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Haemagglutinin-neuraminidase from HPIV3 mediates human NK regulation of T cell proliferation via NKp44 and NKp46

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Abstract

HPIV3 is a respiratory virus causing airway diseases, including pneumonia, croup, and bronchiolitis, during infancy and childhood. Currently there is no effective vaccine or anti-viral therapy for this virus. Studies have suggested that poor T cell proliferation following HPIV3 infection is responsible for impaired immunological memory associated with this virus. We have previously demonstrated that NK cells mediate regulation of T cell proliferation during HPIV3 infection. Here we add to these studies by demonstrating that the regulation of T cell proliferation during HPIV3 infection is mediated via NK receptors NKp44 and NKp46 and involves the surface glycoprotein haemagglutinin-neuraminidase but not the fusion protein of the virus. These studies extend our knowledge of the regulatory repertoire of NK cells and provide mechanistic insights which may explain reoccurring failures of vaccines against this virus.

Human parainfluenza virus type 3 (HPIV3) is a major respiratory pathogen which is responsible for pneumonia, croup and bronchiolitis in infants and children [1–3]. Reinfection with this virus throughout life is common, with no development of lifelong immunity. Even in the presence of circulating neutralizing antibodies, individuals have been shown to be susceptible to reinfection [3, 4]. It has been suggested that reinfection with HPIV3 occurs due to the failure of T cell proliferation to the virus [5, 6]. Previously published data from our group demonstrated that human NK cells induce T cell cycle arrest in a contact-dependent manner during infection with this virus and this regulation does not occur in the absence of the NK population. NK regulation was also associated with reduced levels of interleukin-2 (IL-2) production during HPIV3 infection, and the addition of exogenous IL-2 could override the NK-induced inhibition of T cell proliferation [7]. While these studies suggested a mechanism to explain the failed memory associated with HPIV3 infection, the viral components involved and the mode of NK cell regulation remained unclear. Here we demonstrate that HPIV3 surface glycoprotein haemagglutinin-neuraminidase (HN) but not the fusion (F) protein induces NK cell-mediated inhibition of T cell proliferation during HPIV3 infection. In addition, we show that viral interaction with NKp46 and NKp44 but not NKp30 drives T cell cycle arrest in these cultures. NKp46, NKp44 and NKp30 are known collectively as natural cytotoxicity receptors (NCRs) [8]. NKp46 (also known as NCR1/CD335), NKp44 (also known as NCR2/CD336) and NKp30 (also known as NCR3/CD337) are all expressed by NK cells and play an essential role in NK-mediated recognition and killing of target cells [9]. NKp46 is the only NCR to also have a mouse orthologue (mNKp4619) and it directly recognizes the haemagglutinin protein of numerous viruses including influenza, as well as self and tumour ligands [10]. NKp44 is not expressed in mice and to date, has only been found to have orthologues in humans and non-human primates [11]. It is expressed exclusively on activated NK cells and depending on the ligand it binds to, it can have an activating or inhibitory effect [12, 13]. Of additional interest, we found

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Abbreviations: APC, antigen-presenting cell; CD3, cluster of differentiation 3; CD4, cluster of differentiation 4; CD14, cluster of differentiation 14; CD335, cluster of differentiation 335; CD336, cluster of differentiation 336; CD337, cluster of differentiation 337; CD56, neural cell adhesion molecule; CDV, Canine Distemper Virus; CFSE, carboxyfluorescein succinimidyl ester; F protein, fusion protein; HN, haemagglutinin-neuraminidase; HPIV3, human parainfluenza virus type 3; IL-2, interleukin-2; MACS, magnetic-activated cells sorting; ML, mixed lymphocytes; N, HPIV3 nucleoprotein; NCR, natural cytotoxicity Receptor; NCR1, natural cytotoxicity receptor 1; NCR2, natural cytotoxicity receptor 2; NCR3, natural cytotoxicity receptor 3; NP, Influenza virus nucleoprotein; PMA, phorbol myristate acetate; RPMI, Roswell Park Memorial Institute medium; Treg cells, regulatory cells.

