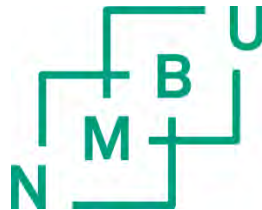# INFLUENZA A(H1N1)PDM09 VIRUS INFECTION IN SWINE

VIRUS CHARACTERIZATION AND NK CELL RESPONSES TO INFECTION

Philosophiae Doctor (PhD) Thesis
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Adamstuen 2016

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ACKNOWLEDGEMENTS

First, I would like to thank the best supervisor team of all time: Anna G. Hauge, Anne K. Storset, Hilde Sindre and Sharon M. Brookes. Anna and Anne, I cannot say how much I appreciate that you picked me for this project! I have been looking forward to come to work every day. Anna, you have been the best support anyone could ask for. Not only do you have great knowledge about influenza A virus, but you have also provided great moral support during this period. You have become a much respected colleague, but also a good friend. I would also like to express my sincere gratitude to Anne. I appreciate that you always have taken the time to discuss matters during a busy workday, and I have felt welcome in your office every time. You are a role model when it comes to knowing something about everything and your enthusiasm for NK cells is an inspiration.

I would like to thank my colleagues at the Section of Virology for providing a good working environment. In particular, I am grateful to Lone T. Engerdahl and Inger Böckerman for all your help in the lab and for your patience with me. Thank you Helena Hauge, for fruitful discussions and for making me look at things from a new angle. My gratitude also goes to the immunology group at Lindern including Grethe M. Johansen, Preben Boysen, Christine Grøndahl-Rosado and Hege Lund. A special thanks to Hege, I have really enjoyed working with you in the lab and appreciate that our talks often have been about completely different things than NK cells and influenza virus. Furthermore, I thank all co-authors, and especially Mette Valheim who took the time to try make me love pathology as much as she does, and for her thorough approach to our results. Mette and Linn Mari, you have been great emotional support and I believe our coffee breaks helped us all through life as PhD students.

I have really appreciated my stays at the University of Veterinary Medicine in Vienna and Animal Health and Veterinary Laboratories Agency in Weybridge, and I would like

ABBREVIATIONS

AFC   Antibody forming cell

ADCC   Antibody-dependent cell-mediated cytotoxicity

AIV   Avian influenza virus

APC   Antigen presenting cell

BAL   Bronchoalveolar lavage

Cal09   Influenza A/California/07/2009

cDC   Conventional dendritic cells

CTL   Cytotoxic T cell

DAMP   Damage-associated molecular pattern

HA   Hemagglutinin

HPAIV   Highly pathogenic avian influenza virus

IFITM   Interferon-inducible transmembrane protein

IFN   Interferon

IL   Interleukin

ILC   Innate lymphoid cell

IRF   Interferon regulatory factor

ISG   Interferon stimulated gene

ITAM   Immunoreceptor tyrosine-based activation motifs

ITIM   Immunoreceptor tyrosine-based inhibition motifs

KIR   Killer immunoglobulin-like receptor

LPAIV   Low pathogenic avian influenza virus

M   Matrix protein

mAb   Monoclonal antibody

MDCK   Madin-Darby Canine Kidney

MHC	Major Histocompatibility complex
Mx	Myxovirus resistance
NA	Neuraminidase
NCR	Natural cytotoxicity receptor
NEP	Nuclear export protein
NK	Natural killer
NLRP3	NOD-like receptor family pryin domain containing 3
NP	Nucleoprotein
NS	Non-structural protein
PA	Polymerase subunit protein
PAMP	Pathogen-associated molecular pattern
PB1	Polymerase subunit protein
PB2	Polymerase subunit protein
pDC	Plasmacytoid dendritic cell
PKR	Protein kinase R
PRRS	Pattern recognition receptor
pH1N1	Influenza A(H1N1)pdm09
RIG-1	Retinoic acid-inducible gene-1
RNP	Ribonucleoprotein
SA	Sialic acid
SIV	Swine influenza virus
T_H	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor

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Paper 1: Swine influenza in Norway: a distinct lineage of influenza A(H1N1)pdm09 virus

Hilde Forberg, Anna G. Hauge, Britt Gjerset, Olav Hungnes, Anette Kilander.

Influenza and Other Respiratory Viruses 2013; Dec;7 Suppl 4:21-6

Paper 2: Early responses of natural killer cells in pigs experimentally infected with 2009 pandemic H1N1 influenza A virus

Hilde Forberg, Anna G. Hauge, Mette Valheim, Fanny Garcon, Alejandro Nunez, Wilhelm Gerner, Kerstin H. Mair, Simon P. Graham, Sharon M. Brookes, Anne K. Storset.

Plos One 2014; Jun 23;9(6):e100619

Paper 3: Porcine CD3+NKp46+ lymphocytes have NK cell characteristics and are recruited to the lung during early influenza infection

Kerstin H. Mair, Maria Stadler, Stephanie C. Talker, Hilde Forberg, Anne K. Storset, Andrea Müllebner, J. Catharina Duvigneau, Sabine E. Hammer, Armin Saalmüller, Wilhelm Gerner.

Submitted to Frontiers in Immunology, 09.03.2016

Paper 4: Influenza A virus leads to degranulation and TNF production in cultured porcine NK cells

Hilde Forberg, Hege Lund, Anna G. Hauge, Hilde Sindre, Carl Andreas Grøntvedt, Preben Boysen, Anne K. Storset.

Manuscript

SUMMARY

Influenza viruses balance on the interface between animals and humans, and swine represents a possible intermediate host for human viruses. It is therefore of great importance for animal welfare and to the public health to monitor the viral reservoir in swine. To better understand the mechanisms deciding clinical outcome, one also needs to study the host immune responses. Natural killer (NK) cells in general and the NKp46 receptor in particular, have been assigned a special role in influenza A virus infections. Little is known about NK cells during influenza A virus infections in swine.

From the first detection of influenza A virus in Norwegian swine in 2009 until 2013, viral hemagglutinin (HA) genes were sequenced and compared to HA sequences from human viruses for the same period (paper 1). The formation of a distinct phylogenetic group was detected in late 2011, showing that the influenza A(H1N1)pdm09 virus (pH1N1) lineage had evolved in Norwegian swine, indicating that these viruses have established themselves in the Norwegian swine population.

Two pH1N1 virus infection experiments were performed in swine. Lymphocytes were isolated from blood and lung and analyzed by flow cytometry and lung tissue sections were stained by immunofluorescence markers. Decreased numbers of NKp46$^+$ NK cells were found in the blood of infected animals, while increased numbers were found in the lung (paper 2). Furthermore, flow cytometric analysis showed the same pattern for CD3$^+$NKp46$^+$ cells (paper 3), indicating a recruitment of cells expressing the NKp46 receptor to the lungs during pH1N1 virus infections in swine. Increased amounts of mRNA encoding tumor necrosis factor (TNF) were found in isolated lung lymphocytes by real-time RT-PCR (paper 2).

To further study porcine NK cell responses to pH1N1 viruses, NKp46$^+$ cells were isolated from blood and spleen and kept in proliferative cultures for 10 days. Subsequently, cells were stimulated with pH1N1 virus *in vitro* and analyzed by flow cytometry. Cultured NK cells degranulated and started the production of TNF upon exposure to the virus (paper 4).

Taken together, these results indicate that NK cells migrate from blood into the lungs following pH1N1 virus infection, and that they participate in viral clearance by killing virus infected cells and by producing TNF.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

Influensa A virus er en viktig årsak til sykdom hos både mennesker og dyr, og gris kan fungere som en mellomvert for virus som kan smitte menneske. Overvåkning av virus reservoaret i gris er derfor viktig også for menneske. For å forstå hva som bestemmer det kliniske utfallet av influensa er det viktig å studere vertens immunrespons mot viruset. Naturlige drepeceller (NK celler) og aktiveringsreseptoren NKp46 har en antatt viktig rolle i influensa A virus infeksjoner. Allikevel er det lite som er kjent når det gjelder NK celle responser i gris ved en influensa A virus infeksjon.

Fra influensa A viruset først ble oppdaget i norsk gris i 2009 og frem til 2013, ble HA genet sekvensert og sammenlignet med HA sekvenser fra humane virus fra samme tidsrom (artikkel 1). Virus fra slutten av 2011 dannet en distinkt fylogenetisk gruppe. Dette viser at det «pandemiske» influensa viruset, pH1N1, har utviklet seg i norsk gris, og tyder på at viruset også har etablert seg i den norske svinepopulasjonen.

To infeksjonsforsøk med pH1N1 virus ble utført i gris. Lymfocytter ble isolert fra blod og lunge og analysert ved hjelp av flowcytometri. I tillegg ble lungesnitt analysert ved hjelp av immunofluoriserende markører. Det ble funnet en nedgang av NKp46^{+} NK celler i blod og en økning i lunge hos infiserte dyr (artikkel 2). CD3^{+}NKp46^{+} celler viste det samme distribusjonsmønsteret (artikkel 3). Dette indikerer at celler som uttrykker NKp46 rekrutteres til lunge ved influensa A virus infeksjoner i gris. Ved hjelp av real-time RT-PCR ble det funnet økte nivåer av mRNA som koder for TNF i lymfocytter isolert fra lunge (artikkel 2).

For å studere NK celle responsene i møte med influensa A virus mer inngående, ble NKp46^{+} celler isolert og holdt i prolifererende kulturer i 10 dager. Deretter ble cellene stimulert med influensa pH1N1 virus *in vitro* og analyserte ved hjelp av flowcytometri. NK cellene viste tegn på degranulasjon, samt produksjon av TNF som respons på influensaviruset (artikkel 4).

Kort oppsummert indikerer disse resultatene at NK celler migrerer fra blod og til lunge som en følge av pH1N1 virus infeksjon i gris, og at de deltar i forsvaret mot viruset ved å drepe infiserte celler og produsere TNF.

INTRODUCTION

Throughout history, several influenza A pandemics have struck the world, with 'The Spanish flu' of 1918 being one of the most devastating examples. This pandemic affected around one third of the world's population and caused over 50 million human deaths [1]. Influenza pandemics in humans are caused by influenza A viruses, and occur when a virus 'jumps' from one species to another, most commonly from swine or poultry [2]. The Spanish flu was followed by novel influenza A pandemics in 1957, 1968 and 2009 [3]. The latest pandemic influenza virus from 2009 has been shown to have emerged in swine [4]. Knowledge of influenza A virus infections in swine is therefore of great importance, not only for animal health, but also for understanding how viruses with pandemic potential evolve and affect its host.

INFLUENZA A VIRUS

CLASSIFICATION

Influenza A virus is classified within the genus *Influenzavirus A,* that together with five other genera; *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus*, *Infectious Salmon Anemia virus* and the recently identified *Quaranjavirus* constitute the family *Orthomyxoviridae* [5]. Influenza B viruses are primarily pathogens for humans, while Influenza C viruses infect humans, pigs and dogs, but only rarely cause severe disease. In addition, a novel bovine influenza virus has been identified [6] and proposed to represent a new genus of the *Orthomyxoviridae* family; Influenza D virus [7]. As this thesis focuses on influenza A viruses, these will simply be referred to as influenza viruses hereafter.

Influenza viruses are classified into different subtypes based on antigenic differences in their two surface proteins; HA and neuraminidase (NA). So far, there have been identified 18 different HAs and 11 different NAs in a number of different combinations [8].

VIRION PROPERTIES

The influenza virions are pleomorphic and may be either spherical or filamentous. The genome of the virus consists of eight single-stranded negative-sense RNA segments. Although once believed that each gene segment coded one single protein, the influenza virus genome uses the host cell splicing machinery making it possible for one gene

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segment to encode several proteins. New proteins have been identified the recent years and up until now, 15 viral proteins have been identified, summarized in Table 1 [9–11].

Table 1. Genomic organization and main functions of encoded proteins of influenza viruses.

Seg. no.	Encoded proteins	Main function
1	PB2	Polymerase subunit; cap-binding
2	PB1	Polymerase subunit; RNA synthesis
	PB1-F2	Pro-apoptotic, IFN-antagonist
	PB1-N40	Not known
3	PA	Polymerase subunit; protease activity
	PA-X	Modifies host response
	PA-N15	Not known, probably functional during replication cycle
	PA-N182	Not known, probably functional during replication cycle
4	HA	Surface glycoprotein; major antigen, receptor binding and fusion
5	NP	RNA binding protein; nuclear import regulation
6	NA	Surface glycoprotein; sialidase activity, virus release
7	M1	Matrix protein; vRNP interaction, RNA nuclear export regulation, viral budding
	M2	Ion channel; virus uncoating and assembly
8	NS1	Interferon inhibitor
	NEP/NS2	Nuclear export of RNA

The structure of the influenza virion is schematically shown in Figure 1. Each of the genome segments is covered with nucleoprotein (NP) and together with three associated polymerase proteins (PB1, PB2 and PA) they form the ribonucleoprotein (RNP) complexes. The RNPs and the nuclear export protein (NEP), also called nonstructural protein (NS2), constitute the core of the virion. The core is protected by a lipid envelope derived from the host cell plasma membrane, layered internally by matrix M1 protein. Three different transmembrane proteins are anchored in the envelope; the HA, the NA and the matrix M2 protein. While the M2 protein is small and not visible by electron microscopy, the HA and NA are larger and responsible for the shape of the virus [9]. HA is more abundant than NA on the virion surface and is formed as a homotrimer with a globular head and a stem region. NA is a globular homotetramer with the shape of a mushroom [8].
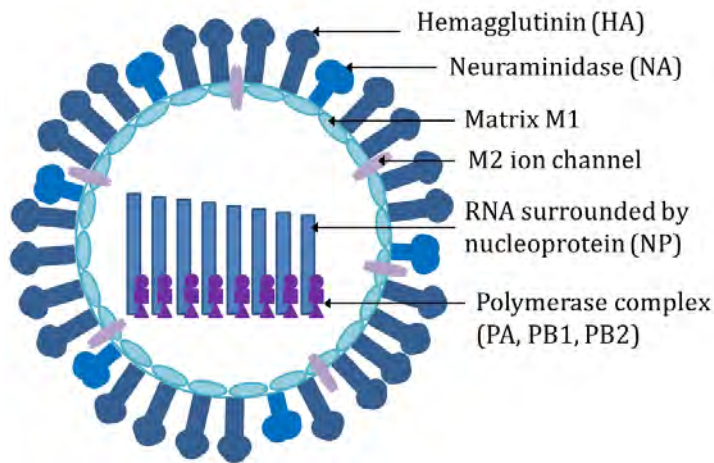
Figure 1. Schematic drawing of the influenza virion.

REPLICATION CYCLE

Influenza viral particles enter the body via aerosols, followed by an infection of epithelial cells of the airways (Fig. 2). Influenza virions recognize and bind to sialic acid (SA) receptors on host cells by the HA proteins on the virion. When meeting a permissive cell, the HA0 molecule is cleaved into two parts; HA1 (globular head) and HA2 (stalk region), and the activated HA allows the virion to attach itself to the host cell membrane. The receptor binding site is found near the top of the HA1 globular head. Following binding, the virion is internalized by receptor mediated endocytosis. The acidic environment in the endosome triggers a new conformational change in the HA, which results in the fusion of the viral envelope with the endosomal membrane. Simultaneously, hydrogen ions are pumped into the virion through M2 ion channels. The acidification of the virion and the fusion of the viral and endosomal membranes result in the release of RNPs into the host cell. The RNPs holding the viral genome are then transported into the host cell nucleus. Here, the negative-sense RNA acts as a template for the viral RNA polymerase to produce two positive-sense RNA species by transcription; 1) mRNA used for viral protein synthesis and 2) cRNA used for transcription of more copies of finished viral RNA. The viral mRNA strands are processed to resemble the host cell's own mRNA. The transportation of viral mRNA from the nucleus into the cytoplasm and the translation into viral proteins will sequentially be performed by the host cell machinery. The cRNA on the other hand, is transcribed into viral RNA by the viral RNA polymerase and associated with NP and polymerase proteins to form new RNPs. After the envelope proteins HA, NA and M2

have been synthesized and folded, they are modified in the endoplasmic reticulum/Golgi apparatus of the cell [9,12]. One important modification event is the glycosylation of HA, which is linked to receptor binding affinity, virulence and antigenicity [13]. After modification, the viral surface proteins are transported and inserted to the cell membrane. The components of the viral core, the RNPs and NEPs, are also transported from the nucleus to the virus forming site, probably by the help of the M1 protein. Each viral particle is packed with the eight different RNPs and all the gene segments have to be present in order for the virus to be infectious. Virions are formed by budding, but stay attached to the host cell by binding of HA to host cell SA receptors. The release of new viral particles is facilitated by the viral NA proteins, which destroy the HA/SA binding by cleavage of the SA residues [9,12].
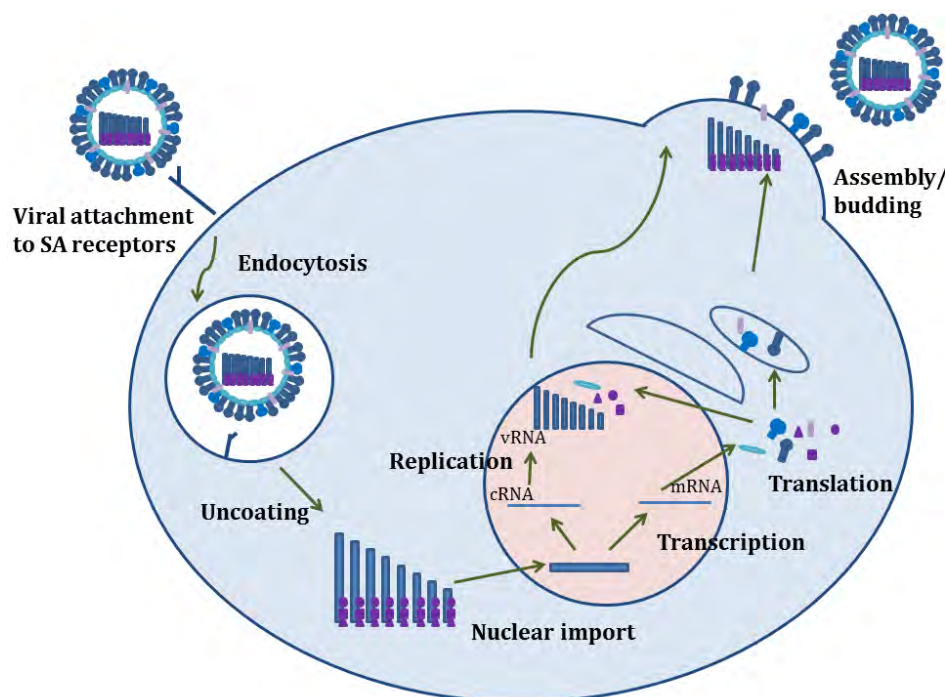
Figure 2. The replication cycle of influenza virus.

The diagram illustrates the replication cycle of influenza virus, including cell attachment, endocytosis, uncoating, nuclear import, viral RNA replication, transcription and translation of viral proteins and assembly/budding of a new viral particle.

ANTIGENIC DRIFT AND ANTIGENIC SHIFT

The genome of influenza viruses is constantly changing by two different mechanisms; 1) antigenic drift and 2) antigenic shift (Fig. 3). The first one involves accumulation of point mutations and causes only minor changes in the genome. The number of

mutations is increased by the lack of proofreading by the viral RNA polymerase. During transcription of viral RNA, 1 in 10^4 bases will be erroneously transcribed in each replication cycle. For comparison, the host cell's DNA polymerase only allows 1 to 10^9 errors per cycle. Thus, influenza viruses exist as populations of quasispecies, where random mutations are selected for or against. Mutations within genes that encode regions of the virus recognized by the immune system, the antigenic sites, will affect its antigenicity [9,14]. The continuous change in the viral genome through antigenic drift is responsible for the seasonal changes of the influenza virus in humans [14].

Although much more rarely occurring, antigenic shift has an immediate and more dramatic effect on the viral genome. It requires that a host is infected with two or more viral strains at the same time. If one host cell harbours two viruses simultaneously, these may exchange gene segments. This reassortment of genes will produce a new virus with a mixture of genes from the two or more original strains. Antigenic shift and the acquirement of a new HA molecule may help a virus to cross the species barrier, since the receptor specificity is determined by HA. Furthermore, viruses that acquire new HA and/or NA genes may have pandemic potential in humans since they hold completely novel antigenic properties, in which the human population has no immunity against [9,12,14].

By both antigenic drift and shift the virus may change in a way that makes it evade the immune system, further described later.
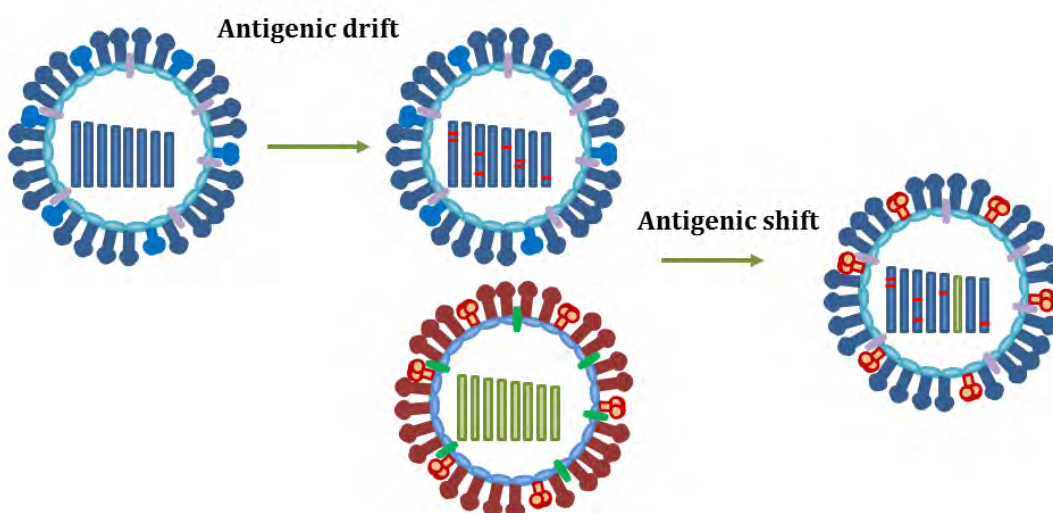
Figure 3. Antigenic drift and shift.
Influenza viruses are constantly changing by accumulation of mutations in their genome (antigenic drift) and by the more rare exchange of gene segments with other influenza viruses (antigenic shift).

Besides the recent findings of H17N10 and H18N11 viruses that have only been isolated from bats, all influenza viruses have been detected in wild waterfowl and shorebirds. Aquatic birds are thus considered as the reservoir of influenza virus [2,14]. Influenza virus infections in wild birds are typically subclinical. The virus mainly replicates in epithelial cells of the intestine and is transmitted by the fecal-oral route. Influenza viruses may cross the species barrier and have established themselves as pathogens of a wide range of species, including swine, horses, dogs, cats, mink, seals, whales, poultry, non-human primates and humans [2]. Successful establishment in a new host is not only dependent on the successful infection of another species, but also an effective transmission between individuals within that species. The mechanisms by which influenza viruses cross species barriers and the properties important for successful onward transmission are poorly understood. Close contact between animals will facilitate the transmission of virus between species. After transmission, the virus will continue to evolve and adapt to the new host. Transmission back to the original species is thereby made more difficult but does occur [14,15].

From 1918 until 2009, there have been five influenza pandemics in humans. The 1918 H1N1 virus has been shown to be of avian descent. The virus was later isolated from swine, but it is not known whether swine were involved as an intermediate host or if the virus was transmitted directly from birds to humans and subsequently onwards to swine. The 1918 H1N1 virus became established in the human population, causing annual outbreaks of seasonal influenza. In 1957 and 1968, a H2N2 and a H3N2 virus, respectively caused new human pandemics. They both seem to descend from reassortant events of the circulating human virus at that time and different avian viruses, but it is not known if an intermediate host was involved. After a period of 11 years, the 1957 H2N2 became extinct, while viruses derived from the 1968 H3N2 still circulate in humans. In 1977 a H1N1 virus closely resembling a H1N1 from the 1950's in humans re-emerged in the human population. From the late 1970's until 2009, descendants from the 1968 H3N2 and the 1977 H1N1 viruses were circulating in humans [2,16].

In domesticated poultry, H5, H6, H7 and H9 viruses occur endemically in some parts of the world. Unlike influenza viruses in water birds, avian influenza viruses (AIVs) in

poultry replicate in the respiratory epithelial cells. Low pathogenic AIVs (LPAIVs) of the H5 and H7 subtype may evolve to become highly pathogenic AIVs (HPAIVs) due to the insertion of a multi-basic cleavage site that enables the virus to replicate throughout the body, thus causing a systemic infection. HPAIV may infect other avian and mammalian species and HPAI H5N1 viruses have infrequently been detected in animals kept in captivity [2]. HPAI H5N1 has also been detected in swine, but has not caused clinical signs after natural or experimental infection in this host [2]. AIVs may infect humans, and HPAIV H5N1 and LPAIV H7N9 have been of particular concern for the human health. HPAIV H5N1 occurs endemically in poultry in many parts of the world, and since the first detection in 1997 until 2015, almost 700 people had been infected, with a case fatality rate close to 60 %. Infection with LPAI H5N1 was reported in 500 human cases of influenza during the same period, with a case fatality rate of 36 %. This virus causes mild or no clinical signs in poultry, and is therefore difficult to detect prior to transmission to humans. However, in most cases AIVs are transmitted through close contact with domestic birds, and no sustained human-to-human transmission has been reported for either of these viruses [2].

