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Controlled infection with a therapeutic virus defines the activation kinetics of human natural killer cells *in vivo*

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Abstract

Human natural killer (NK) cells play an important role in antiviral immunity. However, studying their activation kinetics during infection is highly problematic. A clinical trial of a therapeutic virus provided an opportunity to study human NK cell activation *in vivo* in a controlled manner. Ten colorectal cancer patients with liver metastases received between one and five doses of oncolytic reovirus prior to surgical resection of their tumour. NK cell surface expression of the interferon-inducible molecules CD69 and tetherin peaked twenty-four to forty-eight hours post-infection, coincident with a peak of interferon-induced gene expression. The interferon response and NK cell activation were transient, declining by ninety-six hours post-infection. Furthermore, neither NK cell activation nor the interferon response were sustained in patients undergoing multiple rounds of virus treatment. These results show that reovirus modulates human NK cell activity *in vivo* and suggest that this may contribute to any therapeutic effect of this oncolytic virus. Detection of a single, transient peak of activation, despite multiple treatment rounds, has implications for the design of reovirus-based therapy. Furthermore, our results suggest the existence of a post-infection refractory period when the interferon response and NK cell activation are blunted. This refractory period has previously been observed in animal models and may underlie the enhanced susceptibility to secondary infections that is seen following viral infection.

Keywords: Natural killer cells, Innate immunity, Viral infection, Interferon response, Human infection

Introduction

Infection induces the rapid activation of innate immunity. Innate immune activation serves two purposes, it limits pathogen replication whilst the clonal selection of B and T cells occurs and it favours the development of the appropriate adaptive response [1]. Current information on the kinetics of innate immune activation stems largely from animal models, yet there is a need to define these processes in humans; such knowledge promises to enhance the efficacy of vaccines and other immunotherapeutic strategies. However, studying the early stages of infection in humans presents both logistical and ethical problems.

Natural killer (NK) cells are important in the innate immune response to infected cells and to tumours [2, 3]. Early animal studies revealed that NK cell activation occurred within two to three days of viral infection [4, 5] and NK cells are known to be critical in antiviral immunity [3, 6-9]. Activated NK cells destroy infected cells directly and produce cytokines, such as IFN-γ, that favour the development of a cytotoxic T cell response [2, 10]. Rare human NK cell deficiencies are associated with increased susceptibility to viral infection, revealing the importance of human NK cells in antiviral immunity [11, 12]. However, analysing the timeline of human NK cell activation in response to viral infection *in vivo* remains difficult. Virus-infected patients show evidence of NK cell activation compared to uninfected controls but whilst vaccination allows controlled studies to be performed, the analysis of pre-infection status and very early post-infection events remains challenging [3, 13-18]. Hence, our view of the early stages of NK cell activation is largely based on studies performed using model species.

Reovirus, a non-enveloped dsRNA virus, is pathogenic in mice and induces a type I interferon (IFN-I) response [19]. Whilst it is not a significant human pathogen, reovirus has the interesting property of preferentially killing tumour cells leading to its evaluation as a therapeutic agent [20]. The anti-cancer effects of reovirus and other oncolytic viruses appear to be linked to a two-fold mode of action, namely the direct killing of tumour cells and the induction of innate and adaptive anti-tumour immunity [21-24]. Intravenous delivery of reovirus into patients is associated with its rapid loss from the circulation; in eight out of ten treated patients, the virus was undetectable in the bloodstream after one hour post-infection [25]. Despite the presence of neutralising antibodies, reovirus reached the tumour and was associated with tumour

cell apoptosis [25]. This same trial allowed us to study infection-induced human NK cell activation under controlled conditions. Our results define the kinetics of human NK cell activation in response to viral infection *in vivo*.

Materials and methods

Ethical approval and the clinical trial

This study was undertaken following institutional and national ethical and regulatory approval. Patients were enrolled in the trial and provided blood samples following informed consent. The patient group and the trial are described elsewhere [25].

Antibodies

The following antibodies were used in this study; CD69 (clone FN50); CD56 (clone B159), CD16 (clone 3G8), CD3 (clone SK7), CD107a (clone H4A3), NKG2D (clone 1D11), DNAM-1 (clone DX11), NKp44 (Clone p44-8.1), NKp46 (clone 9E2), CD158a (HP-3E4), CD158b (CH-L), all from BD Biosciences; CD158e (clone DX9) from Miltenyi Biotec and Tetherin/CD317 (clone 26F8) from eBiosciences. For the IFN-I blocking experiments, we used a cocktail of anti-IFNα (clone MMHA-2), anti-IFNβ (clone 76703.111) and rabbit anti-IFNα antiserum, all from PBL Assay Science.