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that although the overall CD3+ T cell numbers are reduced, the levels of regulatory T (Treg) cells increased in line with NK cells during HPIV3 infection.

To conduct these experiments, CD14+ monocytes were purified from human PBMCs (obtained from buffy coats of healthy donors from the Irish blood transfusion service) to >90 % using magnetic-activated cells sorting (MACS) via magnetic anti-CD14 microbeads. These cells (1×10^6 ml^{-1}) were cultured in RPMI (with 10 % FCS, 1 % L-glutamine and 1 % penicillin/streptomycin) and were untreated, infected with HPIV3 (strain C-243, ATCC; Accession number: D10025.1; 50 % $TCID_{50}$ ml^{-1} of 10^6) or influenza virus (A/Puerto-Rico/8/34(H1N1), a generous gift from NIBSC, UK; $TCID_{50}$ ml^{-1} of 10^7) (a respiratory virus associated with acute as opposesd to persistent or recurrent infection with the same strain of influenza) for 2 h at 37 °C. Cells were washed to remove extracellular virions and incubated for a further 20–24 h to allow maturation to antigen-presenting cells (APCs). Reverse transcription PCR for nucleoprotein was performed to confirm that infection had occurred in HPIV3 and influenza virus-infected cells. PCR parameters consisted of 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing (temperature varied with primers) for 45 s, and extension at 72 °C for 10 min. Amplification was performed in 25 µl reactions containing 400 pmol of each primer, 2 mM deoxyribonucleotide triphosphates, 10X PCR buffer (Promega), 2.5 mM Mg^{2+}, 1.25 U of Taq DNA Polymerase (Promega) and 1 µl of cDNA. The primer sequences for the genes examined were as follows: HPIV3 forward, TTGGAAGTGACCTGGATTAT; reverse, GGATACAGA