Swine have been considered a 'mixing vessel' for influenza viruses, meaning that they are susceptible for both avian and mammalian strains of the virus and thereby a potential host for reassortment of the viral genome (Fig. 4). The predilection site of influenza viruses is determined by which SA residues the HA molecule preferentially binds. SAs are monosaccharides terminally attached to galactose present on glycoproteins or glycolipids on the host cell surface by α-2,6 or α-2,3 linkages. Different HA subtypes show specificity towards SAs with different linkages. Human influenza viruses typically prefer SAs with a 2,6-galactose (SAα2,6gal) linkage, while avian influenza viruses predominantly bind SAs with 2,3-galacose (SAα2,3gal) linkages. The theory has been that swine is the only species with receptors for both avian and human influenza strains in the trachea, and that this facilitates a switch in host specificity from avian to human [15]. However, recent studies have contributed to a more nuanced picture. Both SA receptor variants were found in the airways of swine, but whereas SAα2,6gal receptors were found abundantly both in the upper airways including trachea and in the lower respiratory tract, SAα2,3gal receptors could mainly be found in the alveoli and to some extent in the bronchioles of the lower respiratory tract. In fact, the receptor distribution of the porcine airways closely resembles that of humans

[17,18]. Influenza virus has also been shown to cross the species barrier without the involvement of pigs both experimentally and in nature [18]. Nevertheless, the large numbers of swine living in close proximity to humans and other animal species and a relatively frequent transmission of virus from humans to swine and vice versa makes swine an important intermediate host.
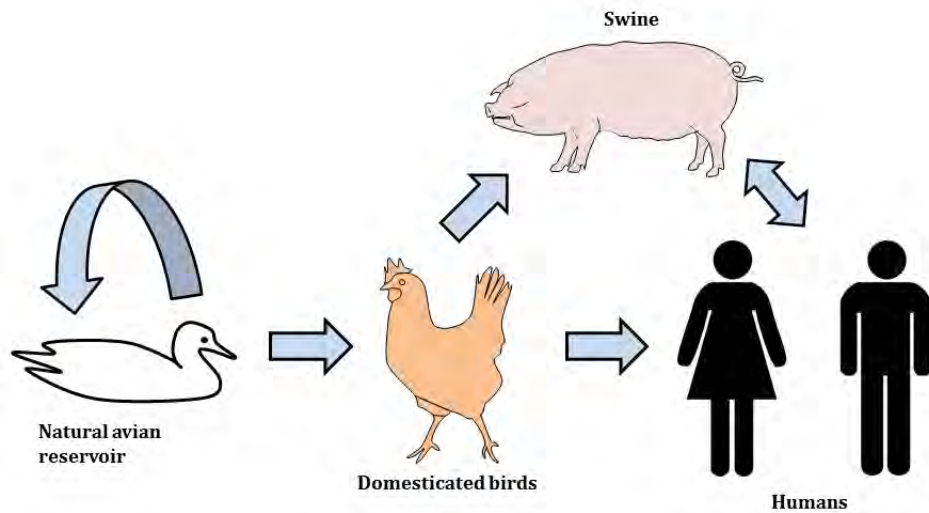
Figure 4. Influenza virus between species.

Aquatic birds are considered the reservoir of influenza viruses, but virus may cross the species barrier and be transmitted to domesticated birds. Subsequently, virus may be further transmitted directly to humans or through infections in swine.

INFLUENZA VIRUS IN SWINE

Influenza as a disease in swine was first described in 1918, after the 'Spanish flu' in humans, and continued to appear in swine in the following years. The virus was most likely transmitted from humans to swine and not vice versa [15]. The first successful isolation of influenza virus from pigs was not performed before 1930, an influenza virus of H1N1 subtype [15]. Swine separate from other mammalian influenza viral hosts in the high number of successful adaptations of influenza virus from other hosts (mainly humans) [19]. This greatly influences the diversity of swine influenza viruses (SIVs).

Swine have become infected with H4 and H9 viruses, but only H1 and H3 viruses are endemic in swine today [2]. Several influenza viruses within these subtypes have established themselves in swine at different time points, and SIVs may therefore be divided into different lineages. The classical swine H1N1 lineage has circulated in swine in the North America and Asia since 1918. In 1998, a triple reassortment (North

American) swine H3N2 emerged, containing genes from the classical lineage, mixed with genes from both North American avian and human H3N2. The triple reassortant virus has later reassorted with the classical H1N1 and human viruses a number of times. Prior to 2009, the triple reassortant H3N2 co-circulated with a reassortant H1N1 and a human-like H1N2 in North American swine (Fig. 5) [19,20].
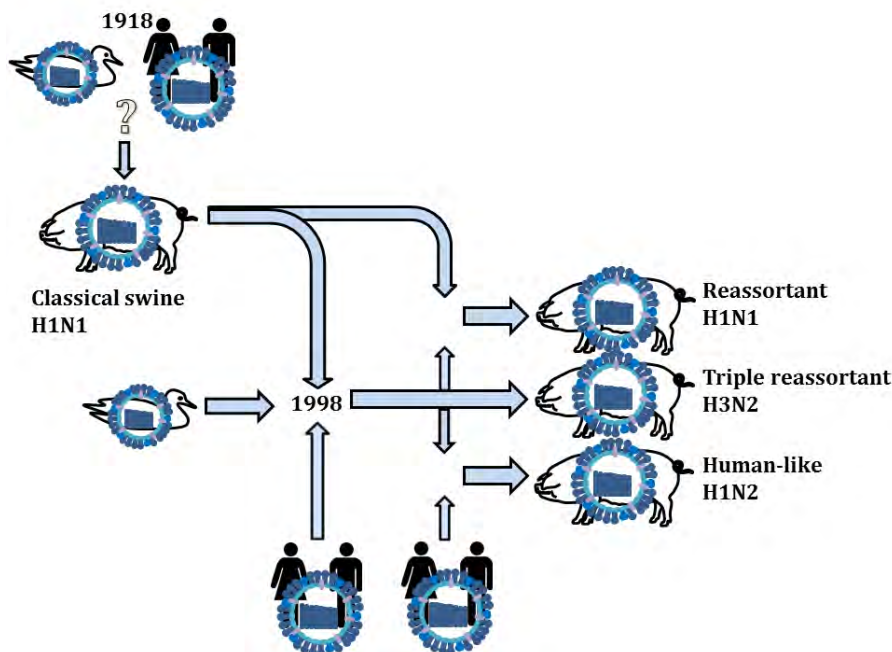
Figure 5. SIVs in North America prior to 2009

The diagram shows the origin of influenza viruses circulating in North American swine prior to 2009; the reassortant H1N1, the triple reassortant H3N2 and the human-like H1N2.

In Europe, the classical swine H1N1 was not detected in swine until 1976 after import of infected pigs from the United States to Italy. This virus circulated until 1979, when a H1N1 virus was transmitted from birds to swine as a whole avian-origin virus. This virus, named Eurasian avian-like swine H1N1, replaced the classical swine H1N1 in Europe. Over the last few decades, this virus has reassorted with human seasonal viruses a number of times, resulting in three lineages circulating in European swine; Eurasian avian-like H1N1 and reassortant human-like H3N2 and H1N2 (Fig. 6) [21]. In Asia, the classical swine H1N1 virus, the Eurasian H1N1 and the triple reassortant H3N2 circulate [4].
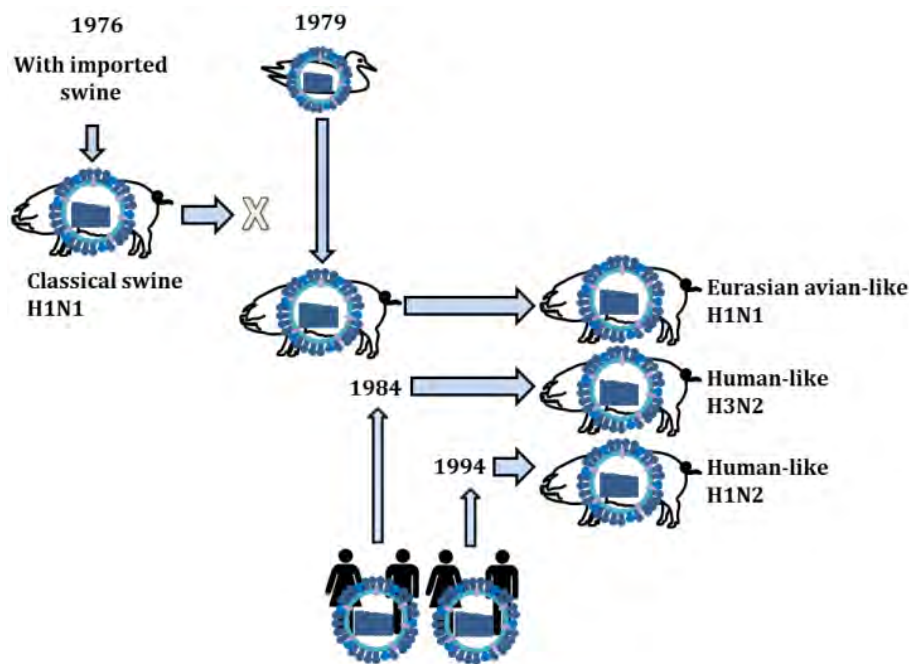
Figure 6. SIVs in Europe prior to 2009

The diagram shows the origin of influenza viruses circulating in swine in Europe prior to 2009; the Eurasian avian-like H1N1, the human-like H3N2 and the human-like H1N2.

In the past years, a number of new introductions of influenza viruses in swine have occurred, including seasonal human viruses of H3N2, H1N1 and H1N2 subtypes [22]. Following the introduction of a new virus, the virus seems to rapidly acquire the internal genes from SIVs by reassortment events, while HA and NA are maintained. Consequently, the onward transmission of HA and NA from human viruses occur in much higher frequencies than onward transmission of the six internal gene segments. Apart from the single introduction of a human gene segment to the triple reassortment H3N2, it seems like either the full human virus genome is conserved, or all the internal genes are being replaced in influenza viruses that successfully establish themselves in swine [19,21]. The emergence of the 2009 pandemic virus further complicated the situation, as described later.

Influenza in swine can range from mild clinical signs to acute respiratory tract disease with signs of nasal discharge, coughing, fever, heavy breathing and conjunctivitis. Subclinical disease is common [14,23]. Even if subclinical, an influenza virus infection may reduce the growth rate of pigs for slaughter, and consequentially be of economic importance for the farm industry [24].

INFLUENZA A(H1N1)PDM09

In spring 2009, a novel influenza virus of putative swine origin with gene segments derived from human, swine and avian influenza viruses, spread in the human population causing a pandemic. Phylogenetic analyses showed that the pH1N1 virus had originated in swine, and had circulated in swine for several years before being transmitted to humans. Moreover, it has been shown that the virus comes from a reassortment event between a virus of the triple reassortant lineage and a virus of the Eurasian swine lineage (Fig. 7). The NA and M gene segments are of Eurasian swine genetic lineage, the HA, NP an NS gene segments are of classical swine lineage, the PB2 and PA of North American avian lineage and the PB1 of human H3N2 origin [4].
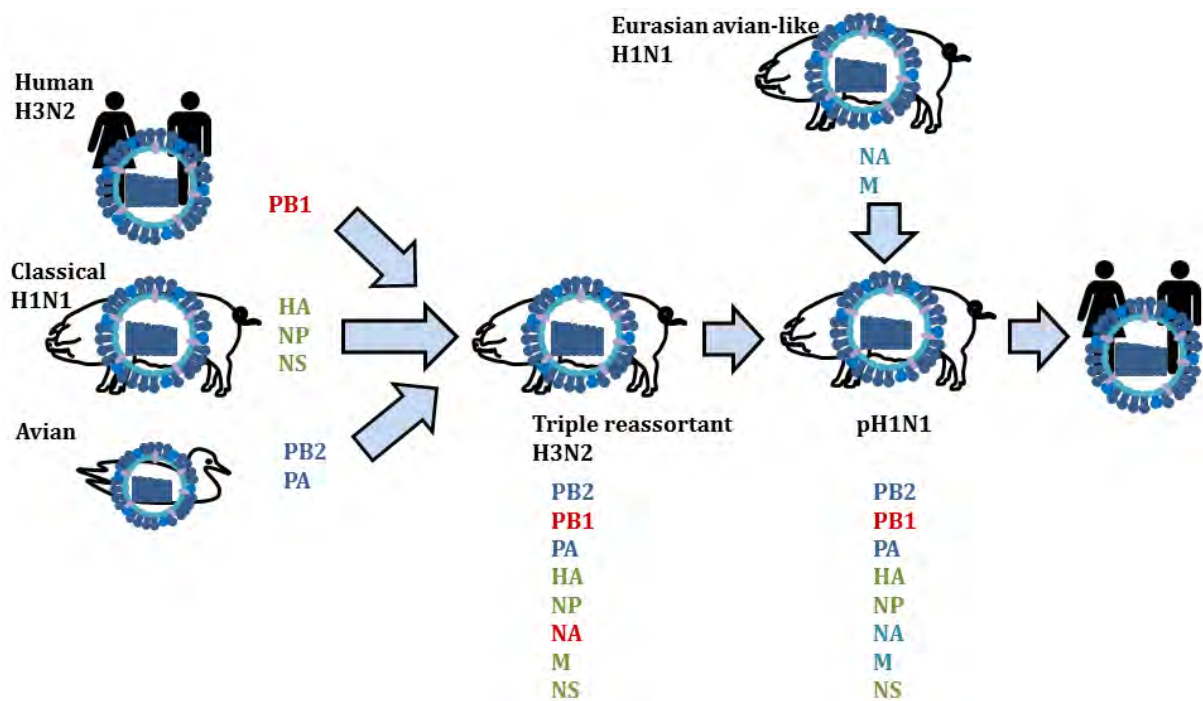
Figure 7. The origin of the pH1N1 virus.

The diagram shows the origin of gene segments in the pH1N1 virus. The virus emerged from a reassortment event between a triple reassortant virus and a virus of the Eurasian swine lineage.

PH1N1 VIRUS IN HUMANS

The pH1N1 virus rapidly replaced the previous H1N1 virus in seasonal outbreaks in humans [25], and was the most commonly detected influenza virus in the 2015/2016 influenza season in humans in Norway and the rest of Europe, according to the European Center of Disease Prevention and Control [26]. Most often, this virus causes a

benign and self-limiting respiratory disease in humans, but severe cases have been reported [27].

The pH1N1 virus had most likely pre-adapted to humans while still circulating in swine, and continued adapting to its new host during the pandemic period and immediately thereafter. This caused greater diversity in the viral genome, and even two separate lineages of pH1N1 viruses in humans from 2011. Later, one of these went extinct and as of today all pH1N1 viruses in humans belong to one lineage. The post-pandemic period (2011 and onwards) has been characterized by a ladder-like phylogeny, typical in situations with strong selective pressure due to increased protective immunity in the human population. The mean substitution rates of HA and NA genes have been higher than for the H1N1 virus previously circulating in humans. Still, the antigenic changes have not been substantial, and the influenza A/California/07/2009 (Cal09) virus isolated during the 2009 outbreak was still the WHO-recommended vaccine strain for the 2015/2016 flu season [25]. The small degree of human adaption is also reflected in the relatively large numbers of human to swine transmissions of the virus that still takes place [28]. Genomic reassortment events with other influenza viruses in humans have been rare [25].

PH1N1 VIRUS IN SWINE

Also after the pH1N1 virus had acquired the ability to transmit efficiently among humans, it retained its ability to infect and be maintained in pigs [22,29,30]. The pH1N1 virus is frequently detected in swine today, and there is evidence of endemic circulation in swine independently of human to swine transmission in Europe [21].

In contrast to humans, a number of reassortment events between pH1N1 and other SIVs have occurred in swine [21,31]. In Europe, viruses with the internal genes from pH1N1 have acquired H1 and N2 from the human-like H1N2 in the United Kingdom, and N2 from the human-like H3N2 in Germany. At least four different sub-lineages of H1N2 in the United Kingdom have replaced their internal genes with the ones from pH1N1, indicating that the internal genes from pH1N1 are well adapted to swine. Moreover, a triple reassortant virus with the internal genes from Eurasian H1N1, the matrix gene from pH1N1 and the H3 and N2 segments from human-like H3N2 have been detected in Spain. This virus is of special zoonotic importance, since the H3N2 lineage has been evolving in swine since the 1970s, and the level of protected immunity in the human

population is expected to be low [21]. Similarly, SIVs of the triple reassortant lineage in the United States that have acquired the matrix gene from pH1N1 have been detected. In the United States, these H3N2 variant viruses have also caused disease in humans. Most frequently there is a suspected transmission directly from contact with swine. However, unlike previously reports of other SIV variants infecting humans, there is also evidence of human to human transmission of the H3N2 virus with a pH1N1 M gene [31].

Before 2009, Norway was in a unique situation, with a population of pigs that were documented to be free of all influenza viruses. However, following the worldwide spread of pH1N1 in humans, influenza virus was detected in Norwegian pigs for the first time in the autumn of 2009 [30]. Serological testing of slaughter pigs showed that two years after the virus was first introduced, more than 50% of the tested pigs had antibodies against the pH1N1 virus [32]. By the end of 2015, around half of the pigs included in the national surveillance program still tested positive (Carl Andreas Grøntvedt, unpublished results). However, antibodies against any other subtypes of influenza viruses have not been detected in Norwegian swine [33].

D222G SUBSTITUTION

As described earlier, HA is important for receptor binding specificity, which is determined by specific amino acids at the antigen binding sites. Single mutations in HA may be enough to change receptor binding specificity. An example of this is the change in amino acid 222 (H1 numbering, 225 in H3 numbering), which is part of the antigenic site [34] and the receptor binding site [35]. Already prior to the detection of pH1N1, a specific substitution in this position of the 1918 H1N1 influenza virus from 222D (aspartic acid) to 222G (glycine) was reported to correlate with increased binding to SAα2,3Gal receptors [36]. The pH1N1 virus normally contains 222D at this position, similar to the 1918 H1N1 virus. However, a more severe infection has been associated with findings of the 222G variant, often in combination with the wild-type 222D variant as a 222D/G quasispecies [37–39]. While the 222D variant is often found in both upper and lower respiratory tract, the 222G variant is only detected in the lungs [40]. While the 222D variant predominantly binds SAα2,6gal linkages, the 222G variant binds with increased affinity to receptors with SAα2,3gal linkages [41], explaining the difference in tissue tropism. The D222G substitution is not fond in all severe cases of pH1N1, but it is

only found in severe and never in mild cases [39,40]. Other substitutions in position 222 in HA, including 222N have also been associated with more severe disease [37,42].

THE IMMUNE RESPONSE AGAINST INFLUENZA VIRUS

The immune system of mammalian species is traditionally divided into the innate and the adaptive immune system.

THE INNATE IMMUNE SYSTEM

Influenza virus enters mammalian hosts through the respiratory tract, where the immune responses are initiated. The innate immune system consists of several components that recognize and react to microbes: The epithelial cell barriers, phagocytes (macrophages and neutrophils), NK cells, the complement system, type I IFNs, cytokines and other plasma proteins. Some of the important cytokines of the innate immune system and their role in infectious disease is summarized in Table 2 [43–46].

Table 2. Cytokines of the innate immune system

Cytokine	Main function(s)	Produced by
IL-1β	Pro-inflammatory effect; leukocyte recruitment, synthesis of acute-phase proteins, fever. Also involved in antimicrobial resistance and T_H17 cell responses.	Macrophages, DCs, endothelial cells and other.
IL-6	Pro-inflammatory effect; recruitment of immune cells, synthesis of acute-phase proteins, proliferation of antibody producing B cells. Also involved in T_H17 cell responses.	Macrophages, endothelial cell, T cells.
IL-10	Regulatory cytokine with inhibitory effect on a number of innate and adaptive immune cells (anti-inflammatory).	T cells (CD8+)
IL-12	Increased activation of NK cells and T cells. T_H1 differentiation.	Macrophages, DCs
IL-18	Induces IFN-γ synthesis in NK cells and T cells and the expression of pro-inflammatory cytokines in monocytes. Activation and cytokine release from neutrophils.	Monocytes, macrophages, DCs and others
IFN-α	Type I IFN: Induces antiviral state and increased MHC I expression in all cells. Activation of NK cells.	pDCs, macrophages
IFN-β	Type I IFN: Induces antiviral state and increased MHC I expression in all cells. Activation of NK cells.	pDCs, fibroblasts
IFN-γ	Pro-inflammatory effect; activation of macrophages, induces increased MHC I and II expression and increased antigen presentation to T cells. Involved in isotype switching of B-cells and T_H1 differentiation.	NK cells, T cells
TNF	Pro-inflammatory effect; activation of endothelial cells and neutrophils, synthesis of acute-phase proteins, fever, cachexia.	Macrophages, NK cells, T cells

Cells of the innate immune system recognize structures that are shared by classes of microbes, so called pathogen-associated molecular patterns (PAMPs). The main PAMP of influenza viruses is viral RNA, both in its single-stranded form and in double-stranded from during viral replication [47–49]. Cells of the innate immune system are also able to recognize damaged or dying host cells by structures that are not present in healthy tissue, so called damage-associated molecular patterns (DAMPS) [50]. The receptors of the innate immune system, the pattern recognition receptors (PRRs), are encoded in the germline, which means that there is limited diversity in the specificity of these receptors. The main PRRs that recognize influenza viral RNA include Toll-like receptor (TLR) 3 and TLR 7, retinoic acid-inducible gene-1 (RIG-1) and the NOD-like receptor family pryin domain containing 3 (NLRP3). The TLRs are expressed by epithelial cells of the respiratory system, as well as in the endosomes of phagocytes and dendritic cells and recognize virus or virus infected cells. RIG-1 is crucial for the detection of virus present in the cytosol of epithelial cells, dendritic cells and alveolar macrophages. Stimulation through TLRs or RIG-1 ultimately leads to activation of transcription factors, including NF-κB and IFN regulatory factors (IRF3/7), that stimulates the expression of pro-inflammatory cytokines, including TNF, interleukin (IL)-1, IL-6 and type I IFNs (IFN-α and IFN-β). NLRP3 is also located in the cytosol, and forms a multiprotein inflammasome complex that is activated by host cell damage in dendritic cells, neutrophils, monocytes/macrophages and human bronchial epithelial cells and is responsible for the production of active IL-1β and IL-18 [48,49].

Type I IFNs are produced by a number of cells, including epithelial cells, macrophages, neutrophils and most importantly; plasmacytoid dendritic cells (pDCs). They are secreted from virus infected cells and affect gene expression in nearby cells by binding to the promotor sequence in IFN-stimulated genes (ISGs) (Fig. 8). Several ISGs have been highlighted in connection with influenza virus infection, including genes coding for myxovirus resistance (Mx) proteins, IFN-inducible transmembrane (IFITM) proteins and protein kinase R (PKR). Common to these ISGs is that they induce an antiviral state in the cells with reduced protein synthesis and limited viral replication. IFITM block viral entry to the cell, while Mx proteins block viral entry into the nucleus. PKR is located in the cytosol and prevents viral translation by binding viral RNA [48].
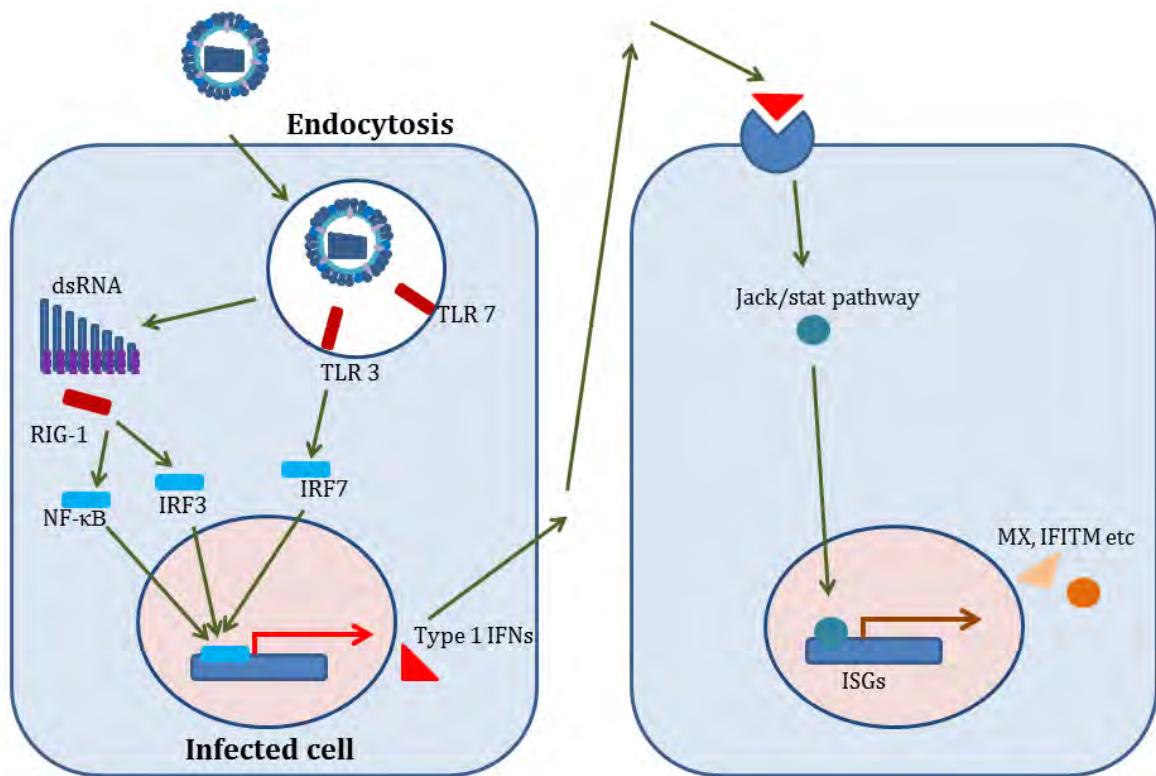
Figure 8. The induction of type I IFNs.