Flow cytometry and gene expression analysis

Cell surface expression of CD69 and tetherin was determined using flow cytometry on either purified NK cells (P1-P4) or by gating on CD56+CD3^{neg} NK cells in Peripheral Blood Mononuclear Cells (PBMC; P5-P10). NK cells were purified by indirect magnetic immunoselection (Miltenyi Biotech). Flow cytometry was performed using a Becton Dickinson (BD) LSRII or BD FACSCalibur flow cytometer using BD FACSDiva software and BD CellQuest Pro software, respectively. For gene expression studies, mRNA was converted to cDNA (using random hexamer priming) and expression of IFIT1, IFI44L, 18S RNA or ABL1 was analysed by quantitative (q)RT-PCR using Taqman reagents from Applied Biosystems. Data was normalised to either 18S RNA or ABL1 mRNA (as indicated) and the fold-change induced during infection calculated using the ΔΔCt method.

***In vitro* studies**

PBMCs from healthy donors were co-incubated with reovirus (REOLYSIN®; Oncolytics Biotech Inc.) at a multiplicity of infection (MOI) of 0.2-1 in the presence of either the anti-human IFN-I antibody cocktail or matched serum/IgG controls. Degranulation assays were performed forty-eight hours post-infection using the K562 target cell line in the presence of GolgiStop (from BD Biosciences) and the anti-CD107a antibody [26]. For analysis of isolated NK cells and fractionation of PBMC, the NK cells were purified using indirect magnetic immunoselection reagents (Miltenyi Biotec) and the NK cell depleted PBMC (PBMCΔNK) were eluted from the column.

Results

Ten patients (P1-10; aged 50-74) with colorectal cancer liver metastases were enrolled in a clinical endpoint trial to assess the delivery of reovirus to the metastatic tumour [25]. Each patient received between one and five intravenous infusions of 10^{10} units of reovirus prior to planned surgical resection of their tumour. Seven of the ten patients received reovirus daily for five days, P7 received four doses, P8 a single dose and P1 received three doses with an altered timing (Fig. 1a). Six of the ten patients experienced fever and several experienced flu-like symptoms during treatment, consistent with viral infection [25].

Blood samples taken before and during treatment were used to analyse the NK cell phenotype. Infection induced rapid expression of the lymphocyte activation marker CD69 on the NK cells, peaking forty-eight hours post-infection (Fig. 1b, c). A single dose of reovirus was sufficient to induce this activation, as shown in P8 who received just one dose and in P1, in whom more than 60% of peripheral blood NK cells were CD69+ before the second dose was administered (Fig. 1b). With the exception of P1 and P8, all patients received two doses of virus before the forty-eight hour sample (when NK cell activation peaked) and a further two doses between the forty-eight hour and ninety-six hour samples (Fig. 1a). However, NK cell activation declined after forty-eight hours in all patients, suggesting that NK cells were refractory to further stimulation within this period.

Reovirus dsRNA, and indeed other viral nucleic acids, induce type I interferon (IFN-I) responses in animals via pathogen-associated molecular pattern receptor recognition. The cytoplasmic RNA sensor RIG-I recognises the 5'-diphosphate

present on reovirus dsRNA and induces IFN gene expression [27]. It is long established that IFN treatment activates human NK cells *in vivo* [19, 28-30]. CD69 is induced via IFN-I responses and we have previously shown that reovirus treatment of peripheral blood mononuclear cells (PBMC) *in vitro* induces CD69 expression by NK cells in an IFN-I dependent manner [23]. Expression of the interferon-stimulated genes (ISGs) IFIT1 and IFI44L in the reovirus-treated patients showed similar kinetics to the induction of NK cell CD69 expression, peaking forty-eight hours post-infection (Fig. 2a). Like CD69, expression of the ISGs was transient and declined after this initial post-infection peak. Collectively, these results are consistent with the virus-mediated induction of an IFN-I response *in vivo* and the IFN-I dependent activation of human NK cells within twenty-four to forty-eight hours post-infection.

Tetherin is an IFN-I inducible antiviral restriction factor and its expression at the cell surface provides a convenient marker for IFN-I responses during viral infection [31-33]. Tetherin was constitutively expressed at the NK cell surface and expression was significantly enhanced following reovirus treatment *in vivo*, exhibiting similar induction kinetics to CD69 and the ISGs (Fig. 2b, c). Human NK cells express several activating receptors that have been implicated in the detection of virus-infected cells, including NKG2D (CD314), DNAM-1 (CD226), NKp30 (CD337) and NKp44 (CD336) [3, 34]. Expression of these molecules was not significantly altered on patient NK cells at the peak of the IFN-I response and did not show further alterations in expression during the course of treatment (Fig. 2b, c).

