subsequently been identified in many mRNAs encoding chemokines and inflammatory cytokines (53). However, the major TNFSF14 transcript has no readily discernible ARE and its expression was dependent upon new transcription, consistent with data showing induction in T cells via the NFAT pathway (54). With the exception of CD16, co-induction of TNFSF14 expression and granule exocytosis required the synergistic action of multiple NK cell activation receptors. This mirrors the findings of Bryceson *et al*, where similar combinations were required to induce a calcium flux, granule exocytosis and production of TNF and IFN- $\gamma$ , with CD16 being the only single receptor sufficient to induce these responses (55). Such synergy is not required for all NK cell responses, with chemokine production requiring fewer activation signals than granule exocytosis or cytokine production (56). Thus, TNFSF14 more closely resembles TNF and IFN- $\gamma$  rather than CCL3/MIP1 $\alpha$  production in terms of its induction via cell surface receptors. Whether this reflects the requirement for activation-induced gene expression for these cytokines compared to the stabilisation of pre-existing chemokine transcripts is currently under test.

Our identification of NK cell TNFSF14 expression was due to its induction in the R-NK cell population following tumor stimulation. The presence of R-NK and NR-NK populations in a single individual reflects the functional licensing of only those NK cells that express inhibitory receptors against self-MHC molecules (11-14). We were able to demonstrate that TNFSF14 protein production occurred preferentially in these licensed NK cells and that both KIR family and NKG2A/CD94 receptors were involved in this process. Previous studies have shown that self-reactive KIRs, as well as other MHC class I receptors, notably NKG2A/CD94 and ILT2, play a role in functional licensing (12, 13, 19). At high NK:DC ratios, activated NK cells kill DC, an activity which has been suggested to allow NK cells to regulate DC function and the onset of adaptive immunity (3, 43). Restricting target-cell induced TNFSF14 production to licensed NK cells may be important in allowing the activated NK cell to kill the DC if required. Furthermore, restricting the expression of TNFSF14 to this self-MHC regulated population of NK cells clearly provides a safeguard against the release of this pro-inflammatory mediator in response to healthy tissue. The emerging evidence for a role for TNFSF14 in inflammatory disease is

testament to the need for such regulation (57, 58). However, infection may require the host to maximise its responses and utilise unlicensed NK cells that are uninhibited by self-MHC class I molecules; pro-inflammatory cytokines have been shown to override the licensing mechanism and allow unlicensed cells to respond (11, 59). Accordingly, both licensed and unlicensed NK cells produced TNFSF14 when stimulated with IL-2 or IL-15. *In vivo*, such responses are likely to be localised to the lesion, thereby minimizing collateral damage.

In summary, we have shown that human NK cell activation leads to the induction of TNFSF14 expression. The ability of NK cells to produce TNFSF14 confers the potential to modulate many cell types involved in immune and inflammatory responses. In particular, the ability to aid DC maturation provides a key link between innate and adaptive immunity. The broad immunomodulatory activity of TNFSF14 must be efficiently regulated and restricting TNFSF14 expression to the licensed NK cell population provides a safeguard by which potentially damaging activity against healthy tissues is restricted.

## **Materials and Methods**

### **Primary cell isolation and culture.**

Samples were collected following informed consent and ethical review by the Leeds Teaching Hospitals NHS Trust. Peripheral blood mononuclear cells from healthy donors were used to purify NK cells (by indirect selection) or monocytes (via CD14 selection) using reagents from Miltenyi Biotec. Monocytes were cultured for five days in the presence of 800 U/ml GM-CSF and 400 U/ml IL-4 (R&D Systems) to generate immature dendritic cells (iDC). KIR and KIR ligand typing was performed using a KIR HLA ligand kit (from Olerup) that amplifies pertinent alleles of the HLA-C1, -C2, Bw4<sup>Thr80</sup>, Bw4<sup>Ile80</sup> and HLA-A<sup>Bw4</sup> groups to be identified.