RIG-1 and TLR3/TLR7 detect viral RNA in cytoplasm and endocytic vesicles, respectively and induces the transcription of type I IFNs. Type I IFNs are secreted from the cell and activate ISGs in nearby cells, thus promoting an antiviral state.

Activation by influenza virus also leads to responses being more specific to different cell populations of the innate immune cells. Neutrophils and monocytes are circulating in the blood, but respond quickly and enter the site of infection by extravasation in response to inflammatory cytokines and chemokines. The lower respiratory tract also harbors its own tissue-specific macrophages, the alveolar macrophages that are present in healthy lung tissue. Neutrophils and macrophages phagocytose opsonized virus and virus infected cells. Macrophages also produce pro-inflammatory cytokines, including IL-6 and TNF. Conversely, NOS2 and TNF produced by macrophages may also contribute to lung pathology, and blood-derived macrophages have been shown to produce greater amounts of pro-inflammatory cytokines than alveolar macrophages [47,49]. The conventional dendritic cells (cDCs) are the most important professional antigen presenting cells (APCs). Professional APCs transport antigens from the site of infection to the draining lymph node via the afferent lymphatic system. Here, they present fragments of the microbe to cells of the adaptive immune system [47]. In the case of influenza virus infection, directly infection seems to be important for antigens

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entering the DCs. The DCs probably also have the ability to capture free influenza virions and influenza infected cells by phagocytic engulfment, although this has not been demonstrated [43]. Lung tissue harbors a great number of NK cells also in steady state [51]. They provide early control of viral infections by cytotoxic activity against infected cells and production of cytokines, and by modulating the adaptive immune responses [52,53]. As NK cells are the main focus of this thesis, their role in influenza virus infections will be extensively elaborated later.

THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune system consists of lymphocytes (B cells and T cells) and their products, such as antibodies. The adaptive immune system separates from the innate immune system by a much more advanced way of recognizing microbes. The receptors of the adaptive immune system are produced by a random combination of genes that results in great variability in the antigen binding site between different lymphocytes. The total lymphocyte repertoire of one individual can recognize over a billion different antigens [46]. During steady state, naïve B and T cells are found in lymph nodes draining the respiratory tract and interspersed within the interstitium of the lung. In addition, bronchial associated lymphoid tissue is formed along the bronchus following influenza virus infection. The priming process of B and T cells during influenza virus infections mainly takes place in the lymph nodes. After priming, the process of clonal expansion takes place and results in thousands of cells with exactly the same specificity. These cells include effector cells that contribute to the clearance of virus and memory cells that have the capacity to react more rapidly and more efficient during a second immune response against the same microbe. The capacity to remember and mount a more effective secondary response are important features that distinct the adaptive immune system from the innate [54,55].

Humoral immunity is mediated by antibodies produced by effector B cells, the antibody forming cells (AFC). B cells can also capture virus and display antigens to CD4 T cells in the process of receptor maturation that increases the affinity of the B cell receptor (Fig. 9). In influenza virus infections, this process is most likely crucial for the clearance of virus. A second signal from CD4 T cells is also necessary for isotype switching from IgM isotype to IgG. The upper parts of the respiratory tract (trachea and larger bronchi) are covered by mucosa, and AFCs here produce IgA antibodies while AFCs of the lung

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generate IgG and IgM antibodies. Antibodies against all the viral proteins are produced, but the most important antibodies are targeted against HA and correlate with protective immunity. Antibodies that efficiently bind HA inhibit virus attachment, thereby neutralizing the virus (Fig. 9). Most antibodies are directed against the receptor binding site of the globular head of HA. Because of the great variability in this region, these antibodies are subtype- and even often strain-specific and offer only homosubtypic protection. Antibodies against the highly conserved proximal part of HA are also produced and are the predominant neutralizing antibodies in heterosubtypic or cross-reactive protective immunity. However, these antibodies are produced in much lower titers. When bound to virus, there are several ways that antibodies facilitate viral clearance: 1) Complement activation, 2) Induction of phagocytoses by immune cells expressing Fc receptors or 3) Antibody-dependent cell-mediated cytotoxicity (ADCC) described later (Fig. 9). Antibodies against viral NA also have protective effect, as they inhibit release of the virus and thereby viral spread. Antibodies against NA also have the capacity to mediate ADCC. Antibodies against M2 or NP may also contribute, but are not able to limit an infection alone. Influenza virus specific AFCs reside transiently in the spleen and long term in the lung and in bone marrow. Also, IgG and IgA memory B cells to influenza virus are generated and can be detected locally in the respiratory tract and within most other organs of the body. Together, these long lived AFCs and memory B cells provide long-term protection against antigenically similar influenza viruses [47,54].
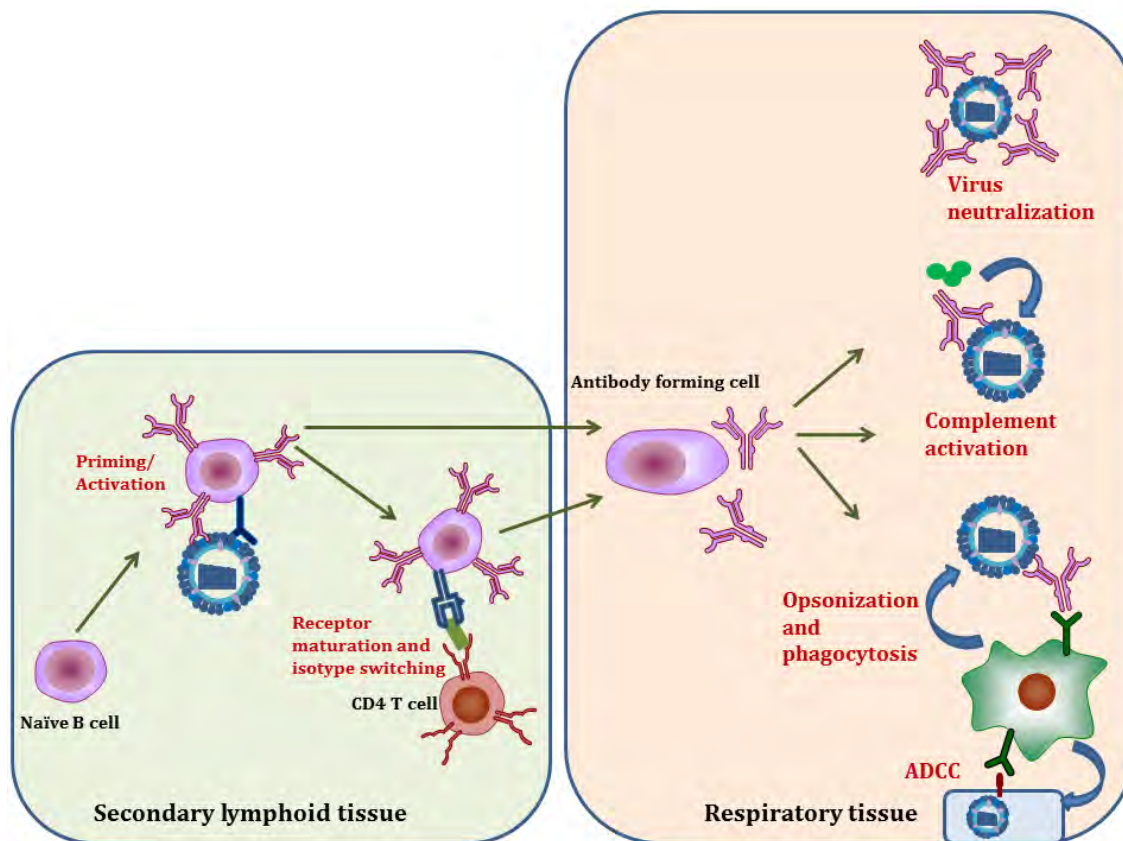
Figure 9. B cell responses

B cells are activated through priming in secondary lymphoid organs, but receptor maturation in response to CD4 T cell stimulation is required for an effective response. Activated antibody forming cells migrate to the respiratory organs where they excrete their effector molecules; the antibodies. Antibodies neutralize free virus, activate complement and induce phagocytosis and ADCC.

T cells are dependent on antigens being displayed on receptor molecules, called major histocompatibility complex (MHC)s. MHC I is expressed on all nucleated cells and presents protein antigens present in the cytoplasm to CD8 T cells. These are cytotoxic T cells (CTL) that kill target cells by cytotoxic molecules, including perforin and granzymes [47] and produce pro inflammatory cytokines during influenza virus infections (Fig. 10) [43]. High amounts of influenza virus specific CD8 T cells have been found to correlate with milder signs and symptoms and decreased viral spread when humoral immunity is lacking [56]. Long lived CD8 T cells have been found in the lungs following influenza virus infection [43]. MHC II is expressed on professional APCs and is up regulated on a number of cells during inflammation. MHC II presents epitopes ingested from the extracellular space to CD4 T cells. CD4 cells include different subsets of T cells, including T helper (T_H)1 cells which produce IFN-γ and IL-2. IFN-γ produced by T_H1 cells has been shown to be the main inducer of B cell isotype switching to IgG,

described above (Fig. 10). In addition, IFN-γ increase phagocytosis and killing of ingested material in macrophages [46]. T_H2 cells are most important in helminthic infections [47]. CD4 T cells have also been suggested to kill influenza virus infected cells in a perforin dependent manner, but the *in vivo* role of this is poorly understood [43]. Also regulatory T cells and T_H17 cells have been identified in influenza virus infections and regulate cellular immune responses. Human CTLs induced by influenza virus are mainly targeted against NP, M1 and PA proteins that are broadly conserved among influenza viruses [47]. Cellular immunity thus offers a broader protection than the humoral response, and T cells may cross-react with influenza viruses of various subtypes, if directed against more conserved epitopes [55,56].

Currently used influenza vaccines contain inactivated virus which mainly stimulates a humoral response with antibodies directed against HA and to a lesser extent NA, and are therefore also highly strain-specific. Live attenuated virus vaccines contain whole virus and result in both humoral- and cellular immunity, but are only used in some countries and for special groups of patients [55,56].
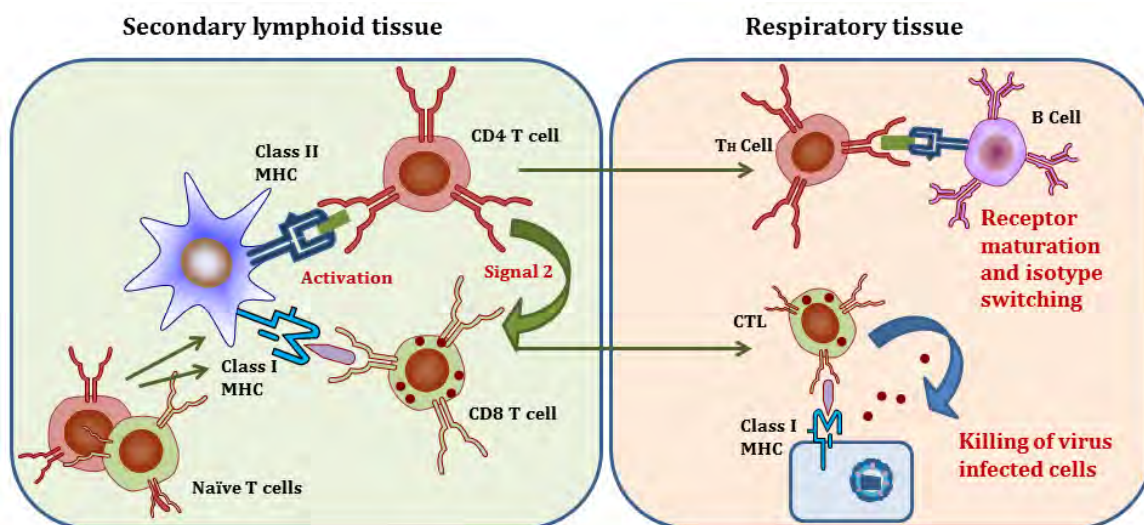
Figure 10. T cell responses
T cells are activated through priming by DCs in secondary lymphoid organs. Activated CD4 T cells are become T_H cells that promote B cell responses, while activated CD8 T cells become CTLs that kill virus infected cells.

INNATE LYMPHOCYTES

In the recent years a number of lymphocytes with innate properties have been described; invariant Natural Killer T cells, mucosa-associated invariant T cells, γδ T cells

and B-1 cells. Common for these is that they all come from the lymphocyte lineage, but express simpler versions of the T cell or B cell receptor than the conventional T and B cells and respond immediate without prior recognition of antigen. These cells are typically found in greatest numbers in mucosal tissue, were antigens first are encountered [57,58]. B-1 cells are the main producers of natural IgM, and produce large amounts of antibodies in response to influenza virus infection. In line with their innate-like qualities, only a small fraction of these antibodies are influenza virus specific, and their specificity does not increase over time. Nonetheless, the antibodies that are specific for influenza virus are neutralizing and have a rapid effect [54].

Another set of lymphocytes with innate properties has also been described, the innate lymphoid cells (ILCs). These cells are defined as lymphoid cells that do not express a functional T cell or B cell receptor, thus NK cells may be classified into this group. ILCs resemble T cells and are often categorized as killer-ILCs and helper-ILCs, in parallel to cytotoxic and helper T cells, respectively. The only known ILCs with cytotoxic function described as of today are the NK cells, which constitute the killer-ILCs. Helper-ILCs are further classified in three groups; ILC1, ILC2 and ILC3, based on cytokine profiles and the transcription factors that regulate their development and function (Table 3). ILC1 contribute in protozoaen and intracellular bacterial infections, while ILC2 are part of the defense against helminths and play a role in allergic responses. ILC3 express RORγt and produce IL-17 and IL-22. They include fetal lymphoid inducer cells that drive the development of secondary lymphoid organs during embryogenesis and post-natal ILC3 that contribute in the immune response against extracellular pathogens. NK cells and the other ILCs share a lot of the same features, and the phenotypically distinction between these is not always easy [59].

Table 3. Overview of ILC groups and important cell functions

Killer-ILCs	Important in:
NK cells	Intracellular bacterial and viral infections
Helper-ILCs	**Important in:**
ILC1	Protozoan and intracellular bacterial infections
ILC2	Helminthic infections and allergic responses
ILC3	Lymphoid inducer cells: Development of secondary lymphoid tissue
	Post-natal ILC3s: Infections with extracellular pathogens

Influenza virus's constant changes by antigenic drift (and the more rare occasions of antigenic shift) prevent the host from generating long-term protective immunity. Selective pressure most dramatically affect antigenic sites in the HA targeted by humoral immunity, but also have an influence on epitopes of the NP targeted by CTLs [47].

In addition, the virus has evolved a number of specific mechanisms that contribute to immune evasion of the host once infected. Viral NS1 protein is the most important IFN-antagonist protein encoded by the virus. It can bind viral RNA and thereby mask it from recognition by TLRs and RIG-1 and inhibit PKR activity by direct binding to the protein. In addition, NS1 causes a general inhibition of host mRNA processing and gene expression by a number of methods, causing an efficient suppression of IFN expression and activation of ISGs. The viral polymerase complex is involved in cap-snatching of host mRNA. In this process, cellular mRNA is cleaved from the 5' end and used as a primer for viral mRNA synthesis, thereby reducing host cell gene expression including that of IFNs. PB1 and PB2 of some strains of influenza virus also have additional ways of inhibit IFN production. Influenza virus NP and M2 proteins interact with different regulatory proteins which ultimately also inhibit PKR and thereby ensure sustained viral protein synthesis. It is also suggested that M2 may inhibit activation of TLRs through interfering with cellular autophagy [47,49].

NK CELLS

As already mentioned, NK cells are lymphoid cells like B and T cells, but belong to the innate immune system [57]. They were first described as large granular lymphocytes that could kill a target cell without prior stimulation, hence the name 'natural killer'.

CLASSIFICATION OF NK CELLS AND NKP46

Only a couple of years ago, NKp46 (NCR1, CD335) was considered an exclusive marker for NK cells in several species including humans [60], mice [61], cattle [62] and sheep [63]. However, the expression of NKp46 on NK cells in humans has been shown to vary between individuals [64] and between organs within the same individual [65]. NKp46+ NK cells may be further classified as NKp46int or NKp46high. Moreover, as other innate lymphocytes are being discovered, NKp46 is found on small, but distinct populations of T cells with innate properties. These include minor fractions of Natural Killer T cells in humans and mice [66] and γδ T cells in mice [67], as well as RORγt expressing ILC3s in the gut of mice and humans [68]. A population of previously unidentified CD3+NKp46+ cells has also been found in cattle [69] and dogs [70]. Furthermore, NKp46 may be up regulated on γδ T cells isolated from cattle following IL-15 stimulation *in vitro* [71].

NK cells are therefore best defined as CD3-CD56+ cells in humans and by their expression of DX5/CD49b or NKR-P1C in mouse [72]. In contrast to human NK cells, porcine NK cells express CD8α and may be identified as CD3-CD8α+ cells [73]. The lack of a specific marker for NK cells hampers the identification of these cells *in vivo*.

ACTIVATION OF NK CELLS

Activation of NK cells is regulated through a balance between activating and inhibitory signals mediated by a number of germline encoded receptors in the cell surface. Recognition of different molecules, including MHC I, expressed on healthy cells, will result in a strong inhibitory signal and no activation of the NK cell. A reduced inhibitory signal, for example caused by a loss of MHC I, will result in a domination of activating signals. In this way, NK cells recognize cells that have down regulated MHC I as a result of infection or malignancy, a hypothesis known as the 'the missing self' hypothesis. Consequently, NK cells act as a control system that recognizes damaged cells that are prevented from activating T cells [74]. Activating signals may come from recognition of cellular stress ligands (DAMPS) that are up regulated by infected or transformed cells

[75]. There is also increasingly evidence that NK cells may recognize viral antigens expressed on the cell surface, such as the HA molecule of influenza virus [53]. Furthermore, activation of NK cells may be enhanced by pro-inflammatory cytokines, such as IL-12, IL-18, IFN-γ and Type I IFNs. If the activating signal exceeds the negative inhibitory signal, the NK cell becomes activated [74].

The activating and inhibitory receptors of NK cells may be divided into families, based on their structure. Several of the NK cell receptor families include both activating and inhibitory receptors. The natural cytotoxicity receptors (NCRs) are activating receptors and include NKp30, NKp44 and NKp46. These receptors have been suggested to be the main receptors for activation in tumor immunity [76], but are also believed to play a key role in recognizing viral infections [53]. NKp46 and NKp44 recognize influenza viral HA, which will be addressed in further details later. CD16 was the first receptor described on NK cells, and belong to the Ig superfamily. It is a Fc receptor, important in ADCC described later, but may also be involved in direct lysis of virus infected and tumorous cells [77]. The most extensively studied inhibitory receptors in humans belong to the killer immunoglobulin-like receptors (KIRs) and recognize mainly MHC I and their associated proteins. This is a large family that also includes activating receptors and has evolved from the Ig superfamily. The murine equivalent to the KIRs are the receptors of the Ly49 family, which are functionally similar but structurally different from the human KIRs [76]. Other receptors are NKG2D (activating), the C94-NKG2 heterodimer receptors (activating/inhibitory), 2B4 (activating/inhibitory) and Killer cell lectin-like receptor G1 (inhibitory). In addition, there are several receptors which are considered to be co-stimulatory. These are receptors that do not have the potential of activating the NK cell alone, but may provide further stimulation of the cell [76].

Inhibitory receptors signal through immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic part of the receptor. During receptor recognition, the ITIMs become phosphorylated and an inhibiting signal is delivered to the cell. Exactly how this signal blocks activation is not known. Some activating receptors, including NCRs and CD16 signal through phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM), which are not part of the receptor itself, but located in membrane bound activation molecules associated with the receptor. Engagement of activating receptors

signaling through ITAMs, result in both cytotoxicity and production of cytokines/chemokines. In contrast, some activating receptors signal through DAP-10 or DAP-12. And while signaling through DAP-12 also leads to activation of ITAMs, DAP-10 uses an alternative route, which only results in cytotoxicity [76].

Different NK cells within an individual express various combinations of activating and inhibitory receptors, ensuring some kind of heterogeneity within the NK cell population [76]. NK cells also change the expression of some surface receptors after maturation status, tissue distribution or upon activation.

MATURATION OF NK CELLS

Increasingly evidence suggests that the lifecycle of NK cells resemble that of the adaptive lymphocytes, with different phases including priming, expansion and memory.

NK cells develop from a common lymphoid progenitor cell, which in turn originate from a common $CD34^+$ hematopoietic stem cell. NK cells evolve and differentiate through intrinsic signals, influenced by a complex combination of homeostatic cytokines and other environmental cues, which will not be further addressed here. The differentiation status of human NK cells is linked to CD56 expression, where $CD56^{bright}$ NK cells are thought to represent less differentiated cells than $CD56^{dim}$ NK cells. Immature $CD56^{bright}CD16^{neg/dim}$ NK cells are mainly cytokine producers, and constitute the majority of NK cells in lymph nodes and extra-lymphatic tissues during steady state. The more mature $CD56^{dim}CD16^+$ NK cells are the main NK cell population in blood and have cytolytic capacity in addition to producing cytokines [59]. Murine NK cells do not express CD56, but CD11b and CD27 can be used to divide murine NK cells into different subsets which represent different maturation stages: $CD11b^-CD27^-$ are the most immature cells, which progress through $CD11b^-CD27^+$ cells, before $CD11b^+CD27^+$cells, and then finally becomes $CD11b^+CD27^-$ cells. $CD27^+$ cells are often compared to the $CD56^{bright}$ NK cells in humans. However, murine $CD27^+$ NK cells are more reactive both when it comes to cytotoxicity and cytokine production [72].

In addition, NK cell function is influenced by extrinsic signals from other cells. NK cells seem to need inhibitory signals through a self MHC receptor in order to become fully responsive NK cells in a process called licensing. KIRs in humans and Ly49 in rodents are important in this educational process of the cells. Evidence suggests that there is

great diversity both in the individual genetic material and the expression pattern of inhibitory receptor genes. Moreover, the numbers of KIRs expressed on each cell accumulate with time. The result is a highly diverse NK cell repertoire. The effector potency of the cell seems to correlate with the number and strength of inhibitory signals, rather than being an on/off situation [78]. Education of NK cells was believed to be an initial process happening in the bone marrow before NK cells entered the circulation [79]. However, education of NK cells probably occurs alongside differentiation of the cell, but is more important for NK cell function in the early stages of development (Fig. 11). It is not known where, or in contact with which cell types education is most important [78].

In contrast to the idea of a 'natural killer' that does not need prior sensitization, several studies suggest that naïve NK cells in fact need priming by toll-like receptor agonists, cytokines or pathogens to become effector cells (Fig. 11) [80]. Lucas et al [81] has shown that murine NK cells do not acquire effector functions unless primed by IL-15 produced by dendritic cells. They found that NK cells recognize pathogens in the periphery, before homing to the draining lymph node where they interact with DCs and become activated. On the other hand, human NK cells isolated from blood respond without the addition of cytokines. This difference might be due to the constant priming by environmental pathogens in humans [80]. Supporting this theory is findings of more responsive NK cells in mice captured from nature compared to laboratory mice kept under strictly hygienic conditions [82].

Recent studies have also shown features of antigen-specific memory in NK cells from humans, mice and non-human primates (Fig. 11). This implies that NK cells have a role in long-term experience-based immunity. The possibility of memory in NK cells has been studied the most in cytomegalovirus infections in humans and mic. It has been shown that murine Ly49H+ NK cells generate long-lived memory NK cells in response to a murine cytomegalovirus infection. These cells reside in spleen and non-lymphatic organs and are able to undergo clonal expansion upon a second and third exposure to murine cytomegalovirus months after the first activation. Similarly, several studies have shown that human NK cells expressing NKG2C (CD94) receptor may expand following a HCMV infection and can persist for years [83].
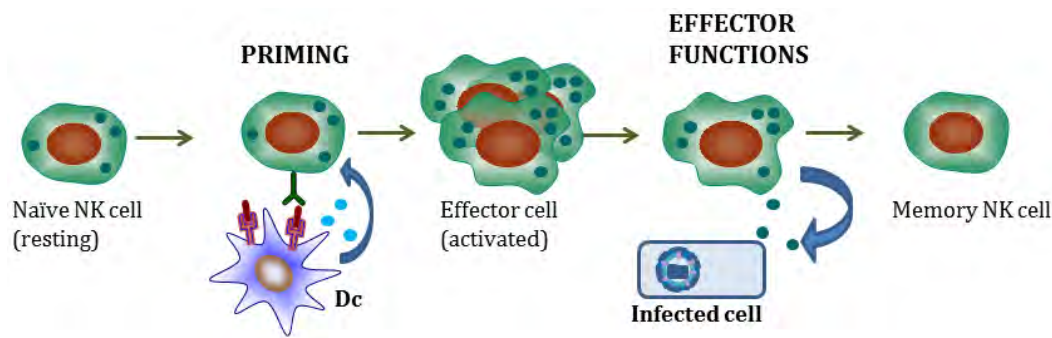
Figure 11. Suggested life cycle of NK cells

Recent studies propose that the NK cell life cycle share many features with T cells, including different phases of priming, effector function and memory.