We then performed experiments to analyse the response to reovirus *in vitro*. We treated PBMC with reovirus in the presence or absence of antibodies that block the IFN-I response. Treated PBMC were then co-cultured with tumour target cells and the tumour-mediated degranulation of the NK cells in the PBMC analysed using flow cytometry [26]. This demonstrated that reovirus treatment of PBMC resulted in the IFN-I dependent, functional activation of the NK cells (Fig. 3a), consistent with previously published data [23]. We then treated PBMC with reovirus for forty-eight hours, purified the NK cells (using immunomagnetic selection) and analysed the expression of IFIT1 mRNA in the NK cell population and in the PBMC depleted of NK cells (PBMCΔNK). Both the NK cells and the PBMCΔNK fraction demonstrated substantial induction of IFIT1 mRNA (Fig. 3b). Furthermore, flow cytometry of the reovirus-treated PBMC showed the induction of CD69 and tetherin expression on the NK cell surface (Fig. 3c), as we observed in the reovirus treated patients (Fig. 1 and

2). Similar to the situation observed *in vivo*, the *in vitro* reovirus treatment did not result in substantial changes in the cell surface expression of NKG2D, DNAM-1, NKp30, NKp44 or NKp46 on NK cells (Fig. 3c). We did observe a significant increase in NKp46 expression *in vitro* but this only represented a ~1.4 fold increase compared to a ~5 fold increase in tetherin expression (Fig. 3c). Cytokines such as IL-2 and IL-15 increase the cell surface expression of NKG2D and DNAM-1 *in vitro* [26] and a comparison of IL-15 and IFN-I stimulation of purified NK cells showed that IL-15 induced expression of NKG2D, DNAM-1, CD69 and tetherin, whereas IFN-I only induced CD69 and tetherin, similar to the effects of reovirus treatment we observed *in vitro* and *in vivo* (Fig. 3d). In conclusion, reovirus treatment, both *in vivo* and *in vitro*, was associated with the induction of CD69 and tetherin expression at the NK cell surface, but with little change in the expression of other NK cell activation receptors analysed. The induction of CD69 and tetherin *in vivo* coincided with the peak of IFN-I induced gene expression and both CD69 and tetherin were IFN-I inducible in NK cells *in vitro*.

These results, showing that reovirus treatment modulates NK cell activation in the early post-infection period, are consistent with a role for NK cells in controlling viral infection whilst adaptive immunity is developing. Recently, NK cells have been shown to have a more durable role in the immune response. The identification of so-called memory NK cells and the ability of activated NK cells to limit T cell responses have revealed that NK cell activity persists beyond this initial wave of activation [14, 35-37]. Interestingly, we found a significant increase in the absolute numbers of NK cells in the pre-surgery samples; in two patients (P9 and P7) we detected a six-fold and a thirteen-fold increase respectively (Fig. 4). Expression of KIR molecules is clonal and maintained following cell division and KIRs thus provide markers for analysis of putative clonal expansions. Cell surface expression of CD158a, CD158b and CD158e antigens identified eight distinct populations constituting between ~2% through to ~50% of total NK cells (Supplementary Table 1). However, we did not detect clonal expansions that could account for the changes in absolute numbers seen between the ninety-six hour and pre-surgery samples; this suggests that the increase in the absolute numbers of NK cells was due to polyclonal expansion.

Discussion

The use of a therapeutic virus within the context of a clinical trial has allowed us to analyse the kinetics of human NK cell activation in response to viral infection under controlled conditions. Our results demonstrate that human viral infection results in the rapid and transient activation of NK cells in the bloodstream and that this activation, which occurred within twenty-four to forty-eight hours post-infection, was associated with an IFN-I response. Blood samples from healthy volunteers given a poly IC-like molecule (a mimic of dsRNA) exhibited similar kinetics of ISG induction and other gene expression responses that were consistent with the activation of innate immune responses, including those involving NK cells [38]. This work, together with the results presented here, are consistent with early studies using mouse models in which viral infection resulted in IFN production and the induction of NK cell activation within two to three days of infection [4, 5, 39]. In the absence of an IFN-I response, viral pathology is enhanced and this is associated with a reduction in infection-induced NK cell activity [39-41]. However, the effect of IFN-I on NK cells is largely indirect, with IFN-I inducing IL-15 production and expression of the IL-15 receptor on NK cells; IL-15 then acts upon NK cells [41, 42]. Indeed, several viruses (including reovirus) induce IL-15 mRNA in PBMC and activate NK cells in an IL-15 dependent manner [43]. The early *in vivo* activation of NK cells in response to reovirus treatment is highly suggestive of IFN-I and IL-15 mediated events. However, the contribution of other NK cell activating cytokines, such as IL-12 and IL-18 or indeed IL-2 (produced predominantly by activated T cells during adaptive immunity) cannot be discounted.