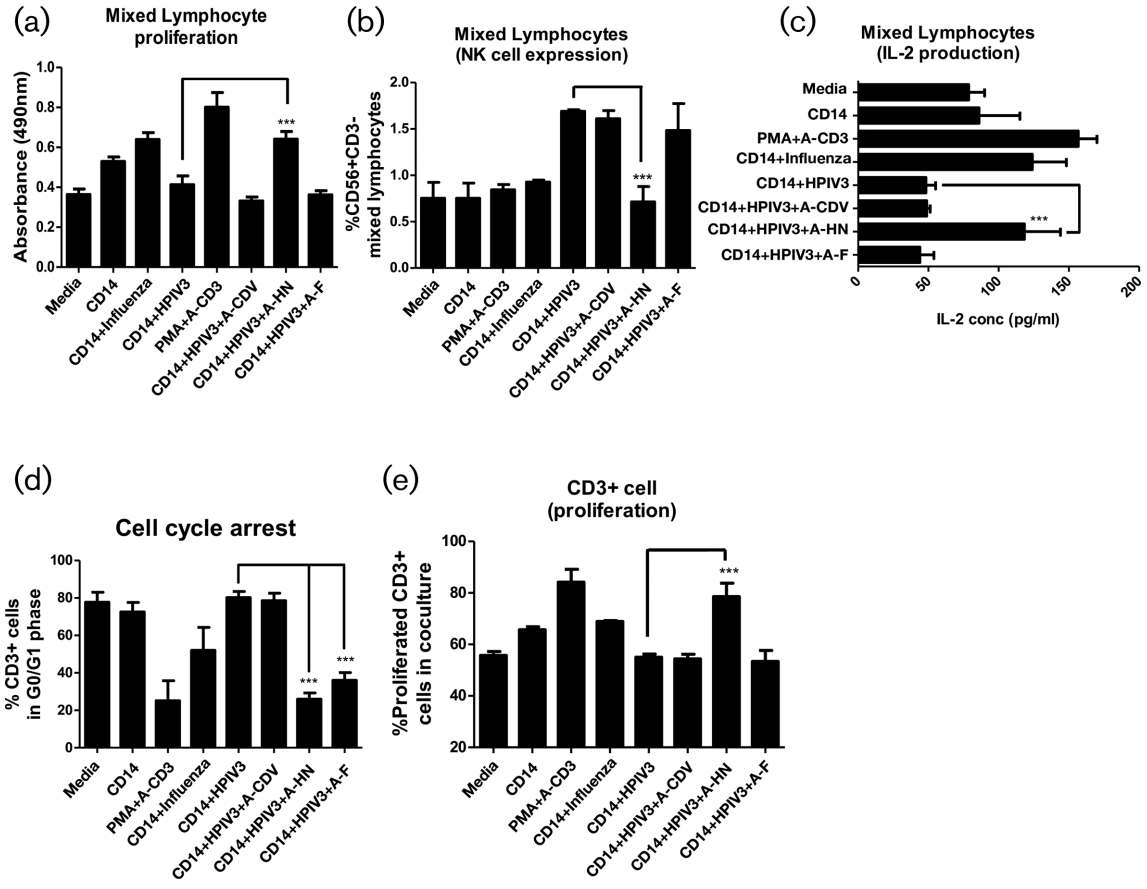

Fig. 1. Effect of neutralizing antibodies to HPIV3 surface glycoproteins on NK cell-mediated T cell inhibition. Isolated CD14+ cells were infected with HPIV3 or influenza, washed after 2 h, and co-cultured with allogeneic mixed lymphocytes after 24 h. Prior to co-culture, HPIV3-infected CD14+ cells were cultured with Anti-Canine Distemper Virus (A-CDV), Anti-HN (A-HN) or Anti-F (A-F) antibodies in some cases. Media samples, and PMA and Anti-CD3 (A-CD3) stimulated mixed lymphocytes (MLs) were also included. After 5 days, these were assessed for overall ML proliferation by MTS (a), or the percentage of cells expressing NK cell markers (CD56+CD3) were determined by flow cytometry (b). Additionally, IL-2 levels in supernatants were determined by ELISA (c). The percentage of CD3+ cells in the G0/G1 phase of the cell cycle was determined by PI staining and flow cytometry analysis (d). These experiments were repeated, isolated CD56+ and CD3+ cells, with CFSE incorporated into the latter (e). These were again co-cultured with allogeneic-infected and antibody-treated CD14+ cells. All experiments represent normalized results from three donors in triplicate. Significance was determined using the Newman–Keuls test to compare all parameters, with comparisons indicated on graphs. ***$P\leq0.001$.

TAAAAGGAGC; influenza virus forward, CTTACGAACAGATGGAGACT; reverse, TGTTTTCCGCCCATTCTCAC; human β-actin forward, TACAATGAGCTGCGTGTG; reverse, TGTTGGCGTACAGGTCTT. DNA electrophoresis was performed on a 1 % agarose gel containing 2 µl of ethidium bromide at 100 V for 45 min. Infected or control APCs were then co-cultured with allogeneic (CD14-depleted) mixed lymphocytes (2×10^5 