A peptide:MHC based DNA vaccination strategy to activate natural killer cells by targeting killer cell immunoglobulin-like receptors.

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ABSTRACT

Background: Natural killer (NK) cells are increasingly being recognized as agents for cancer immunotherapy. The killer cell immunoglobulin-like receptor (KIR) are expressed by NK cells and are immunogenetic determinants of the outcome of cancer. In particular KIR2DS2 is associated with protective responses to several cancers and also direct recognition of cancer targets *in vitro*. Due to the high homology between activating and inhibitory KIR genes to date it has been challenging to target individual KIR for therapeutic benefit.

Methods: A novel KIR2DS2-targeting therapeutic peptide:MHC DNA vaccine was designed and used to immunize mice transgenic for KIR genes (KIR-Tg). NK cells were isolated from the livers and spleens of vaccinated mice and then analysed for activation by flow cytometry, RNA profiling and cytotoxicity assays. *In vivo* assays of NK cell function using a syngeneic cancer model (B16 melanoma) and an adoptive transfer model for human hepatocellular carcinoma (Huh7) were performed.

Results: Injecting KIR-Tg mice with the vaccine construct activated NK cells in both liver and spleens of mice, with preferential activation of KIR2DS2-positive NK cells. KIR specific activation was most marked on the CD11b+CD27+ mature subset of NK cells. RNA profiling indicated that the DNA vaccine upregulated genes associated with cellular metabolism and downregulated genes related to histone H3 methylation, which are associated with immune cell maturation and NK cell function. Vaccination led to canonical and cross-reactive peptide:MHC specific NK cell responses. *In vivo*, DNA vaccination led to enhanced anti-tumour responses against B16F10 melanoma cells and also to enhanced responses against a tumour model expressing the KIR2DS2 ligand HLA-C*0102.

Conclusion: We show the feasibility of a peptide-based KIR-targeting vaccine strategy to activate NK cells and thence generate functional anti-tumour responses. This approach does

not require detailed knowledge of the tumour peptidomes, nor HLA-matching with the patient. It therefore offers a novel opportunity for targeting NK cells for cancer immunotherapy.

INTRODUCTION

The potential of natural killer (NK) cells for treating cancer is becoming increasingly recognized. Current NK cell therapeutics include adoptive transfer of NK cells, CAR-NK cells, cytokine-based activation of NK cells and checkpoint inhibitors, with methods to specifically target endogenously expressed activating receptors being less common [1]. In order to develop new methods to target NK cells requires a detailed understanding of the receptor:ligand interactions between NK cells and their cancer targets [2 3]. One important family of NK cell receptors are the killer cell immunoglobulins like receptors (KIR). These form a polymorphic gene family of receptors with MHC class I ligands [4]. KIR have been implicated in susceptibility to and the outcome of many different cancers [5-11]. Thus targeting the KIR could form part of a therapeutic strategy to treat cancer [12].

The KIR can be activating or inhibitory with the MHC class I ligand specificities of the inhibitory KIR being relatively well defined. However the ligand specificities of the activating KIR have been much harder to identify. Recent work has shown that activating KIR can have an HLA class I-restricted peptide specificity [13-16]. Whilst T cell receptors have a tight restriction on the peptide:MHC complexes that they bind, the KIR recognize families of peptide:MHC complexes in a motif-based manner allowing recognition of multiple peptides and HLA class I allotypes [17 18]. Utilising this cross-reactive specificity of KIR offers novel opportunities for immunotherapy in which precise peptide:HLA matching to the target is not required.

KIR2DS2 is an activating receptor that recognizes group 1 HLA-C molecules in combination with different viral and synthetic peptides, and we have recently shown that KIR2DS2 recognizes highly conserved flaviviruses and hepatitis C peptides with an alanine-threonine

sequence at the C-terminal -1 and -2 positions of the peptide in the context of HLA-C [14 19]. Activating KIR have been associated with protective responses against cancer. Specifically, KIR haplotypes containing activating KIR confer protection against relapse of acute myeloid leukaemia (AML) following bone marrow transplantation, and this has been mapped to the region of the KIR locus that contains KIR2DS2 [20-22]. In cord blood transplantation the benefit of KIR2DS2 is augmented if the recipient of the transplant possess group 1 HLA-C allotypes, the putative ligands for KIR2DS2 [23]. KIR2DS2 has also been associated with protection against a number of solid tumours including cervical neoplasia, breast cancer, lung cancer, colorectal cancer and hepatocellular carcinoma [6 7 24-26]. *In vitro*, recognition of cancer cell lines for KIR2DS2 has been observed, but is not specific and also encompasses inhibitory KIR2DL2/3 [27]. Additionally, KIR2DS2-positive NK cells appear to express higher levels of FcγRIII (CD16), have enhanced functionality and confer enhanced protection against glioblastoma in a xenograft model [28]. Consistent with this enhanced functionality, in a clinical trial of an anti-GD2 antibody in neuroblastoma, KIR2DS2-positive patients had improved survival compared to KIR2DS2-negative patients [29].

Targeting KIR2DS2 in an immunotherapeutic strategy is challenging as it shares more than 98% sequence homology with the inhibitory receptors KIR2DL2 and KIR2DL3 making antibody-based therapeutics challenging. However KIR2DS2 does have a distinct peptide:MHC specificity suggesting that there is potential for developing peptide-based approaches to activate NK cells in a manner analogous to targeting cytotoxic T cells [30]. Furthermore as it has a broad peptide:MHC class I specificity, it has the potential to recognize multiple different peptide:MHC combinations, consistent with observations of protection in both viral diseases and cancer [14 23]. To investigate the therapeutic potential of targeting

activating KIR in a peptide-dependent manner, we have tested a DNA vaccine strategy to activate NK cells through KIR2DS2.

METHODS

Mice vaccination and tumour models

KIR transgenic mouse expressing a complete human KIR B haplotype, on a C57BL6 background MHC class I–deficient $Kb^{-/-}$ $Db^{-/-}$ were a kind gift from J Van Bergen and kept under specific pathogen-free conditions [31]. DNA constructs were made expressing HLA-C*0102 alone or linked with the T2A self-cleaving peptide, and peptide and cloned into pIB2 [14]. For the B16 model, mice were injected intramuscularly with 50µg DNA. $2.5x10^5$ B16F10 cells into the mice left flank, and 50ug of DNA plasmid on days 0 and 7. For the Huh7 model, NSG mice were injected with $2x10^6$ Huh7-C*0102 cells subcutaneously and then10^6 purified NK cells from 2 weeks vaccinated KIR-Tg mice spleen were injected intravenously on days 0 and 14. NK cells were purified using MACS technology (Miltenyi Biotec, NK cell isolation kit II). Purity was > 90% NK cells with < 3% $CD3^+$ T cells.

Histology

Formalin-fixed paraffin embedded sections were generated form muscle tissue isolated 1 week after the second vaccination. Five micrometre sections were cut, dewaxed and incubated with 0.5% hydrogen peroxidase to block endogenous peroxidase activity. Following blocking with avidin-biotin blocking solution (Vector Labs) and then with 1% bovine serum albumin 20% foetal calf serum in Dulbecco's modified Eagle's medium (DMEM), sections were stained with 1:100 anti-HLA class I antibody (Invitrogen), followed by 1:800 biotinylated goat anti-rabbit secondary antibody (Vector Labs) and then avidin biotin-peroxidase (Vector Labs). Slides were developed with DAB substrate (BioGenex) then counterstained with haematoxylin blue for 30 seconds.

Cell lines

HLA class I-deficient 721.221 lymphoblastoid EBV-B cells were cultured in R10 medium (RPMI 1640 supplemented with 1% penicillin-streptomycin [Life Technologies] and 10% heat inactivated foetal bovine serum [FBS; Sigma]). 721.221 cells were transduced with the pIB2 constructs to express HLA-C*0102 alone or together with peptides LNPSVAATL, IVDLMCHATF. B16F10 cells were cultured in DMEM with 1% penicillin-streptomycin (Life Technologies) and 10% FBS.

Flow cytometry analyses

Murine lymphocytes were stained using anti-mouse CD3ε-PE, NK1.1-BV421, CD11b-APC-Cy5, CD27-BV510, KLRG1-PECy7, (Biolegend). The 1F12-FITC antibody was used to selectively stain KIR2DS2 [32]. For CD107a assays, freshly isolated splenocytes were cultured with anti-CD107a AF647 (Biolegend) and GolgiStop™ (BD Biosciences) prior to staining. Cells were stained with 1F12-FITC, CD3-PE, NK1.1-BV421 (Biolegend). For the IFN-γ secretion assay, splenocytes were surface stained with 1F12-FITC, CD3-PE, NK1.1-BV421 antibodies, fixed and permeabilised using BD Cytofix/Cytoperm buffers and then stained with anti-mouse IFNγ-APC (Biolegend). Events were acquired on Aria II (BD Biosciences) using the FACSDiva software (BD Biosciences) and analysed with FlowJo software (Treestar). For Huh7-HLA-C*0102 cytotoxicity assays target cells were co-incubated with the indicated NK cell population for 4 hours. Cells were then stained with LIVE/DEAD™ stain (ThermoFisher Scientific) and analysed by flow cytometry, gating on the target cell population identifiable by GFP within the HLA-C*0102 construct.

RNA Seq data analysis and processing

RNA was isolated from murine splenic NK cells using the RNeasy Kit (QIAGEN) and prepared the QIAseq UPX 3' Transcriptome Kit (QIAGEN). 10ng purified RNA was used for the NGS libraries. The library pool was sequenced on a NextSeq500 instrument. Raw data was de-multiplexed and FASTQ files generated using bcl2fastq software (Illumina inc.). FASTQ data were checked using the FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). For differential gene expression analysis, raw counts from RNA-Seq were processed in Bioconductor package EdgeR[33], variance was estimated and size factor normalized using TMM. Genes with minimum 4 reads at minimum 40% samples were included in the downstream analyses. Differentially expressed genes (DEG) were identified applying significance threshold FDR $p<0.05$. Blind, normalized log2 values calculated by EdgeR were used for principal component analysis and to calculate Euclidean distances for hierarchical clustering using Ward's method. For heatmaps the normalized log2 values of all high-fold change peaks were used to hierarchically cluster peak regions into seven clusters, with the top 100 most variable genes (based on calculated variance across all samples). Gene ontology and pathway enrichment analysis were done using CAMERA [34], Ensemble of Gene Set Enrichment Analyses (EGSEA) [35] and ToppGene [36] . All analyses used default settings considering mouse orthologues from the MSigDB v5.2 databases retrieved from http://bioinf.wehi.edu.au/software/MSigDB/. Only pathway terms with a minimum of 25 genes were considered and used for multiple-hypothesis correction. Enriched pathways were filtered for those that showed Padj < 0.05 for both P values as calculated for the contrast C*0102-IVDL vs C*0102-AAA in KIR2DS+ and KIR2DS2- NK cell populations. Pathway results were further filtered on those that showed the lowest p.adj values.

Immunoblotting

NK cells were obtained from murine spleens using an NK cell isolation kit (Miltenyi Biotech Ltd) and lysed in NP40 Cell Lysis Buffer (Fisher Scientific UK Ltd). Primary antibodies from Cell Signalling Technology against Di-Methyl-Histone H3 (Lys4) (#9725), Tri-Methyl-Histone H3 (Lys27) (#9733), Histone H3 (#14269) and actin (#3700) were used in conjunction with HRP-conjugated secondary antibodies. Proteins were visualised using ChemiDoc-It Imaging system and VisionWorks software.

Statistical analysis

Experimental statistical analyses were performed using Graph-Pad Prism 7.0 software. Student two-tailed t test was used for comparison between two groups and two-way ANOVA with post-hoc analysis were used to compare more than two groups. Data were considered statistically significant at $p< 0.05$. For the tumour model statistical comparisons between survival to the humane end point was performed by Log-rank test (Mantel-cox).

RESULTS

Activation of NK cells using peptide:MHC DNA vaccine to target KIR2DS2

We developed a DNA construct that expresses the peptide:MHC (pMHC) ligand for KIR2DS2 as a single ORF, building on the model that KIR2DS2 is a cross-reactive receptor that recognises different pMHC ligands, and that in order to target KIR2DS2 for therapeutic benefit it is therefore not required to know the peptide ligand of the tumour being targeted, or to match for the HLA of the patient. These constructs contained HLA-C*0102 linked to a viral peptide and separated by a T2A self-cleaving sequence and with an E3/19K endoplasmic reticulum (ER) targeting sequence upstream of the peptide [14]. The constructs were made with two previously described KIR2DS2-binding peptides: LNPSVAATL (C*0102-LNP) and IVDLMCHATF (C*0102-IVDL) and cloned in to the pIB2 vector (Fig. 1A). They were used as naked DNA vaccines to immunize KIR transgenic (KIR-Tg) mice [31]. These mice express a human KIR haplotype B locus that includes KIR2DS2 and are backcrossed onto an MHC class I negative background. An MHC class I deficient mouse was used to avoid presentation of peptides onto endogenous murine MHC class I and hence confounding T cell or NK cell responses. In these animals KIR2DS2 expression is unaffected by the absence of an endogenous HLA-C ligand [31]. KIR-Tg mice were injected intramuscularly with 50µg of DNA and expression of MHC class I analysed by immune histochemistry one week after the final injection. This demonstrated expression of the construct within muscle tissues (Fig. S1). KIR-Tg mice were the injected intramuscularly with 50µg of DNA, without additional adjuvant, weekly for two weeks. Mice were sacrificed one week after the final injection, and splenocytes and hepatic lymphocytes isolated using KLRG1 expression as a marker of NK cell activation that is also associated with NK cell proliferation and maturation [37 38]. Results were compared to DNA constructs encoding HLA-C*0102 alone, or DNA encoding HLA-C*0102 in combination with a control peptide, IVDLMCHAAA (C*0102-AAA), in which the P9 and P10

residues of the peptide IVDLMCHATF had been mutated to alanine to abrogate binding to KIR2DS2 and HLA-C*0102 respectively. Injection with DNA encoding viral peptides induced activation of splenic NK cells as indicated by KLRG1 expression, but was greatest in the C*0102-IVDL group (40%) compared to C*0102-AAA (28%), $p<0.001$ (Fig. 1B and 1C). However we did not observe a specific increase in the frequency of KIR2DS2-positive NK cells (data not shown).