Expression of both CD69 and tetherin is IFN-I inducible. Tetherin was originally identified as an IFN-I inducible antiviral restriction molecule with the ability to prevent release of HIV [31]. This activity extends to a number of enveloped viruses and IFN-I induction of tetherin allows it to act as a broad defense against viral spread. Tetherin provides a convenient cell surface marker of an IFN-I induced antiviral response [32, 33]. However, our *in vitro* data show that both tetherin and CD69 are inducible in NK cells by IFN-I and IL-15. Others have shown that several cytokines can induce tetherin and that its induction can precede IFN-I responses [44-46]; it remains possible that other cytokines or signals induce CD69 and tetherin in response to reovirus infection. The actual role of CD69 in NK cell activity is poorly defined. Activated mouse NK cells traffic from the periphery to the lymph nodes

where NK cell derived IFN-γ helps to promote cytotoxic T cell responses [10]. In mouse B and T lymphocytes, IFN-I induction of CD69 decreases the activity of the sphingosine-1-phosphate receptor 1 ($S1P_1$) thereby inhibiting egress from mouse secondary lymphoid tissue (SLT) [47]. It is possible that human NK cell expression of CD69 causes similar effects, allowing NK cells that traffic from the blood to other tissues (such as the SLT) to remain there. However, whilst CD69 inhibits $S1P_1$ responses in B and T lymphocytes, mouse and human NK cells preferentially express $S1P_5$, and this receptor is not inhibited by CD69 [25, 48, 49]. Thus, the role of NK cell CD69 remains unclear. Reovirus-activated NK cells may traffic to the liver (the site of the colorectal metastases in these patients) where they would be able to attack the tumour directly. Whether reovirus-activated NK cells participate directly in tumour lysis or whether these activated NK cells mediate other pathways of anti-tumour immunity via cytokine secretion for example remains unknown. A limitation of our study is the inability to analyse NK activation and trafficking beyond the peripheral blood. However, the trial did establish that reovirus reaches the tumour [25], suggesting that liver-resident NK cells might be activated. Indeed, we have previously shown that reovirus can activate liver-derived NK cells *in vitro* and enhance their response to colorectal tumour cell lines [23].

Nine of the ten patients in the trial received multiple doses of reovirus, yet CD69, tetherin, IFI44L and IFIT1 all exhibited just a single peak of expression approximately forty-eight hours after the first dose. For example, all patients except P1 and P8 received two further doses of virus between the forty-eight hour and the ninety-six hour time points, yet we did not observe a second peak of activation (or IFN-I response) and in all cases, responses declined in all patients after forty-eight hours post-infection. Furthermore, P1 and P8 revealed that a single dose of virus gave a similar magnitude of response to those patients receiving multiple doses; P1 also showed that a strong response could be detected within twenty-four hours of treatment. The results suggest that the initial IFN-I response (and NK cell activation) was followed by a refractory period during which the patients were unable to respond to further exposure to reovirus. Most adults have been exposed to reovirus and all patients in the trial had neutralising antibodies that increased in titre around days 3-5 post-infection [25]. Whether this boost in antibody titre blocks the IFN-I response to subsequent doses of reovirus seems unlikely, but nevertheless remains unclear. An intriguing alternative is that the refractory period is related to that observed in mouse

viral infection models [50, 51]; the initial viral infection induces an IFN response which is followed by a refractory period in which further IFN responses to unrelated pathogens are blunted. This refractory period has been suggested to contribute to the enhanced susceptibility to unrelated, secondary infections that can follow viral infection. In mice, the mechanisms underlying this refractory period include a reduced capacity of plasmacytoid dendritic cells (pDC) to produce IFN [51] and the induction of OASL1, a negative regulator of IFN production [52]. However, other homeostatic control mechanisms that halt responses, including molecules that target IFN production and downstream signalling pathways, may also influence responses [53-57]. Interestingly, tetherin was proposed to act as a feedback inhibitor of IFN production by engaging the receptor ILT7 on pDC [58]. However, whilst ILT7 ligation was confirmed to halt IFN production, a role for tetherin in this process was subsequently called into question [59]. To the best of our knowledge, the data presented here is the first demonstration of this refractory period in humans. However, the constraints of working within a clinical trial make these conclusions speculative. Furthermore, applying these findings to the general population also warrants caution because all of the patients in the trial have metastatic cancer, presumably associated with alterations in immune status. Notwithstanding the limitations of our study, the clinical importance of opportunistic infections following acute viral infection (e.g. with influenza), or chronic infections (such as HIV) cannot be understated. From a cancer therapy perspective, our results indicate that the scheduling of oncolytic viruses will require optimisation, if IFN-I and NK cell responses are to be maximised.