ml^{-1}) isolated using MACS via magnetic anti-CD14 microbeads (Miltenyi Biotec) as previously shown [14]. These co-cultures were incubated at 37 °C for a further 5 days. Additional mixed lymphocytes were activated with phorbol myristate acetate (PMA) and anti-CD3 as a positive control. Proliferation was evaluated using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay [MTS (Promega)] by adding 20 µl MTS solution to 100 µl cell suspension (6×10^5 cells ml^{-1}) for the last 4 h of incubation. Carboxyfluorescein succinimidyl ester (CFSE) incorporation was also used to track proliferation of CD3+ T cells according to manufacturer's guidelines (Invitrogen). Flow cytometric analysis of the cell cycle was performed on alcohol-fixed cells with propidium iodide staining. To establish the involvement of HPIV3 surface glycoproteins F and HN, neutralizing antibodies [Anti-HN, Anti-F or Anti-Canine Distemper Virus (CDV) (control) antibodies; a generous gift from Professor Claes Orvell, Karolinska Institute] were co-incubated with HPIV3 infected CD14+ cells at a 1 : 100 vol ratio for 30 min prior to co-culture. To identify the NK receptors involved, 1 µg Anti-NKp44 or Anti-NKp46 or Anti-NKp30 were cultured with mixed lymphocytes prior to co-culture as described for previous studies [15, 16]. Human IL-2 ELISA kits (R&D) were used to quantify the cytokine levels in supernatants. Fluorescence conjugated antibodies to CD56, CD3, NKp30, NKp44 and NKp46 were used to stain cells for detection by flow cytometry (Becton Dickinson FACs Calibur). To further prove the involvement of HN and not F, these glycoproteins were purified as previously described [17] and substituted for HPIV3 viral infection in these cultures.