In paired analyses KLRG1 expression was upregulated to a significantly greater extent on KIR2DS2-positive (KIR2DS2+) versus KIR2DS2-negative (KIR2DS2-) splenic NK cells ($p<0.01$ for C*0102-IVDL and $p<0.05$ for C*0102-LNP), but not by control constructs (Fig. 1D). Activation was more marked in mice injected with C*0102-IVDL compared to C*0102-LNP, consistent with the stronger binding *in vitro* noted previously in tetramer binding experiments [14]. Furthermore, activation of hepatic NK cells was noted only in experiments using the C*0102-IVDL construct, with the expression of KLRG1 being significantly higher on the KIR2DS2+ versus KIR2DS2- population ($p<0.01$) (Fig. 1E).

Peptide:MHC vaccination upregulates KLRG1 expression on KIR2DS2-positive mature splenic NK cells

Maturation of NK cells can be characterized by expression of CD11b and CD27, with CD11b-CD27+ NK cells being classified as the least mature, CD11b+CD27+ NK cells as mature, and CD11b+CD27- NK cells terminally differentiated. Overall there were no substantial changes in the relative proportions of the different CD11b/CD27 subsets induced by these constructs (Fig. S2). KLRG1 was upregulated by C*0102-IVDL vaccination on CD11b+CD27+ splenic 2DS2+ NK cells compared to C*0102-AAA: 27% versus 18% ($p<0.05$) respectively and on terminally mature CD11b+CD27- splenic 2DS2+ NK cells compared to both C*0102-AAA

and C*0102: 62% versus 40% ($p<0.001$) and 46% ($p<0.005$) respectively (Fig. 2A and 2B). However effects on hepatic lymphocytes were less clear, with upregulation of KLRG1 only noted on terminally differentiated CD11b+CD27- NK cells in the C*0102-IVDL group only ($p<0.05$ vs control vaccinations) (Fig. 2C and 2D). Conversely we observed up regulation of CD69 on hepatic, but not splenic CD11b+CD27+ and CD11b+CD27- 2DS2+ NK cells (Fig. 2E). This difference may reflect the kinetics of CD69 versus KLRG1 expression and also the observation that CD69 can mark tissue resident NK cells, in addition to being a marker of activation.

Specific activation of KIR2DS2+ versus KIR2DS2- NK cells was most marked on the CD11b+CD27+ splenic NK cells ($p<0.001$), as compared to terminally differentiated CD11b+CD27- NK cells (Fig. 2F and 2G). Analysis of draining lymph nodes demonstrated a trend towards an increase in KIR2DS2-positive NK cells and also towards expression of KLRG1 on NK cells in the C*0102-IVDL group as compared to the C*0102-AAA group (Fig.S3). After four weekly injections we observed activation of NK cells, with upregulation of KLRG1 on splenic and hepatic NK cells with both peptide-containing constructs, and KIR2DS2-specific activation in the CD11b+CD27+ double-positive population with C*0102-IVDL (Fig. S4). However at this timepoint there was no significant difference between the C*0102-LNP and C*0102-IVDL constructs. This lack of difference may reflect that a ceiling for activation using this strategy may have been reached, or that after four doses more apoptosis is induced by the C*0102-IVDL than the C*0102-LNP construct.

Education of NK cells in the mouse is determined by interactions between the Ly49 inhibitory receptors and their MHC class I ligands. Therefore to confirm that activation of NK cells could be induced on educated NK cells, we crossed the KIR-Tg mice with wild type C57BL/6 mice

to provide MHC ligands for the murine Ly49 receptors, and immunized with 2 doses of DNA. Consistent with findings in the MHC deficient mouse, we observed KLRG1 upregulation on mature CD11b+ CD27+ KIR2DS2+, and terminally differentiated CD11b+ CD27- KIR2DS2+NK cells ($p<0.01$ and $p<0.05$ respectively) (Fig 2G).

NK cells from C*0102-IVDL and C*0102-AAA vaccinated mice were profiled by RNA seq one week following vaccination (see Fig. S5 for gating strategy). Principal component analysis (PCA) showed that KIR2DS2+ NK cells from C*0102-IVDL vaccinated mice formed a discrete cluster to KIR2DS2+ NK cells from C*0102-AAA vaccinated mice, in contrast to KIR2DS2- NK cells (Fig. 3A). Those mice receiving C*0102-IVDL had upregulation of genes in pathways associated with cellular metabolism, as compared to those receiving control C*0102-AAA vaccination (Fig. 3B). Differential gene expression analysis identified 42 differentially expressed genes (FDR<0.05) between the KIR2DS2+ NK cells from the C*0102-IVDL versus control groups (Table S1). These included upregulation in genes associated with: RNA binding and splicing; metabolism, especially glutathione metabolism; and regulation of IFN alpha (Fig. 3C). Additionally, we observed downregulation of genes related with histone H3 dimethylation at K4 (H3K4me2) and trimethylation at K27 (H3K27me3), consistent with a change in transcriptional regulation induced by vaccination (Fig. 3D). Purified NK cells from the spleens of vaccinated mice had upregulation of both H3K4me2 and H3K27me3 in IVDL mice as compared to control (Fig. 3E). Changes in H3K4 and H3K27 methylation have been associated with both NK cell activation and maturation [39 40]. In particular promoters with H3K4 and H3K27 methylation marks are considered to be in a poised, ready to transcribe state, which is consistent with our analysis at 7 days post-vaccination [41]. Whilst H3K4me2 and H3K4me3 marks are generally concordant in T cell analyses, H3K4me2 marks are associated with genes that are rapidly transcribed after stimulation consistent with such a poised state [42 43]. Detailed

temporal analysis by chromatin immunoprecipitation is required to further clarify the changes in histone methylation induced by vaccination.

A KIR targeting vaccine augments NK cell functions

To test for functional effects of our DNA vaccination strategy, KIR-Tg mice were inoculated subcutaneously in the flank with B16F10 melanoma cells and injected intramuscularly with DNA on the same day and one week later. Growth of B16F10 cells was significantly attenuated by day 12 in mice given C*0102-IVDL as compared to those given C*0102-AAA or unvaccinated ($p<0.02$) (Fig. 4A). In vitro, KIR2DS2+ NK cells, but not KIR2DS2- NK cells, from C*0102-IVDL-vaccinated mice had increased degranulation to B16F10 cells as compared to the control vaccinated mice ($p<0.05$) (Fig. 4B). As B16F10 cells do not express HLA-C these data indicate that DNA vaccination with C*0102-IVDL activates NK cells and induces MHC class I unrestricted responses, which is relevant as many cancers can down-regulate MHC class I.

To identify if peptide-specific NK cell responses were generated using this strategy, NK cells from vaccinated mice were tested against the MHC class I-negative target human cell line 721.221 transfected with either HLA-C or a construct of HLA-C in combination with the peptides LNPSVAATL and IVDLMCHATF [14]. KIR2DS2+ NK cells from mice vaccinated with C*0102-IVDL demonstrated increased activity against 721.221 cells expressing HLA-C*0102 in combination with both KIR2DS2-binding peptides as compared to 721.221 cells transfected with HLA-C*0102 alone ($p<0.01$ for both LNP and IVDL targets) (Fig. 4C). No effect was observed for KIR2DS2- NK cells. Similarly, we observed an increase in IFNγ secretion for the KIR2DS2+ NK cells from IVDL vaccinated mice when incubated with the

C*0102-LNP target as compared to KIR2DS2+ NK cells from AAA vaccinated mice (42.5% vs 30.5%, $p<0.05$) (Fig. 4D). Thus activation using a peptide:MHC strategy can generate both specific and cross-reactive peptide responses.

To identify if this strategy could recognize a human MHC class I target in vivo, we used the Huh7 hepatocellular carcinoma cell line transfected with the KIR2DS2 ligand HLA-C*0102. In vitro killing assays demonstrated that NKL cells transfected with KIR2DS2 killed Huh7-C*0102 cells to a greater extent than untransfected NKL cells ($p<0.0001$) (Fig. 4D), thus validating the cell line as KIR2DS2-specific target. As the KIR-Tg mice are not permissive for human tumours we used an adoptive transfer model to test the effects of our DNA vaccine on Huh7-C*0102 cells. KIR-Tg mice were vaccinated with two doses of the DNA vaccine C*0102-IVDL or the control vaccine weekly, and then purified splenic NK cells containing <1% CD3+ T cells, were adoptively transferred into immunodeficient NSG mice, which had been inoculated with Huh7-C*0102 cells. We observed a significantly delayed growth of the tumour in mice that received C*0102-IVDL-stimulated NK cells compared to control vaccine concomitant (Fig. 4E). Thus stimulation of NK cells via KIR2DS2 can generate anti-cancer reactivity against HLA-C expressing human tumour cells.

DISCUSSION

We have developed a novel strategy to activate NK cells through KIR2DS2 in a peptide:MHC dependent manner using a construct that expresses both the cognate MHC and peptide. This strategy may be relevant for other activating KIR, such as KIR2DS1and KIR2DS4 which have been convincingly shown to have peptide:MHC specificities [13 15 44]. Additionally KIR3DS1, which is associated with protection from HIV, is more controversially thought to have a peptide dependent HLA-B specificity [45]. Thus our work may be relevant for a number of different KIR in addition to KIR2DS2.

KIR2DS2-mediated activation generated a cytotoxic immune response against targets both with and without a cognate KIR2DS2-ligand, and thus utility of this strategy as a therapeutic may not be critically dependent on MHC class I expression by the target. Furthermore, previous work has also shown that KIR2DS2+ NK cells can recognize several different cancer targets *in vitro*, including cell lines derived from prostate, breast and ovarian carcinomas [27]. This recognition was beta-2-microglobulin independent, suggesting that there may also be non-peptide:MHC class I ligands for KIR2DS2 and is consistent with our observation that KIR2DS2+ murine NK cells are more cytotoxic against B16F10 melanoma cells even in unvaccinated mice. Additionally, KIR2DS2+ NK cells appear to have a greater potential to mediate antibody-dependent cellular cytotoxicity (ADCC) *in vitro* and *in vivo* [28 29]. Therefore KIR2DS2 may be an attractive target for cancer immunotherapy in combination with antibody based therapeutics.

In our experimental model KIR2DS2 expression is unaffected by the absence of an endogenous HLA-C ligand as NK cell education is driven by inhibitory, rather than activating, receptors for MHC class I [31]. This could be further investigated in humanised mouse models, with the

caveat that this requires administration of human IL-15 to maintain NK cells, and so will require careful interpretation [46 47]. Despite the absence of a known ligand for KIR2DS2, we observed activation of both KIR2DS2+ and KIR2DS2- NK cells *in vivo*. This is not unexpected as a DNA vaccine may generate an inflammatory response thus activating NK cells, and CpG sequences within the DNA vaccine may activate NK cells either directly or indirectly via dendritic cells. Importantly, NK cells are well known to undergo reciprocal activation with dendritic cells which could lead to cytokine release and further activation of NK cells in a non-specific manner through release of IL-12 [48]. This is consistent with our observations that whilst there was activation of both KIR2DS2+ and KIR2DS2- NK cells *in vivo*, activation was preferential for KIR2DS2+ NK cells. The presence of antigen non-specific responses may be important for generating more effective anti-tumour responses, and is consistent with a mode of action of NK cells in which they are activated through one receptor and recognize a target through a different one. For instance, killing of the B16F10 melanoma cell line is mediated by NKp46, and hence not MHC class I restricted [49].

Furthermore, we demonstrated peptide cross reactivity of KIR2DS2, as mice vaccinated with IVDLMCHATF were able to recognize the hepatitis C virus peptide LNPSVAATL, consistent with KIR2DS2 recognizing peptides with an AT motif at the carboxy-terminal -1 and -2 positions. Thus, in order to take our findings to the clinic it is not necessary to identify the ligand on the cancerous cell, or to match for HLA class I as KIR2DS2 may bind other group 1 HLA class I allotypes such as HLA-C*0304 [14 16]. The broad specificity of KIR2DS2 also provides an advantage for peptide-based NK cell therapy over T cell pMHC therapeutics which require more precise pMHC matching. Additionally, as the strategy provides both the peptide and the MHC class I ligand, then no HLA class I matching is required.

Plasmid DNA vaccination is a novel strategy to activate NK cells. To understand its effects in detail would be interesting in additional studies. In particular we have noted differences between splenic and hepatic NK cells. Baseline KLRG1 expression appears relatively elevated within the liver which may be the result of homeostatic proliferation or more ready activation of KIR2DS2-positive NK cells which has been observed in humans [28 38] . Additionally we formally tested only splenic NK cells *ex vivo* but not hepatic or lymph-node NK cells, which could also have anti-cancer effects.

Based on our work we propose the following model based. As there is expression of MHC class I in the muscle tissue we envisage that circulating NK cells can be activated directly within the tissues. NK cells circulate in a primed state, in comparison to T cells, so uptake by professional antigen presenting cells may not be required for NK cell activation. The activated NK cells circulate to the periphery and generate anti-cancer responses. Potentially, some may develop a "memory" phenotype within the liver. We envisage that this strategy could be readily translated to the clinic as a DNA or RNA therapeutic that activates NK cells *in vivo* to generate anti-cancer responses or augment other immunotherapeutic strategies, including antibody-dependent cellular cytotoxicity. It would however only be relevant for the approximately 50% of individuals who express KIR2DS2 as part of a KIR B haplotype. An intramuscular approach has advantages over other delivery methods, such as intra-tumoral injection as it is less invasive and could be administered in primary care settings. To harness the functional activation advantage of KIR2DS2+ NK cells, cell lines expressing peptide:MHC combinations designed to specifically activate KIR2DS2-positive NK cells could also be used in *ex vivo* expansion protocols for adoptive therapy. This strategy may offer a more controlled activation of NK cells but would be more cumbersome than an injection therapeutic.