### **Gene expression analysis.**

A scaled up NK cell degranulation assay (60-62) provided the material for the microarray analysis. After four hours of co-culture, cells were sorted on a Beckman Coulter MoFlo cell sorter and responding (R) and non-responding (NR) NK cells purified according to the CD56<sup>dim</sup>CD107<sup>neg</sup> and CD56<sup>dim</sup>CD107<sup>+</sup> phenotype respectively. cDNA was synthesised from fractions pooled from three donors

(collected from six experiments) and a separate sample from one donor (collected from three experiments), each sample containing approximately one million cells. Array analysis was performed using Illumina Sentrix HumanRef-8 v3 Beadchips and an Illumina BeadArray reader. The data is available from the Gene Expression Omnibus ([ncbi.nlm.nih.gov/geo/](http://ncbi.nlm.nih.gov/geo/)); accession number GSE55977. For validation, R-NK and NR-NK fractions were sorted from repeat degranulation assays and analysed using quantitative RT-PCR with Taqman probes (from ABI/Life Technologies). Samples were normalised to 18S rRNA and compared using  $\Delta\Delta\text{CT}$  method, as previously described (63).

### **TNFSF14 expression.**

TNFSF14 (and TNF) expression by responding and non-responding NK cells was assayed using antibodies against CD56, CD3, CD107, TNF and TNFSF14 and a BD Biosciences LSRII flow cytometer (details of antibody clones are provided in the supplementary information). Analysis was performed on NK cells treated with 50U/ml IFN- $\gamma$ , 20ng/ml IL-12 plus 20ng/ml IL-18, 300U/ml of IL-2, 30ng/ml IL-15 or 50ng/ml PMA plus 500ng/ml ionomycin (Miltenyi Biotech) for eight hours before analysis. All degranulation assays used an Effector:Target ratio of 1:1 with either tumor target cells or antibody loaded P815 cells. For the latter, P815 cells were loaded with combinations of anti-NK receptor antibodies (or an IgG control) at 7 $\mu$ g/ml for 20 mins at room temperature and washed once before NK cell co-culture. Soluble cytokine was detected by ELISA using supernatants from NK cells stimulated with IL-2, IL-15 or PMA/ionomycin (or in media alone) for 24 hours. Supernatants were diluted and assayed using either a TNFSF14 ELISA (R&D Systems) or TNF ELISA (Peprotech).

### **NK-DC Co-culture.**

Immature DC were prepared from monocytes (as described above) and fresh autologous NK cells were purified on day 5 of the iDC culture. NK cells were co-cultured with K562 and R-NK and NR-NK fractions collected by FACS based on CD107 expression. These sorted NK cells were co-cultured with iDC at a 1:1 ratio for 48 hours in either media alone or 12 $\mu$ g/ml of neutralizing anti-TNFSF14 antibody (goat polyclonal, R&D Systems), anti-TNF (goat polyclonal, R&D Systems) or normal goat IgG. IL-2 activated NK cells were co-cultured at an NK:DC ratio of 1:3

for 15 hours. The IL-2 activated NK cells were co-cultured with iDC at lower NK:iDC ratios and for less time than those used for tumor-stimulated NK cells because of the propensity of the more cytotoxic IL-2 activated NK cells to kill iDC (64). Induction of CD86 expression was analysed on the DCs in the co-culture by gating on CD11c<sup>+</sup> cells.

Further details are provided in supplementary information.

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**Author contributions:** The study was designed by TDH, EBW and GPC. Gene expression profiling and analysis was performed by TDH, CV, BT and VMT, functional studies were performed by TDH, EVIB, AVB and EBW. GPC, TDH and EBW wrote the paper with input from all authors.

**Conflict of interest disclosure:** None.

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## Figure Legends

### Figure 1

#### Gene expression profiling of tumor stimulated human NK cells

(A) Primary human NK cells (isolated from healthy donors) were co-cultured for 4 hours with K562 tumor cells and the responding (R-NK) and non-responding (NR-NK) cell populations were identified by staining for cell surface expression of CD56 (as a marker of NK cells) and CD107 (as a marker of NK cell degranulation). The R-NK and NR-NK populations were purified by FACS and used as a source of mRNA for cDNA synthesis and gene expression profiling using Illumina arrays. The inset shows CD107 display (x-axis) versus CD56 expression (y-axis) on NK cells in the absence of tumor targets.