Whilst profound effects were observed in the first forty-eight hours post-infection, we also observed a later change in NK cells, namely a significant increase in the absolute numbers of NK cells. In HIV infection there is an expansion of particular KIR expressing cells [16]. We did not detect particular clonal expansions using antibodies that detect CD158 family molecules that include KIR2DL1, KIR2DL3 and KIR3DL1 (as well as related short form KIRs). This panel identified approximately 30-50% of total NK cells, consistent with more detailed KIR phenotyping where approximately 50% of NK cells lack KIR expression [60]. However, we did not analyse expression of NKG2C expressing cells; this population expands in both cytomegalovirus and hantavirus infection and for the latter, expanded cells expressed at least one KIR molecule against a self-MHC class I molecule, indicating that the expanded NK cells were functionally licensed [14, 61]. The KIR

and MHC haplotype of the patients within our study was unavailable to us and we do not know whether the expansions we detected were confined to licensed populations. The significance of this relatively late post-infection phenotype is unclear. There is emerging data that suggest a role for NK cells beyond the immediate post-infection stage [14]. The significance of these more durable NK cell responses, for example whether they represent the formation of a memory-like NK cell population [35], or a role for NK cells in the cessation of T cell responses currently remains unclear [36, 37].

The ability of reovirus to induce NK cell activation is likely to contribute to its oncolytic activity *in vivo*. However, effective oncolytic virus treatment will depend upon achieving the correct balance of antiviral and anti-tumour activity [20, 62]. For example, depletion of NK cells limits the efficacy of both vesicular stomatitis virus and reovirus virus treatment, consistent with stimulation of the anti-tumour effector function of this population [63, 64]. However, the antiviral activity of NK cells has been shown to impede the action of oncolytic herpes simplex virus against glioblastoma [65]. In the patients treated here, replication competent reovirus was recovered from the colorectal liver metastases but not from surrounding healthy tissue, suggesting effective targeting of the virus to the tumour [25]. Furthermore, *ex vivo* studies show that reovirus activates NK cells from the liver and enhances their cytotoxic activity towards colorectal cancer cell lines [23]. However, viral infection in the liver can induce potent immunosuppressive activity (via IL-10 and TGF-β) that limits NK cell production of IFN-γ; similar effects would be expected to blunt oncolytic virus induced NK cell activation and anti-tumour immunity [66].

In summary, use of a therapeutic virus in a clinical trial has enabled us to study the kinetics of NK cell activation in response to viral infection. The increasing use of therapeutic viruses promises to provide new opportunities to study the activation and resolution of the human immune response *in vivo* and provide key information that is currently inferred from studies performed in other species.

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Figure legends

Figure 1. Human NK cell activation by reovirus *in vivo*.

(a) Schedule of patient infection and sampling during the reovirus clinical trial. Four different schedules were employed. Patients (P)2, 3, 4, 5, 6, 9 and 10 were treated according to the schedule shown at the top. The variations for P7, P8 and P1 are shown below. The time of infection is shown above the horizontal line (grey vertical arrow) and the time of blood sampling below (black vertical arrow). Times of blood sampling (time post-infection) are shown in hours (0, 1, 24, 48, 72, 96), immediately prior to surgery (Sgy) and in months (1Mo, 3Mo). The indicated variations were made according to clinical parameters, which together with patient details and timing of the surgery, have previously been reported [25]. Each infusion consisted of 10^{10} units of reovirus, with one unit defined as the dose of virus required to kill 50% of cultured cells *in vitro* (the Tissue Culture Infective Dose 50% or $TCID_{50}$).

(b) NK cell surface expression of CD69 in selected patients representing key variations to the schedule, and in a healthy control (HC); P3 represents the patients with repeated doses, P8 had a single dose and P1 underwent treatment with altered timing to the other patients. The time post-infection is shown with 0 hour immediately prior to the first infusion. The values in grey indicate the percentage of CD69 expressing NK cells compared to isotype control stains that were performed for all analyses (not shown).

(c) Summary of CD69 expression in patients P2-10 and in healthy control (HC) donors (n=7). The P values were calculated using the Mann-Whitney test; P<0.001***. P1 data is omitted from this analysis because of the altered timing of therapy.

Figure 2. Interferon responses and changes in the NK cell surface phenotype *in vivo*.

(a) Expression of the interferon stimulated genes (ISGs) IFIT1 and IFI44L following reovirus treatment, as determined by quantitative RT-PCR. The analyses were performed using NK cells purified from P5 or from whole PBMC isolated from P7. The data shows expression in the treated patient (black lines and squares) and in an

uninfected control (grey lines and diamonds). Expression was calculated as the fold-change in expression compared to the pre-infection (0 hour) timepoint (assigned a value of 1 unit of expression).