As NK cells are involved in the early immune response to viral infections, and KIR2DS2 recognizes peptides derived from many different viruses, then targeting KIR2DS2 by DNA vaccination may also form part of an anti-viral therapeutic strategy to reduce infection and transmission in the early stages of infection. In conclusion, our work identifies a novel mechanism for activating NK cells and we propose that this may have potential as a novel therapeutic.

DECLARATIONS

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Competing interests:

The University of Southampton has applied for patents covering the use of the DNA vaccine constructs described in this manuscript

Author contributions:

SIK, PR, MDB designed experiments; PR, MDB, RF, LBL, LE-S performed experiments; AVP, PR, MDB, and SIK analysed data; CR provided key reagents; SIK, AA-S, MP supervised experiments and interpreted data; PR, MDB, AVP, AA-S and SIK wrote the manuscript.

Data availability:

Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

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FIGURE LEGENDS

Figure 1: A peptide:MHC DNA vaccine that targets KIR2DS2 activates NK cells

A) The conformation of the constructs used in this study to inoculate the mice. **B)** Gating strategy for KLRG1 on KIR2DS2+ NK cells derived from the spleens (top panels) and livers (lower panels) of KIR-Tg mice. KIR2DS2+ NK cells were identified using the antibody 1F12 and the numbers indicate the percentage positive cells in the gate. **C)** The frequency of KLRG1 expression on KIR2DS2+ NK cells in the spleen and livers of KIR-Tg mice vaccinated with DNA plasmids containing HLA-C*0102 (C*0102, light grey bars), HLA-C*0102 plus IVDLMCHATAAA (C*0102-AAA, grey bars), HLA-C*0102-LNPSVAATL (C*0102-LNP, dark grey bars), HLA-C*0102-IVDLMCHATF (C*0102-IVDL, black bars) and compared to PBS alone control mice (white bars). **D, E)** Comparison of KLRG1 frequencies on KIR2DS2+ (filled circles) or KIR2DS2- (open circles) CD3-NK1.1+ NK cells in the spleens (**D**) and livers (**E**) following vaccination. All analyses were performed one week following the second vaccination. Comparisons between two groups were made by paired t test (2 groups) and 2-way ANOVA (more than 2 groups) (*p < 0.05, **p< 0.01, ****p<0.001).

Figure 2: Vaccination activates mature and terminally differentiated NK cells.

KIR-Tg mice were injected with two doses of the indicated DNA construct one week apart and then assessed for expression of KLRG1 on CD11b, CD27 NK cell subsets from the spleens and livers one week after the final injection. **A-D)** KLRG1 on splenic CD11b+CD27+ (**A**) and CD11b+CD27- (**B**) NK cells and on hepatic CD11b+CD27+ (**C**) and CD11b+CD27- (**D**) NK cells following vaccination. N=7-14 mice per group. **E)** CD69 expression on KIR2DS2-positive CD11b CD27 NK cell subsets (n=3 per group). **F, G)** Comparison of KLRG1 expression on KIR2DS2+ and KIR2DS2- splenic NK cells in the CD11b+CD27+ (**F**) and CD11b+CD27- (**G**) sub-populations. N=7-14 mice per group. **H**) KIR-Tg mice crossed with

C57BL/6 mice were injected with two doses of the indicated DNA construct one week apart and then assessed for expression of KLRG1 on total NK cells, and CD11b, CD27, NK cell 2DS2+ and 2DS2- subsets from their spleens. N=4-6 mice per group. Comparisons between two groups were made by paired t test (2 groups) and 2-way ANOVA (more than 2 groups) (*p < 0.05, **p< 0.01, p<0.005 ****p<0.001).

Figure 3: Transcriptomic analysis of NK cells following DNA vaccination

A) Principal component analysis (PCA) of whole NK cell transcriptomes from C*0102-IVDL and C*0102-AAA vaccinated mice taken one week after the second vaccination. KIR2DS2-negative NK cells from both groups are shown in the left panel and KIR2DS2-positive NK cells in the right panel. Counts were normalized and filtered using EdgeR. The first two components of the PCA are shown. **B)** Heatmap of the top 100 differentially expressed genes derived from the comparison of KIR2DS2 positive NK cells from C*0102-IVDL and C*0102-AAA vaccinated mice. **C, D)** EGSEA analysis of C5 gene ontology (**C**) and C2 canonical pathway (**D**) signatures comparing KIR2DS2+ and KIR2DS2- NK cell populations in both C*0102-IVDL and control vaccinated mice. Effect significances were calculated individually for each arm of the study and the plots indicate the overall effects of vaccination on KIR2DS2+ NK cells in the C*0102-IVDL vaccinated mice ("positive") as compared to the other three groups ("negative"). The colour denotes the direction of the change and the size of the bar represents the -Log10(FDR). All categories shown were significant at FDR<0.05. E) Western blot analysis of histone 3 marks (H3K4me2 and H3K27me3) on purified NK cells from the spleens of vaccinated mice one week after the second vaccination with either C*0102-AAA (AAA) or C*0102-IVDL (IVDL).

Figure 4: DNA vaccination induces functional NK cell responses

A) KIR-Tg mice were injected subcutaneously with B16F10 melanoma cells on day 0 and then vaccinated intramuscularly with C*0102-IVDL (squares) or C*0102-AAA (triangles) on days 0 and 7 or untreated (circles) and tumour volume measured. (n=4 mice per group: one of two independent experiments). **B**,**C,D)** Mice were injected intramuscularly with C*0102-IVDL (black bars) or C*0102-AAA (grey bars) on days 0 and day 7 and then NK cells purified from the spleens on day 14 for *in vitro* assays of activation. **B)** shows degranulation of KIR2DS2+ and KIR2DS2- KIR-Tg NK cells to B16F10 melanoma cells (n=4 mice per group), **C)** shows degranulation of NK cells from KIR-Tg mice to human 721.221 cells expressing HLA-C*0102 alone (221-C*0102) or HLA-C*0102 in combination with the peptide: LNPSVAATL (221-C*0102-LNP) or IVDLMCHATF (221-C*0102-IVDL) and **D)** shows IFNγ expression following incubation with 221-C*0102 and 221-C*0102-LNP cells (n=7-8 mice per group). **E)** Killing of Huh7-C*0102 hepatoma cells by NKL cells either untransfected (NKL) or transfected with KIR2DS2 (NKL-2DS2) or the inhibitory receptor KIR2DL2 (NKL-2DL2) at the indicated effector to target (E:T) ratios. Cytotoxicity was determined by flow cytometry using the LIVE/DEAD™ stain. Shown are the results of two independent experiments performed in triplicate. **F**) NK cells from KIR-Tg mice vaccinated either with C*0102-IVDL or C*0102-AAA as a peptide control, were adoptively transferred into NSG mice inoculated subcutaneously with Huh7-C*0102 hepatoma cells and tumour volume was measure (n=4 mice per group, one of two independent experiments). The control mice (n=3) did not receive any NK cells. Comparisons were by Students t-test (2 groups) or two-way ANOVA (more than 2 groups). For all plots *p < 0.05, **p<0.01, ***p< 0.005, ****p< 0.0001.

A peptide:MHC based DNA vaccination strategy to activate natural killer cells by targeting killer cell immunoglobulin-like receptors.

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ABSTRACT

Background: Natural killer (NK) cells are increasingly being recognized as agents for cancer immunotherapy. The killer cell immunoglobulin-like receptor (KIR) are expressed by NK cells and are immunogenetic determinants of the outcome of cancer. In particular KIR2DS2 is associated with protective responses to several cancers and also direct recognition of cancer targets *in vitro*. Due to the high homology between activating and inhibitory KIR genes to date it has been challenging to target individual KIR for therapeutic benefit.

Methods: A novel KIR2DS2-targeting therapeutic peptide:MHC DNA vaccine was designed and used to immunize mice transgenic for KIR genes (KIR-Tg). NK cells were isolated from the livers and spleens of vaccinated mice and then analysed for activation by flow cytometry, RNA profiling and cytotoxicity assays. *In vivo* assays of NK cell function using a syngeneic cancer model (B16 melanoma) and an adoptive transfer model for human hepatocellular carcinoma (Huh7) were performed.

Results: Injecting KIR-Tg mice with the vaccine construct activated NK cells in both liver and spleens of mice, with preferential activation of KIR2DS2-positive NK cells. KIR specific activation was most marked on the CD11b+CD27+ mature subset of NK cells. RNA profiling indicated that the DNA vaccine upregulated genes associated with cellular metabolism and downregulated genes related to histone H3 methylation, which are associated with immune cell maturation and NK cell function. Vaccination led to canonical and cross-reactive peptide:MHC specific NK cell responses. *In vivo*, DNA vaccination led to enhanced anti-tumour responses against B16F10 melanoma cells and also to enhanced responses against a tumour model expressing the KIR2DS2 ligand HLA-C*0102.

Conclusion: We show the feasibility of a peptide-based KIR-targeting vaccine strategy to activate NK cells and thence generate functional anti-tumour responses. This approach does

not require detailed knowledge of the tumour peptidomes, nor HLA-matching with the patient. It therefore offers a novel opportunity for targeting NK cells for cancer immunotherapy.

INTRODUCTION

The potential of natural killer (NK) cells for treating cancer is becoming increasingly recognized. Current NK cell therapeutics include adoptive transfer of NK cells, CAR-NK cells, cytokine-based activation of NK cells and checkpoint inhibitors, with methods to specifically target endogenously expressed activating receptors being less common [1]. In order to develop new methods to target NK cells requires a detailed understanding of the receptor:ligand interactions between NK cells and their cancer targets [2 3]. One important family of NK cell receptors are the killer cell immunoglobulins like receptors (KIR). These form a polymorphic gene family of receptors with MHC class I ligands [4]. KIR have been implicated in susceptibility to and the outcome of many different cancers [5-11]. Thus targeting the KIR could form part of a therapeutic strategy to treat cancer [12].

The KIR can be activating or inhibitory with the MHC class I ligand specificities of the inhibitory KIR being relatively well defined. However the ligand specificities of the activating KIR have been much harder to identify. Recent work has shown that activating KIR can have an HLA class I-restricted peptide specificity [13-16]. Whilst T cell receptors have a tight restriction on the peptide:MHC complexes that they bind, the KIR recognize families of peptide:MHC complexes in a motif-based manner allowing recognition of multiple peptides and HLA class I allotypes [17 18]. Utilising this cross-reactive specificity of KIR offers novel opportunities for immunotherapy in which precise peptide:HLA matching to the target is not required.

KIR2DS2 is an activating receptor that recognizes group 1 HLA-C molecules in combination with different viral and synthetic peptides, and we have recently shown that KIR2DS2 recognizes highly conserved flaviviruses and hepatitis C peptides with an alanine-threonine

sequence at the C-terminal -1 and -2 positions of the peptide in the context of HLA-C [14 19]. Activating KIR have been associated with protective responses against cancer. Specifically, KIR haplotypes containing activating KIR confer protection against relapse of acute myeloid leukaemia (AML) following bone marrow transplantation, and this has been mapped to the region of the KIR locus that contains KIR2DS2 [20-22]. In cord blood transplantation the benefit of KIR2DS2 is augmented if the recipient of the transplant possess group 1 HLA-C allotypes, the putative ligands for KIR2DS2 [23]. KIR2DS2 has also been associated with protection against a number of solid tumours including cervical neoplasia, breast cancer, lung cancer, colorectal cancer and hepatocellular carcinoma [6 7 24-26]. *In vitro*, recognition of cancer cell lines for KIR2DS2 has been observed, but is not specific and also encompasses inhibitory KIR2DL2/3 [27]. Additionally, KIR2DS2-positive NK cells appear to express higher levels of FcγRIII (CD16), have enhanced functionality and confer enhanced protection against glioblastoma in a xenograft model [28]. Consistent with this enhanced functionality, in a clinical trial of an anti-GD2 antibody in neuroblastoma, KIR2DS2-positive patients had improved survival compared to KIR2DS2-negative patients [29].

Targeting KIR2DS2 in an immunotherapeutic strategy is challenging as it shares more than 98% sequence homology with the inhibitory receptors KIR2DL2 and KIR2DL3 making antibody-based therapeutics challenging. However KIR2DS2 does have a distinct peptide:MHC specificity suggesting that there is potential for developing peptide-based approaches to activate NK cells in a manner analogous to targeting cytotoxic T cells [30]. Furthermore as it has a broad peptide:MHC class I specificity, it has the potential to recognize multiple different peptide:MHC combinations, consistent with observations of protection in both viral diseases and cancer [14 23]. To investigate the therapeutic potential of targeting

activating KIR in a peptide-dependent manner, we have tested a DNA vaccine strategy to activate NK cells through KIR2DS2.

METHODS

Mice vaccination and tumour models

KIR transgenic mouse expressing a complete human KIR B haplotype, on a C57BL6 background MHC class I–deficient $Kb^{-/-}$ $Db^{-/-}$ were a kind gift from J Van Bergen and kept under specific pathogen-free conditions [31]. DNA constructs were made expressing HLA-C*0102 alone or linked with the T2A self-cleaving peptide, and peptide and cloned into pIB2 [14]. For the B16 model, mice were injected intramuscularly with 50µg DNA. $2.5x10^5$ B16F10 cells into the mice left flank, and 50ug of DNA plasmid on days 0 and 7. For the Huh7 model, NSG mice were injected with $2x10^6$ Huh7-C*0102 cells subcutaneously and then10^6 purified NK cells from 2 weeks vaccinated KIR-Tg mice spleen were injected intravenously on days 0 and 14. NK cells were purified using MACS technology (Miltenyi Biotec, NK cell isolation kit II). Purity was > 90% NK cells with < 3% $CD3^+$ T cells.