(B) Venn diagram of the number of genes exhibiting a significant difference in expression between the R-NK and NR-NK cell populations (greater than 1.5 fold change;  $P < 0.05$ ) of samples from a single donor and three pooled donors. A list of the 541 genes differentially expressed in both the single donor and pooled donor datasets is provided in supplementary dataset 1. The array data is available from the Gene Expression Omnibus ([ncbi.nlm.nih.gov/geo/](http://ncbi.nlm.nih.gov/geo/)); accession number GSE55977. The graph shows validation (using quantitative RT-PCR) of a selection of genes exhibiting differential expression in the array analysis (in order of increasing expression in the R-NK population, as determined by the array analysis).

(C) Summary of differential expression between the R-NK and NR-NK samples. Selected genes exhibiting  $>1.5$  fold upregulation ( $P < 0.05$ ) in the R-NK cell fraction are shown according to function. Genes with a defined AU rich element (ARE) in the 3' untranslated region (52, 53, 65, 66) are indicated (\*), as are genes encoding molecules in the HVEM regulatory axis (\*\*); TNFSF6 (FASL) has an ARE (67) and it is linked to the HVEM axis via its binding to DcR3(\*\*\*). Additional details (gene ID, function and references) are provided in the supplementary information.

(D) The HVEM regulatory axis. The top row indicates ligands, the middle row receptors. Both TNF (TNFSF2) and TNFSF14 (LIGHT) activate NF- $\kappa$ B. This panel is based on information presented in published reviews (20, 21).

## Figure 2

### **Tumor cell stimulation of NK cells induces production of TNFSF14.**

(A) Expression of cell surface molecules detected by gene expression profiling versus CD107 display on unstimulated NK cells (No target) and NK cells stimulated with tumor cells (+K562). For the latter, the cell surface expression of molecules analysed on the y-axis is expressed as R/NR; the ratio of expression in the tumor responding (CD107+) versus non-responding (CD107<sup>neg</sup>) populations (based on mean fluorescence intensity on the y-axis; the corresponding gates are shown). This data is representative of three separate experiments performed.

(B) Sustained expression of TNFSF14 (and CD69) in the continued presence of tumor cells. NK cells and K562 cells were co-cultured for the times indicated and cell surface TNFSF14 and CD69 were analysed by flow cytometry. The values indicate the percentage of cells in each quadrant. cAb is an isotype control antibody for the anti-CD69 and anti-TNFSF14 staining. This experiment is representative of three separate experiments performed.

(C) Induction of TNFSF14 expression requires *de novo* gene expression. NK cells alone (no target) or NK cells co-cultured with K562 cells (+K562) were cultured for 5 hours in the presence of 5 $\mu$ M actinomycin D (ActD) to inhibit transcription or 50 $\mu$ M cycloheximide (CHX) to inhibit translation (or with DMSO, the solvent for these compounds) and the cell surface expression of TNFSF14 and CD69 analysed by flow cytometry. The values indicate the percentage of cells in each quadrant. This data is representative of two separate experiments performed.

## Figure 3

### **Induction of TNFSF14 by NK cell activation receptor cross-linking.**

Cell surface expression of TNFSF14 and CD107 was analysed on NK cells after co-culture with P815 cells displaying antibodies against NK cell activation receptors. P815 cells were loaded with antibodies against a single receptor, or combinations of receptors as indicated (or without antibody, labelled Media). K562 tumor cells were used in place of antibody loaded P815 cells as a positive control for TNFSF14 expression and degranulation. The values indicate the percentage of cells in the each quadrant. This experiment is representative of four separate experiments performed.

## **Figure 4**

### **Cytokine induction of immunomodulatory TNFSF14.**

(A) Expression of TNF or TNFSF14 in response to cytokine treatment or a combination of PMA and ionomycin (PMA/I), as indicated. NK cells ( $2.5 \times 10^5$ ) were cultured without stimulation (unstimulated) or in the presence of 50U/ml IFN- $\gamma$ , 20ng/ml IL-12 and IL-18, 300U/ml of IL-2, 30ng/ml IL-15, or 50ng/ml PMA plus 500ng/ml ionomycin (PMA/I) for eight hours. Cells were stained for cell surface TNFSF14 expression or processed for intracellular staining for TNF. The values indicate the percentage of expressing cells. This data is representative of three donors for TNF and six donors for TNFSF14.

(B) Release of soluble TNF or TNFSF14 by IL-2, IL-15 or PMA/I treated NK cells. NK cells ( $2.5 \times 10^5$ ) were stimulated with the treatments shown in panel A for twenty four hours and the supernatants analysed for TNF or TNFSF14 by ELISA (performed using at least three different donors). Media is the culture media without any added cells as a background control.