(b) Expression of NK cell receptors in patients at pre-infection (0hr) timepoint (-) and 48 hours post-infection (+). Data shows the change in mean fluorescence intensity (MFI) with the expression at the 0 hour timepoint assigned a value of 1. The number of patients in each group is indicated (*n*). Data (where $n>2$) was analysed using the Student's T test and statistically significant differences are shown; $P<0.05$*.

(c) Flow cytometric analysis of expression of patient NK cell surface molecules throughout the treatment course. The grey dotted line shows the approximate position of the median fluorescence intensity of the signal at the pre-infection timepoint. The plots are from individual patients (shown) and are representative of data collected across the treatment group; only tetherin (and CD69, Fig.1) showing substantial alterations in expression. Tetherin expression was not determined at the three-month (3Mo) timepoint.

Figure 3. Analysis of NK cell responses to reovirus treatment *in vitro*.

(a) Reovirus and IFN-I mediated activation of NK cell granule exocytosis. The left hand panel shows the display of cell surface CD107 (gated on CD56+CD3^{neg} NK cells within PBMC) in the presence or absence of K562 target cells. The experiment was performed using PBMC from healthy donors without further treatment (untreated), in the presence of 0.2 MOI reovirus (+virus) and in the presence of reovirus and an anti-IFN antibody or a control antibody (cAb). The percentage values indicate the proportion of CD107+ NK cells for each treatment. Statistical analysis was performed between the indicated pairs of treatments using the Student's T test; $P<0.05$*. Limitations in the size of samples available from the clinical trial made cytotoxicity assays from the *in vivo* study difficult to perform. However, of three patients analysed, one showed increased cytotoxic activity 48 hours post-infection (data not shown).

(b) Expression of IFIT1 mRNA in NK cells and NK cell-depleted PBMC (PBMCΔNK) with (+) and without (-) reovirus treatment *in vitro*. Whole PBMC (from healthy donors) were treated with reovirus (at an MOI of 1), cultured for 48 hours and fractionated into NK cells and NK-depleted PBMC (using magnetic indirect selection of NK cells). RT-PCR for IFIT1 and ABL1 was performed using

mRNA isolated from these fractions; IFIT1 expression was normalised to ABL1 mRNA and the fold-change induced during infection calculated via the ΔΔCt, with the untreated cells (-) assigned an expression value of 1 unit. The data shown is from two different donors.

(c) The expression of NK cell surface markers +/- reovirus treatment *in vitro*, analysed 48 hours post-infection. The right hand panel indicates the percentage of CD69 expressing NK cells in the PBMC population, the left hand panel indicates the change in mean fluorescence intensity of the indicated markers. Statistical analysis was performed using the Student's T test; $P<0.05$*, $P<0.01$**, $P<0.001$***.

(d) Expression of NK cell surface molecules following cytokine treatment *in vitro*. Purified NK cells (from healthy donors) were treated with 20ng/ml of IL-15 or 100 IU IFN-I for 48 hours and expression of the indicated markers was analysed by flow cytometry. The dotted grey line shows the approximate position of the mean fluorescence intensity of the isotype control for CD69 or for expression of the other markers in untreated cells.

Figure 4. Absolute numbers of NK cells (cells per μl) in patients during the treatment course. The values at each timepoint were compared to the pre-treatment 0 hour timepoint using the Mann-Whitney test, only the statistically significant differences are shown; $P<0.05$*. The normal range for the absolute number of NK cells in healthy donors is ~90-600 cells per μl.