Histology

Formalin-fixed paraffin embedded sections were generated form muscle tissue isolated 1 week after the second vaccination. Five micrometre sections were cut, dewaxed and incubated with 0.5% hydrogen peroxidase to block endogenous peroxidase activity. Following blocking with avidin-biotin blocking solution (Vector Labs) and then with 1% bovine serum albumin 20% foetal calf serum in Dulbecco's modified Eagle's medium (DMEM), sections were stained with 1:100 anti-HLA class I antibody (Invitrogen), followed by 1:800 biotinylated goat anti-rabbit secondary antibody (Vector Labs) and then avidin biotin-peroxidase (Vector Labs). Slides were developed with DAB substrate (BioGenex) then counterstained with haematoxylin blue for 30 seconds.

Cell lines

HLA class I-deficient 721.221 lymphoblastoid EBV-B cells were cultured in R10 medium (RPMI 1640 supplemented with 1% penicillin-streptomycin [Life Technologies] and 10% heat inactivated foetal bovine serum [FBS; Sigma]). 721.221 cells were transduced with the pIB2 constructs to express HLA-C*0102 alone or together with peptides LNPSVAATL, IVDLMCHATF. B16F10 cells were cultured in DMEM with 1% penicillin-streptomycin (Life Technologies) and 10% FBS.

Flow cytometry analyses

Murine lymphocytes were stained using anti-mouse CD3ε-PE, NK1.1-BV421, CD11b-APC-Cy5, CD27-BV510, KLRG1-PECy7, (Biolegend). The 1F12-FITC antibody was used to selectively stain KIR2DS2 [32]. For CD107a assays, freshly isolated splenocytes were cultured with anti-CD107a AF647 (Biolegend) and GolgiStop™ (BD Biosciences) prior to staining. Cells were stained with 1F12-FITC, CD3-PE, NK1.1-BV421 (Biolegend). For the IFN-γ secretion assay, splenocytes were surface stained with 1F12-FITC, CD3-PE, NK1.1-BV421 antibodies, fixed and permeabilised using BD Cytofix/Cytoperm buffers and then stained with anti-mouse IFNγ-APC (Biolegend). Events were acquired on Aria II (BD Biosciences) using the FACSDiva software (BD Biosciences) and analysed with FlowJo software (Treestar). For Huh7-HLA-C*0102 cytotoxicity assays target cells were co-incubated with the indicated NK cell population for 4 hours. Cells were then stained with LIVE/DEAD™ stain (ThermoFisher Scientific) and analysed by flow cytometry, gating on the target cell population identifiable by GFP within the HLA-C*0102 construct.

RNA Seq data analysis and processing

RNA was isolated from murine splenic NK cells using the RNeasy Kit (QIAGEN) and prepared the QIAseq UPX 3' Transcriptome Kit (QIAGEN). 10ng purified RNA was used for the NGS libraries. The library pool was sequenced on a NextSeq500 instrument. Raw data was de-multiplexed and FASTQ files generated using bcl2fastq software (Illumina inc.). FASTQ data were checked using the FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). For differential gene expression analysis, raw counts from RNA-Seq were processed in Bioconductor package EdgeR[33], variance was estimated and size factor normalized using TMM. Genes with minimum 4 reads at minimum 40% samples were included in the downstream analyses. Differentially expressed genes (DEG) were identified applying significance threshold FDR $p<0.05$. Blind, normalized log2 values calculated by EdgeR were used for principal component analysis and to calculate Euclidean distances for hierarchical clustering using Ward's method. For heatmaps the normalized log2 values of all high-fold change peaks were used to hierarchically cluster peak regions into seven clusters, with the top 100 most variable genes (based on calculated variance across all samples). Gene ontology and pathway enrichment analysis were done using CAMERA [34], Ensemble of Gene Set Enrichment Analyses (EGSEA) [35] and ToppGene [36] . All analyses used default settings considering mouse orthologues from the MSigDB v5.2 databases retrieved from http://bioinf.wehi.edu.au/software/MSigDB/. Only pathway terms with a minimum of 25 genes were considered and used for multiple-hypothesis correction. Enriched pathways were filtered for those that showed Padj < 0.05 for both P values as calculated for the contrast C*0102-IVDL vs C*0102-AAA in KIR2DS+ and KIR2DS2- NK cell populations. Pathway results were further filtered on those that showed the lowest p.adj values.

Immunoblotting

NK cells were obtained from murine spleens using an NK cell isolation kit (Miltenyi Biotech Ltd) and lysed in NP40 Cell Lysis Buffer (Fisher Scientific UK Ltd). Primary antibodies from Cell Signalling Technology against Di-Methyl-Histone H3 (Lys4) (#9725), Tri-Methyl-Histone H3 (Lys27) (#9733), Histone H3 (#14269) and actin (#3700) were used in conjunction with HRP-conjugated secondary antibodies. Proteins were visualised using ChemiDoc-It Imaging system and VisionWorks software.

Statistical analysis

Experimental statistical analyses were performed using Graph-Pad Prism 7.0 software. Student two-tailed t test was used for comparison between two groups and two-way ANOVA with post-hoc analysis were used to compare more than two groups. Data were considered statistically significant at $p< 0.05$. For the tumour model statistical comparisons between survival to the humane end point was performed by Log-rank test (Mantel-cox).

RESULTS

Activation of NK cells using peptide:MHC DNA vaccine to target KIR2DS2

We developed a DNA construct that expresses the peptide:MHC (pMHC) ligand for KIR2DS2 as a single ORF, building on the model that KIR2DS2 is a cross-reactive receptor that recognises different pMHC ligands, and that in order to target KIR2DS2 for therapeutic benefit it is therefore not required to know the peptide ligand of the tumour being targeted, or to match for the HLA of the patient. These constructs contained HLA-C*0102 linked to a viral peptide and separated by a T2A self-cleaving sequence and with an E3/19K endoplasmic reticulum (ER) targeting sequence upstream of the peptide [14]. The constructs were made with two previously described KIR2DS2-binding peptides: LNPSVAATL (C*0102-LNP) and IVDLMCHATF (C*0102-IVDL) and cloned in to the pIB2 vector (Fig. 1A). They were used as naked DNA vaccines to immunize KIR transgenic (KIR-Tg) mice [31]. These mice express a human KIR haplotype B locus that includes KIR2DS2 and are backcrossed onto an MHC class I negative background. An MHC class I deficient mouse was used to avoid presentation of peptides onto endogenous murine MHC class I and hence confounding T cell or NK cell responses. In these animals KIR2DS2 expression is unaffected by the absence of an endogenous HLA-C ligand [31]. KIR-Tg mice were injected intramuscularly with 50µg of DNA and expression of MHC class I analysed by immune histochemistry one week after the final injection. This demonstrated expression of the construct within muscle tissues (Fig. S1). KIR-Tg mice were the injected intramuscularly with 50µg of DNA, without additional adjuvant, weekly for two weeks. Mice were sacrificed one week after the final injection, and splenocytes and hepatic lymphocytes isolated using KLRG1 expression as a marker of NK cell activation that is also associated with NK cell proliferation and maturation [37 38]. Results were compared to DNA constructs encoding HLA-C*0102 alone, or DNA encoding HLA-C*0102 in combination with a control peptide, IVDLMCHAAA (C*0102-AAA), in which the P9 and P10

residues of the peptide IVDLMCHATF had been mutated to alanine to abrogate binding to KIR2DS2 and HLA-C*0102 respectively. Injection with DNA encoding viral peptides induced activation of splenic NK cells as indicated by KLRG1 expression, but was greatest in the C*0102-IVDL group (40%) compared to C*0102-AAA (28%), $p<0.001$ (Fig. 1B and 1C). However we did not observe a specific increase in the frequency of KIR2DS2-positive NK cells (data not shown).

In paired analyses KLRG1 expression was upregulated to a significantly greater extent on KIR2DS2-positive (KIR2DS2+) versus KIR2DS2-negative (KIR2DS2-) splenic NK cells ($p<0.01$ for C*0102-IVDL and $p<0.05$ for C*0102-LNP), but not by control constructs (Fig. 1D). Activation was more marked in mice injected with C*0102-IVDL compared to C*0102-LNP, consistent with the stronger binding *in vitro* noted previously in tetramer binding experiments [14]. Furthermore, activation of hepatic NK cells was noted only in experiments using the C*0102-IVDL construct, with the expression of KLRG1 being significantly higher on the KIR2DS2+ versus KIR2DS2- population ($p<0.01$) (Fig. 1E).

Peptide:MHC vaccination upregulates KLRG1 expression on KIR2DS2-positive mature splenic NK cells

Maturation of NK cells can be characterized by expression of CD11b and CD27, with CD11b-CD27+ NK cells being classified as the least mature, CD11b+CD27+ NK cells as mature, and CD11b+CD27- NK cells terminally differentiated. Overall there were no substantial changes in the relative proportions of the different CD11b/CD27 subsets induced by these constructs (Fig. S2). KLRG1 was upregulated by C*0102-IVDL vaccination on CD11b+CD27+ splenic 2DS2+ NK cells compared to C*0102-AAA: 27% versus 18% ($p<0.05$) respectively and on terminally mature CD11b+CD27- splenic 2DS2+ NK cells compared to both C*0102-AAA

and C*0102: 62% versus 40% ($p<0.001$) and 46% ($p<0.005$) respectively (Fig. 2A and 2B). However effects on hepatic lymphocytes were less clear, with upregulation of KLRG1 only noted on terminally differentiated CD11b+CD27- NK cells in the C*0102-IVDL group only ($p<0.05$ vs control vaccinations) (Fig. 2C and 2D). Conversely we observed up regulation of CD69 on hepatic, but not splenic CD11b+CD27+ and CD11b+CD27- 2DS2+ NK cells (Fig. 2E). This difference may reflect the kinetics of CD69 versus KLRG1 expression and also the observation that CD69 can mark tissue resident NK cells, in addition to being a marker of activation.

Specific activation of KIR2DS2+ versus KIR2DS2- NK cells was most marked on the CD11b+CD27+ splenic NK cells ($p<0.001$), as compared to terminally differentiated CD11b+CD27- NK cells (Fig. 2F and 2G). Analysis of draining lymph nodes demonstrated a trend towards an increase in KIR2DS2-positive NK cells and also towards expression of KLRG1 on NK cells in the C*0102-IVDL group as compared to the C*0102-AAA group (Fig.S3). After four weekly injections we observed activation of NK cells, with upregulation of KLRG1 on splenic and hepatic NK cells with both peptide-containing constructs, and KIR2DS2-specific activation in the CD11b+CD27+ double-positive population with C*0102-IVDL (Fig. S4). However at this timepoint there was no significant difference between the C*0102-LNP and C*0102-IVDL constructs. This lack of difference may reflect that a ceiling for activation using this strategy may have been reached, or that after four doses more apoptosis is induced by the C*0102-IVDL than the C*0102-LNP construct.

Education of NK cells in the mouse is determined by interactions between the Ly49 inhibitory receptors and their MHC class I ligands. Therefore to confirm that activation of NK cells could be induced on educated NK cells, we crossed the KIR-Tg mice with wild type C57BL/6 mice

to provide MHC ligands for the murine Ly49 receptors, and immunized with 2 doses of DNA. Consistent with findings in the MHC deficient mouse, we observed KLRG1 upregulation on mature CD11b+ CD27+ KIR2DS2+, and terminally differentiated CD11b+ CD27- KIR2DS2+NK cells ($p<0.01$ and $p<0.05$ respectively) (Fig 2G).

NK cells from C*0102-IVDL and C*0102-AAA vaccinated mice were profiled by RNA seq one week following vaccination (see Fig. S5 for gating strategy). Principal component analysis (PCA) showed that KIR2DS2+ NK cells from C*0102-IVDL vaccinated mice formed a discrete cluster to KIR2DS2+ NK cells from C*0102-AAA vaccinated mice, in contrast to KIR2DS2- NK cells (Fig. 3A). Those mice receiving C*0102-IVDL had upregulation of genes in pathways associated with cellular metabolism, as compared to those receiving control C*0102-AAA vaccination (Fig. 3B). Differential gene expression analysis identified 42 differentially expressed genes (FDR<0.05) between the KIR2DS2+ NK cells from the C*0102-IVDL versus control groups (Table S1). These included upregulation in genes associated with: RNA binding and splicing; metabolism, especially glutathione metabolism; and regulation of IFN alpha (Fig. 3C). Additionally, we observed downregulation of genes related with histone H3 dimethylation at K4 (H3K4me2) and trimethylation at K27 (H3K27me3), consistent with a change in transcriptional regulation induced by vaccination (Fig. 3D). Purified NK cells from the spleens of vaccinated mice had upregulation of both H3K4me2 and H3K27me3 in IVDL mice as compared to control (Fig. 3E). Changes in H3K4 and H3K27 methylation have been associated with both NK cell activation and maturation [39 40]. In particular promoters with H3K4 and H3K27 methylation marks are considered to be in a poised, ready to transcribe state, which is consistent with our analysis at 7 days post-vaccination [41]. Whilst H3K4me2 and H3K4me3 marks are generally concordant in T cell analyses, H3K4me2 marks are associated with genes that are rapidly transcribed after stimulation consistent with such a poised state [42 43]. Detailed

temporal analysis by chromatin immunoprecipitation is required to further clarify the changes in histone methylation induced by vaccination.

A KIR targeting vaccine augments NK cell functions

To test for functional effects of our DNA vaccination strategy, KIR-Tg mice were inoculated subcutaneously in the flank with B16F10 melanoma cells and injected intramuscularly with DNA on the same day and one week later. Growth of B16F10 cells was significantly attenuated by day 12 in mice given C*0102-IVDL as compared to those given C*0102-AAA or unvaccinated ($p<0.02$) (Fig. 4A). In vitro, KIR2DS2+ NK cells, but not KIR2DS2- NK cells, from C*0102-IVDL-vaccinated mice had increased degranulation to B16F10 cells as compared to the control vaccinated mice ($p<0.05$) (Fig. 4B). As B16F10 cells do not express HLA-C these data indicate that DNA vaccination with C*0102-IVDL activates NK cells and induces MHC class I unrestricted responses, which is relevant as many cancers can down-regulate MHC class I.