FUNCTION OF NK CELLS

The important functions of NK cells during killing of virus infected and cancerous cells have been well documented, as well as their immunoregulatory effect as cytokine producers. NK cells have also been shown to be important in immune tolerance and maintains of pregnancy.

NK cells can kill target cells spontaneously by inducing apoptosis by one of the following mechanisms: 1) Ca^{2+}-dependent secretion of intracytoplasmic granules containing perforin and granzymes (degranulation) to the intercellular space or by 2) Binding of Fas ligand or TRAIL to Fas/TRAIL receptors (TNF family death receptors) on the target cell. Perforin forms a pore in the target cell membrane, leading to disrupted membrane function and cell death. Both granzymes and binding of TNF family death receptors trigger key target cell caspases, thereby activating the target cell's own caspase-dependent apoptotic pathway. Granzymes also have the capacity to induce apoptosis in a caspase-independent pathway (Fig. 12) [74,84].
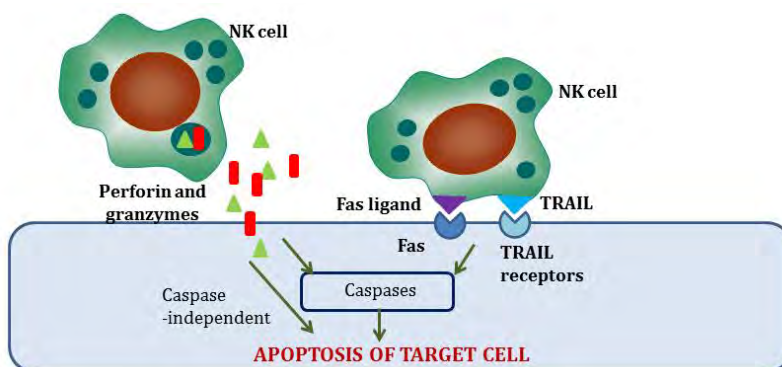

Figure 12. Activation of NK cell cytotoxicity.
NK cell may induce apoptosis in target cells by forming a pore in the cell membrane (perforin), by activation of host caspases (granzymes and Fas/TRAIL) or by a caspase-independent pathway (granzymes).

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In addition to the 'natural' ways of killing, NK cells can also kill target cells by the help of ADCC. ADCC involves killing of target cells coated with monoclonal antibodies (mAbs) produced by B-cells. NK cells recognize the Fc portion of the IgG1 antibodies by the low-affinity Fc receptor CD16. This leads to killing of the target cell by release of perforin and granzymes or Fas/TRAIL activation, as well as the production of cytokines, including IFN-γ (Fig. 13). ADCC has received much attention in connection with cancer immunotherapy and is considered to be crucial for the antitumor-effect of many mAbs used in cancer therapy [85].
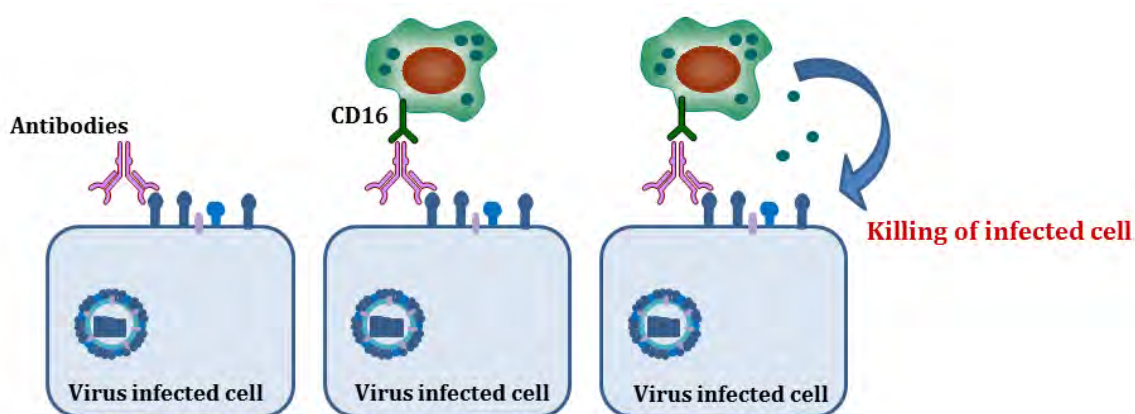
Figure 13. ADCC

Antibodies produced by B cells bind antigens on the surface of virus infected cells. NK cells recognize antibodies through CD16 and are activated to kill the infected cell (adapted from [86]).

NK cells also modulate their surrounding cells by the release of cytokines, including IFN-γ and TNF. These are especially important when a large number of target cells are affected. Both IFN-γ and TNF have antiviral effect. IFN-γ is produced mainly by NK cells during the initial phase of an immune response, and later also by CD4 and CD8 T cells. IFN-γ production is stimulated by IL-12 and IL-18 secreted by APCs, and inhibited by a number of cytokines including IL-10. IFN-γ causes up regulation of MHC I on many cell types, thus increasing the potential of CD8 T cell recognition and cell mediated immunity. IFN-γ also up regulates MHC II expression, not only on professional APCs, but also on cells not constitutively expressing MHC II (non-professional APCs), thus promoting the activation of CD4 T cells. Furthermore, IFN-γ enhances the killing of intracellular microbes by leading the immune response towards a T_H1 response promoting cellular immunity and by activation of macrophage killing, as mentioned above [87].

TNF (formerly known as TNF-α) is also produced by activated macrophages and T cells and was first described as a cytokine involved in the killing of tumor cells, but has a broad range of effects. It has a strong pro-inflammatory effect and is required for inflammatory cell recruitment, promotes monocyte/macrophage differentiation and enhances B cell proliferation. It has also been shown to inhibit viral replication in a number of different virus infections by inducing apoptosis in infected cells prior to maximal virus production being reached. TNF enhances the effect of itself by upregulation of the TNF receptor and promotes IL-6 production. TNF often works together with IL-1 and IL-6, and combined these cytokines are the central mediators of endotoxic shock. TNF is also involved in a syndrome of heritable autoimmune disease (TRAPS) and has a yet undefined role in neuroscience [88].

NK cells can interact directly with other immune cells, thus shaping the immune response. Cross-talk between NK cells and DCs occurs both in secondary lymphoid organs and in peripheral tissue and is mediated through direct cell contact and the production of cytokines and other soluble factors. NK cells are important in the maturation process of DCs and have a stimulatory effect. On the other hand, NK cells have a negative regulatory effect on DCs by killing immature DCs of particular subsets in peripheral tissues. Visa versa, the proliferation and activation of NK cells are greatly influenced by dendritic cells [89].

TISSUE DISTRIBUTION AND LUNG NK CELLS

NK cells develop primarily in the bone marrow before they enter the blood and are found in a number of peripheral tissues, including the peritoneal cavity, spleen, liver, lung, lymph nodes, thymus, and in the uterus during pregnancy. Many of these tissue-resident NK cells form distinct subsets with separate phenotype and functional levels. It is not known where or how these subsets differentiate [51].

The lung is an important site of exposure to antigens and pathogens, and harbors more NK cells than any other non-lymphatic organ, around 10 % of lymphocytes in humans [90] and mice [91] and around 20 % in swine (own results). In mice, it has been shown that lung NK cells have a more mature phenotype, which might reflect that they are ready to respond to infections. At the same time, lung NK cells express more inhibitory-than activating receptors compared to other tissues, ensuring pulmonary homeostasis in steady-state. For example, only 89 % of murine lung NK cells express NKp46,

whereas 98 % of spleen NK cells and 95 % of NK cells from bone marrow express NKp46 in the same study [91]. Also human lung NK cells display a more mature phenotype, where 80 % of the cells belong to the mature CD56^{dim}CD16^{+} subset [90].

NK CELLS IN SWINE

As already mentioned, porcine NK cells may be defined as CD3-CD8α+ cells, and like human NK cells, they express CD16 and perforin. With regards to MHC II expression, porcine NK cells can be negative, but may up regulate MHC II after stimulation in vivo or in vitro. Both the expression of CD8α and MHC II separates porcine NK cells from those in humans, and the function of these receptors on NK cells are currently not known [73]. Furthermore, both NKp46- and NKp46+ NK cells can be found, and the expression seems to be linked to the expression of CD8α. In blood, almost all cells can be categorized as CD8α[+]NKp46^{+} or CD8α[+]NKp46^{-} (Fig. 14) [92]. The proportion of NKp46^{+} versus NKp46^{-} cells in the blood differs between individual animals, and may vary at different time points in the same individual without any clear connection to age or infection status [93]. In organs such as lymph nodes, liver, spleen and lung, a third NK cell population is found, displaying a CD8α[dim]NKp46^{high} phenotype (Fig. 14) [92,94]. The tissue distribution of NK cell populations might be explained by differences in the expression of chemokine receptors. A higher degree of CXCR3 expression have been found on NKp46^{high} cells, compared to NKp46^{-} and NKp46^{+} cells in the spleen [95].
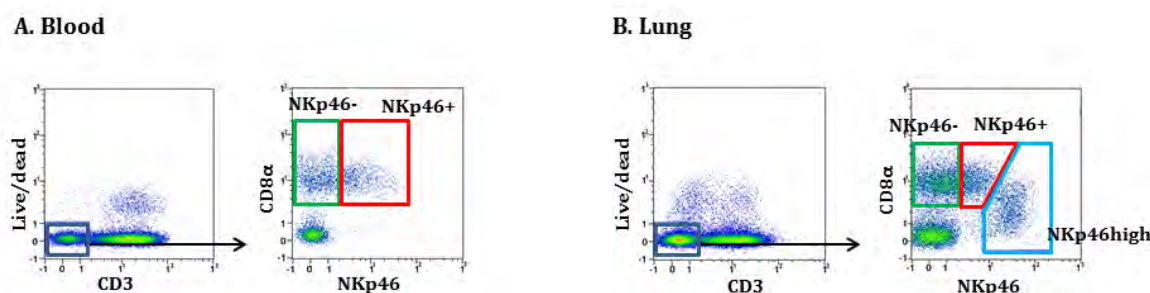
Figure 14. NK cell phenotype in blood (A) and lung (B)
Lymphocytes were isolated from blood and lung of swine, and gated as live CD3^{-} cells, before classified as A) NKp46^{-} or NKp46^{+} NK cells in blood, and B) NKp46^{-}, NKp46^{int} or NKp46^{high} NK cells in lung based on their expression of CD8α and NKp46 (own results).

Although it has been shown that NKp46- an NKp46+ NK cells in swine show comparable ability to kill target cells [92], NKp46 expression seems to correlate with activation status. NKp46+ NK cells have both increased cytolytic capacity after ligation of CD16 and

NKp46 receptors, and produce more IFN-γ and TNF compared to NKp46$^-$ NK cells. Moreover, IFN-γ and TNF production and the cytotoxic potential of NKp46^{high} cells greatly exceed NKp46$^-$ and NKp46$^+$ cells [95]. In humans NKp46^{high} NK cells are also regarded as more activated as they have greater cytotoxic potential than NKp46^{dim} NK cells [64,96]. Furthermore, expression of NKp46 in swine correlates with expression of CD27, the latter has been connected with cytokine production in humans [95]. NKp46 expression may be induced in CD8α$^+$NKp46$^-$ cells, thus indicating that the NKp46$^-$ and NKp46$^+$ cells represent different stages of NK cells, rather than defined subsets [92].

Other NK cell receptors are poorly described in swine. NKp30 and NKGD2 have been identified at the mRNA level, but mAbs against these receptors have not yet been developed. Moreover, one single KIR gene has been identified, as well as one Ly49 gene, but mutations in the Ly49 gene indicate that the latter may not be expressed as a functional protein [97].

NK CELLS IN INFLUENZA VIRUS INFECTIONS

NK CELL MIGRATION TO THE LUNGS

The bone marrow has been suggested to be main site of NK cell proliferation during influenza virus infections, with some proliferation also occurring in the spleen [98]. Subsequently, NK cells are hypothesized to be released into the blood.

Several studies in humans show that following influenza virus infection, the population of NK cells in the blood is reduced and it has been proposed that NK cells migrate from blood into the lungs [99–103]. Consistent with this, an increase in the percentages of NK cells in the upper airways [104] and lungs [98,105,106] of mice is observed shortly after influenza virus infection.

There are few reports on the role of NK cells in swine during influenza virus infection. It has been shown that NK cells in blood were decreased at day 3 and 6 following influenza virus infection, and that NK cells in lung tissue on day 3 post infection (pi) and in bronchoalveolar lavage (BAL) fluid on day 6 pi were decreased [107]. In contrast to this study, an increase in the number of NK cells in BAL fluid from pigs on day 2 pi has also been observed [108].

ACTIVATION OF NK CELLS THROUGH NKP46

Human NK cells have been shown to bind the HA of influenza and parainfluenza virus infected cells via NKp46 [109] and NKp44 [110]. NKp46 belongs to the Ig superfamily and contains two extracellular IgC2-like domains, connected by a short peptide to the transmembrane segment and a small cytoplasmic domain. The NKp46 receptor, like other NCRs, is associated with ITAM proteins that becomes phosphorylated upon receptor activation [111]. Binding of NKp46 to HA is mediated mainly through SA residues in the membrane proximal domain of the NKp46 protein, though a SA independent triggering pathway is also likely to be involved [112,113]. Human NK cells express both α-2,3 and α-2,6 linked SAs. There are conflicting results whether one of them is of greater importance in interactions between human NKp46 and influenza virus. Arnon et al has shown that SAα2,6gal linkages are of greater importance for the binding of NK cells to virus infected cells [113]. Contradictory to this, Owen et al demonstrated that NK cells still have killing potency after removing of SAα2,6gal linkages, indicating that SAα2,3gal linkages are of significant importance [112].

Reduced NK cell capacity to lyse influenza virus infected cells has been linked to the introduction of additional glycosylation sites on HA, which reduce HA-SA interactions, showing the importance of HA-SA interactions in NK cell mediated killing of influenza virus infected cells [112].

EFFECTOR FUNCTIONS OF NK CELLS DURING INFLUENZA

NK cells have been shown to efficiently lyse influenza infected cells *in vitro* [112,114]. Not surprisingly, the CD56^{dim} subset in humans is the most cytolytic active against influenza virus [115,116].

NK cells have been shown to produce IFN-γ in response to influenza virus *in vitro* with the CD56^{bright} subset being the main producers [115,116]. One study has reported that there was no elevation of TNF detected in the media of human NK cells incubated with viral particles from a reovirus system expressing HA and NA from influenza virus [116]. Although the cellular source of cytokine production rarely is identified in human cases of influenza, it is likely that NK cells contribute in early phases of the disease. Elevated levels of TNF have been found in cases of human influenza in a number of studies [101,102,117–119], and has also been found to correlate with clinical symptoms and pathological changes in swine [120,121]. Moreover, TNF has been shown to exert anti-viral effect on epithelial cells infected with influenza virus *in vitro* [122], strengthening the hypothesis that this is an important cytokine in influenza virus infections. Analyses of IFN-γ production in human cases of influenza have given conflicting results. Significant production of IFN-γ was found in nasopharyngeal lavage in one study [119] and in the blood of severely ill patients in another study [103]. In contrast to this, no increase of IFN-γ was found in the blood of influenza virus infected humans in other studies [101,102,117].

Influenza virus binding antibodies are found in humans that lack neutralizing antibodies against the same virus. This suggests that ADCC of NK cells represent a way of killing influenza virus infected cells in the absence of neutralizing antibodies. Indeed, human NK cells were found to be strongly activated by antibodies bound to influenza virus coated plates, and efficiently reduced the number of influenza virus infected epithelial cells. This implies that NK cells may provide a level of protection through cross-protective antibodies against new influenza viral strains [86].

NK cells are also proposed to be important in the recruitment of CD8 T cells and DCs into lymph nodes during influenza viral infections, thus being key players in initiating T cell responses to influenza viruses [123].

MEMORY

As already mentioned, recent studies suggest that NK cells may acquire memory following infections. Long-lived NK cells have been shown to migrate back to the bone marrow and proliferate in response to influenza virus infection in mice [98]. An increase in IFN-γ production has also been observed when NK cells from vaccinated humans were co-cultured with influenza virus, compared to NK cells from unvaccinated controls. The increased responsiveness lasted six months following vaccination. Moreover, the increased production seemed to be linked to activation through NKp46, followed by internalization of this receptor [124].

INFLUENZA VIRUS ESCAPING NK CELLS

Several studies have shown that influenza virus isolates from the past stimulate NK cells more efficiently than more recent isolates, indicating that NK cells participate in driving the evolution of influenza virus [23,112]. One study linked this to the addition of potential glycosylation sites in the HA of influenza viruses, raising the question whether influenza viruses that acquire additional glycosylation sites are being selected through evolution [112]. Moreover, influenza virus has been shown to evade NK cell binding by removing SAs on NKp46 and thereby reducing recognition of virus infected cells. This process is mediated through NA, and NA inhibitors like Tamiflu will counteract this effect [125].

Several viruses cause a down regulation of MHC I expression on host cells in order to escape recognition by T cells. The lack of inhibitory signals from MHC I will usually trigger NK cells. In response to influenza virus, MHC I is re-organized into lipid rafts in the cell surface. These gatherings of receptors deliver a strong inhibitory signal to the NK cell which prevents killing of the infected cell.

It has also been hypothesized that NK cells may be directly infected and killed by the influenza virus in lung tissue [99,126,127]. Fatal cases of influenza in humans have been associated with diminished numbers of NK cells [99,127]. Simultaneously findings of apoptotic markers on other innate immune cells could reflect that NK cells had

undergone apoptosis [127]. But whether this was initiated by the influenza virus as a way of escaping the immune system, or by the immune system itself in order to limit the infection is impossible to say.

Taken together, the fact that influenza virus has evolved all of these specific ways of avoiding NK cell activation, strengthens the idea that NK cells are important in the immune response against influenza virus infection.

AIMS OF THE STUDY

Main objective

The overall aim of the present study was to characterize pH1N1 viruses in Norwegian swine and porcine NK cell responses to pH1N1 virus infections.

Sub-goals

The following sub-goals were set to reach the main objective:

1. Characterize pH1N1 viruses in Norwegian swine and compare these to pH1N1 viruses found in humans during the same period (**paper 1**).
2. Determine the role of porcine NK cells in general and the NKp46 receptor in particular in pH1N1 virus infected pigs with emphasis on the organ distribution of NKp46$^-$ and NKp46$^+$ NK cells (**paper 2**) and CD3$^+$NKp46$^+$ cells (**paper 3**).
3. Determine the effector functions of porcine NK cells in pH1N1 virus infected pigs (**paper 2**) and in response to pH1N1 virus *in vitro* (**paper 4**).
4. Determine whether the 222D/G polymorphism of pH1N1 viruses influences NK cell responses against the virus *in vivo* (**paper 2**) and *in vitro* (**additional material**).

SUMMARY OF PAPERS

Paper 1: Swine influenza in Norway: a distinct lineage of influenza A(H1N1)pdm09 virus

Influenza pH1N1 virus was introduced to Norwegian pigs in September 2009, thus ending the status of the Norwegian swine population as influenza virus free. We isolated influenza viruses from Norwegian pigs from 2009 to 2011, followed by sequencing of the HA protein gene. Sequences obtained from these viruses were compared to those found in humans in Norway during the same period. Until early 2011 the viruses circulating in pigs closely resembled those in humans. In late 2011, however, virus persisted in pigs after cessation of human virus circulation and the sequence analysis showed that viruses in pigs were forming a distinct lineage.

Paper 2: Early responses of natural killer cells in pigs experimentally infected with 2009 pandemic H1N1 influenza A virus

In two independent infection experiments, we explored the role of NK cells in piglets experimentally infected with pH1N1 virus by flow cytometric analyses of cells isolated from blood and lung tissue. Additionally, samples from lung tissue were analysed by immunostaining in the second experiment. In the first experiment, animals were infected with a mixture of the two different virus variants; 222D and 222G, while only the 222G virus was used in the second experiment. The number of NKp46$^+$ NK cells was reduced while NKp46$^-$ NK cells remained unaltered in the blood 1-3 days after infection in both experiments, but a more uniform reduction in all infected animals was seen when the animals were infected solely with the 222G variant. In the lungs, the intensity of NKp46 expression on NK cells was increased during the first 3 days, and areas where influenza virus NP was detected were associated with increased numbers of NKp46$^+$ NK cells when compared to uninfected areas. NKp46$^+$ NK cells in the lung were neither found to be infected with influenza virus nor to be undergoing apoptosis. The binding of porcine NKp46 to influenza virus infected cells was verified in an *in vitro* assay. These data supported the involvement of porcine NKp46$^+$ NK cells in the local immune response against influenza virus, and a possible role of the NKp46 receptor.

Paper 3: Porcine CD3+NKp46+ lymphocytes have NK cell characteristics and are recruited to the lung during early influenza infection

The NKp46 receptor is not only expressed on NK cells, but also on different innate lymphocytes, including a small but distinct population of CD3+NKp46+ cells. We reported a minor population of CD3+NKp46+ lymphocytes in blood, lymph nodes, spleen, liver and lung of swine. Phenotypic analyses and functional tests showed that the majority of CD3+NKp46+ cells mainly displayed typical NK cell traits. However, both the NKp46 and the CD3 receptor were shown to most likely be functional, leading to degranulation of the CD3+NKp46+ cells. To determine if the CD3+NKp46+ cells were involved in pH1N1 influenza virus infection in swine, we analyzed blood and lung samples from pH1N1 virus infected swine by flow cytometry. We found reduced frequencies of CD3+NKp46+ lymphocytes in blood and increased frequencies in the lung in the early phase of infection. Thus, CD3+NKp46+ cells appear to also be involved in the early phase of influenza virus infection.

Paper 4: Influenza A virus leads to degranulation and TNF production in cultured porcine NK cells

To study the responses of NK cells to influenza virus *in vitro*, NKp46+ cells were isolated from swine blood and spleen, and cultured for 10 days. Subsequently, cultured cells were analyzed for the expression of CD3 and several NK cell markers. This is the first report of cultivation of NK cells in proliferating cultures. Phenotyping by flow cytometry showed a high purity of cells with a typical NK cell phenotype. However, a substantial proportion of the cells did not express NKp46. Cultured cells degranulated and produced IFN-γ and TNF in response to ligation of CD16 and NKp46 similar to freshly isolated porcine NK cells. Cultured cells were also incubated with pH1N1 virus and degranulated and produced TNF in response to the virus. However, there was no increase in the production of IFN-γ. These results resemble those obtained from the *in vivo* infection experiments.

DISCUSSION

INFLUENZA VIRUS IN NORWEGIAN SWINE

Influenza viruses that cause more severe disease in swine and/or humans may appear first in swine. After the first detection of influenza virus in Norwegian swine in 2009, it was of great importance to monitor the evolution of this virus. Not only to see if it established itself in the Norwegian swine population, but also in an attempt to detect possible mutations in the virus that could be of concern to public health. In the initial study (paper 1), we compared HA from swine viruses collected between 2009 and 2013 to the publically available human sequences. Phylogenetic analysis showed that viruses circulating in Norwegian swine by the end of 2011 formed a distinct phylogenetic group.

ENDEMIC OCCURRENCE OR SIMPLY SPILLOVER FROM HUMANS?

Based on the occurrence of a distinct clade of pH1N1 viruses and the high seroprevalence of antibodies against pH1N1 in Norwegian swine, paper 1 concludes that pH1N1 most likely is endemically occurring in Norwegian swine.

The most recent results from the Norwegian surveillance program show that still around half of the Norwegian pigs have evidence of exposure to pH1N1 infection (Carl Andreas Grøntvedt, unpublished results). In the Norwegian human population, around one third had antibodies against pH1N1 (natural infections and/or vaccination) prior to the 2015-2016 influenza season [128]. People working with swine have been encouraged to vaccinate themselves by The Norwegian Pig Health Service, and one could therefore assume that the seroprevalence in swine workers is even higher than in the general population. As antibodies provide good protection against influenza virus infections [54], transmission from seropositive individuals is not likely. Norwegian swine are kept in closed facilities, and contact with humans is often limited to a few persons. Because of the high turnover of animals in swine production, it would require many encounters with virus infected humans in order to keep up a seroprevalence of nearly 50 %, if transmission from humans was the only source of virus infection. SIVs generally are maintained through the continuous production of new, susceptible piglets [15]. Phylogenetic clustering of pH1N1 viruses seen in other European countries is

indicative of circulation of the pH1N1 virus as well, independently of virus circulation in humans [21]. In a study based on sequencing data of SIVs from 18 countries worldwide, continuous re-introductions of pH1N1 to swine was reported [22]. The phylogenetic analysis from paper 1 suggests that Norwegian swine are also being seeded with virus from the human population. Taken together, it is more than likely that pH1N1 now circulates endemically in Norwegian pigs, but that transmission from humans still is an important source for new introduction of the virus.