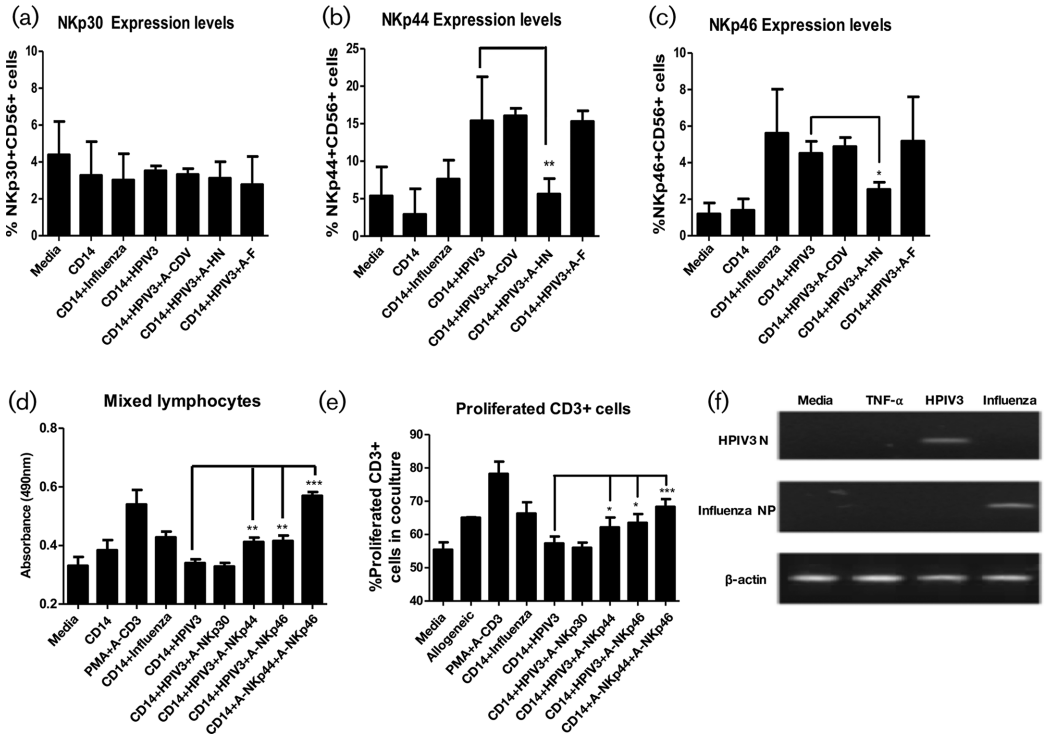

Fig. 2. The involvement of NCRs in NK cell activation by HPIV3. Isolated CD14+ cells were infected with HPIV3 or influenza, washed after 2 h, and then co-cultured with allogeneic-mixed lymphocytes after 24 h. Prior to co-culture, HPIV3-infected CD14+ cells were cultured with Anti-Canine Distemper Virus (A-CDV), Anti-HN (A-HN) or Anti-F (A-F) in some cases. Media samples, and PMA and Anti-CD3 stimulated MLs were also included. After 5 days, the percentage of CD56+cells expressing NKp30 (a), NKp44 (b), or NKp46 (c) were determined by flow cytometry. Similarly, HPIV3-infected CD14+ cells were co-cultured with Anti-NCR (A-NCR) (neutralizing antibody)-treated mixed lymphocytes. The effect of these antibodies on mixed lymphocyte proliferation was determined by MTS assay (d). Similarly, CD56+ and CD3+ cells were isolated, with CFSE incorporated into the latter, and treated with Anti-NCR antibodies. These were again co-cultured with infected CD14+ cells and CD3+ cell proliferation determined by flow cytometry (e). After 24 h incubation, mRNA expression of HPIV3 N, influenza NP and β-actin from infected cells was determined by RT-PCR (f). All experiments represent three donors in triplicate. Significance was determined using the Newman–Keuls test to compare all parameters, with comparisons indicated on graphs. ***$P\leq0.001$, **$P\leq0.01$, *$P\leq0.05$.

We had previously found that HPIV3-infected monocytes induced NK cell-mediated inhibition of T cell proliferation in an allogeneic mixed lymphocyte reaction [7]. We now show that blocking HPIV3 HN but not F on virally infected monocytes restored all the associated indicators of NK regulation; firstly mixed lymphocyte and purified CD3 + T cell (+NK cells) proliferation and IL-2 concentrations were restored to levels comparable with those induced by influenza-infected monocytes (Fig. 1a, c). In addition, Anti-HN antibody treatment resulted in significantly less lymphocytes retained in the G0/G1 phase of cell cycle during HPIV3 infection (Fig. 1d). Concomitantly, the percentage NK cell CD56+CD3 in Anti-HN-treated cultures dropped to similar levels detected following polyclonal activation or influenza infection (Fig. 1c). Neither Anti-HPIV3 F or a control antibody to CDV has a significant impact on NK-induced inhibition of T cell proliferation in HPIV3-infected co-cultures (Fig. 1a–c). Although treatment with Anti-F did prevent cell cycle arrest, this unlike HPIV3 HN, did not translate into restoration of actual T cell proliferation (Fig. 1e). Coincidentally, published data have shown that the HN from HPIV binds human NKp46 and NKp44 but not human NKp30 [2, 18], although no functional explanation has been proposed. We therefore examined the expression of the NCRs; NKp44, NKp46 and NKp30 during HPIV3 infection. We demonstrate no change to NKp30 expression during HPIV3 infection (Fig. 2a) however, we observe an up-regulation of both NKp44 and NKp46 (Fig. 2b, c) during HPIV3 infection which is abrogated when HPIV3 HN but not F was blocked (Fig. 2b, c). Additionally, when either NKp44 or NKp46 were blocked prior to co-culture with HPIV3-infected CD14 cells, mixed lymphocyte proliferation was restored to levels similar to influenza responses during infection (Fig. 2d). This restoration was further enhanced when both receptors were blocked simultaneously, suggesting that these receptors act synergistically. We also detected significant enhancement of CD3+ T cell proliferation in Anti-HN treated, HPIV3-infected co-cultures when CD3+ proliferation was measured using CFSE incorporation (Fig. 1e). We obtained confirmation that infection had occurred in both HPIV3 and influenza virus-infected cells by detecting for the presence of viral nucleoproteins (Fig. 2f). Finally, to provide proof of the involvement of HN we demonstrate that purified HN but not F can mimic viral infection by inducing NK regulation of T cell proliferation which could be restored by blocking NK receptors NKp44 and NKp46 (Fig. 3a–f).