To identify if peptide-specific NK cell responses were generated using this strategy, NK cells from vaccinated mice were tested against the MHC class I-negative target human cell line 721.221 transfected with either HLA-C or a construct of HLA-C in combination with the peptides LNPSVAATL and IVDLMCHATF [14]. KIR2DS2+ NK cells from mice vaccinated with C*0102-IVDL demonstrated increased activity against 721.221 cells expressing HLA-C*0102 in combination with both KIR2DS2-binding peptides as compared to 721.221 cells transfected with HLA-C*0102 alone ($p<0.01$ for both LNP and IVDL targets) (Fig. 4C). No effect was observed for KIR2DS2- NK cells. Similarly, we observed an increase in IFNγ secretion for the KIR2DS2+ NK cells from IVDL vaccinated mice when incubated with the

C*0102-LNP target as compared to KIR2DS2+ NK cells from AAA vaccinated mice (42.5% vs 30.5%, $p<0.05$) (Fig. 4D). Thus activation using a peptide:MHC strategy can generate both specific and cross-reactive peptide responses.

To identify if this strategy could recognize a human MHC class I target in vivo, we used the Huh7 hepatocellular carcinoma cell line transfected with the KIR2DS2 ligand HLA-C*0102. In vitro killing assays demonstrated that NKL cells transfected with KIR2DS2 killed Huh7-C*0102 cells to a greater extent than untransfected NKL cells ($p<0.0001$) (Fig. 4D), thus validating the cell line as KIR2DS2-specific target. As the KIR-Tg mice are not permissive for human tumours we used an adoptive transfer model to test the effects of our DNA vaccine on Huh7-C*0102 cells. KIR-Tg mice were vaccinated with two doses of the DNA vaccine C*0102-IVDL or the control vaccine weekly, and then purified splenic NK cells containing <1% CD3+ T cells, were adoptively transferred into immunodeficient NSG mice, which had been inoculated with Huh7-C*0102 cells. We observed a significantly delayed growth of the tumour in mice that received C*0102-IVDL-stimulated NK cells compared to control vaccine concomitant (Fig. 4E). Thus stimulation of NK cells via KIR2DS2 can generate anti-cancer reactivity against HLA-C expressing human tumour cells.

DISCUSSION

We have developed a novel strategy to activate NK cells through KIR2DS2 in a peptide:MHC dependent manner using a construct that expresses both the cognate MHC and peptide. This strategy may be relevant for other activating KIR, such as KIR2DS1and KIR2DS4 which have been convincingly shown to have peptide:MHC specificities [13 15 44]. Additionally KIR3DS1, which is associated with protection from HIV, is more controversially thought to have a peptide dependent HLA-B specificity [45]. Thus our work may be relevant for a number of different KIR in addition to KIR2DS2.

KIR2DS2-mediated activation generated a cytotoxic immune response against targets both with and without a cognate KIR2DS2-ligand, and thus utility of this strategy as a therapeutic may not be critically dependent on MHC class I expression by the target. Furthermore, previous work has also shown that KIR2DS2+ NK cells can recognize several different cancer targets *in vitro*, including cell lines derived from prostate, breast and ovarian carcinomas [27]. This recognition was beta-2-microglobulin independent, suggesting that there may also be non-peptide:MHC class I ligands for KIR2DS2 and is consistent with our observation that KIR2DS2+ murine NK cells are more cytotoxic against B16F10 melanoma cells even in unvaccinated mice. Additionally, KIR2DS2+ NK cells appear to have a greater potential to mediate antibody-dependent cellular cytotoxicity (ADCC) *in vitro* and *in vivo* [28 29]. Therefore KIR2DS2 may be an attractive target for cancer immunotherapy in combination with antibody based therapeutics.

In our experimental model KIR2DS2 expression is unaffected by the absence of an endogenous HLA-C ligand as NK cell education is driven by inhibitory, rather than activating, receptors for MHC class I [31]. This could be further investigated in humanised mouse models, with the

caveat that this requires administration of human IL-15 to maintain NK cells, and so will require careful interpretation [46 47]. Despite the absence of a known ligand for KIR2DS2, we observed activation of both KIR2DS2+ and KIR2DS2- NK cells *in vivo*. This is not unexpected as a DNA vaccine may generate an inflammatory response thus activating NK cells, and CpG sequences within the DNA vaccine may activate NK cells either directly or indirectly via dendritic cells. Importantly, NK cells are well known to undergo reciprocal activation with dendritic cells which could lead to cytokine release and further activation of NK cells in a non-specific manner through release of IL-12 [48]. This is consistent with our observations that whilst there was activation of both KIR2DS2+ and KIR2DS2- NK cells *in vivo*, activation was preferential for KIR2DS2+ NK cells. The presence of antigen non-specific responses may be important for generating more effective anti-tumour responses, and is consistent with a mode of action of NK cells in which they are activated through one receptor and recognize a target through a different one. For instance, killing of the B16F10 melanoma cell line is mediated by NKp46, and hence not MHC class I restricted [49].

Furthermore, we demonstrated peptide cross reactivity of KIR2DS2, as mice vaccinated with IVDLMCHATF were able to recognize the hepatitis C virus peptide LNPSVAATL, consistent with KIR2DS2 recognizing peptides with an AT motif at the carboxy-terminal -1 and -2 positions. Thus, in order to take our findings to the clinic it is not necessary to identify the ligand on the cancerous cell, or to match for HLA class I as KIR2DS2 may bind other group 1 HLA class I allotypes such as HLA-C*0304 [14 16]. The broad specificity of KIR2DS2 also provides an advantage for peptide-based NK cell therapy over T cell pMHC therapeutics which require more precise pMHC matching. Additionally, as the strategy provides both the peptide and the MHC class I ligand, then no HLA class I matching is required.

Plasmid DNA vaccination is a novel strategy to activate NK cells. To understand its effects in detail would be interesting in additional studies. In particular we have noted differences between splenic and hepatic NK cells. Baseline KLRG1 expression appears relatively elevated within the liver which may be the result of homeostatic proliferation or more ready activation of KIR2DS2-positive NK cells which has been observed in humans [28 38]. Additionally we formally tested only splenic NK cells *ex vivo* but not hepatic or lymph-node NK cells, which could also have anti-cancer effects.

Based on our work we propose the following model based. As there is expression of MHC class I in the muscle tissue we envisage that circulating NK cells can be activated directly within the tissues. NK cells circulate in a primed state, in comparison to T cells, so uptake by professional antigen presenting cells may not be required for NK cell activation. The activated NK cells circulate to the periphery and generate anti-cancer responses. Potentially, some may develop a "memory" phenotype within the liver. We envisage that this strategy could be readily translated to the clinic as a DNA or RNA therapeutic that activates NK cells *in vivo* to generate anti-cancer responses or augment other immunotherapeutic strategies, including antibody-dependent cellular cytotoxicity. It would however only be relevant for the approximately 50% of individuals who express KIR2DS2 as part of a KIR B haplotype. An intramuscular approach has advantages over other delivery methods, such as intra-tumoral injection as it is less invasive and could be administered in primary care settings. To harness the functional activation advantage of KIR2DS2+ NK cells, cell lines expressing peptide:MHC combinations designed to specifically activate KIR2DS2-positive NK cells could also be used in *ex vivo* expansion protocols for adoptive therapy. This strategy may offer a more controlled activation of NK cells but would be more cumbersome than an injection therapeutic.

As NK cells are involved in the early immune response to viral infections, and KIR2DS2 recognizes peptides derived from many different viruses, then targeting KIR2DS2 by DNA vaccination may also form part of an anti-viral therapeutic strategy to reduce infection and transmission in the early stages of infection. In conclusion, our work identifies a novel mechanism for activating NK cells and we propose that this may have potential as a novel therapeutic.

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Competing interests:

The University of Southampton has applied for patents covering the use of the DNA vaccine constructs described in this manuscript

Author contributions:

SIK, PR, MDB designed experiments; PR, MDB, RF, LBL, LE-S performed experiments; AVP, PR, MDB, and SIK analysed data; CR provided key reagents; SIK, AA-S, MP supervised experiments and interpreted data; PR, MDB, AVP, AA-S and SIK wrote the manuscript.

Data availability:

Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

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FIGURE LEGENDS

Figure 1: A peptide:MHC DNA vaccine that targets KIR2DS2 activates NK cells

A) The conformation of the constructs used in this study to inoculate the mice. **B)** Gating strategy for KLRG1 on KIR2DS2+ NK cells derived from the spleens (top panels) and livers (lower panels) of KIR-Tg mice. KIR2DS2+ NK cells were identified using the antibody 1F12 and the numbers indicate the percentage positive cells in the gate. **C)** The frequency of KLRG1 expression on KIR2DS2+ NK cells in the spleen and livers of KIR-Tg mice vaccinated with DNA plasmids containing HLA-C*0102 (C*0102, light grey bars), HLA-C*0102 plus IVDLMCHATAAA (C*0102-AAA, grey bars), HLA-C*0102-LNPSVAATL (C*0102-LNP, dark grey bars), HLA-C*0102-IVDLMCHATF (C*0102-IVDL, black bars) and compared to PBS alone control mice (white bars). **D, E)** Comparison of KLRG1 frequencies on KIR2DS2+ (filled circles) or KIR2DS2- (open circles) CD3-NK1.1+ NK cells in the spleens (**D**) and livers (**E**) following vaccination. All analyses were performed one week following the second vaccination. Comparisons between two groups were made by paired t test (2 groups) and 2-way ANOVA (more than 2 groups) (*p < 0.05, **p< 0.01, ****p<0.001).

Figure 2: Vaccination activates mature and terminally differentiated NK cells.

KIR-Tg mice were injected with two doses of the indicated DNA construct one week apart and then assessed for expression of KLRG1 on CD11b, CD27 NK cell subsets from the spleens and livers one week after the final injection. **A-D)** KLRG1 on splenic CD11b+CD27+ (**A**) and CD11b+CD27- (**B**) NK cells and on hepatic CD11b+CD27+ (**C**) and CD11b+CD27- (**D**) NK cells following vaccination. N=7-14 mice per group. **E)** CD69 expression on KIR2DS2-positive CD11b CD27 NK cell subsets (n=3 per group). **F, G)** Comparison of KLRG1 expression on KIR2DS2+ and KIR2DS2- splenic NK cells in the CD11b+CD27+ (**F**) and CD11b+CD27- (**G**) sub-populations. N=7-14 mice per group. **H**) KIR-Tg mice crossed with

C57BL/6 mice were injected with two doses of the indicated DNA construct one week apart and then assessed for expression of KLRG1 on total NK cells, and CD11b, CD27, NK cell 2DS2+ and 2DS2- subsets from their spleens. N=4-6 mice per group. Comparisons between two groups were made by paired t test (2 groups) and 2-way ANOVA (more than 2 groups) (*p < 0.05, **p< 0.01, p<0.005 ****p<0.001).

Figure 3: Transcriptomic analysis of NK cells following DNA vaccination

A) Principal component analysis (PCA) of whole NK cell transcriptomes from C*0102-IVDL and C*0102-AAA vaccinated mice taken one week after the second vaccination. KIR2DS2-negative NK cells from both groups are shown in the left panel and KIR2DS2-positive NK cells in the right panel. Counts were normalized and filtered using EdgeR. The first two components of the PCA are shown. **B)** Heatmap of the top 100 differentially expressed genes derived from the comparison of KIR2DS2 positive NK cells from C*0102-IVDL and C*0102-AAA vaccinated mice. **C, D)** EGSEA analysis of C5 gene ontology (**C**) and C2 canonical pathway (**D**) signatures comparing KIR2DS2+ and KIR2DS2- NK cell populations in both C*0102-IVDL and control vaccinated mice. Effect significances were calculated individually for each arm of the study and the plots indicate the overall effects of vaccination on KIR2DS2+ NK cells in the C*0102-IVDL vaccinated mice ("positive") as compared to the other three groups ("negative"). The colour denotes the direction of the change and the size of the bar represents the -Log10(FDR). All categories shown were significant at FDR<0.05. E) Western blot analysis of histone 3 marks (H3K4me2 and H3K27me3) on purified NK cells from the spleens of vaccinated mice one week after the second vaccination with either C*0102-AAA (AAA) or C*0102-IVDL (IVDL).

Figure 4: DNA vaccination induces functional NK cell responses

A) KIR-Tg mice were injected subcutaneously with B16F10 melanoma cells on day 0 and then vaccinated intramuscularly with C*0102-IVDL (squares) or C*0102-AAA (triangles) on days 0 and 7 or untreated (circles) and tumour volume measured. (n=4 mice per group: one of two independent experiments). **B**,**C,D)** Mice were injected intramuscularly with C*0102-IVDL (black bars) or C*0102-AAA (grey bars) on days 0 and day 7 and then NK cells purified from the spleens on day 14 for *in vitro* assays of activation. **B)** shows degranulation of KIR2DS2+ and KIR2DS2- KIR-Tg NK cells to B16F10 melanoma cells (n=4 mice per group), **C)** shows degranulation of NK cells from KIR-Tg mice to human 721.221 cells expressing HLA-C*0102 alone (221-C*0102) or HLA-C*0102 in combination with the peptide: LNPSVAATL (221-C*0102-LNP) or IVDLMCHATF (221-C*0102-IVDL) and **D)** shows IFNγ expression following incubation with 221-C*0102 and 221-C*0102-LNP cells (n=7-8 mice per group). **E)** Killing of Huh7-C*0102 hepatoma cells by NKL cells either untransfected (NKL) or transfected with KIR2DS2 (NKL-2DS2) or the inhibitory receptor KIR2DL2 (NKL-2DL2) at the indicated effector to target (E:T) ratios. Cytotoxicity was determined by flow cytometry using the LIVE/DEAD™ stain. Shown are the results of two independent experiments performed in triplicate. **F**) NK cells from KIR-Tg mice vaccinated either with C*0102-IVDL or C*0102-AAA as a peptide control, were adoptively transferred into NSG mice inoculated subcutaneously with Huh7-C*0102 hepatoma cells and tumour volume was measure (n=4 mice per group, one of two independent experiments). The control mice (n=3) did not receive any NK cells. Comparisons were by Students t-test (2 groups) or two-way ANOVA (more than 2 groups). For all plots *p < 0.05, **p<0.01, ***p< 0.005, ****p< 0.0001.