First spillover event from humans to swine in Norway

The pH1N1 virus is the first influenza virus that has established itself in the Norwegian swine population. Transmission of a whole new influenza virus from one species to another, followed by successful onward transmission is dependent on both host and viral genetic factors and is a rare event. Most new influenza viruses emerge from reassortment events with already circulating strains [1,2]. For example, the 1918 Spanish flu affected a great proportion of the world population, but the virus only managed to establish itself in North American Swine. The virus was not transmitted to European swine before infected pigs were imported to Italy nearly 60 years later [15,21]. The pH1N1 virus was originally a swine adapted virus that was later transmitted to humans [4]. The original source of other endemic human influenza viruses is not known [16], but they all have contained an avian derived HA [14]. Since HA is determining receptor specificity, the fully adapted swine pH1N1 was therefore probably more easily transferred to the naïve swine population in Norway than previous human influenza viruses.

Challenges with virological sampling

Even though the influenza seroprevalence in Norwegian swine is reported to be high, the Norwegian Veterinary Institute has only received one virus positive sample since 2011 and no positive samples since paper 1 was published in 2013. Clinical signs of pH1N1 in Norwegian swine herds have been reported to be mild or subclinical [129] in line with reports of SIV infections in other countries [15]. Thus, it might not be of interest for the farmers to correctly suspect and diagnose the disease by virological investigation. Norwegian veterinarians in areas with high density of swine herds were asked to submit samples for viral detection, when there were sign of respiratory disease. Still, no virus positive samples were obtained. Influenza virus is only shed

during the initial phase of infection, and usually peaks four to six days following infection [29,130]. Sampling may therefore have been carried out too late for viral detection. Recent studies have suggested that collection of oral fluids by the use of ropes might represent a better way of sampling at herd level than the traditional individual animal sampling of virus from nasal swabs. After experimental infection, virus was reported to be detected for a longer time period in oral fluids than in nasal swabs [131]. This represents a potential new detection method of SIVs.

THE *IN VIVO* IMPORTANCE OF PORCINE NK CELLS

Little is known about virulence markers deciding the clinical outcome of influenza virus infections. To better understand the mechanisms that decide disease severity, one also needs to study the immune responses. NK cells in general and the NKp46 receptor in particular, have been assigned a special role in influenza virus infections. Swine serve as a valuable model for influenza virus infections in humans. Still, little is known about NK cells during influenza virus infections in swine. In order to advance our knowledge on NK cells during influenza virus infections in swine, we conducted two separate infection experiments and the results were presented in paper 2 and 3.

SWINE – THE OPTIMAL ANIMAL MODEL FOR INFLUENZA STUDIES?

Most of the studies of immune responses to influenza virus infections are conducted in mice. The mouse is a practical and inexpensive animal model and the availability of species specific reagents is great [132]. However, there are a number of significant drawbacks in using the murine model in influenza studies. Mice are not naturally infected with influenza virus. Although most inbred mice are highly susceptible to certain mouse adapted viruses, wild mice are resistant to even high doses of the virus [132,133]. The reason for this difference is the lack of functional Mx protein coding genes in most inbred mice that results in reduced viral protection [48,132]. Genomic studies have also revealed other differences in the immune system between humans and mice. As a consequence of these differences, results from studies of vaccines or anti-viral drugs obtained from mice have often not been transferable to humans [134]. Moreover, NKp46 in mice are substantially different from human NKp46 with regard to glycosylation patterns [135]. Since influenza viral HA binds NKp46 through specific glycosylation sites [113], this is likely to influence HA-NKp46 interactions. NK cells in mice raised under sterile conditions might also have different abilities to react against microbes than human NK cells [82].

There are species differences between swine and humans as well, and extrapolation of results from one species to another should always be done with care. Negative aspects to the swine model are the practical, economical and ethical considerations that may limit the number of animals used in one experiment. Also, the use of outbred animals results in greater variations because of more pronounced individual differences that are often difficult to predict when calculating the numbers of animal needed in an

experiment. In the *in vivo* infection experiments in our study (paper 2 and 3) some of the results did not provide statistically significant answers because of greater variation within the study population. Nonetheless, to better understand infectious disease and in particular zoonotic diseases such as influenza, it is important to undertake studies in natural hosts [134]. In addition to being a natural host for influenza viruses, swine are anatomically and physiologically similar to humans and respond to an influenza virus infection in much the same manner as humans [27,29,119,120,136–138]. Pigs may therefore serve as a better model than mice for studying the pathogenesis of influenza virus infections, as well as the immune response against it.

THE ROLE OF THE NKP46 RECEPTOR

As already mentioned, NKp46 has been assigned a special role in recognition of influenza viral HA and activation of NK cells. Although once stated as an exclusive NK cell marker in humans [60], up to 40 % of NK cells in the blood of some individuals are reported to display a NKp46- or NKp46dim phenotype during steady state in some studies [115,124]. NKp46 expression on porcine NK cells was recently shown to vary, and some pigs have quite high proportions of NKp46- NK cells in blood and other organs [92]. Moreover, NKp46 has been found on small subsets of T cells, including CD3+NKp46+ cells in humans [66] and swine [92]. In paper 2 and 3 the connection between NKp46 expression and tissue distribution during influenza virus infection was studied in swine.

A reduction of NKp46+ NK cells was seen in blood of infected animals (Fig. 15A) (paper 2), similar to the reduction of NK cells observed in cases of influenza in humans [99–103]. The reduction of NKp46+ cells in blood could either represent down regulation of the receptor or specific migration out of the blood. Reduced percentages of NKp46+ cells in total NK cells have also been found in the blood of influenza virus vaccinated humans [115]. The reduction of NKp46+ cells was proposed to be caused by a down regulation of NKp46, based on the fact that NKp46 was down regulated upon exposure to influenza virus *in vitro* [115]. However, if this was the case, one would expect a simultaneously relative increase in the number of NKp46- cells. In the second experiment, absolute cell numbers were obtained, and no increase in the numbers of NKp46- NK cells were seen.

The numbers of NKp46- NK cells in blood remained unaltered during the first couple of days, before they increased on day three post infection (Fig. 15B) (paper 2). Mair et al

has suggested that NKp46^{-} and NKp46^{+} NK cells represent different maturation stages, rather than separate subsets [92]. Studies in mice have shown that bone marrow is the primary site of NK cell proliferation during influenza virus infection in mice and that all four NK cell subsets proliferate, but that the more immature CD27^{-}CD11b^{-} and CD27^{+}CD11b^{-} NK cell subsets had the greatest proportions of proliferating cells [98]. In the present studies, no increase in the expression of the proliferation marker Ki67 was detected in the peripheral tissues sampled. This indicates that there is neither an increased proliferation of NK cells in peripheral tissue during influenza virus infections in swine. In light of these results, the NKp46^{-} NK cells in blood of swine might represent more immature NK cells released from the bone marrow after the initial recruitment of NKp46^{+} NK cells from the blood.
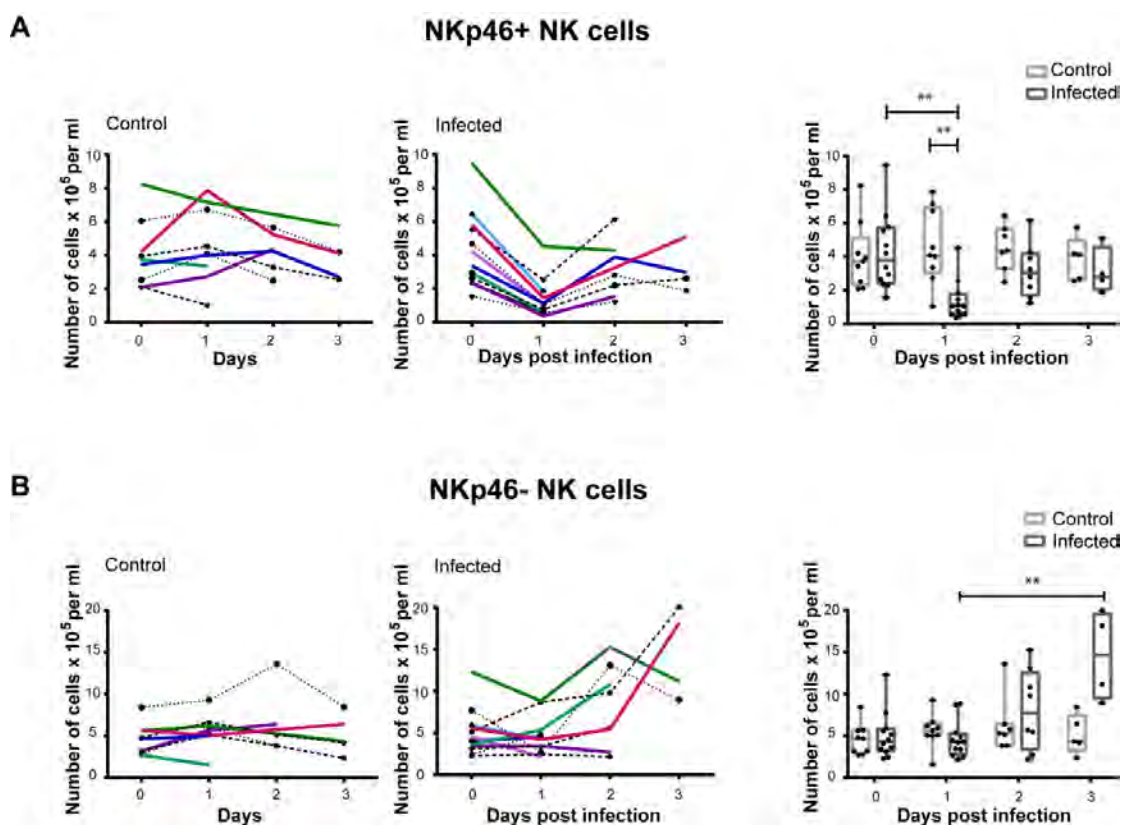
Figure 15. NK cell numbers in the blood of influenza virus infected pigs
Blood was collected from pH1N1 virus infected pigs (n=12) and control pigs (n=9) on day 0-3 following infection. Isolated blood lymphocytes were analyzed by flow cytometry and live CD3^{-} lymphocytes were gated as **A)** NKp46^{+} or **B)** NKp46^{-} according to CD8α and NKp46 expression. *$p \leq$ 0,05, **$p \leq$0,01 (modified from Fig. 2, paper 2).

To investigate whether NK cells were recruited to the site of infection, cells needed to be isolated from the respiratory tract (paper 2). During the first experiment, BAL was

attempted post mortem followed by flow cytometric analyses of isolated cells. However, only a few NK cells were obtained by this method and the results showed inconsistency between days. In the second experiment, lung tissue samples were processed and analyzed by flow cytometry. Percentages of NK cells in lymphocytes only showed moderate increase of both NKp46$^-$ and NKp46$^+$ NK cells. The infiltration of other lymphocytes is likely to cause an underestimation of the absolute numbers of NK cells in lung tissue. Therefore, lung tissue sections were stained with immunofluorescence markers against NKp46 and influenza NP (Fig. 16). Tissue sections showed that NKp46$^+$ cells were located in the connective tissue of the upper and lower respiratory tract and not in the outer epithelial layer. This may explain why few cells were obtained by BAL. Increased numbers of NKp46$^+$ cells were found in infected animals compared to uninfected controls. There were also more NKp46$^+$ cells in areas where influenza virus was detected (Fig. 16A), than in areas without virus (Fig. 16B) in the same animal. The number of NKp46$^+$ stained cells would also represents some CD3$^+$ cells. However, double staining with CD3 and NKp46 was attempted and only a few double positive cells were detected. The relative contribution of CD3$^+$NKp46$^+$ to the total number of NKp46$^+$ is thus considered to be negligible. The increase of NKp46$^+$ cells observed in lungs strengthens the hypothesis that NKp46$^+$ NK cells recruited from the blood migrate into the lungs.
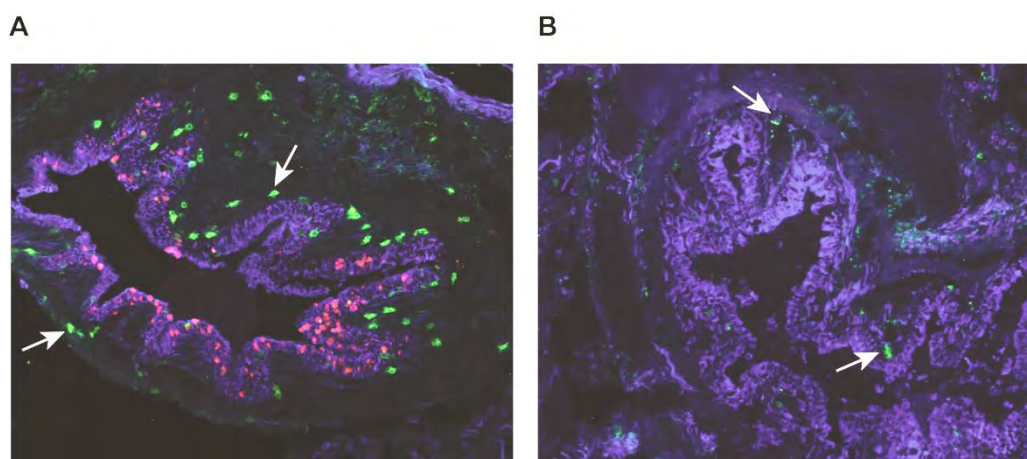
Figure 16. NKp46$^+$ cells in the lungs of influenza virus infected pigs

Lung tissue sections from pigs infected with pH1N1 virus were stained with immunofluorescence markers against influenza virus NP (red) and NKp46 (green) together with the epithelial cell marker cytokeratin (blue). Pictures shown are representative for areas where influenza virus could **A)** be detected and **B)** not be detected from the same animal (modified from Fig. 4, paper 2).

Although flow cytometric data from the second experiment could not be used to detect alterations in the number of NK cells in the lung, interesting information was obtained on the different NK cell populations (paper 2). As already mentioned, three populations of NK cells can be found in the lungs of swine; NKp46-, NKp46int and NKp46high NK cells. In paper 2, the greatest proportions of cells expressing the activation marker CD25 were found in the NKp46high population, indicating that this population is in a more activated state. NKp46high NK cells have later been verified to represent more activated cells by Mair et al [95]. If NKp46 expression also is linked to differentiation of NK cells as suggested above, one could speculate that less differentiated NKp46- cells up regulate NKp46 upon differentiation and activation in lung tissue. In paper 2, we compared median fluorescence intensity values of different markers between the different NK cell populations as a measure of expression levels of the relevant marker. An increase in NKp46 expression was detected in the NKp46int NK cell population from infected animals on day two post infection, compared to day one and three, and compared to uninfected controls. Is some animals, there were also elevated levels of NKp46 expressed in the NKp46high population on day two, but the differences between groups were not significant. This implies that NKp46int cells up regulate NKp46 and may become NKp46high cells in response to influenza viral infections of pigs. No increase in the relative proportion of NKp46high cells was detected, but this could be caused by the influence of other cell types as mentioned above.

What triggers the differentiation, and whether the activation occurs in the lung in response to influenza virus alone or if migration to lymph nodes and priming by DCs are involved in pigs could not be definitively determined. In mice, an increase of NK cells is found in lymph nodes draining the lung [123]. In the first pig experiment, lymph nodes draining the respiratory tract were sampled and processed, followed by flow cytometric analysis of lymphocytes. However, the results obtained were inconsistent between days, and no conclusions could be drawn. Suggested migration and differentiation patterns of porcine NK cells based on the preliminary results of our infection experiments are presented in figure 17.
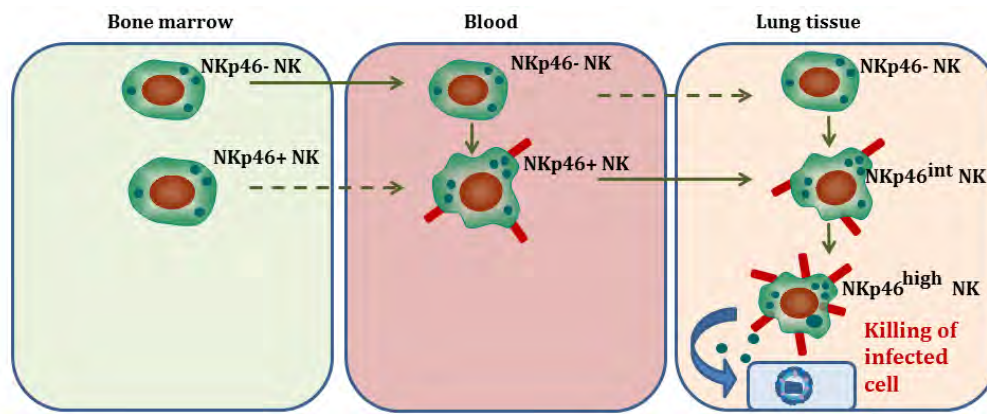
Figure 17. Suggested migration and development of porcine NK cells in response to pH1N1 infection. A schematic diagram showing the hypothesized route of migration and differentiation of different porcine NK cell subsets based on the findings in two infection experiments in swine. NK cells are suggested to mainly proliferate as NKp46- cells in the bone marrow and released to the blood. Here, the more mature NKp46+ NK cells are recruited to the lungs, where they further up regulate NKp46 and become NKp46high NK cells proposed to be the main effector subset. Note that results from lymph nodes and possible involvement of priming could not be obtained.

Since a selective recruitment of NKp46+ NK cells was found, we wanted to determine if CD3+NKp46+ cells were also involved in influenza virus infections (paper 3). Indeed, we found a reduction of CD3+NKp46+ cells in the blood and an increase in the percentages of CD3+NKp46+ cells in the lungs. This indicates a recruitment of several cell types expressing the NKp46 receptor and suggests a universal role of this receptor. However, one would expect a general recruitment of immune cells, regardless of NKp46 expression. The role of CD3+NKp36+ cells in influenza virus infections thus need to be further elucidated.

THE *IN VIVO* FUNCTION OR DYSFUNCTION OF NK CELLS

In order to determine the functional role of NK cells in influenza virus infections in swine, lymphocytes were analyzed for intracellular IFN-γ and analyzed by flow cytometry in the second experiment (paper 2). Cells from lung and lymph nodes draining the lung were kept in culture for four hours in presence of protein transport inhibitors (GolgiPlug/GolgiStop) to prevent IFN-γ to be excreted from the cells. No differences between cells isolated from infected and control animals could be detected. Isolated mononuclear cells were frozen and analyzed later for mRNA encoding IFN-γ and TNF and adjusted according to the expression levels of a 'housekeeping' gene. Although there were some individual differences between animals, no clear increase

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could be detected in IFN-γ mRNA, confirming the results obtained by flow cytometry. In contrast to this, all animals had increased mRNA levels encoding TNF on day 1. Since these data are based on mRNA levels, they do not necessarily reflect the amount of expressed protein. However, it is widely acknowledged that mRNA may be used as an indication of the amount of protein that potentially is produced. Unfortunately, the production of TNF was not investigated during the experiment. The functional data does not say anything about NK cell contribution to the cytokine production compared to other lymphocytes. But it is known that NK cells are important producers of TNF in the early course of infections [46], and it is likely that they have contributed to the increased levels of TNF seen in the present study.

Based on *in vitro* results, it has been suggested that NK cells can be directly infected by influenza virus and killed by the induction of apoptosis [126]. In search of infected or apoptotic NK cells, tissue sections from the second experiment were stained with mAbs against NKp46, the apoptotic marker caspase-3 and influenza virus NP (paper 2). We did not find any infected or apoptotic NKp46^{+} cells indicating that this mechanism is not frequently occurring *in vivo* in swine.

IMPORTANCE OF THE D222G SUBSTITUTION

The 222D/G polymorphism of pH1N1 has been associated with more severe disease, but there is no report on this in connection with NK cell responses. While HA bearing the wild type 222D variant preferentially bind SAs with a α2,6Gal linkage, the 222G substitution leads to increased affinity for SAα2,3Gal receptors. Human NKp46 has been shown to bind influenza virus infected cells in a SA dependent manner, but there are conflicting results whether one of the linkages is of greater importance than the other [112,113]. Since changes in glycosylation sites of influenza virus alter the activation of NK cells [112], one could hypothesize that the 222G substitution also affect NK cell activation.

In order to study NK cell responses in connection with the D222G substitution, two different pH1N1 viruses were used in two infection experiments described in paper 2 and 3: A/Hamburg/05/2009 and A/Hamburg/05/2009-e. The two viruses differ only in amino acid position 222 in HA. While Ham expresses the wild-type 222D, Ham-e carries the 222G substitution associated with more severe disease. In the first experiment, the pigs were infected solely with the 222D variant, while both viruses were used in the

second experiment, thus mirroring an infection with a 222D/G quasispecies. Higher viral loads were detected by real-time RT-PCR in the lower respiratory tract in the second experiment, compared to the first experiment. Moreover, sequence analysis showed that while the 222D variant was the main virus detected in the upper respiratory tract, the 222G was selected for in the lungs (Sharon M. Brookes, unpublished results). These findings show that swine resemble humans in the dynamics of influenza virus infections with 222D/G quasispecies and that they represent a good model for studying this substitution.

In the first experiment (222D), there were individual differences regarding which day the proportions of NKp46$^+$ cells declined in blood. A more uniform and marked response was seen in the second experiment (222D/G) (paper 2). This indicates an effect of virus variant on NK cell responses. Unfortunately, tissue sections from the first experiment were not stained for NKp46$^+$ cells. Since the sampling of BAL fluid failed, there is no data from lung tissue from the first experiment. If this was to be further elucidated, it is important to make a distinction of samples obtained from the upper- versus the lower respiratory tract. As the virus cell tropism and tissue preference differ between the two virus variants, they might also elicit different NK cell responses in the two different compartments of the respiratory tract.

ELUCIDATING THE ROLE OF PORCINE NK CELLS *IN VITRO*

Based on the *in vivo* experiments, we hypothesized that NKp46+ NK cells were recruited to the lungs. As the *in vivo* experiments told us little about NK cell functions, we wanted to study NKp46+ NK cell responses to influenza virus further *in vitro*. The results are presented in paper 4.

CULTURING OF PORCINE NK CELLS

In order to get the best comparative results using fewer animals and reduce inter-assay variation, it is better to compare cells isolated from the same animal and organ. To increase cell numbers obtained from one animal/organ, NKp46+ cells were isolated and kept in proliferating cultures for 10 days. To our knowledge, this is the first report of proliferating NK cell cultures from swine.

Proliferating cells grew as non-adherent cells with prominent lamellipodia. After 10 days, the cells were phenotypically analyzed, and were found to be CD3-CD16+perforin+, thus displaying a typical porcine NK cell phenotype [73]. To our surprise, although selected for their NKp46 expression, a substantial number of cells were found to be NKp46- (median value; 15 %, range; 9-31 %). The engagement of the NKp46 receptor during the sorting process would be expected to induce temporarily down regulation of the receptor. However, based on our previous experiments with bovine NK cells [62], we find it unlikely that the receptor had not been up regulated after 10 days in culture. A more likely explanation is a contaminating proportion of NKp46- NK cells in the primary cultures that respond to IL-2 treatment. IL-2 is a cytokine mainly produced by T cells that activates NK cells to proliferate [139]. Another explanation for the reduction in NKp46 expression is that the cultured NK cells have down regulated NKp46. The NKp46 receptor has been reported to be deficiently expressed on NK cells isolated from patients with acute myeloid leukemia [140], in response to phagocytes *in vitro* [140] and after *in vitro* exposure to influenza virus [115]. Culturing may induce stress responses in cells and one could hypothesize that stress may have lead to alterations in the expression of different surface receptors on the NK cells, including NKp46. To study NK cell cultures from swine more thoroughly, a proportion of the cells could have been analyzed at different time points following isolation in order to detect changes in the phenotype. Since the main goal of our *in vitro* assays was to characterize NK cell responses to influenza virus, this was not attempted in the present study.

Since cells were selected for NKp46 expression, small populations of other innate lymphocytes expressing NKp46 also may have been isolated. However, only 3 % (range; 0-5 %) of the cultured cells were CD3$^+$. The possible contamination by CD3$^+$NKp46$^+$ cells is therefore not likely to affect the results substantially.

Cultured cells were incubated in wells coated with anti-CD16 or anti-NKp46 mAbs. Subsequently, the activation status was evaluated by their expression of CD107a and intracellular IFN-γ and TNF by flow cytometry. CD107a (LAMP-1) is a protein lining the inside of cytolytic granules containing perforin and granzymes in the NK cell. Following the release of these cytotoxic mediators, CD107a appears on the NK cell surface. CD107a has been shown to be a valid marker for degranulation and cytotoxicity in T cells and NK cells [141].