(C) Maturation of DC in response to recombinant TNFSF14. Immature (i) DC (differentiated from CD14<sup>+</sup> monocytes) were left untreated or treated with recombinant TNF or TNFSF14 (100ng/ml of each) for 24 hours and analysed for cell surface markers of DC maturation, CD86, CD40 and HLA-DR using flow cytometry.

(D) Left panel;  $1 \times 10^5$  IL-2 stimulated NK cells were applied to  $3 \times 10^5$  iDC for 15 hours in the presence of antibodies against TNFSF14 or TNF or both cytokines (or a control antibody, cAb). The induction of CD86 on the iDC was analysed by flow cytometry, the percentage of cells expressing CD86 is shown for each treatment. The right panel summarises the data collected from five donors. Expression of CD86 on the DC co-cultured with IL-2 stimulated NK cells in the presence of a control antibody (cAb) was assigned an expression level of 1. The reduction in expression of CD86 in the presence of the different anti-cytokine antibodies is indicated;  $P < 0.05^*$  and  $P < 0.005^{**}$ , as determined by the paired Student's T test.

## **Figure 5**

### **TNFSF14 is produced by functionally licensed NK cells.**

Five donors were typed for KIR ligands and self-KIR molecules as well as NKG2A expression by flow cytometry. NK cells from these donors were then co-cultured with K562 cells for six hours and cell surface TNFSF14 expression was analysed on NK

cells expressing none of these receptors, individual receptors or combinations thereof (see Fig. S3 and supplementary information for details of the gating strategy). (A) Cell surface TNFSF14 expression on NK cells expressing no detectable NKG2A or self-reactive KIRs versus NK cells expressing NKG2A and two self-reactive KIR molecules. The difference in TNFSF14 between these unlicensed and licensed NK cells is approximately ten-fold based on the mean fluorescence intensity of TNFSF14 expression. (B) Cell surface expression of TNFSF14 in response to tumor cell stimulation on NK cells expressing none (0 KIR), one (1KIR) or two self-reactive KIRs (2KIR) with or without NKG2A expression. The data show that any one of the inhibitory receptors (either NKG2A or KIR) confers a statistically significant effect on TNFSF14 expression ( $P < 0.05^*$ ,  $P < 0.005^{**}$  as determined by a two tailed Student's T test). (C) Tumor stimulated NK cells induce CD86 expression on iDC in a TNFSF14 dependent manner. NK cells were co-cultured with K562 tumor cells for four hours and the R-NK and NR-NK cells isolated by FACS based on CD107 display alone. Samples from these sorted populations were reanalysed for expression of cell surface TNFSF14 and CD107 molecules (centre panel; Post-sort NK) and the remaining cells co-cultured with iDC (for forty eight hours at a ratio of 1:1) in the presence or absence of 10 $\mu$ g/ml of anti-TNFSF14 antibody or a control antibody (cAb). CD86 expression of the iDC was analysed by flow cytometry and the percentage of CD86 expressing cells with the different treatments is shown. The top panel of the iDC analysis shows iDC plus control antibody (cAb), without added NK cells. (D) Induction of cell surface CD86 expression on iDC following co-culture with NR-NK or R-NK cells in the presence of either a control Ab (cAb) or anti-TNFSF14 antibody. This data is the fold change in mean fluorescence intensity of CD86 expression from three independent experiments ( $P < 0.05^*$ ,  $P < 0.005^{**}$ , as determined by a two tailed Student's T test).