FIGURE 1

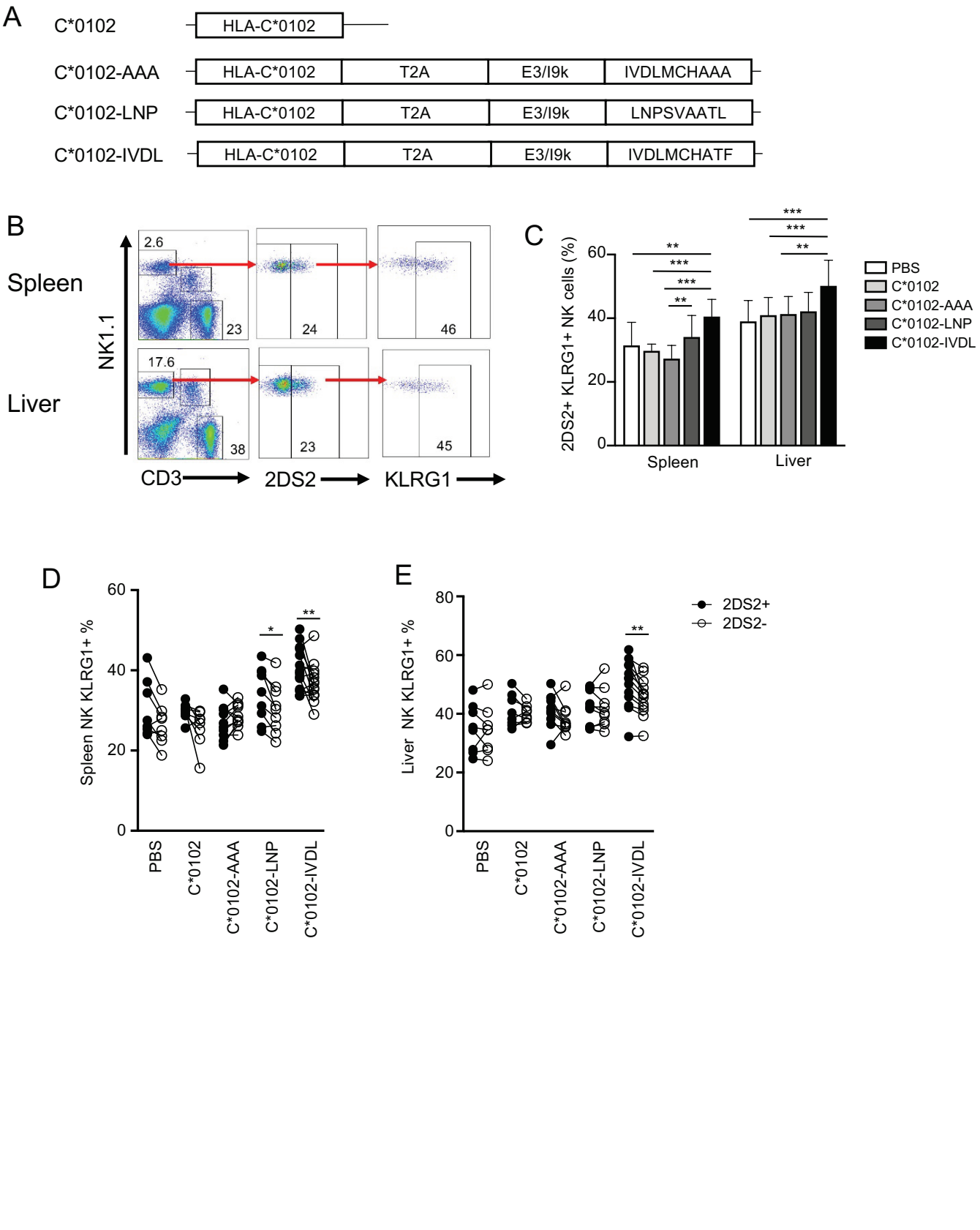

A
C*0102
HLA-C*0102
C*0102-AAA
HLA-C*0102
T2A
E3/I9k
IVDLMCHAAA
C*0102-LNP
HLA-C*0102
T2A
E3/I9k
LNPSVAATL
C*0102-IVDL
HLA-C*0102
T2A
E3/I9k
IVDLMCHATF
B
Spleen
Liver
NK1.1
2.6
23
24
46
17.6
38
23
45
CD3
2DS2
KLRG1
C
2DS2+ KLRG1+ NK cells (%)
60
40
20
0
Spleen
Liver
PBS
C*0102
C*0102-AAA
C*0102-LNP
C*0102-IVDL
D
Spleen NK KLRG1+ %
60
40
20
0
PBS
C*0102
C*0102-AAA
C*0102-LNP
C*0102-IVDL
E
Liver NK KLRG1+ %
80
60
40
20
0
2DS2+
2DS2-
PBS
C*0102
C*0102-AAA
C*0102-LNP
C*0102-IVDL

FIGURE 2

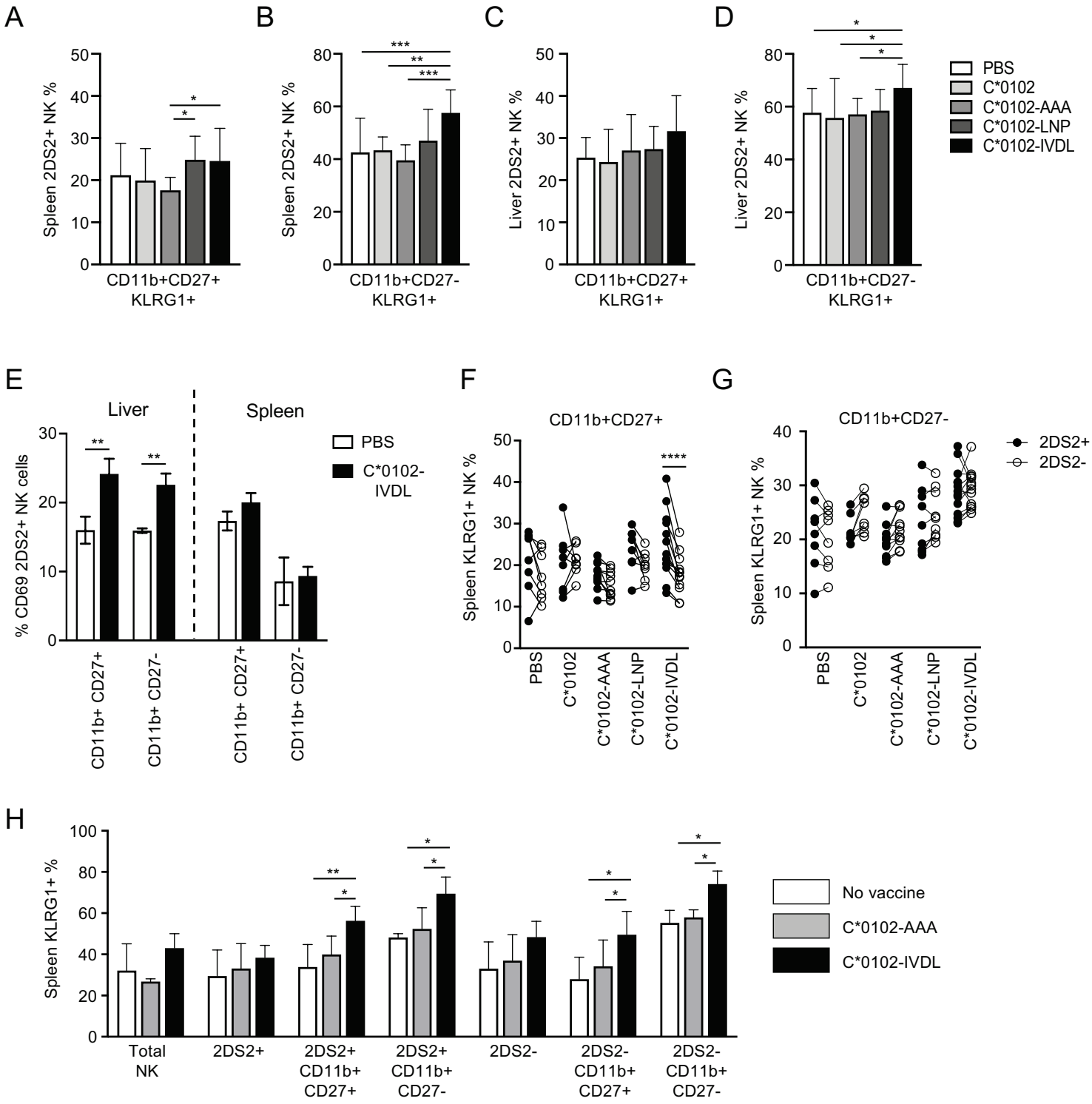

A
Spleen 2DS2+ NK %
CD11b+CD27+ KLRG1+
B
Spleen 2DS2+ NK %
CD11b+CD27- KLRG1+
C
Liver 2DS2+ NK %
CD11b+CD27+ KLRG1+
D
Liver 2DS2+ NK %
CD11b+CD27- KLRG1+
PBS
C*0102
C*0102-AAA
C*0102-LNP
C*0102-IVDL
E
Liver
Spleen
% CD69 2DS2+ NK cells
PBS
C*0102-IVDL
CD11b+ CD27+
CD11b+ CD27-
CD11b+ CD27+
CD11b+ CD27-
F
CD11b+CD27+
Spleen KLRG1+ NK %
PBS
C*0102
C*0102-AAA
C*0102-LNP
C*0102-IVDL
G
CD11b+CD27-
Spleen KLRG1+ NK %
2DS2+
2DS2-
PBS
C*0102
C*0102-AAA
C*0102-LNP
C*0102-IVDL
H
Spleen KLRG1+ %
Total NK
2DS2+
2DS2+ CD11b+ CD27+
2DS2+ CD11b+ CD27-
2DS2-
2DS2- CD11b+ CD27+
2DS2- CD11b+ CD27-
No vaccine
C*0102-AAA
C*0102-IVDL

FIGURE 3

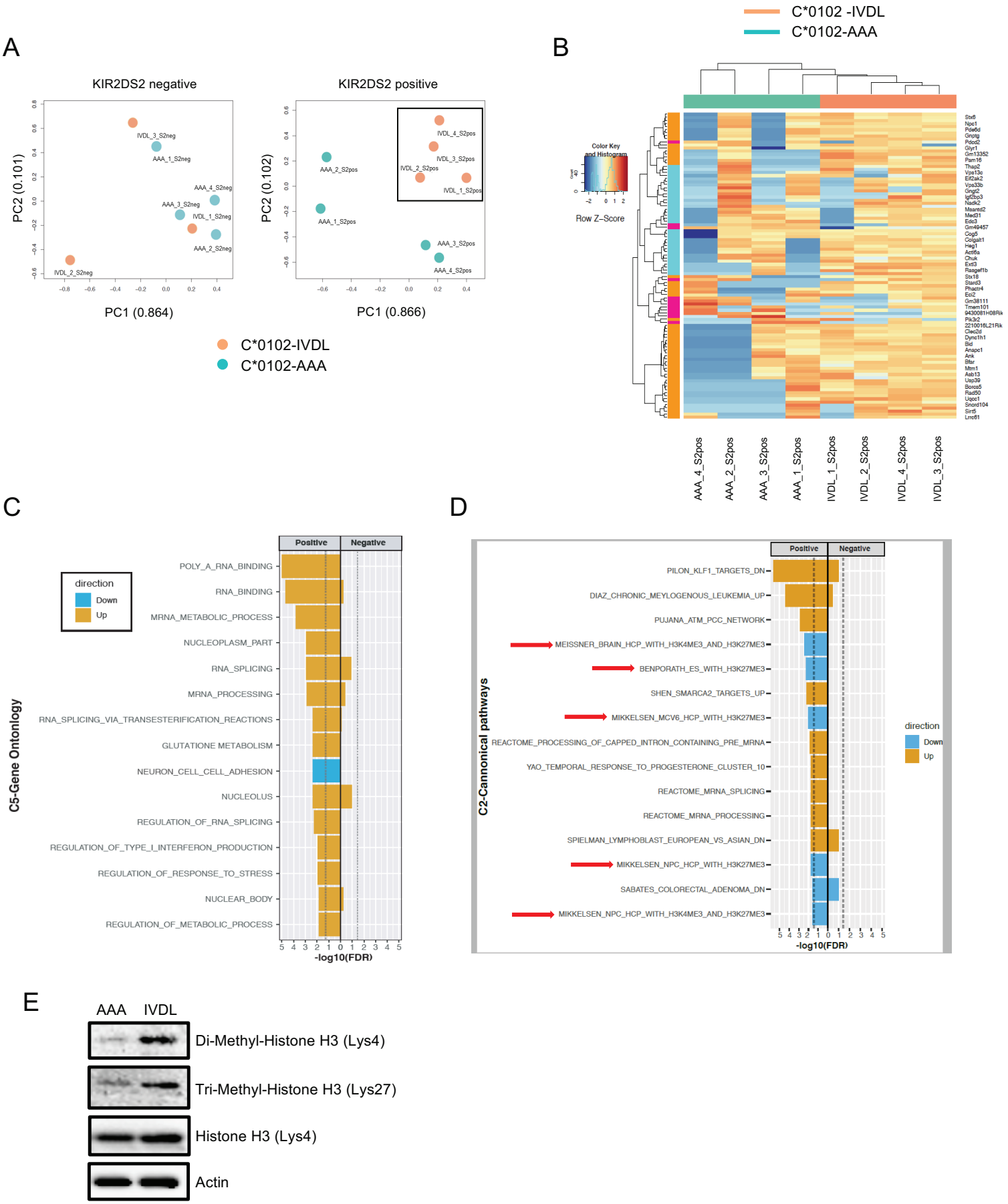
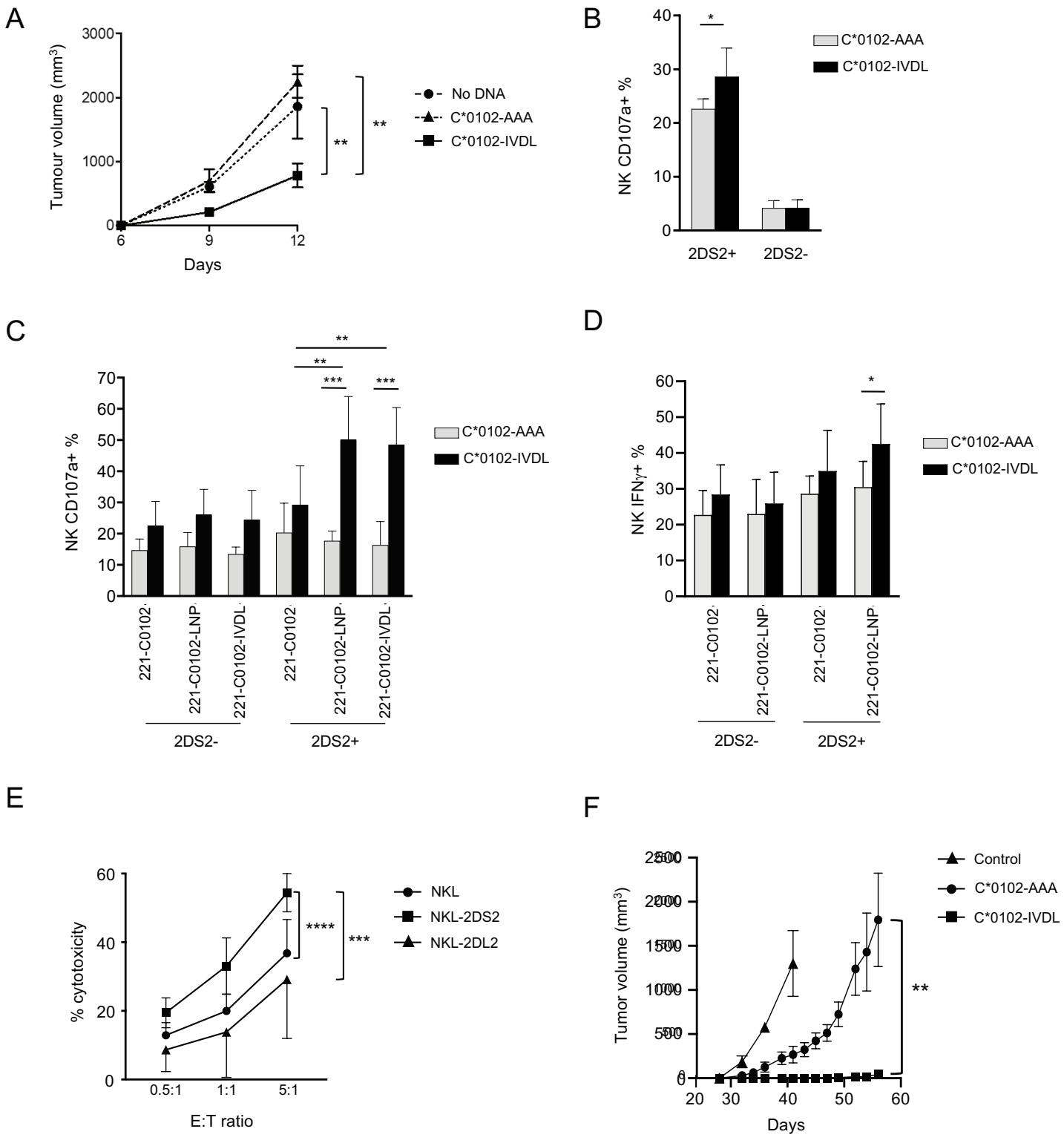