In the present study, cultured NK cells were shown to up regulate CD107a and produce IFN-γ and TNF in response to ligation of the NKp46 and the CD16 receptors. Based on phenotypical and functional traits we concluded that the cultured cells represented NK cells.
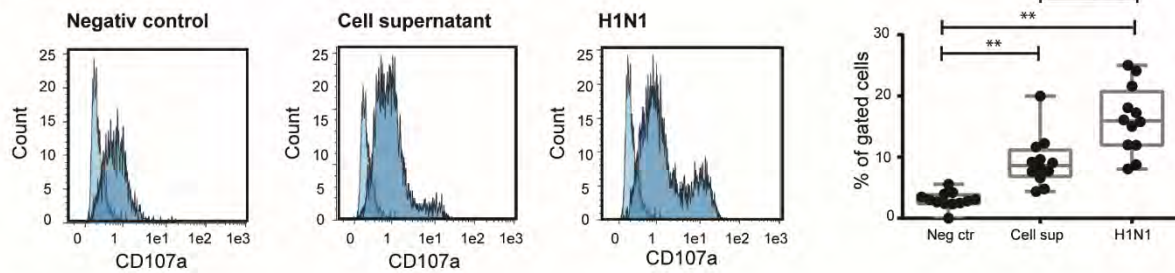
NK CELL ACTIVATION BY INFLUENZA VIRUS

In order to study NK cell responses to influenza virus, cultured NK cells were incubated in wells coated with pH1N1 (Cal09) virus, and analyzed for the expression of CD107a, IFN-γ and TNF by flow cytometry.
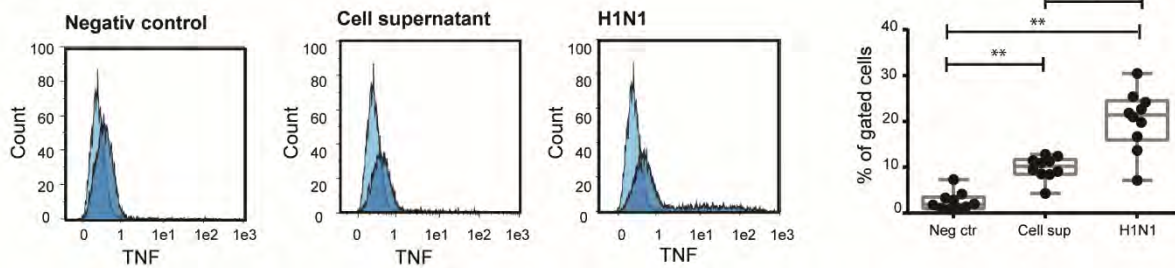
NK cells kept in wells coated with cell supernatant displayed moderate increase in CD107a expression, compared to NK cells kept in coating buffer alone. Protein measurements showed high amounts of proteins in cell supernatant that potentially could activate NK cells. For future studies it would be beneficial to purify the virus or use recombinant HA protein in order to eliminate the effect of proteins in cell supernatants. One could also attempt to use HA-rosettes in order to get an efficient stimulation of NK cells.

Although cell supernatant elicited NK cell activation, cultured NK cells responded to influenza virus with a higher degree of degranulation (Fig. 18A) and production of TNF (Fig. 18B) compared to cell supernatant. The amount of IFN-γ$^+$ cells in some animals were elevated compared to controls, but differences between groups were not significant (Fig. 18C).
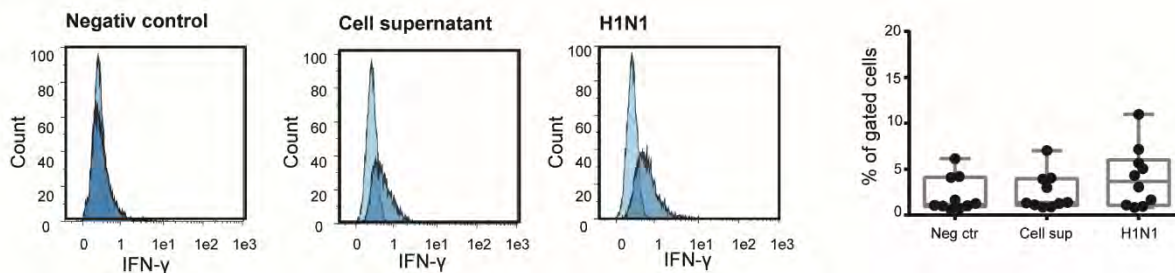
Figure 18. Stimulation with influenza virus resulted in activation of cultured NK cells.

NK cells were cultivated for 10 days before incubation in wells coated with coating buffer alone (negative control), cell supernatant or pH1N1 (Cal09) virus. Cells were subsequently analyzed for their expression of **A)** CD107a (n=12) and intracellular amounts of **B)** TNF (n=10) and **C)** IFN-γ (n=10). Percentages of positive cells in live cultured cells were compared between different stimulations. **$p \leq 0{,}01$. (Modified from Fig. 4, paper 4)

PRIMING IN INFLUENZA VIRUS INFECTIONS

Recent studies indicate a need for priming of NK cells [80], but only a few studies have linked NK cell priming to the encounter with influenza virus. Draghi et al hypothesized that activation of human NK cells in response to influenza viral infections required both IFN-α and IL-12 produced by DCs and direct contact between these cells [142]. In contrast to this, NK cells have been shown to become activated without the presence of dendritic cells in a number of studies [109,112,116]. In the present study, NK cells degranulated and produced cytokine in response to influenza virus, without the presence of DCs.

NK cells in the present study were cultured with IL-2. This cytokine is necessary for the survival of murine NK cells *in vitro* [80] and leads to recognition of a broader range of targets and killing with increased cytolytic potential [46,143]. Human NK cells may survive up to four days without the addition of IL-2, but are less efficiently activated to kill than IL-2 activated NK cells [144]. As already mentioned, the difference between murine and human NK cells has been hypothesized to be linked to human NK cell constant priming through environmental factors including microbes [80]. Porcine NK cells in the present study were not attempted cultured without IL-2. Although the levels of IL-2 were kept at a minimum during the assays, NK cells were shown to be in an activated state prior to assays by increased expression of CD25. IL-2 may therefore substitute the need for DCs by inducing an activated state in NK cells. Interestingly, blocking of IL-2 has been shown to have minimal effect on NK cell effector functions against influenza virus infected cells, questioning the need of IL-2 in NK cell activation in influenza virus infections [115].

RESULTS NOT INCLUDED IN THE PAPERS

THE *IN VITRO* ROLE OF THE NKP46 RECEPTOR

The selective *in vivo* recruitment of NKp46+ NK cells (paper 2) and the involvement of CD3+NKp46+ cells (paper 3) indicated a role of the NKp46 receptor. In paper 2, we also showed that a porcine NKp46 fusion protein bound influenza virus infected cells, similar to human NKp46 [109]. To follow up on this, we attempted to prove the involvement of the NKp46 receptor in our *in vitro* system with cultured NK cells.

Blocking of the NKp46 receptor was attempted by incubating cultured NK cells with anti-NKp46 mAbs prior to stimulation with influenza virus. Binding was confirmed by the absence of staining by the same mAbs afterwards. The binding had no effect on degranulation or cytokine production of NK cells (results not shown). While NK cells have been shown to bind influenza virus only through glycosylation sites in their more proximal Ig-like domain [113], the specific binding site of the anti-NKp46 mAb is not known. Thus, anti-NKp46 mAbs and HA may very well bind different epitopes. It has previously been shown that a mAb directed against the distal Ig-like domain does not block activation of human NK cells by influenza virus infected cells [113]. Others that have shown little effect of NKp46 blocking on NK cells effector functions in response to influenza HA, have at the same time shown that NKp46 was internalized, indicating engagement of this receptor [116]. Staining for intracellular NKp46 was not attempted in the present study.

Ligation of NKp46 using anti-NKp46 mAbs in coated plates caused a loss in NKp46 expression (results not shown), typical for NKp46 receptor engagement [145]. However, stimulation with influenza virus only caused minor changes in NKp46 expression (results not shown). This might be due to the short incubation period of one hour used in the degranulation assay in the present study. Stimulation of human NK cells with influenza virus infected cells causes an initial up regulation of NKp46 during the first two hours of incubation, followed by a loss of NKp46 expression at six hours [115].

Although NKp46 is important in activation of NK cells during influenza, other receptors are also likely to be involved. NK cell responses are generally governed by the combination of several signals. Human 2B4 has been suggested to act as a co-receptor

to NKp46 and the proportion of 2B4+ NK cells has been shown to expand in response to influenza virus infection [115]. Moreover, the combination of KIR genes in humans have been shown to correlate with influenza disease severity [146]. The role of other receptors needs to be further elucidated, but is also limited by the number of mAbs available in swine.

IMPORTANCE OF THE D222G POLYMORPHISM *IN VITRO*

During the isolation of virus from Norwegian swine (paper 1), the D222G substitution associated with more severe disease was detected in one isolate (A/swine/Hedmark/A161/2011) following propagation in embryonated chicken eggs and Madin-Darby Canine Kidney (MDCK) cells. It has been reported that this substitution may occur during *in ovo* culture, presumable due to adaptation to avian receptors [147]. Since more marked reductions of NKp46+ cells were seen when the 222G virus variant was used in the infection experiments (paper 2), we speculated that the D222G substitution affects NK cell responses. In order to further study the importance of the D222G substitution, one field isolate of the 222D variant (Field-D) and one isolate of the 222G (Field-G) variant were originally included in the *in vitro* assays. However, a substitution in the Field-D virus occurred during a second round of propagation, from glutamine (Q) to arginine (R) in position 223 in HA (Q223R). Unfortunately, sequencing of viruses obtained after the second propagation was not performed until after the NK cell assays were completed and the new mutation thus not revealed. The Q223R substitution is also commonly detected in pH1N1 viruses following *in ovo* culture, but is less studied than the D222G substitution. It has been reported to be associated with altered receptor preference to SAα2,3gal receptors [148]. The Cal09 virus which naturally occurs in a 222D/G quasispecies was also included.

Five NK cell cultures were stimulated with all three viruses (Field-D, Field-G and Cal09) separately. In three of five cultures, Field-G caused less NK cell degranulation, than Field-D (Fig. 19A). Influenza virus and influenza viral HA have been shown by Mao et al to inhibit human NK cell function by direct infection and reduction of NK cell cytotoxicity [149]. As already mentioned, influenza virus infected NK cells were not found in the infection experiments (paper 2). One could speculate that more virulent influenza virus strains impair NK cell function and that this contributes to more severe

disease. To support this, diminished numbers of NK cells have been found in fatal cases of influenza in humans [99,127]. Moreover, the Field-G virus caused greater amounts of TNF producing NK cells in three of five cultures (Fig. 19B) and greater amounts of IFN-γ producing NK cells in four of five cultures (Fig. 19C). Remarkably high amounts of TNF have been found in the lungs in fatal cases of influenza [118]. Furthermore, large amounts of IFN-γ produced by NK cells during respiratory syncytial virus infections in mice have been shown to result in acute lung immune injury [150]. These studies suggest that excessive cytokine production might be associated with pulmonary damage and severe disease in influenza virus infections.
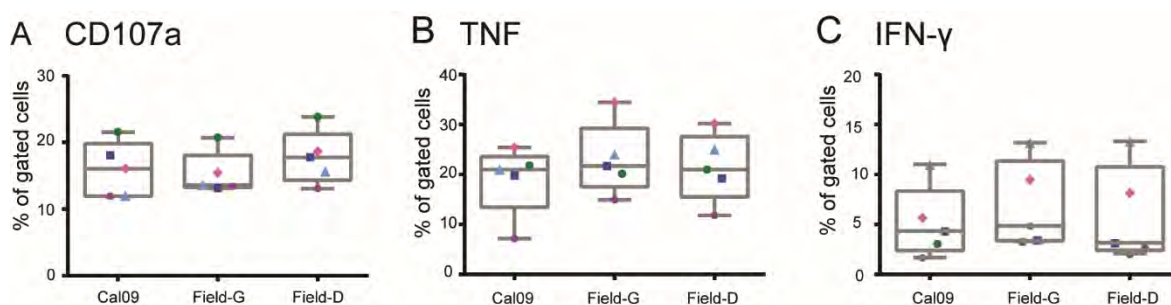

Figure 19. Stimulation of NK cells using different virus isolates.
Cultured NK cells (n=5) were incubated with Cal09 virus, a swine field isolate with the wild-type 222D (Field-D) or field virus with the 222G substitution (Field-G). Cells were subsequently analyzed for their expression of **A)** CD107a and intracellular amounts of **B)** TNF and **C)** IFN-γ. Percentages of positive cells in live cultured cells were compared between different stimulations.

In the present study, there were individual differences between cultures and significantly differences could not be detected. Moreover, since a Q223R substitution was detected in the Field-D virus, it is not possible to determine if the effects were caused by the D222G or the Q223R substitution. The effect of the D222G mutation on NK cell response *in vitro* needs further investigation.

PRIMARY CULTURES OF EPITHELIAL CELLS

In parallel with the isolation of NKp46+ cells, epithelial cells from the upper respiratory tract were isolated and cultured from some of the animals. The primary epithelial cells were infected with influenza virus and the infection was confirmed by immunofluorescence staining (Fig. 20) and real-time RT-PCR on cell supernatant.
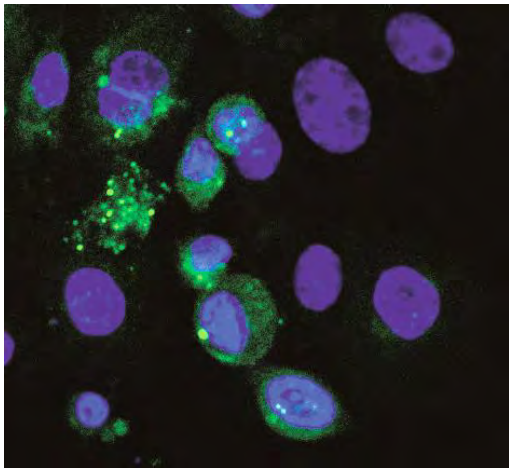
Figure 20. Influenza pH1N1 virus infected primary epithelial cells.

Influenza infected (Cal09) primary cultures of epithelial cells were stained with fluorescent markers against influenza NP (green) and nuclear staining with DAPI (blue).

Cultured NK cells were incubated alone, or together with uninfected or infected epithelial cells isolated from the same individual. NK cells were then analyzed for the expression of CD107a (Fig. 21A), TNF (Fig. 21B) and IFN-γ (Fig. 21C) by flow cytometry. All NK cells incubated with infected epithelial cells showed increased numbers of degranulating cells compared to NK cells incubated alone. However, incubation with uninfected epithelial cells also resulted in activation of NK cells. Interestingly, NK cells from one culture were also incubated with MDCK cells (results not shown). NK cells incubated with both infected and uninfected MDCK cells showed reduced signs of activation, compared to NK cells incubated with swine epithelial cell. This could be linked to NK cell recognition of DAMPS up regulated on epithelial cells in the culture. Activation by DAMPs could be initiated through NKp46, as this receptor is suggested to play a role in recognition of altered cells in diabetes [151]. We have previously seen binding of bovine NKp46 fusion protein to cultured primary kidney cells isolated from cattle 24 hours after isolation (Anne K. Storset, unpublished results). It would have been interesting to further investigate activation of NK cells by DAMPS, but this was beyond the scope of the present study.
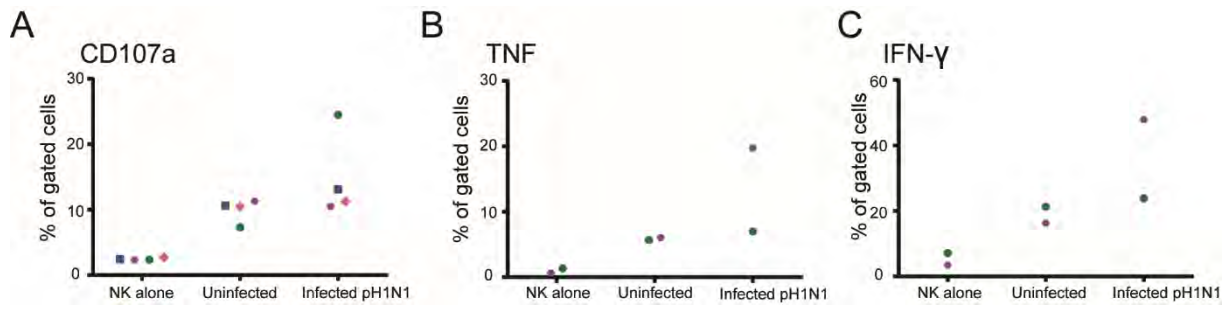
Figure 21. Cultivated NK cells are activated by infected primary epithelial cells

Primary epithelial cells from the upper respiratory tract of swine and NK cells from the same animals were cultivated separately for 10 days. Subsequently, NK cells were incubated alone (NK alone), with uninfected primary cells or primary cells infected with pH1N1 (Cal09). Cells were analyzed for their expression of **A)** CD107a (n=4) and intracellular **B)** TNF (n=2) and **C)** IFN-γ (n=2). Percentages of positive cells in live cultured cells were compared between different stimulations.

MAIN CONCLUSIONS

The detection of a distinct clade of pH1N1 influenza virus in Norwegian swine, together with serological results from the surveillance program strongly suggest that this subtype of influenza virus is circulating in Norwegian swine. Seeding from the human population was also shown to occur.

NKp46+ NK cells in the blood of pH1N1 infected animals were reduced, showing that NK cell responses in swine resemble that of humans. The increased numbers of NKp46+ cells in the lungs strengthens the hypothesis that NK cells migrate from the blood to the lungs to participate in the immune response against pH1N1 virus in swine and most likely also in humans.

The selective recruitment of NKp46+ NK cells and CD3+NKp46+ cells from blood and the increase of these cells in the lungs suggest a common role for cells expressing the NKp46 receptor. Moreover, a porcine NKp46 fusion protein was shown to bind pH1N1 virus infected cells *in vitro* strengthening the hypothesis that this receptor is involved in recognizing virus infected cells in swine.

Like human NK cells, porcine NK cells degranulated in response to pH1N1 virus in vitro, suggesting that NK cells participate in viral clearance by killing of virus infected cells. Increased levels of mRNA encoding TNF were found in pH1N1 infected pigs, followed by findings of elevated levels of TNF producing NK cells in response to pH1N1 virus *in vitro.* Taken together, these results show that TNF is produced in the early course of pH1N1 infections in swine and that NK cells most likely are important contributors to this production. Based on these results, it is also likely that NK cells participate in the early production of TNF seen in human cases of influenza.

The infection experiments suggest that the influenza 222G virus variant resulted in a more pronounced NK cell response than the 222D wild-type virus, but this needs to be further elucidated.

FUTURE PERSPECTIVES

Continued attempts to isolate influenza virus in swine are important to monitor the evolution of SIVs in Norway. Collection of virus positive samples through saliva in ropes could be attempted as an alternative to nasal swabs. In the absence of virological detection, it is of great importance to continue subtyping virus-specific antibodies in order to look for HA/NA reassortment events.

The present studies support the role of NK cells as important immune cells in influenza virus infections and provide evidence that NK cells migrate in to the lungs in response to infection. Further *in vivo* studies should be performed with the purpose of describing porcine NK cells from the respiratory tract and the draining lymph nodes to increase our understanding of NK cell migration during influenza virus infections. These are matters that are difficult to address in humans and based on the findings of the presents study and others, swine should be considered used as an animal model.

The role of the porcine NKp46 receptor in pH1N1 infections needs further investigation. Future studies may compare responses between NK cells expressing NKp46 and those that do not, using purified cultures of NKp46$^-$ and NKp46$^+$ NK cells. NKp46$^-$ NK cells could either be isolated by negative selection for CD3 expression followed by positive selection for CD8α expression by the use of MACS beads or by selection of the different NK cell populations by FACS sorting.

The present studies suggest an impact of the D222G substitution on NK cell responses that may influence the severity of clinical outcome. As there are no reports on this substitution in connection with NK cell response, this should be further investigated.

To summarize, NK cells are likely to be important players in the early defense against pH1N1 virus and may contribute to determine disease severity. Future studies of NK cell responses in influenza virus infections in swine may provide further insight to the role of NK cells and the NKp46 receptor in swine and perhaps also in humans.

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ENCLOSED PAPERS 1-4

Swine influenza in Norway: a distinct lineage of influenza A(H1N1)pdm09 virus

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Background Since the influenza A(H1N1)pdm09 virus was first introduced to the Norwegian pig population in September 2009, it has repeatedly been detected in pigs in Norway. No other subtypes of influenza virus are circulating in Norwegian pigs.

Objective To follow the diversity of A(H1N1)pdm09 viruses circulating in pigs in Norway and to investigate the relationship between viruses circulating in Norwegian pigs and in humans.

Methods Between January 2011 and January 2013, nasal swabs from 507 pigs were tested for A(H1N1)pdm09 virus by real-time RT-PCR. The hemagglutinin (HA) gene of virus-positive samples was sequenced and compared with publically available sequences from viruses circulating in humans at the time.

Results Sequencing and phylogenetic analysis of the HA gene showed that the A(H1N1)pdm09 virus circulating in Norwegian pigs early in 2011 resembled the A(H1N1)pdm09 virus circulating in humans during this time. Viruses detected in pigs by the end of 2011 had acquired four characteristic amino acid substitutions (N31D, S84I S164F, and N473D) and formed a distinct phylogenetic group.

Conclusions A(H1N1)pdm09 virus detected in Norwegian pigs by the end of 2011 formed a distinct genetic lineage. Also, our findings indicate that reverse-zoonotic transmission from humans to pigs of the A(H1N1)pdm09 virus is still important.

Keywords Influenza A(H1N1)pdm09 virus, Swine, Public health, Norway.

Introduction

Influenza A viruses of three subtypes (H1N1, H1N2, and H3N2) occur endemic within most pig populations throughout the world. However, these swine influenza subtypes differ in origin and genetic characteristics in different continents and regions. In Norway, a swine influenza surveillance program based on serological screening of blood samples has been in place since 1997. Results from this program have shown that influenza A virus was not present in the Norwegian pig population prior to 2009. However, following the worldwide spread of the pandemic influenza A(H1N1) strain (A(H1N1)pdm09) in humans, influenza A virus was detected in Norwegian pigs for the first time in the autumn of 2009.[1] Serological testing of slaughter pigs showed that 2 years after the virus was first introduced, about 60% of the tested pigs had antibodies against the influenza A(H1N1)pdm09 virus,[2] and by the end of 2012, 49% of the pigs included in the program tested positive.[3] Together, this indicates that the pandemic subtype has established itself within the Norwegian pig population.

The introduction of the A(H1N1)pdm09 virus to commercial swine herds was first described from Canada in April 2009 and was most likely caused by human-to-pig transmission.[4] The virus was subsequently isolated from pigs throughout the world.[1,5–9] It is efficiently transmitted between pigs, as confirmed by experimental studies,[10,11] and is now co-circulating with endemic swine influenza viruses in most countries. Different reassortant viruses containing genes from A(H1N1)pdm09, and other swine influenza viruses are regularly isolated, and the new viruses are typically also efficiently transmitted between pigs.[4,6–8] Some of these reassortant viruses have also caused infections in humans.[12]

In order to follow the diversity of the A(H1N1)pdm09 virus in the Norwegian pig population, where no other influenza subtype is circulating, a limited target surveillance at a boar testing station was carried out. In addition, diagnostic samples submitted to the Norwegian Veterinary Institute were included in the study. The hemagglutinin (HA) gene of virus-positive samples was sequenced, and their relationship to contemporary viruses circulating in humans examined.

Material and methods

Study material

Nasal swab (Copan Innovation LTD, Brescia, Italy) samples from a total of 316 pigs were collected from pigs at a boar testing station at eight different time points between April and November 2011. The test station receives piglets aged 10–12 weeks from closed nucleus herds located in different parts of Norway every week. The test station recruits boars from a total of 46 closed nucleus herds. Sampling was performed based on suspicion of influenza virus infection at the station, but the symptoms were generally mild, and the majority of sampled pigs did not display any symptoms of respiratory illness. Furthermore, lung tissue was collected from seven pigs with clinical symptoms at the station in November 2011.

In addition, 191 nasal swabs from 17 herds with suspected influenza virus infection submitted to the Norwegian Veterinary Institute for diagnostic purposes between January 2011 and January 2013 were available for the study. The nasal swab samples were placed in 1 ml of transport media (EMEM 2% IBFS/Tris) before they were shipped to the laboratory.

Virological investigation

RNA was extracted from lung tissue samples and nasal swabs. Approximately 20 mg of lung tissue in 650 μl of NucliSens lysis buffer (bioMériux, Norge AS, Oslo, Norway) was homogenized using Qiagen tissue lyser (Qiagen, Valencia, CA) at 25 Hz for 10 min. 200 μl of lung tissue homogenate or transport media from nasal swabs was used for RNA extraction using the automatic extraction instrument NucliSens easyMag (bioMériux, Norge AS, Oslo, Norway) according to the manufacturer's instructions. Detection of influenza A virus by real-time RT-PCR was performed as described by the World Health Organization Collaborating Centre for the Surveillance, Epidemiology, and Control of Influenza at CDC, Atlanta, USA.[13] Specific detection of the HA gene of the A(H1N1)pdm09 subtype was carried out on influenza-A-positive samples, also by real-time RT-PCR.[14] Amplification was performed on a Stratagene Mx3500P (LaJolla, CA, USA) using the SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen, Paisley, UK).