This study demonstrates that HN from HPIV3 induces NK regulation of conventional T cell proliferation in humans. We also show that HN requires access to NK receptors NKp44 and NKp46 to drive this regulatory response providing a

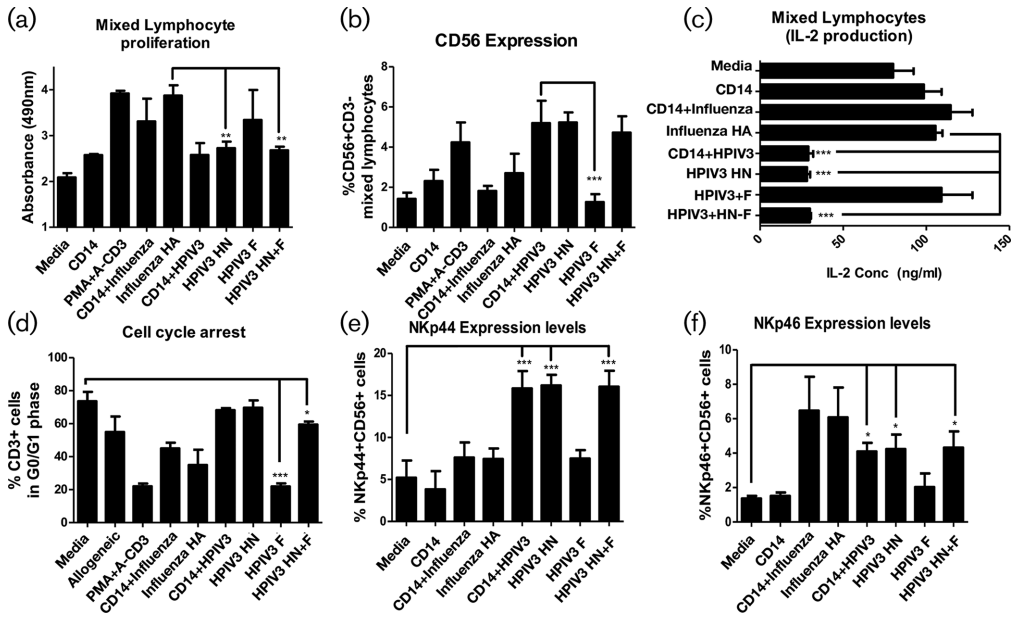

Fig. 3. Effect of isolated HPIV3 surface glycoproteins on NK cell-mediated T cell inhibition. Mixed lymphocytes were cultured with HPIV3 HN or F, or Influenza HA, in addition to media and PMA and A-CD3 controls. Allogeneic HPIV3 or Influenza infected CD14+ cells were also co-cultured with mixed lymphocytes for comparison. After 5 days, these cultures were assessed for overall ML proliferation by MTS assay (a). The percentage of cells expressing NK cell markers (CD56+CD3-) was investigated by flow cytometry (b). Additionally, IL-2 levels in supernatants were determined by ELISA (c). The percentage of CD3+ cells in the G0/G1 phase was determined by PI staining (d). The percentage of CD56+ cells expressing NKp44 (e) or NKp46 (f) was analysed by flow cytometry. All experiments represent three donors in triplicate. Significance was determined using the Newman–Keuls test to compare all parameters, with comparisons indicated on graphs. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

functional rationale for previous studies which demonstrate HPIV3 HN binding to these NK receptors [18, 19]. A recent study has shown expansion of human CD4+ T cells in *ex vivo* human PBMC with overlapping pools of 15-mer peptides encompassing HPIV3 proteins including HN. It may be that the peptides chosen for HN may not have interacted with NKp44/Nkp46 in this study. Furthermore, the authors used additional cytokines to culture the T cells which included additional IL-2 for intracellular staining studies. Interestingly however, in the baseline study on the healthy donors, only the immune response to HPIV3-F protein reached significance suggesting that the healthy donors had been exposed to HPIV3 but had only mounted responses to F and not HN [20]. We suggest that HPIV3-HN induced NK regulation of human T cell proliferation is a plausible mechanism for failed immunological memory in humans. Indeed, this may also explain the successive failures of vaccines that have involved the use of this component. We would further suggest that the success of future vaccines against this important pathogen will require the generation of modified HN versions that retain immunogenicity but do not bind/interact with human NKp44 and NKp46.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Anonymous blood samples from the Irish Blood Transfusion Service at St. James' Hospital were used. The donor information is not available to the researchers. The university's research ethics committee has granted ethical approval.

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