A
KIR2DS2 negative
KIR2DS2 positive
PC2 (0.101)
PC1 (0.864)
PC2 (0.102)
PC1 (0.866)
IVDL_3_S2neg
AAA_1_S2neg
AAA_4_S2neg
AAA_3_S2neg
IVDL_1_S2neg
AAA_2_S2neg
IVDL_2_S2neg
IVDL_4_S2pos
IVDL_3_S2pos
IVDL_2_S2pos
IVDL_1_S2pos
AAA_2_S2pos
AAA_1_S2pos
AAA_3_S2pos
AAA_4_S2pos
C*0102-IVDL
C*0102-AAA
B
C*0102 -IVDL
C*0102-AAA
Color Key and Histogram
Row Z-Score
AAA_4_S2pos
AAA_2_S2pos
AAA_3_S2pos
AAA_1_S2pos
IVDL_1_S2pos
IVDL_2_S2pos
IVDL_4_S2pos
IVDL_3_S2pos
C
Positive
Negative
direction
Down
Up
C5-Gene Ontology
POLY_A_RNA_BINDING
RNA_BINDING
MRNA_METABOLIC_PROCESS
NUCLEOPLASM_PART
RNA_SPLICING
MRNA_PROCESSING
RNA_SPLICING_VIA_TRANSESTERIFICATION_REACTIONS
GLUTATIONE METABOLISM
NEURON_CELL_CELL_ADHESION
NUCLEOLUS
REGULATION_OF_RNA_SPLICING
REGULATION_OF_TYPE_I_INTERFERON_PRODUCTION
REGULATION_OF_RESPONSE_TO_STRESS
NUCLEAR_BODY
REGULATION_OF_METABOLIC_PROCESS
-log10(FDR)
D
Positive
Negative
C2-Cannonical pathways
PILON_KLF1_TARGETS_DN
DIAZ_CHRONIC_MEYLOGENOUS_LEUKEMIA_UP
PUJANA_ATM_PCC_NETWORK
MEISSNER_BRAIN_HCP_WITH_H3K4ME3_AND_H3K27ME3
BENPORATH_ES_WITH_H3K27ME3
SHEN_SMARCA2_TARGETS_UP
MIKKELSEN_MCV6_HCP_WITH_H3K27ME3
REACTOME_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_10
REACTOME_MRNA_SPLICING
REACTOME_MRNA_PROCESSING
SPIELMAN_LYMPHOBLAST_EUROPEAN_VS_ASIAN_DN
MIKKELSEN_NPC_HCP_WITH_H3K27ME3
SABATES_COLORECTAL_ADENOMA_DN
MIKKELSEN_NPC_HCP_WITH_H3K4ME3_AND_H3K27ME3
direction
Down
Up
-log10(FDR)
E
AAA
IVDL
Di-Methyl-Histone H3 (Lys4)
Tri-Methyl-Histone H3 (Lys27)
Histone H3 (Lys4)
Actin

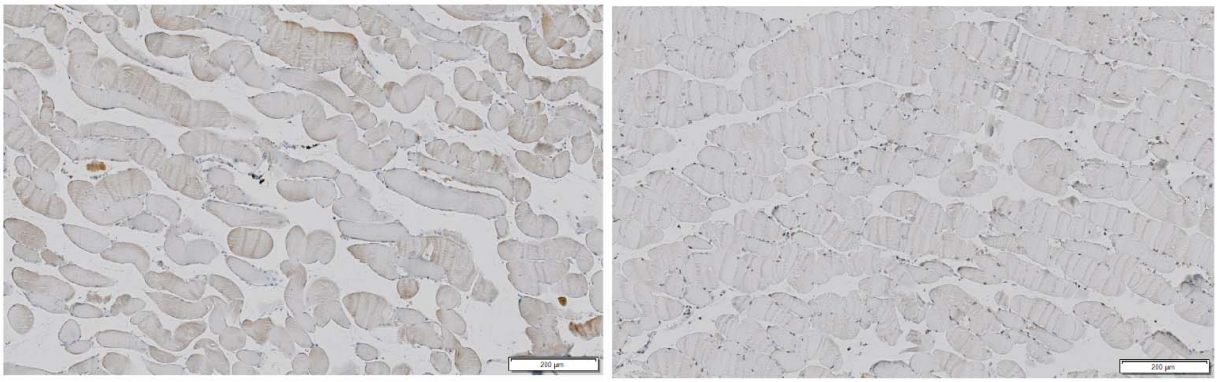
FIGURE 4


A
Tumour volume (mm3)
3000
2000
1000
0
6
9
12
Days
No DNA
C*0102-AAA
C*0102-IVDL
**
**
B
NK CD107a+ %
40
30
20
10
0
*
C*0102-AAA
C*0102-IVDL
2DS2+
2DS2-
C
NK CD107a+ %
70
60
50
40
30
20
10
0
**
**
***
***
C*0102-AAA
C*0102-IVDL
221-C0102
221-C0102-LNP
221-C0102-IVDL
221-C0102
221-C0102-LNP
221-C0102-IVDL
2DS2-
2DS2+
D
NK IFNγ+ %
60
50
40
30
20
10
0
*
C*0102-AAA
C*0102-IVDL
221-C0102
221-C0102-LNP
221-C0102
221-C0102-LNP
2DS2-
2DS2+
E
% cytotoxicity
60
40
20
0
0.5:1
1:1
5:1
E:T ratio
****
***
NKL
NKL-2DS2
NKL-2DL2
F
Tumor volume (mm3)
2500
2000
1500
1000
500
0
20
30
40
50
60
Days
**
Control
C*0102-AAA
C*0102-IVDL

Supplemental Figure 1: Immunohistochemistry staining showing expression of C*0102-IVDL construct in muscle

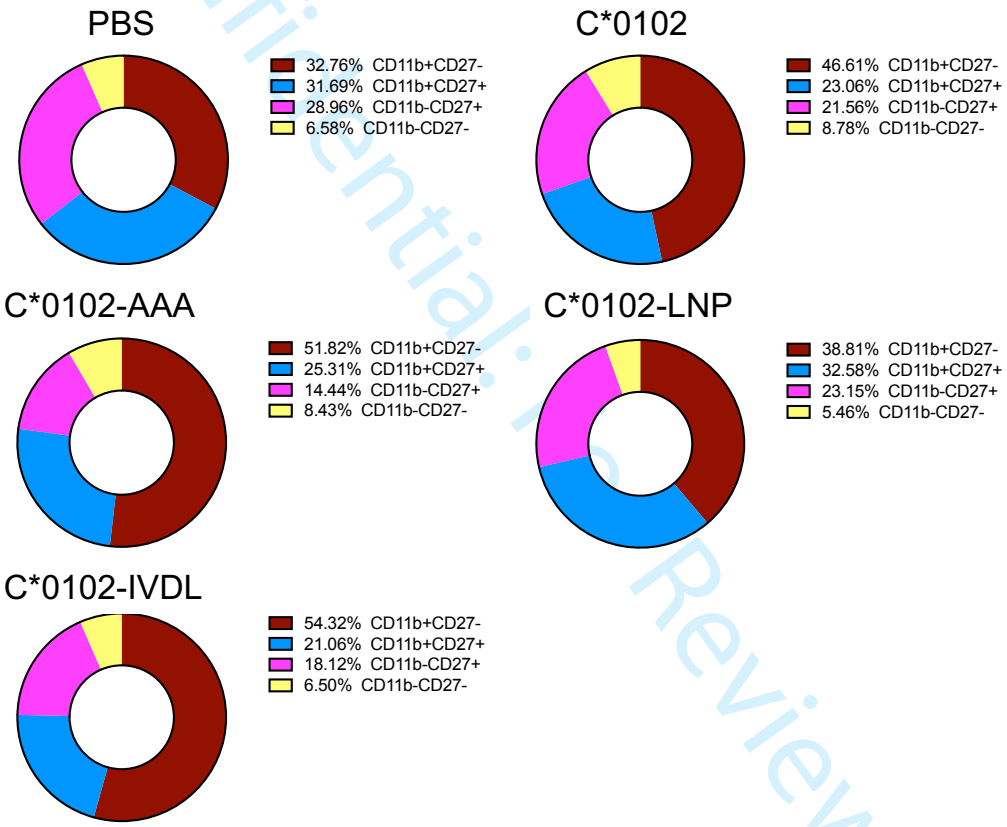
KIR-Tg mice were injected with two doses of C*0102-IVDL construct (left panel) or PBS (right panel) one week apart and thigh muscle removed 7 days after the second injection. Expression of HLA-C was tested by immunostaining with anti-HLA antibody using an immunoperoxidase technique and counterstained with haematoxylin. Magnification was x100, and a 200mM scale bar is indicated.

C*0102-IVDL **PBS**



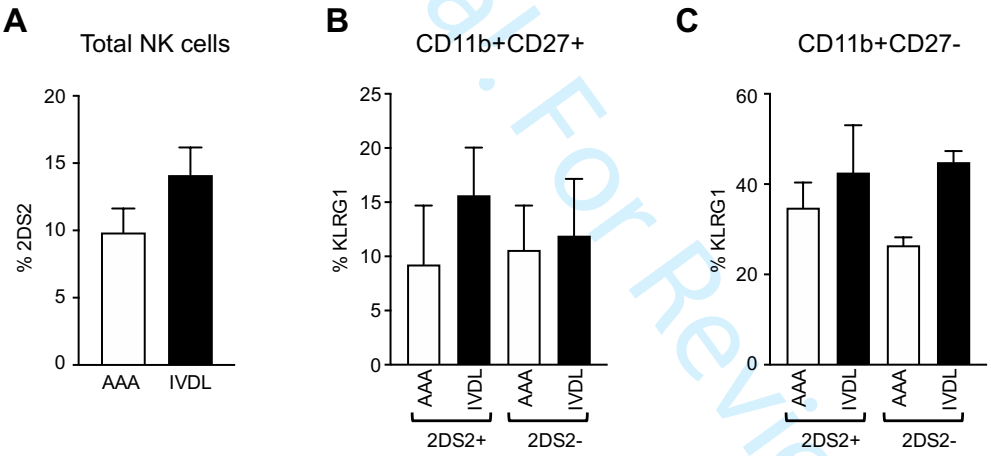
Supplemental Figure 2: CD11b/CD27 subsets from vaccinated mice

KIR-Tg mice were injected with two doses of the indicated DNA construct one week apart and then assessed for maturity as defined by the different CD11b, CD27 NK cell subsets from the spleens one week after the final injection.