Sequencing and phylogenetic analysis

Altogether, 10 positive samples, including nasal swabs and lung tissue samples, from five different time points (April, July, October, November, and December) in 2011 and one positive nasal swab sample from 2013 (January) were selected for HA gene sequencing. From 2012, no samples were available for sequencing. The HA gene was reverse transcribed and amplified using the OneStep RT-PCR Kit (Qiagen).[15] RT-PCR products were excised from agarose gel and purified using the Qiaquick Gel extraction Kit (Qiagen). DNA was sequenced using the Prism BigDye Terminator v3.1 Cycle Sequencing Kit on 3130 Genetic Analyzer (Applied Biosystem, Warrington, USA) according to manufacturer's instructions. Sequence assembly and multiple sequence alignment were performed using Sequencher version 4.5 (Gene codes Corporation, Ann Arbor, MI; http://www.genecodes.com), and MEGA version 5.0. Sequences obtained in this study have been submitted to the EpiFlu database provided by the Global Initiative on Sharing All Influenza Data (GISAID) (accession numbers are provided in Table 1 and Figure 1). For comparison, the HA sequences from one pig virus from Norway 2009, human viruses occurring in Norway and obtained by the Norwegian Institute of Public Health, as well as a selection of representative international reference strains and the closest matching sequences available in the publicly accessible EpiFlu database, were included in the analysis. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.0.

Results

Detection of virus

At the boar testing station, 160 of the 316 nasal swabs and all seven lung tissue samples collected in 2011 were positive for influenza A virus. Of the 191 diagnostic samples submitted to Norwegian Veterinary Institute between January 2011 and January 2013, 59 samples from seven herds tested positive for influenza A virus. In 2012, no samples were found to be positive. All positive samples were confirmed to be A(H1N1)pdm09 virus positive by the HA subtype-specific real-time RT-PCR.

Characterization of A(H1N1)pdm09 in Norwegian pigs

Results from sequencing analysis of the HA gene of A(H1N1)pdm09 viruses detected in this study, together with one Norwegian pig isolate from 2009, and selected human isolates are summarized in Table 1. Up until now, the A(H1N1)pdm09 viruses in humans have evolved and diversified to some extent, but are not yet shown to have changed substantially in antigenic or other important characteristics. The HA sequences of the swine viruses detected between April and July 2011 were similar to the virus sequence found in 2009[1] and showed only minor nucleotide substitutions which did not result in any amino acid changes of known importance (Table 1). The changes that were observed corresponded to changes seen in human viruses and phylogenetic analysis showed that the swine viruses closely clustered with contemporary A(H1N1)pdm09 viruses circulating in humans in Norway (Figure 1). However, in October, November, and December 2011, viruses detected in pigs on two well-separated production sites in Norway

Table 1. Substitutions in the HA gene of A(H1N1)pdm09 viruses detected in humans and pigs in Norway between October 2009 and January 2013

|	HA0 Amino acid position																
	31	56	83	84	97	141	143	164	185	197	203	205	216	321	374	451	473
A/California/7/2009* (EPI176620)	N	N	P	S	D	A	S	S	S	T	S	R	I	I	E	S	N
A/Norway/3721/09 Nord-Trondelag October 2009 (EPI381831)	-	-	S	-	-	-	-	-	-	A	T	-	-	V	-	-	-
A/swine/Norway/02-11342/2009[1] October 2009 (EPI352385)	-	-	S	-	-	-	-	-	-	A	T	-	-	V	-	-	-
A/Norway/324/2011 Akershus January 2011 (EPI304461)	-	-	S	-	-	-	G	-	T	A	T	-	-	V	K	?	?
A/swine/Hedmark/P249_1/2011[2] April 2011 (EPI 378462)	-	-	S	-	-	-	G	-	T	A	T	-	-	A	K	N	-
A/Norway/1404/2011 Vest-Agder April 2011 (EPI381832)	-	-	S	-	N	-	-	-	-	A	T	K	V	V	K	?	?
A/swine/Hedmark/A64/2011 April 2011 (EPI378461)	-	-	S	-	N	-	-	-	-	A	T	K	V	V	K	-	-
A/swine/Hedmark/P249_3/2011[1] April 2011 (EPI378464)	-	-	S	-	N	-	-	-	-	A	T	K	V	V	K	?	?
A/swine/Hedmark/A66_1/2011[1] April 2011 (EPI378466)	-	-	S	-	N	-	-	-	-	A	T	K	V	V	K	-	-
A/swine/Hedmark/A66_2/2011[1] April 2011 (EPI378467)	-	-	S	-	N	T	-	-	-	A	T	K	V	V	K	-	-
A/Norway/231/2011 Oslo January 2011 (EPI304457)	-	-	S	-	N	-	-	-	T	A	T	-	-	V	K	N	-
A/swine/Hedmark/A114/2011[1] July 2011 (EPI378468)	-	S	S	-	N	-	-	-	T	A	T	-	-	V	K	N	-
A/swine/Oppland/A2/2013[1] January 2013 (EPI440999)	-	-	S	-	N	-	-	S	T	A	T	-	-	V	K	N	-
A/Norway/331/2011 Akershus January 2011 (EPI304465)	-	-	S	I	N	-	-	-	T	A	T	-	-	V	?	?	?
A/swine/Nord_Trøndelag/A148/2011[1] October 2011 (EPI378469)	D	-	S	I	N	-	-	F	T	A	T	-	-	V	K	N	D
A/swine/Hedmark/P907/2011[2] November 2011 (EPI378470)	D	-	S	I	N	-	-	F	T	A	T	-	-	V	K	N	D
A/swine/Hedmark/A161/2011[1] November 2011 (EPI378471)	D	-	S	I	N	-	-	F	T	A	T	-	-	V	K	N	D
A/swine/Hedmark/A173/2011[1] December 2011	D	-	S	I	N	-	-	F	T	A	T	-	-	V	K	N	D

*Reference strain, [1] nasal swab, [2] lung tissue, (-) amino acids identical to California/7/2009, (?) virus sequence not determined, (green) amino acid changes characteristic for the distinct lineage seen in Norwegian pigs in late 2011.

were forming a distinct phylogenetic group (Figure 1). No sequences from human-derived viruses fell into this clade. The most closely related available human virus was a Norwegian isolate from January 2011. The distinct group is characterized by amino acid substitutions N31D, S84I, S164F, and N473D in the viral HA0 protein, with the substitutions at positions 31, 164, and 473 (position 146 in the HA2 subunit) appearing to be unique to this group (Table 1). Amino acid position 164 is within a known antigenic site,[16] but antigenic characterization of one of the isolates showed that the virus was not distinguishable from viruses lacking the S164F substitution (data not shown). The

HA gene of the virus detected in pigs in January 2013 was again similar to the viruses detected in humans during the 2012/2013 season.

Discussion

In Norway, pigs are free of endemic swine influenza viruses of other subtypes than A(H1N1)pdm09. Therefore, the Norwegian pigs may serve as a unique population for studying the developing characteristics of an influenza strain where the chance of coinfections with other swine influenza viruses is negligible. Here, we report on the emergence of a
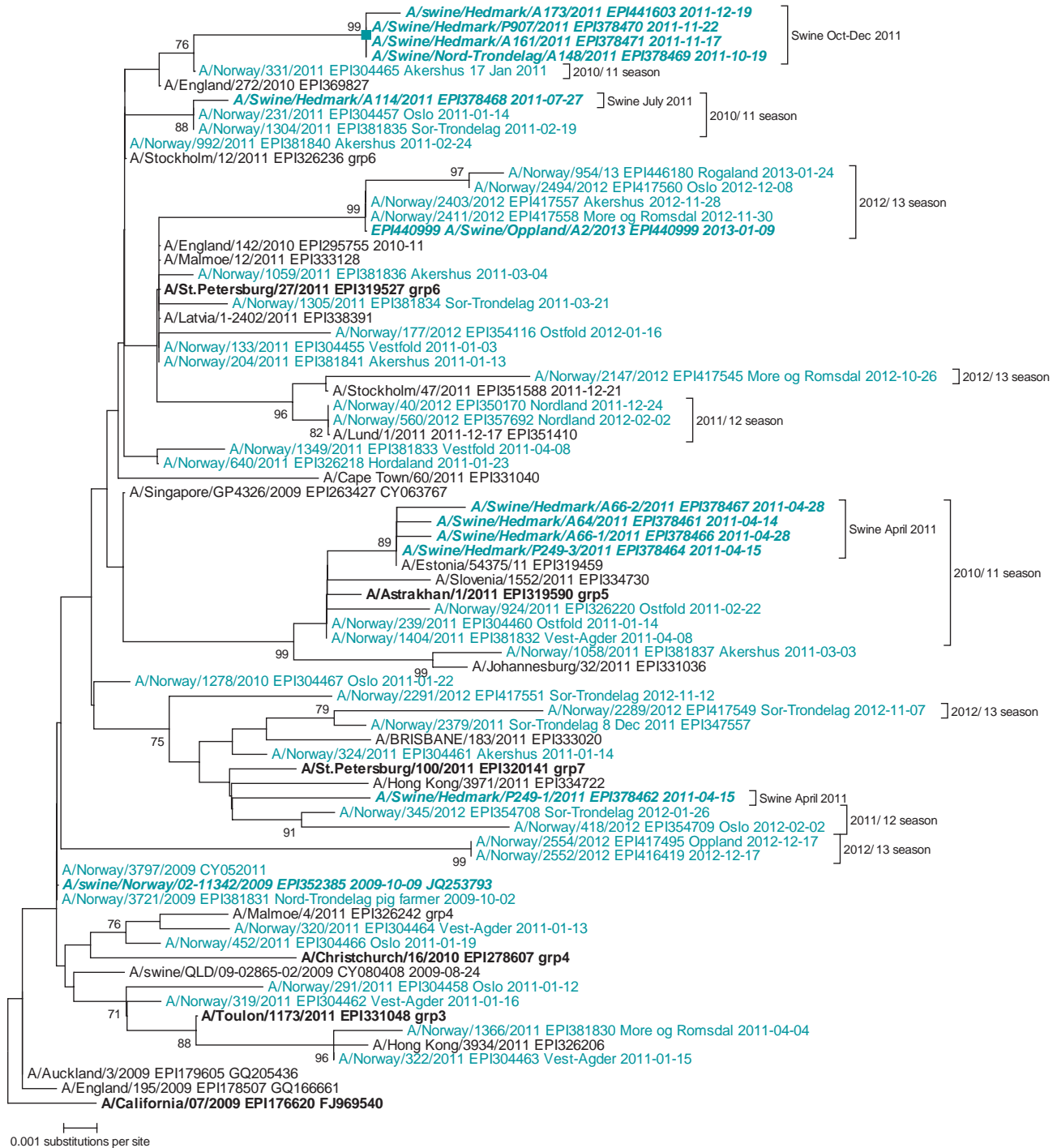
Figure 1. Phylogenetic reconstruction of the hemagglutinin gene (HA1 subunit) of influenza A(H1N1)pdm09 viruses from pigs in Norway, together with viruses from humans in Norway and elsewhere during the corresponding period. An alignment of the 981 nucleotides encoding the HA1 subunit was used. The evolutionary history was inferred using the neighbor-joining method, from pairwise evolutionary distances computed using the Kimura 2-parameter method. Bootstrap scores of 70 or higher (1000 replicates) are shown next to the branches. Norwegian isolates are in color. Viruses from pigs are in bold italics. International reference strains are in bold. The clade emerging in Norwegian pigs in late 2011 is indicated with a square.

distinct clade of influenza A(H1N1)pdm09 viruses in Norwegian swine. Sequence analysis shows that the emergence of the new clade most likely occurred during 2011 and that

human viruses circulating in Norway in early 2011 are the most closely related viruses from which sequence is available. The HA gene of the new clade contains few amino acid

substitutions as compared to other A(H1N1)pdm09 viruses. One of the substitutions, S164F, is located in the HA globular head subunit (HA1) and has not been reported elsewhere. The N473D substitution, located in the HA2 stalk subunit (position 146), has subsequently been found in another A (H1N1)pdm09 genetic lineage. This linage was first seen in some African countries but has also been observed in European countries including Norway during the influenza season 2012/2013[17] and is represented by the viruses A/ Norway/2252/2012 and A/Norway/2254/2012 in Figure 1. During the 2011/12 influenza season in Norway, A(H1N1) pdm09 viruses occurred only sporadically in humans, presumably because the immunity in the human population following the widespread infection and vaccination in 2009 remained high. Viruses resembling the pig viruses from the new group have not been detected in the human influenza surveillance. This may indicate that the few amino acid changes in the HA of this swine lineage have not caused significant antigenic drift and immune evasion enabling the virus to spread more easily in humans.

Viruses representing the new phylogenetic clade originated from swine at three well-separated pig farms, where there were no known contact between at least two of the farms. However, no thorough epidemiological work was performed to determine the source of the viruses in each case, and transmission of virus between the farms cannot be completely ruled out. To determine how commonly viruses that fall into the new group occur, extensive sampling from influenza infected pigs is needed. Even though the serological surveillance shows that infection with influenza viruses of the pandemic subtype frequently occurs in Norwegian pigs, most infections seem to be subclinical or only induce mild symptoms. This, together with the fact that influenza virus only is shed during the initial phase of infection[1,10,11] makes sampling of influenza virus-positive material from Norwegian pigs difficult.

The virus sampled from pigs in January 2013 did not fall into the new group of viruses. It was more closely related to viruses isolated from humans during the 2012/2013 influenza season and is likely to have originated from a new introduction of the virus from humans. Consistent with this, influenza caused by the A(H1N1)pdm09 virus was confirmed in persons who had been in contact with the pigs. The distinct lineage observed in pigs in late 2011 was not detected during the winter of 2012/2013, but as only a few positive samples were available for sequencing, it cannot be ruled out that it is still circulating. New introductions of the A(H1N1)pdm09 virus from humans to swine occur at a higher frequency than what is seen with seasonal influenza viruses.[12] The phylogenetic analysis performed in this study indicates that also the situation in the Norwegian pig population is driven by constant spillover from humans.[1,2] As influenza A(H1N1)pdm09 in general seems to cause only modest or no symptoms at all in Norwegian pigs, it is notable that the herd sampled in January 2013 that most likely got infected by humans, presented with typical clinical symptoms of influenza virus infection. This suggests that new introductions of the virus from humans to pigs could cause more severe infection in pigs, but this needs to be studied in more details.

Pigs can serve as a major reservoir for influenza viruses. Once a herd is infected, the virus is likely to persist through continuous introduction of young susceptible pigs. The selection pressure toward antigenic drift (i.e., amino acid substitutions affecting antigenicity) is lower as compared to humans due to short life span of the pigs.[18] Although molecular data indicate that A(H1N1)pdm09 viruses from humans have continued to seed the pig population, the wide distribution of this virus in Norwegian pigs suggests that A (H1N1)pdm09 also has been transmitting extensively among pigs, suggestive of endemicity. The demonstration of endemic, all-year circulation of a distinct A(H1N1)pdm09 lineage in the farmed pig population in Norway in 2011 has some animal and public health implications. Firstly, a novel natural virus reservoir has arisen, from which the human population can be seeded with virus at any time of year. Hitherto, the direction of cross-species transmission appears to have been primarily humans to animals. However, sporadic cases of humans infected with other swine influenza viruses have been documented in other parts of the world,[19] and as long as the virus remains fully human transmissible, pig-to-human transmission is likely to occur. This may lead to more frequent out-of-season influenza cases in humans. Secondly, reproduction and transmission of the virus in another mammalian species may impose distinct evolutionary selection pressures on the virus, with unpredictable effects on the biological properties of the virus. Further analysis of the virus with full-genome sequencing will reveal whether additional mutations in other gene segments have been acquired. More detailed studies may also reveal whether the mutations described here has any impact on viral fitness.

This study shows the high importance of continuing the surveillance of A(H1N1)pdm09 in pigs, to monitor the circulation and further evolution of this and other genetic groups, as well as to further elucidate the possible impact of this novel lineage for public and animal health.

Acknowledgements

the contribution of submitting and original laboratories for the reference virus sequences accessed in the GISAID sequence database.

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Early Responses of Natural Killer Cells in Pigs Experimentally Infected with 2009 Pandemic H1N1 Influenza A Virus

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Abstract

Natural killer (NK) cells are important players in the innate immune response against influenza A virus and the activating receptor NKp46, which binds hemagglutinin on the surface of infected cells, has been assigned a role in this context. As pigs are natural hosts for influenza A viruses and pigs possess both NKp46$^-$ and NKp46$^+$ NK cells, they represent a good animal model for studying the role of the NKp46 receptor during influenza. We explored the role of NK cells in piglets experimentally infected with 2009 pandemic H1N1 influenza virus by flow cytometric analyses of cells isolated from blood and lung tissue and by immunostaining of lung tissue sections. The number of NKp46$^+$ NK cells was reduced while NKp46$^-$ NK cells remained unaltered in the blood 1–3 days after infection. In the lungs, the intensity of NKp46 expression on NK cells was increased during the first 3 days, and areas where influenza virus nucleoprotein was detected were associated with increased numbers of NKp46$^+$ NK cells when compared to uninfected areas. NKp46$^+$ NK cells in the lung were neither found to be infected with influenza virus nor to be undergoing apoptosis. The binding of porcine NKp46 to influenza virus infected cells was verified in an *in vitro* assay. These data support the involvement of porcine NKp46$^+$ NK cells in the local immune response against influenza virus.

Introduction

Natural killer (NK) cells are innate lymphocytes that provide early protection against a number of viral infections and are thought to participate in the early defence against influenza virus [1,2]. Human NK cells recognize influenza virus infected cells through the activating NK cell receptor NKp46/NCR1 *in vitro* [3,4], but their importance *in vivo* is still poorly documented. Clinical cases of human influenza have shown that the population of NK cells in the blood is reduced. It has been proposed that NK cells migrate from the blood into the lungs [5–7] where they may become infected and killed by the influenza virus [8,9]. Supporting this theory are findings of increased numbers of NK cells in the lungs of influenza infected mice [10–12]. Little is known about the function of NK cells during an influenza virus infection. However, NK cells kill influenza virus infected cells from mice and humans *in vitro* [3,4,13–15]. They are also important early producers of antiviral cytokines such as interferon gamma (IFN-γ) and tumour necrosis factor (TNF), which have been associated with the acute stages of influenza virus infection in both humans and swine. [16–20].

NK cells in swine blood are identified as CD3$^-$CD8α$^+$ cells [21], and a recent study has found that only about half of the NK

cell population in the blood of swine express NKp46 [22]. NKp46$^-$ NK cells show a similar ability to kill target cells but produce less IFN-γ as compared to NKp46$^+$ NK cells. In liver and spleen, a third NK cell population with a higher NKp46 expression and a dim or negative CD8α expression has been described [22]. These CD8$^{-/dim}$NKp46^{high} NK cells appear to represent a more activated state [23].

To better understand infectious disease and in particular zoonotic diseases such as influenza, it is important to undertake studies in natural hosts [24]. In addition to being a natural host to influenza, swine are anatomically and physiologically similar to humans and respond to an influenza virus infection in much the same manner as humans [17–20,25–27]. Swine are therefore proposed to serve as a valuable model for studying the pathogenesis of respiratory diseases, including influenza, as well as the immune response against it [1,28–30]. They also present a unique model for studying the role of NK cells expressing the NKp46 receptor *in vivo*, since they have both NKp46$^-$ and NKp46$^+$ NK cells [22].

In spring 2009 a novel influenza A virus, A (H1N1) pdm09, of putative swine origin with gene segments derived from human, swine and avian influenza viruses, spread in the human population causing a pandemic. Although this virus acquired the ability to

transmit efficiently among humans, it also retained its ability to infect and be maintained in pigs [25,31,32]. The 2009 pandemic influenza A virus is now circulating in both humans and swine in most countries worldwide [32]. Most often, this virus causes a benign and self-limiting respiratory disease in humans and pigs, but severe cases have been reported in humans [26]. Since it occurs naturally in both species, the 2009 pandemic influenza A virus is well suited for comparative studies of influenza in humans and swine.

There are few reports on the role of NK cells in swine during influenza virus infection, and the results are conflicting [29,33]. Furthermore, no studies have distinguished between NKp46^{−} and NKp46^{+} NK cells. Here, we provide evidence that NKp46^{+} NK cells are recruited from the blood to infected parts of the lungs in swine inoculated with the 2009 pandemic influenza virus. Moreover, the NKp46^{+} cells in the lungs are not infected by influenza virus and do not undergo apoptosis. This is the first report on the role of NKp46^{+} NK cells in pigs infected with influenza A virus and further demonstrates the value of the pig as a model for clinical disease in humans.

Material and Methods

Ethical statement

The project was approved by the Animal Health and Veterinary Laboratories Agency Ethics Committee, and all procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under Project License Permit Number 70/7062.

Animals and virus

In two independent challenge experiments, six week-old-pigs were sourced from a high health status UK herd of the large white cross breed and tested negative for influenza A virus by Matrix real-time RT-PCR (rRT-PCR) [34] and pre-existing antibodies (subtypes H1N1, H1N2, H3N2 and H1N1pdm09) by hemagglutinin inhibition test [35]. The animals were kept in approved facilities and were closely monitored throughout the experiments. Two virus isolates were used in the infections; A/Hamburg/05/2009 (Ham) and A/Hamburg/05/2009-e (Ham-e) [36].

Experimental infection and sampling

The pigs were inoculated via intranasal aerosols with 10^6 $TCID_{50}$ in 2 ml per nostril using a mucosal atomization device (MAD 300, Wolfe Tory Medical). In the first study, 12 pigs were inoculated with a 1:1 mixture of the influenza A (H1N1) pdm09 225D (Ham) and 225G (Ham-e) variants. 12 pigs were mock-inoculated with similarly diluted clean cell culture medium and included as control animals. Two pigs from each group were sedated and euthanized by intravenous injection of barbiturates prior to post-mortem examination and sampling at days 1–5 and 8 post-infection (pi). In the second study, 12 pigs were inoculated solely with the 225G (Ham-e) variant. An untreated group consisting of 13 pigs was included as controls. Ten control animals were euthanized whilst three remaining pigs were used for blood analysis only. Four control pigs were euthanized prior to the experiment, while four infected and two control pigs were euthanized daily from day 1 to 3 pi.

For both studies, animals were randomly allocated to each of the two groups and the day of post-mortem examination. Heparinized blood was collected from all animals on each sampling day. At post-mortem, systematic recording of macroscopic findings and blinded estimation of the gross lung lesion score was carried out as previously described [37], as well as the

collection of tissues for further analysis. From the lungs, samples were taken from the cranial, middle, caudal and accessory lung lobes from the right side. Macroscopically affected areas (Fig. 1A) were chosen if present.
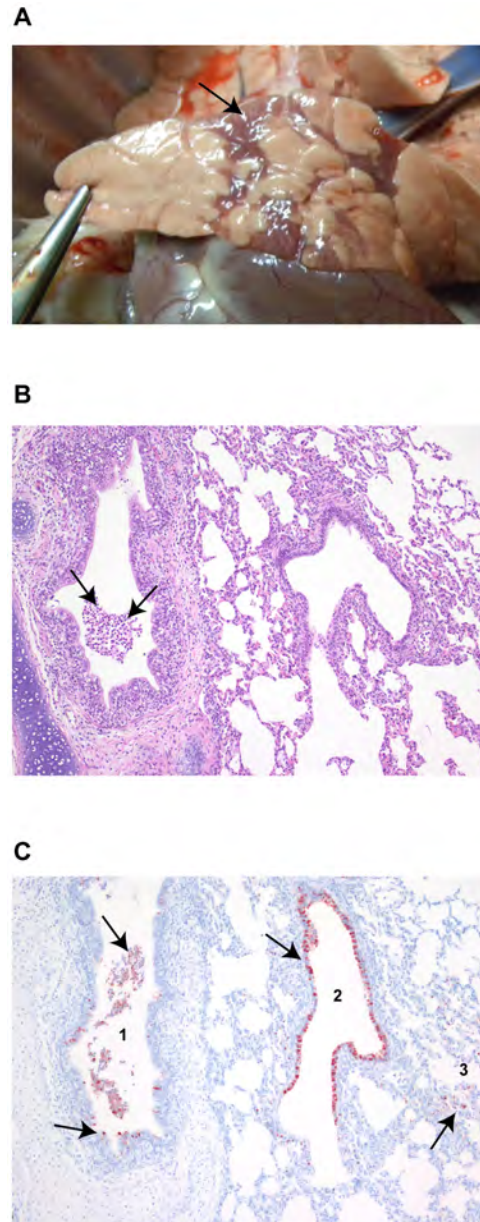
Figure 1. Bronchointerstitial pneumonia in pigs infected with influenza A virus. Pathological changes were recorded in the lungs of twelve pigs inoculated with influenza A virus on days 1–5 and 8 pi ($n = 2$ per day). (**A**) Macroscopic lesions were most prominent on day 3 pi; representative lesions are shown (arrow). (**B**) Histological changes on day 1 pi; representative lesions are shown. Leucocytes and cell debris (arrows) in the lumen of a bronchus. Hematoxylin and eosin, 100x. (**C**) Influenza A virus NP positive staining (arrows) in luminal cells and in the epithelial lining of a bronchus (1), a bronchiole (2) and in the pneumocytes of the parenchyma (3) in the same animal as shown in (C), serial sections (NP stained with AEC and counterstained with hematoxylin, 100x).
doi:10.1371/journal.pone.0100619.g001

Clinical observations and virological monitoring

Pigs were recorded daily for signs of clinical disease. For detection of viral RNA, total RNA was extracted from lung tissue homogenate supernatant and matrix gene rRT-PCR was performed as previously described [34]. Presence of virus was verified by virus re-isolation and titration in MDCK cells [35].