Figure 1

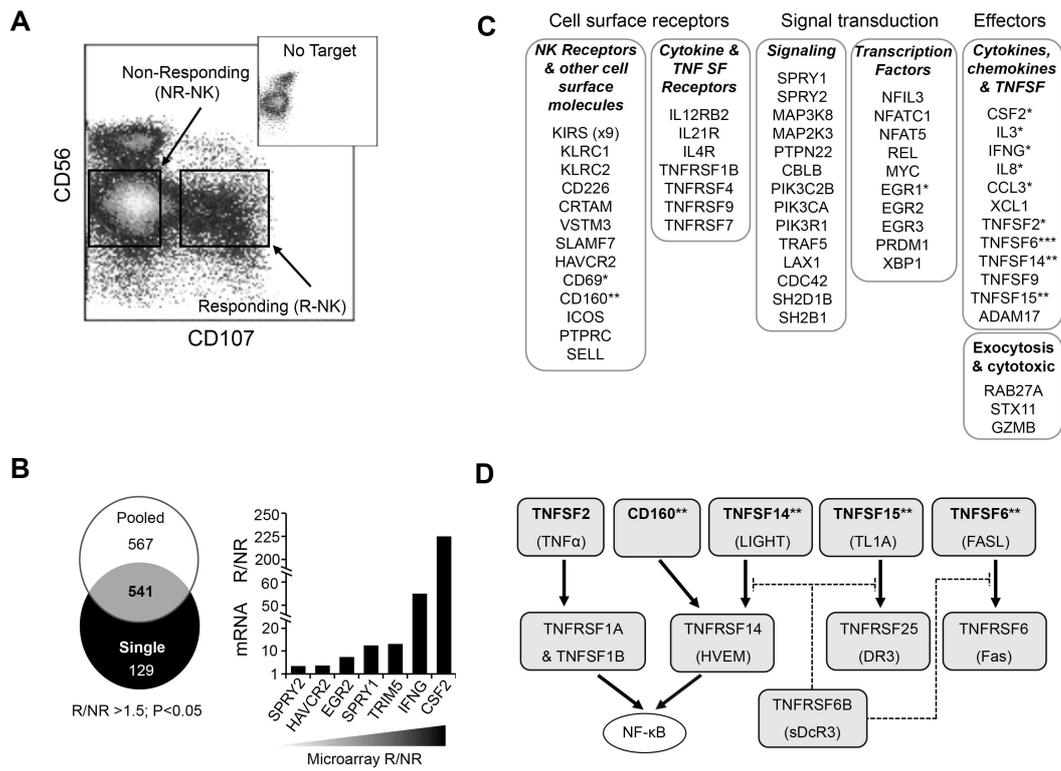


Figure 2

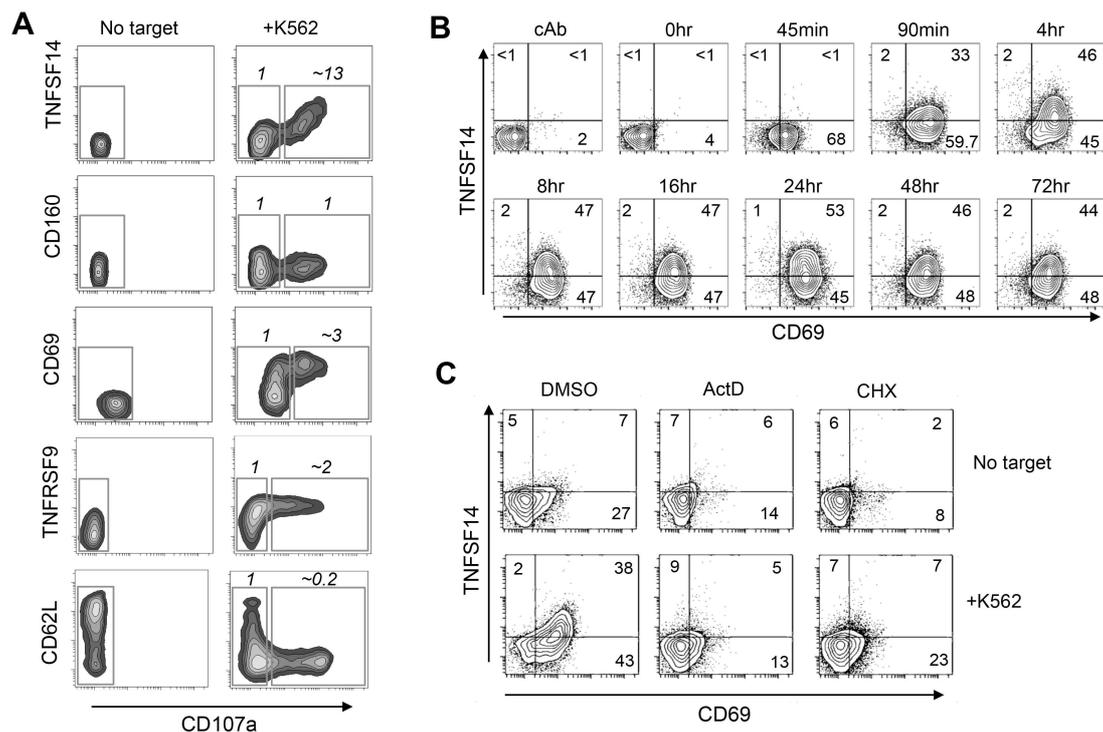