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Figure 1

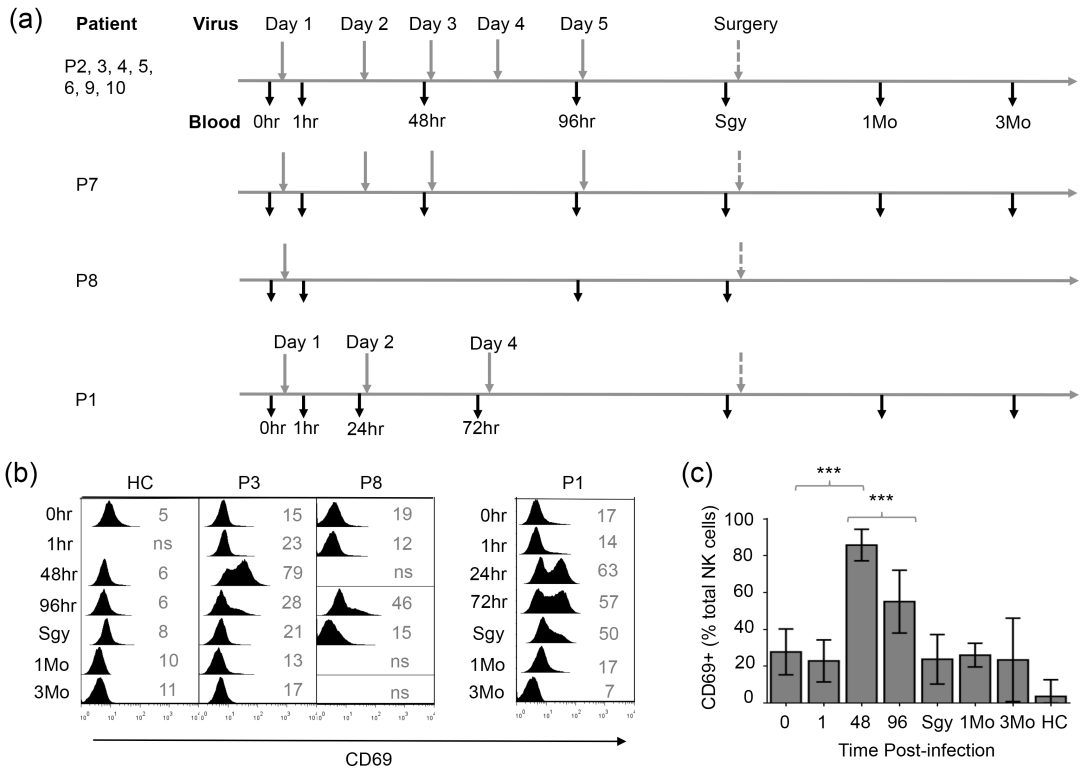


Figure 2

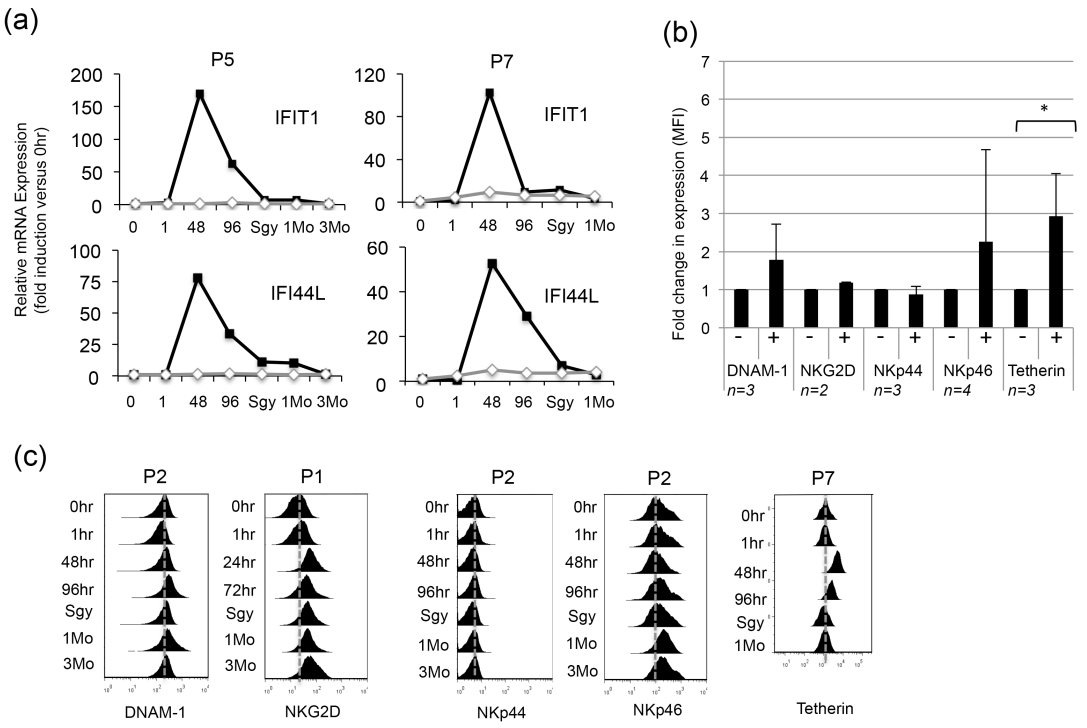


Figure 3

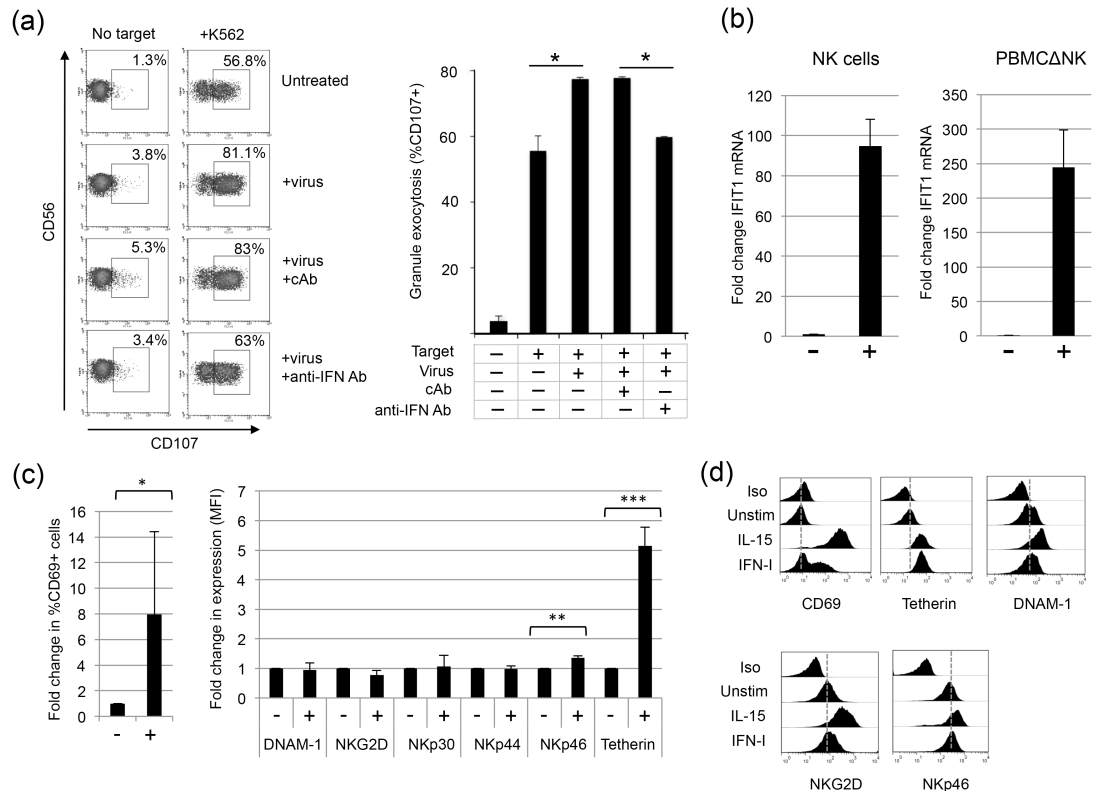
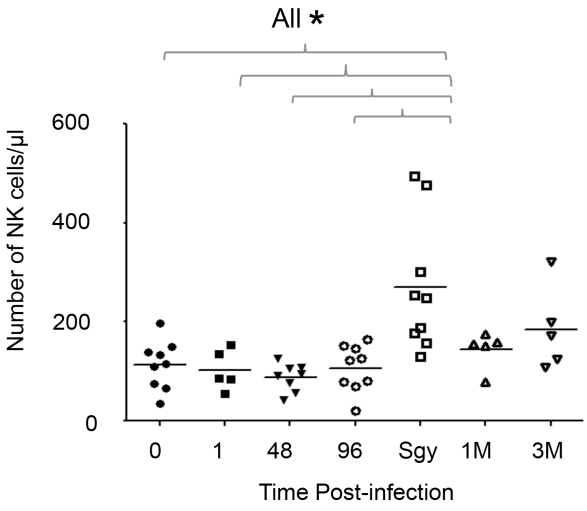


Figure 4



	P7	~13x	P8	~2x	P9	~6x	P10	~2x
CD158	96hr	Sgy	96hr	Sgy	96hr	Sgy	96hr	Sgy
ABE	2.12	2.35	1.19	0.95	0.1	0.06	1.95	2.19
ABe	6.21	6.15	8.65	5.89	9.00	9.35	6.19	5.63
AbE	7.37	9.39	3.81	2.95	0.00	0.02	4.16	3.87
aBE	2.71	3.24	1.94	0.85	0.03	0.02	2.65	2.49
Abe	6.97	6.17	11.38	7.78	5.99	6.36	9.43	8.69
aBe	11.9	12.48	17.41	12.77	26.53	28.14	14.69	16.02
abE	4.78	5.65	2.38	1.39	0.07	0.04	3.83	2.57
abe	57.94	54.56	53.24	67.42	58.29	56.01	57.10	58.54

Supplementary Table 1: Clonal analysis of NK cell populations.

NK cells from P7-10 were analysed for expression of CD158a, b and e at ninety-six hours post-infection and in the pre-surgery sample (Sgy). The fold change in absolute numbers of NK cells between these two samples is shown next to the patient number (e.g. for P7, ~13X). The values indicate the size of the respective populations as a percentage of the total CD56+CD3^{neg} NK cells.
The three markers identify eight distinct populations listed in the left hand column. Expressed CD158 molecules are denoted by upper case letters (e.g. ABC is CD158a+, b+, c+) or lower case for non-expressed (abc denotes the triple negative population of CD158a^{neg}, b^{neg}, c^{neg}).