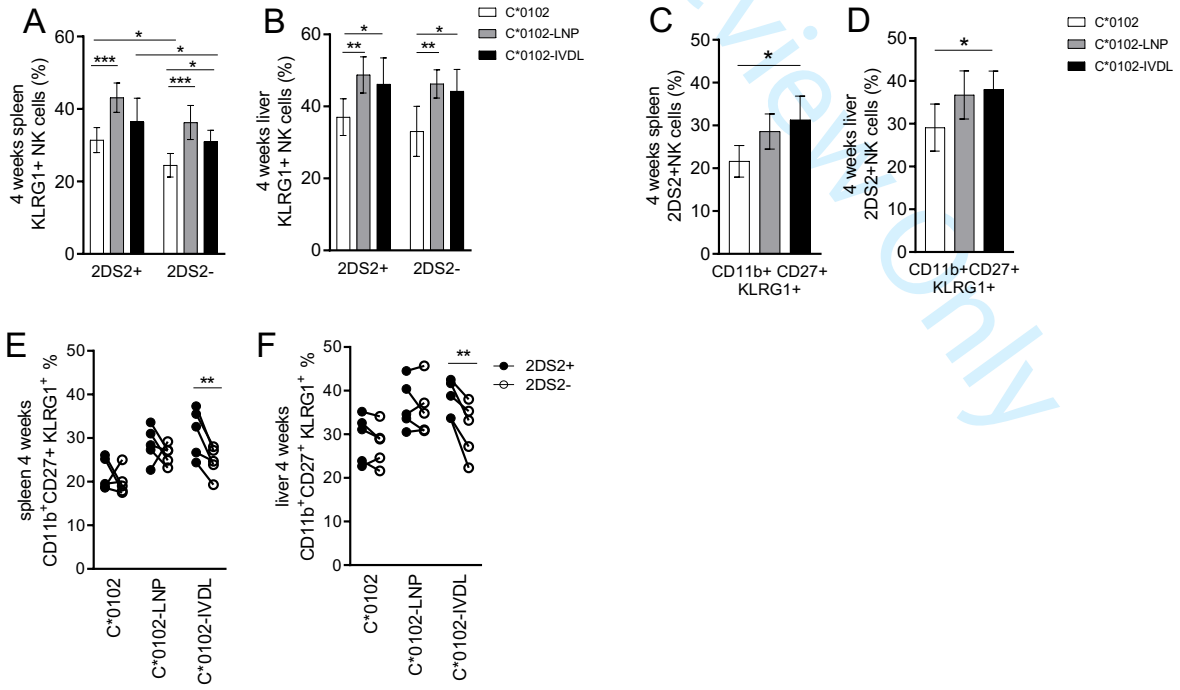
Supplemental Figure 3: Analysis of activation of NK cells in the lymph nodes of KIR-Tg mice

KIR-Tg mice were injected intramuscularly weekly for 2 weeks with DNA constructs encoding HLA-C*0102- IVDLMCHAAA (AAA) or HLA-C*0102-IVDLMCHATF (IVDL). Inguinal lymph nodes were isolated and analysed by flow cytometry. CD3-NK1.1+ NK cells were analysed for KIR2DS2 expression (panel A: n=7 per group) and expression of KLRG1 on KIR2DS2+, or KIR2DS2-, CD11b/CD27 subsets (Panels B and C: 4 mice per group). For all graphs means +/- SEM are shown. There were trends towards increased number of KIR2DS2 –positive cells, and increases in KLRG1 expression in IVDL vaccinated mice, but these did not reach statistical significance.



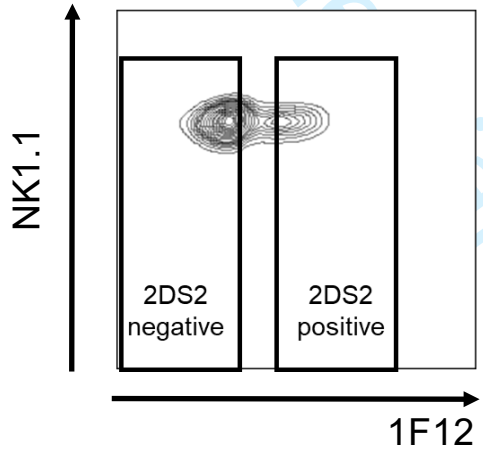
Supplemental Figure 4: Activation of NK cells after DNA vaccination for 4 weeks

KIR-Tg mice were injected intramuscularly weekly for 4 weeks with DNA constructs encoding HLA-C*0102 (white bars), HLA-C*0102-LNPSVAATL (C*0102-LNP; grey bars) or HLA-C*0102-IVDLMCHATF (HLA-C*0102-IVDL; black bars). KLRG1 expression was measured on KIR2DS2+ NK cells and CD11b, CD27 subsets. **A, B**) KLRG1 frequencies on KIR2DS2+ and KIR2DS2- NK cells in spleen **(A)** and livers **(B). C, D).** Frequency of KLRG1 expression on CD11b+CD27+ NK cells in the KIR2DS2+ NK cell subpopulations in the spleens (**C**) and livers **(D)** of vaccinated KIR-Tg mice. **E, F)** Comparison of KLRG1+ expression on CD11b+CD27+ within the KIR2DS2+ and KIR2DS2- NK cells in the spleens (**E**) and livers (**F**) of vaccinated mice. For all experiments n=4 mice per group. A paired t test was used for comparisons between two groups and a 2-way ANOVA with correction used when comparing more than two groups. For all plots *p< 0.05, **p< 0.01, ***p< 0.005.



Supplemental Figure 5: Gating strategy for sorting KIR2DS2-positive and -negative NK cells for analysis by RNA seq

Splenocytes from C*0102:IVDL and C*0102:AAA vaccinated mice were isolated and sorted by flow cytometry for analysis by RNAseq. Shown is a flow cytometry plot of the gating strategy used to delineate KIR2DS2+ from KIR2DS2- NK cells. Cells are gated on the CD3-, NK1.1+ sub-population.



genes	AAA_1_S2pos	AAA_2_S2pos	AAA_3_S2pos	AAA_4_S2pos	IVDL_1_S2pos	IVDL_2_S2pos	IVDL_3_S2pos	IVDL_4_S2pos	logFC	logCPM	LR	PValue	FDR	de	sig
Insc	6.094218	7.617772	60.94218	121.8844	0	3.385677	6.414966	4.68786	-3.31043	4.526329	12.56168	0.000394	0.075459	-1	1
Tstd3	12.18844	22.85332	17.41205	24.37687	5.540198	0	1.603742	2.34393	-2.93167	3.870316	13.51371	0.000237	0.049425	-1	1
A530053G22Rik	127.9786	38.08886	130.5904	146.2612	11.0804	10.15703	20.84864	21.09537	-2.63963	5.826831	34.19271	4.99E-09	1.05E-05	-1	1
Jazf1	24.37687	15.23554	17.41205	36.56531	0	6.771353	1.603742	4.68786	-2.59493	4.072019	12.47533	0.000412	0.076949	-1	1
Palld	152.3554	68.55995	339.535	134.0728	27.70099	33.85677	35.28231	30.47109	-2.40485	6.547134	31.68394	1.81E-08	2.01E-05	-1	1
Gm49457	115.7901	76.17772	121.8844	146.2612	0	37.24244	25.65987	21.09537	-2.26584	5.967197	33.86845	5.90E-09	1.05E-05	-1	1
9330185C12Rik	225.4861	91.41327	121.8844	73.13062	22.16079	30.47109	25.65987	32.81502	-2.22508	6.242058	31.96581	1.57E-08	2.01E-05	-1	1
Foxj2	54.84796	30.47109	52.23615	36.56531	16.62059	3.385677	8.018708	11.71965	-2.20338	4.821986	16.90125	3.94E-05	0.018277	-1	1
Gm15785	30.47109	45.70663	43.53013	36.56531	0	6.771353	14.43367	4.68786	-2.20095	4.657623	13.71316	0.000213	0.049425	-1	1
Dbx2	140.167	15.23554	78.35423	170.6381	27.70099	13.54271	17.64116	28.12716	-2.18912	5.809687	16.81342	4.12E-05	0.018277	-1	1
Epb41l4a	207.2034	198.0621	278.5928	48.75374	22.16079	44.0138	36.88606	58.59825	-2.15415	6.750062	31.50572	1.99E-08	2.01E-05	-1	1
Mir6236	365.6531	373.2709	679.07	402.2184	138.505	40.62812	97.82824	133.604	-2.15237	8.042721	50.13467	1.44E-12	1.02E-08	-1	1
Plcz1	42.65953	45.70663	60.94218	48.75374	0	10.15703	19.2449	14.06358	-1.85948	5.008747	13.75596	0.000208	0.049425	-1	1
Esrrg	365.6531	190.4443	409.1832	268.1456	55.40198	101.5703	78.58334	103.1329	-1.83494	7.556751	46.96522	7.23E-12	2.56E-08	-1	1
Vmn2r84	170.6381	198.0621	365.6531	73.13062	38.78139	37.24244	75.37585	70.3179	-1.81326	6.960175	22.28891	2.35E-06	0.001663	-1	1
Spata5	274.2398	83.7955	252.4747	170.6381	49.86178	77.87056	65.7534	53.91039	-1.652	6.958043	24.24505	8.48E-07	0.000668	-1	1
mt-Tv	73.13062	53.32441	52.23615	73.13062	0	33.85677	17.64116	25.78323	-1.57219	5.416176	12.0062	0.00053	0.091706	-1	1
Irak2	164.5439	83.7955	130.5904	134.0728	60.94218	20.31406	51.31973	44.53467	-1.54149	6.388233	20.25089	6.79E-06	0.004014	-1	1
Ptprj	73.13062	121.8844	87.06026	60.94218	11.0804	47.39947	30.47109	30.47109	-1.46452	5.875759	14.65953	0.000129	0.039704	-1	1
Hexb	152.3554	99.03104	322.123	243.7687	77.56277	88.02759	86.60205	82.03755	-1.24121	7.11058	15.91514	6.62E-05	0.026097	-1	1
Yaf2	121.8844	144.7377	87.06026	60.94218	55.40198	60.94218	46.50851	28.12716	-1.21372	6.269011	12.04265	0.00052	0.091706	-1	1
Cdyl	140.167	129.5021	148.0024	146.2612	49.86178	88.02759	73.77211	53.91039	-1.04747	6.693364	13.85458	0.000198	0.049425	-1	1
Gm42418	45572.56	35117.93	43556.25	57614.74	21767.44	30853.67	23411.42	18896.76	-0.93776	15.07801	13.50349	0.000238	0.049425	-1	1
CT010467.1	2608.325	2826.194	3099.345	3205.559	2116.356	1939.993	1975.81	1858.736	-0.57049	11.2593	20.71358	5.33E-06	0.003438	-1	1
Rpl18-ps1	396.1242	243.7687	426.5953	268.1456	504.158	599.2648	508.3861	585.9825	0.70207	8.843326	13.55266	0.000232	0.049425	1	1
Supt16	201.1092	213.2976	208.9446	158.4497	243.7687	358.8817	370.4643	365.6531	0.783455	8.148406	14.11659	0.000172	0.046861	1	1
Snord15b	237.6745	182.8265	191.5326	134.0728	337.9521	399.5098	274.2398	349.2456	0.809278	8.134131	13.78143	0.000205	0.049425	1	1
Rbms1	85.31905	83.7955	113.1783	146.2612	227.1481	155.7411	224.5238	196.8901	0.966681	7.398969	13.45806	0.000244	0.049425	1	1
Gm14513	146.2612	137.1199	130.5904	60.94218	271.4697	281.0112	213.2976	257.8323	1.017168	7.684824	18.18903	2.00E-05	0.010131	1	1
Rpgrip1	176.7323	144.7377	104.4723	121.8844	210.5275	220.069	344.8044	344.5577	1.027101	7.843913	15.44847	8.48E-05	0.031643	1	1
Vti1b	79.22483	45.70663	95.76628	48.75374	132.9648	189.5979	128.2993	192.2023	1.22329	7.042034	14.95942	0.00011	0.037092	1	1
Vim	109.6959	114.2666	130.5904	60.94218	199.4471	281.0112	259.8061	283.6155	1.275108	7.653901	27.59607	1.49E-07	0.000132	1	1
Srrm1	73.13062	76.17772	52.23615	48.75374	99.72357	226.8403	165.1854	152.3554	1.337051	7.037403	16.34213	5.29E-05	0.022055	1	1
Fbxo11	42.65953	22.85332	52.23615	12.18844	110.804	60.94218	88.20579	98.44506	1.357465	6.256188	11.93019	0.000552	0.093249	1	1
Ccser2	30.47109	15.23554	17.41205	24.37687	88.64317	91.41327	72.16837	42.19074	1.664308	5.949181	12.0971	0.000505	0.091706	1	1
4930470G03Rik	18.28265	38.08886	17.41205	12.18844	66.48238	77.87056	81.79082	98.44506	1.872876	6.100767	19.89932	8.16E-06	0.004453	1	1
Itih5	6.094218	7.617772	43.53013	12.18844	38.78139	60.94218	76.9796	60.94218	1.888569	5.760945	13.56859	0.00023	0.049425	1	1
Pdcd5-ps	18.28265	15.23554	8.706026	12.18844	55.40198	74.48489	48.11225	56.25432	1.981956	5.66119	15.19875	9.68E-05	0.034309	1	1
Cops8	12.18844	7.617772	26.11808	24.37687	127.4246	50.78515	62.54592	51.56646	2.04499	5.858204	14.11756	0.000172	0.046861	1	1
Neurl3	6.094218	30.47109	17.41205	12.18844	116.3442	88.02759	38.4898	53.91039	2.072884	5.852982	13.00435	0.000311	0.061213	1	1
Hmg20b	6.094218	7.617772	0	0	33.24119	40.62812	25.65987	39.84681	2.932458	5.019742	14.82435	0.000118	0.038034	1	1
Extl3	0	0	8.706026	0	16.62059	30.47109	25.65987	30.47109	3.4277	4.752539	14.55328	0.000136	0.040256	1	1