Figure 3

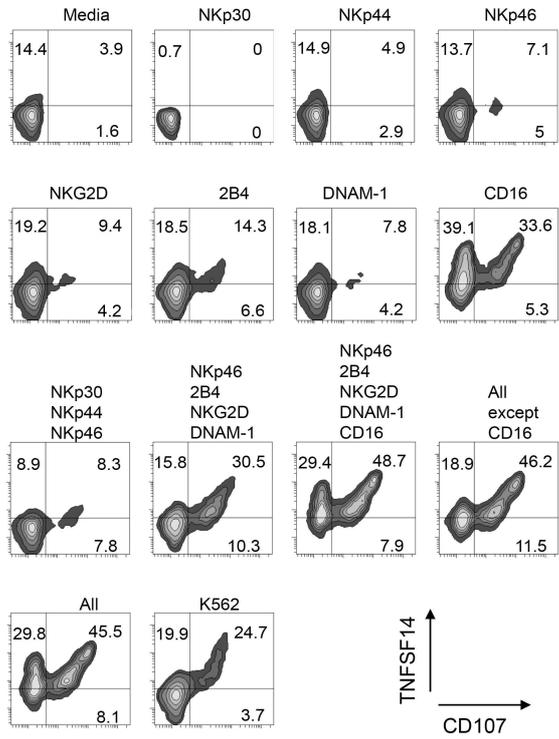


Figure 4

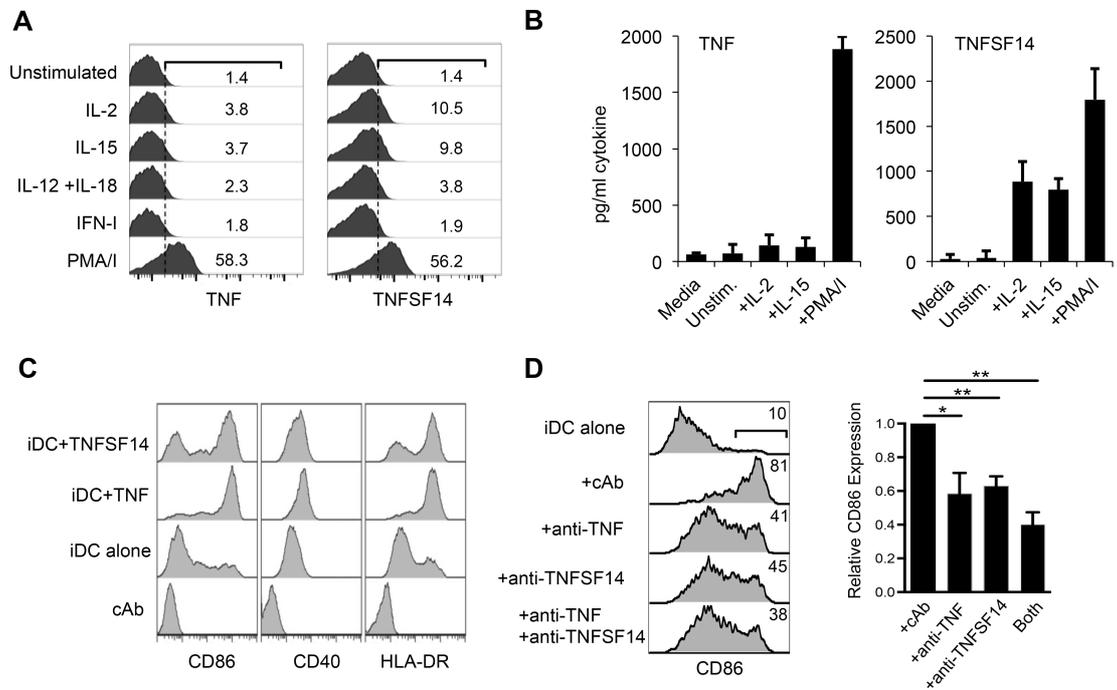


Figure 5