Histopathology and immunohistochemistry

Tissues for histopathological examination and immunohistochemical analysis were collected from the nasal concha, trachea, lungs, lymph node draining the nasal cavity (LN retropharygeales), lung lymph node (LN tracheabronchiale), liver, kidney, spleen and encephalon. Tissue was prepared and stained with hematoxylin and eosin for histopathological evaluation or used for immunohistochemical detection of influenza A virus nucleoprotein (NP) by the avidin-biotin-peroxidase complex method as previously described [25]. Influenza A virus NP was only detected in the respiratory tract and associated lymph nodes.

Isolation of porcine mononuclear cells

For the first study, mononuclear cells from the blood, spleen, liver and lymph nodes draining the spleen (LN lienales), liver (LN hepaticiseuportales), lung (LN tracheobronchiale) and nasal cavities (LN retropharyngeales) were analysed by flow cytometry. For the second study, cells from the four lung lobes were additionally included, while cells from the spleen and LN lineales were omitted.

Pheriperial blood mononuclear cells (PBMC's) were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque Premium with a density of 1,077 g/ml (GE Healthcare). Tissue samples from all organs were homogenized using disposable scalpels. Mononuclear cells from liver and spleen were isolated by forcing homogenized tissue samples through a cell strainer (70 µm, BD Biosciences) before purification by density gradient centrifugation. Homogenized tissue from the lymph nodes was forced through a cell strainer and further sifted through a cotton wool filter to remove any remaining tissue debris. Mononuclear cells from lung tissue were isolated by enzymatic degradation before density gradient centrifugation. Briefly described, homogenized tissue was incubated for 60 min at 37°C in Gibco RPMI1640 Medium with Hepes (Invitrogen) with 2% FBS, gentamicin, 300 U/ml collagenase type 1 (Worthington) and 500 U/ml DNase (Sigma Aldrich). The processed tissue was then sifted through a cotton wool filter before mononuclear cells were isolated by density gradient centrifugation. Isolated mononuclear cells from the lungs not used in the flow cytometric analysis from infected animals were frozen in Gibco Recovery Cell Culture Freezing Medium (Invitrogen) at a concentration of 5×10^6 cells per ml media and stored at $-80°C$ for later use.

Incubation of isolated mononuclear cells for IFN-γ staining

In study 2, cells isolated from lung and lung lymph nodes were analysed for intracellular IFN-γ expression by flow cytometry. Cells isolated from liver were included as controls. Cells (5×10^6 per well in a 24 well plate) from each tissue were incubated in 500 µl Gibco RPMI 1640 Medium Hepes (Invitrogen) with 10% FBS, gentamicin and 1 µl/ml BD Golgi Plug (BD Biosciences) for 4 hours at 37°C. All samples were set up in parallel; one of the two wells was incubated with 100 IU/ml recombinant porcine IL-2 (BioSupply) while the other well was incubated without cytokine. No difference was found between cells incubated with or without

IL-2, and cells incubated with IL-2 were chosen for subsequent analysis.

Flow cytometry and antibodies

In the second study, complete blood counts were obtained by flow cytometry. Whole blood was incubated for 10 min with CD45-FITC mAb (IgG1, clone K252-1E4, AbD Serotec), before 10 min incubation with FACS Lysing solution (BD Biosciences) at room temperature to lyse erythrocytes and fix leukocytes. Samples were diluted in PBS and cell counts were obtained on a volumetric flow cytometer (MACSQuant, Miltenyl Biotec). Lymphocytes were gated as $CD45^{high}$ cells with a low side scatter and granulocytes as $CD45^{dim}$ cells with a high side scatter.

For further analysis, isolated mononuclear cells were used in a concentration of 2.5×10^6 cells/ml in 200 µl. Cells were incubated with LIVE/DEAD Fixable Near-IR Dead Cell Kit (Invitrogen) for 10 min at room temperature, and washed once in Dulbecco's PBS without Mg^{2+} and Ca^{2+} (Invitrogen) supplemented with 10% porcine plasma and.09% sodium azide (FCM buffer) prior to staining. The FCM buffer was also used for all washing steps during the surface staining procedure.

Cells from blood and all tissues were stained with antibodies against CD3, CD8α and NKp46 in combination with CD25 or Ki-67. Stimulated cells from lung, lung lymph nodes and liver were additionally stained with antibodies against CD3, CD8α and NKp46 in combination with IFN-γ. For surface staining, primary antibodies and secondary reagents were used at previously determined optimal concentrations and incubated for 20 min on ice in the dark: CD3-eFluor450 (IgG1, clone PPT3), CD8α (IgG2a, clone 11/295/33), CD8α-AlexaFluor 647 (IgG2a, clone 11/295/33), NKp46-biotin (IgG1, clone VIV-KM1), CD25-AlexaFluor 647 (IgG1, clone 3B2), goat anti-mouse IgG2a-AlexaFluor 488 (Invitrogen), Streptavidin-AlexaFluor 647 (Invitrogen) and Streptavidin-PE (eBioscience). Wells were washed three times following incubation with primary antibodies and twice following incubation with secondary reagents. For intracellular staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), followed by intracellular staining according to the manufacturer's instruction using CD3-Pacific Blue mAb (IgG1, clone CD3-12, AbDSerotec) and Ki-67-FITC mAb (IgG1, clone B56, BD Biosciences) or IFN-γ-PE mAb (IgG1, clone P2G10, BD Biosciences). Appropriate secondary and isotype controls were included. Where not specified, antibodies were produced in-house from hybridoma cultures, purified and biotinylated or conjugated to fluorochromes as described elsewhere [38]. CD3-eFluor450 was obtained by a custom conjugation (eBioscience). Acquisition was performed on a MACSQuant Analyser flow cytometer (Miltenyi Biotec) and data was analysed using MACSQuantify software. A total of 100 000 cells were analysed when possible. Dead cells were excluded and lymphocytes were gated individually according to forward/side scatter characteristics. $CD3^-$ cells were gated according to the expression of CD8α and NKp46, and defined as $NKp46^-$ or $NKp46^+$ NK cells in blood and as $NKp46^-$, $NKp46^{int}$ or $NKp46^{high}$ NK cells in tissues.

Immunofluorescence staining of lung tissue sections

Pulmonary samples were blocked to maximum thickness of 0.5 cm, embedded in cryomoulds containing Tissue-TEK O.C.T compound (Sakura Finetek Europe) and snap frozen by immersion in cooled isopentane over dry ice followed by storage at $-80°C$. The samples were cut on a cryostat, and 7 µm sections were placed on Polysine glass slides (Menzel GmbH & Co Kg, Braunschweig) and stored at $-70°C$ until staining. A triple

indirect fluorescence technique [39] with minor modifications was performed to simultaneously localize influenza A virus infected cells and NKp46+ cells in combination with either cytokeratin+ or caspase-3+ cells. Briefly, frozen sections were air-dried, fixed in acetone for 10 min before incubation with 20% BSA for 10 min to reduce non-specific binding. Antibodies against influenza A virus NP (IgG2a, clone F8, Abnova), NKp46 (IgG1, clone VIV-KM1, produced in-house), cytokeratin 8 (rabbit polyclonal, Antibodies-online) or caspase-3 (rabbit polyclonal, ACTIVE Caspase-3, Promega) were diluted in 2.5% BSA and simultaneously added to the sections. Incubations were carried out for 1 hour at room temperature. Secondary reagents used were goat anti-mouse IgG2a-AlexaFluor 546, goat anti-mouse IgG1-AlexaFluor 488 and goat anti-rabbit AlexaFluor 405. The sections were coverslipped with Fluka Polyvinyl alcohol mounting medium with DABCO antifading (Sigma-Aldrich). The controls included the omission of primary antibodies and known influenza A virus negative sections.

Distribution and quantification of NKp46+ cells in lung tissue sections

The immunofluorescence stained lung tissue sections were examined with a Nikon Eclipse 80i microscope equipped for fluorescence with a Nikon Intenslight C-HGFI and filters for red (ET Cy3), green (FITC EX 465-495) and blue (ET DAPI) fluorescence. Fluorescence micrographs were taken with a Nikon DS-Ri1 camera using the software NIS-Elements D 3.0. Minor adjustments of brightness and contrast were applied to the entire image for publishing, using Adobe Photoshop Elements V9 (Adobe Systems Incorporated).

The quantification of NKp46+ cells followed a determined protocol (Fig. S1) using NIS-Elements D3.0 Annotations and Measurements. Six areas including a small bronchi or bronchioles and the surrounding loose connective tissue and omitting the bronchial or bronchiolar lumina from each of the four lung lobes were defined manually and automatically measured by the software. The NKp46+ cells were then defined as showing a clear fluorescence and having a form indicating a cell, and cells that were within the demarcated area or on the line of demarcation were counted. The numbers of NKp46+ cells per 0.1 mm^2 were calculated. Areas both with and without virus were included in the examination. In total, 95 areas from control animals were analysed and compared to similar areas from infected animals. From infected animals, a total of 60 areas without virus and 36 areas with virus from day 1 pi, 81 areas without virus and 13 areas with virus from day 2 pi and 28 areas without virus and 68 areas with virus from day 3 pi were investigated.

From each area three separate micrographs (magnification 200x) were taken with the respective filters to identify influenza A virus NP (red), NKp46 (green) and cytokeratin (blue). The settings of the Nikon intenslight lamp and NIS-Elements D3.0 software were kept constant for the micrographs of NKp46, but minor modifications were made to optimize the micrographs of influenza A virus NP, due to strong variation of quantity of antigen, and of the cytokeratin micrographs due to varying staining intensity. The three separate micrographs were merged to be able to identify influenza A virus NP in cytokeratin+ epithelial cells and the NKp46+ cells in the same micrograph.

Binding of NKp46 immunoglobulin fusion protein to influenza virus infected cells

MDCK cells were cultivated in MEM (PAA) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM stable glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin at 37°C. For infection,

confluent cells in T25 tissue flasks were inoculated with 180 PFU/ml of the 2009 pandemic H1N1 influenza virus (isolate A/California/07/2009) in medium without serum for 1.5 hours at 34°C. Afterwards, inoculum was removed and cells were cultivated in medium without serum supplemented with 1 µg/ml trypsin for two days at 34°C. Non-infected MDCK cells were treated in an analogous manner.

For the NKp46-binding assay, cells were detached by trypsinization and resuspended in PBS containing 2% FBS. Cells were left on ice overnight for recovery from trypsin-treatment and incubated with different concentrations (40 µg, 20 µg, 10 µg and 5 µg) of a recombinant fusion protein containing the extracellular part of porcine NKp46 and the hinge and Fc part of murine IgG2b [22] for two hours on ice. In addition, cells were stained with anti-H1 mAbs (IgG1, clone C102, AbD Serotec). Anti-H1 mAbs were added for the last 30 min of the NKp46-Ig incubation step to overcome potential competitive effects in the binding to HA antigen. Goat anti-mouse IgG1-PE (Southern Biotech) and Goat anti-mouse IgG2b-AlexaFluor 647 (Invitrogen) antibodies were used as secondary reagents. Mouse IgG1 irrelevant mAbs (clone NCG01, Dianova) were used to control the H1-specific mAb. Cells were analysed by flow cytometry on a FACSCanto II (BD Biosciences). Data for at least 3×10^4 cells were recorded and data was analysed with FACS-Diva (Version 6.1.3, BD Biosciences) and FlowJo software (Version 7.6.3., Tree Star).

Analysis of IFN-γ and TNF mRNA by rRT-PCR in lung tissue

Lung mononuclear cells from all infected animals from the second study were analysed for gene expression of IFN-γ and TNF and compared to that of four control pigs. The control pigs were aged matched with the pigs from the study and purchased from a high health status herd. All pigs were negative for influenza A virus determined by rRT-PCR from nasal swabs [40]. Control pigs were euthanized and mononuclear cells were isolated from the lungs and frozen by the same method as described above.

Total RNA was isolated from frozen mononuclear cells from the lungs using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Amplification and detection of IFN-γ (forward primer: 5′ TTCAGCTTTGCGTGACTTTG-3′. reverse primer: 5′-AAGAAAAGAGGTCCACCATTAGG-3, probe: 5′-TexasRed-GCTCTTACTGCCAGGCGCCCBHQ2-3′), TNF (forward primer: 5′-CCCCTGTCCATCCCTTTATT-3′, reverse primer: 5′-ACACATCCCTGAATCCCTGA-3′, probe: 5′-6FAM-ATGAGGGGCTGGGGACTGGG-BHQ1-3′) and the household gene GADPH (forward primer: 5′-GTTCCACGGCACAGT-CAAG-3′, reverse primer 5′- CATGGTCGTGAAGACACC-CAG-3′, 5′-probe 5′-6FAM-CGGAGAACGGGAAGCTTGTCA-3′) mRNA was performed on a Stratagene Mx3500P using the Qiagen One-Step RT-PCR kit (Qiagen). The RT step condition was 30 min at 50°C followed by 15 min at 95°C. A three-step PCR cycling protocol was used as follows: 45 cycles of 94°C for 10s, 55°C for 20s and 72°C for 10s. Expression of each target gene was compared to that of the household gene and the Ct values of each infected animal were compared to the mean Ct value of the controls as previously described [41].

Statistical analysis

Statistical analyses were performed using JMP V9 (SAS Institute Inc) and Graph Pad Prism V6 (GraphPad Software). Differences among groups were assessed by the Mann-Whitney test. When three groups or more were present, each pair of groups was compared individually. In some figures, statistical difference between groups is displayed by giving these groups different

letters, while groups with the same letter does not differ significantly. Box plots show median values, the 25th and 75th percentiles and the lowest and highest values. Statistical analysis was performed on results from groups with four or more observations only.

Results

Influenza A(H1N1)pdm09 virus infection causes bronchointerstitial pneumonia in pigs

To identify the most relevant time period to study NK cells, pigs were experimentally infected with influenza A(H1N1)pdm09 and pairs of infected and control animals were euthanized and sampled 1−8 days pi. All infected animals showed mild clinical signs of influenza [25] on day 1−3 pi and infection was confirmed by detection of viral RNA by rRT-PCR in lung tissue samples (data not shown). At post-mortem examination, macroscopic (Fig. 1A) and histological changes (Fig. 1B) typically associated with influenza [25] were found restricted to the respiratory tract and associated lymphoid tissue. There was a peak in severity and extension of macroscopic pulmonary changes on day 3 pi (Fig. S2). Histological changes included moderate bronchointerstitial pneumonia and necrotizing bronchiolitis (Fig. 1B), and less frequently, lymphocytic rhinitis and tracheitis. The extension of the histological changes increased from day 1 to 3 pi. From day 4 pi, the number of affected bronchi and bronchioles gradually decreased and the epithelia showed sign of regeneration. Influenza A virus NP was detected by immunohistochemistry (Fig. 1C) in the epithelial cells of both the turbinates and trachea and in the bronchi and bronchioles. Positive pneumocytes could be found in areas close to infected bronchioles. Viral NP was also detected in macrophages in respiratory lymph nodes (data not shown). The highest number of infected cells was observed in the lungs at day 1 pi with a progressive reduction throughout the infection.

Blood was collected daily from all remaining pigs, and the NK cells defined as CD3$^-$CD8α^+NKp46$^+$ and CD3$^-$CD8α $^+$NKp46$^-$ cells in PBMC were analysed by flow cytometry. The percentages of NK cells varied between animals, and there was also some day to day variation in the same animal, especially in the control group. During the first three days pi, there was a decrease in NKp46$^+$ cells in infected animals (median = 4.2%, range = 1.3−12% of lymphocytes) compared to the controls (median = 6.1%, range = 2.3−14% of lymphocytes), but the decrease from one day to the other was not significant. NK cell levels in liver, spleen and all lymph nodes investigated were similar in control and infected animals (data not shown).

A reduction in NKp46$^+$ NK cells in the blood of influenza infected pigs was confirmed in a second experiment

Since the histological findings showed that the lung infection was regressing from day 4 pi, the first three days pi was considered as the most relevant time period to study the early involvement of NK cells. A second experiment was performed, including more animals on each sampling day. Lung lesion score [37] was compared between the two experiments (Fig. S2). The median score of the infected animals in the second experiment was higher compared to the animals in the first study, but the differences were not significant due to low numbers of animals. The histological changes, viral distribution as determined by immunohistochemistry and the presence of virus in lung tissue samples as measured by rRT-PCR were similar between the two experiments (data not shown).

The total numbers of lymphocytes in blood were reduced in the infected animals on the first day after infection (Fig. S3) as assessed by the number of CD45^{high} cells in a defined volume of whole blood. A similar reduction has also been described in cases of human influenza [6,7]. Live CD3$^-$CD8α^+ lymphocytes were gated as NKp46$^-$ or NKp46$^+$ NK cells for further analysis (Fig. 2A), and the proportions of NKp46$^-$ and NKp46$^+$ cells in the blood of individual animals were similar to earlier reports [22]. There was a drop in NKp46$^+$ NK cells on day 1 in all infected animals with a subsequent increase on day 2 pi, both in absolute numbers (Fig. 2B and C) and in percentage of lymphocytes (median = 3.7%, range = 1.7−12% on day 0; median = 1.4%, range = 0.5−6.8% on day 1; median = 3.0%, range = 1.4−5.9% on day 2). No reduction was seen in NKp46$^-$ NK cells in infected pigs, but rather an increase was observed on day 2 and 3 pi (Fig. 2D and E).

To investigate cell activation and proliferation, PBMCs were additionally stained for CD25 and Ki-67 expression, respectively, but no differences in the expression of these markers were found in NK cells (data not shown). No differences in percentages of NK cells among lymphocytes were seen between control and infected animals in liver or lymph nodes (data not shown).

### Influenza A virus infection leads to up regulation of NKp46 on NK cells in the lungs

In the second experiment, mononuclear cells were isolated from lung tissue and analysed by flow cytometry. In the lungs, the CD8$\alpha^{-/dim}$NKp46^{high} population, previously described in swine spleen and liver [22] was found. Thus, the NK cell populations were defined as NKp46$^-$, NKp46^{int} and NKp46^{high} NK cells in lung tissue (Fig. 3A). The distribution of the different NK cell populations in individual animals were as shown in Figure 3B.

The percentages of NKp46$^-$ NK cells were increased in two of the four infected animals sampled on day 1 pi compared to the control animals, while the percentage of NKp46^{high} NK cells exceeded that of the controls in one infected animal on day 1. The greatest differences were found in the NKp46^{int} NK cells, where three animals on day 1 and three animals on day 2 had higher percentages of NKp46^{int} cells than the control animals (Fig. 3C). However, there were no significant differences between the groups. Nevertheless, there was an increase in the NKp46 expression on NKp46^{int} NK cells, evaluated as median fluorescence intensity (MFI), in the infected animals on day 2 compared to control animals (Fig. 3D). Increased NKp46 expression was also evident on the NKp46^{high} NK cells in several of the infected animals, although the differences between groups were not significant (Fig. 3D).

The mononuclear lung cells were also stained for CD25 (Fig. 3E) and Ki67 (data not shown). No significant increase in the expression of either of these markers was found in infected animals compared to the controls in any of the three NK cell populations. However, a higher percentage of the NKp46^{high} cells were found to express CD25 compared to the NKp46$^-$ and NKp46^{int} NK cells in the lungs of both control and infected animals (Fig. 3F).

Influenza virus infected areas of the lungs were associated with more NKp46$^+$ cells

In order to assess the numbers and distribution of NKp46$^+$ cells in lung tissue, sections were stained for influenza A virus NP, NKp46 and cytokeratin 8. The distribution of influenza A virus NP (Fig. 4A) was as shown earlier in Figure 1C. The majority of NKp46$^+$ cells were found in the lamina propria of bronchi and bronchioles, in the surrounding connective tissue and in the interalveolar septa, while few NKp46$^+$ cells were seen in the
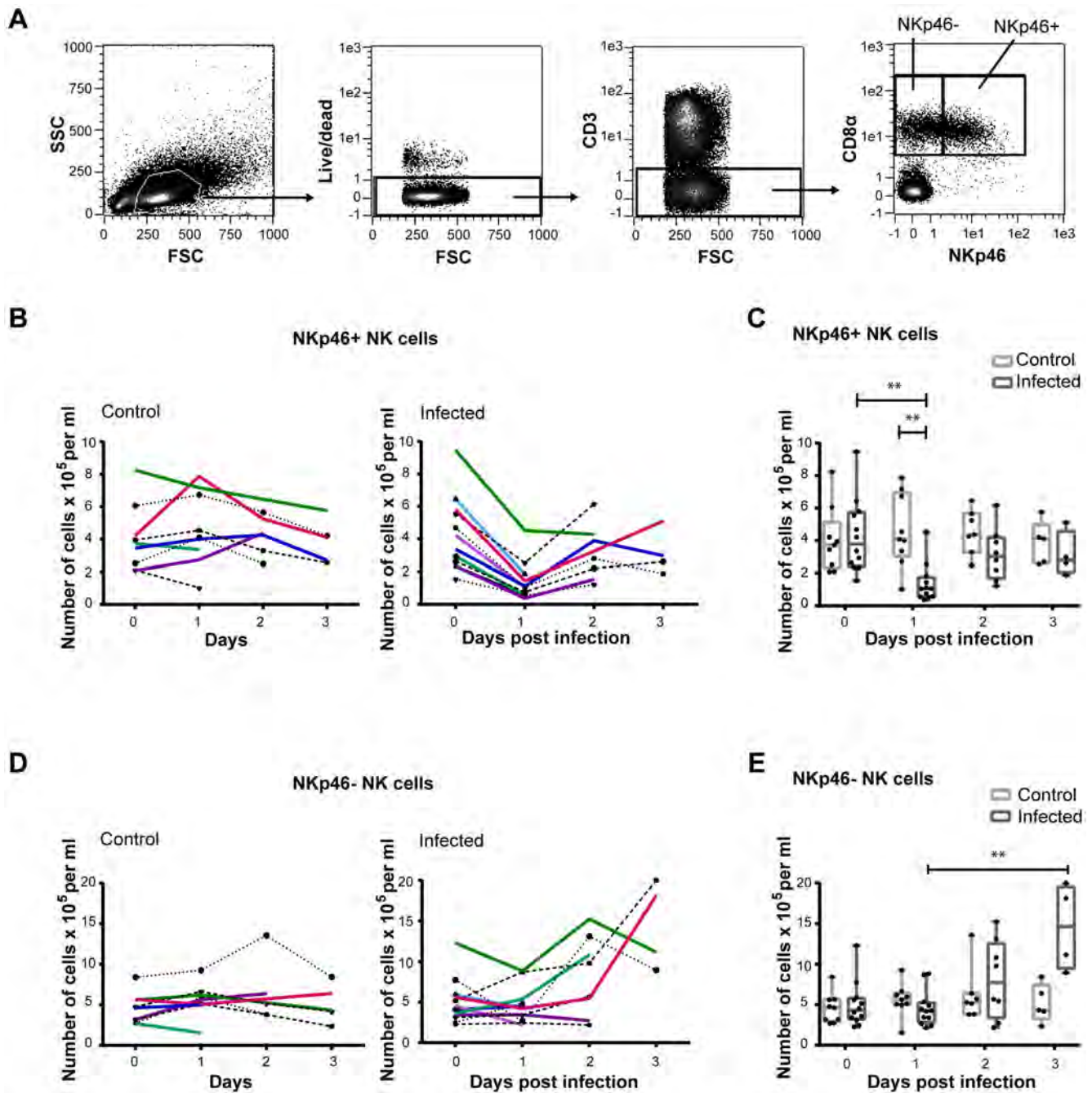
Figure 2. NK cell numbers in the blood of influenza infected pigs. Blood was taken from influenza A virus infected ($n = 12$) and control pigs ($n = 9$) on day $0-3$ pi in a second experiment. (**A**) Isolated PBMCs were analysed by flow cytometry and live CD3$^-$ lymphocytes were gated as NKp46$^-$ or NKp46$^+$ NK cells according to CD8α and NKp46 expression. Plots are taken from a representative control animal. Absolute numbers of (**B**) NKp46$^+$ NK cells in PBMC were analysed by flow cytometry in control (left) and infected animals (right). (**C**) Results for the NKp46$^+$ cells in the two groups were compared on each sampling day. (**D**) NKp46$^-$ NK cells in PBMC in control (left) and infected animals (right). (**E**) NKp46$^-$ NK cells were compared for the two groups. Each line in (B) and (D) represents one animal. **$p \leq 0.01$.
doi:10.1371/journal.pone.0100619.g002

epithelial lining (Fig. 4A and B). The distribution of NKp46$^+$ cells in the tissue was similar in infected and control animals, and these cells were seen as both scattered cells and in clusters.

As the influenza A virus NP$^+$ cells had a multifocal distribution in the lung tissue, areas surrounding small bronchi and bronchioles with and without influenza virus NP$^+$ cells were selected to enumerate NKp46$^+$ cells and compared against similar areas from control animals (Fig. S1). In infected animals, lung areas with

influenza virus NP$^+$ cells contained more NKp46$^+$ cells (Fig. 4A) than areas where influenza virus NP could not be detected (Fig. 4B). There was a peak in the number of NKp46$^+$ cells on day 2 pi in areas with virus, while the number of NKp46$^+$ cells in areas without virus gradually increased from day 1 to 3 pi (Fig. 4C). Similar results were obtained from all lung lobes sampled. In animals that had cellular exudate in the lumen of bronchi and bronchioles, a high number of NKp46$^+$ cells were present in the
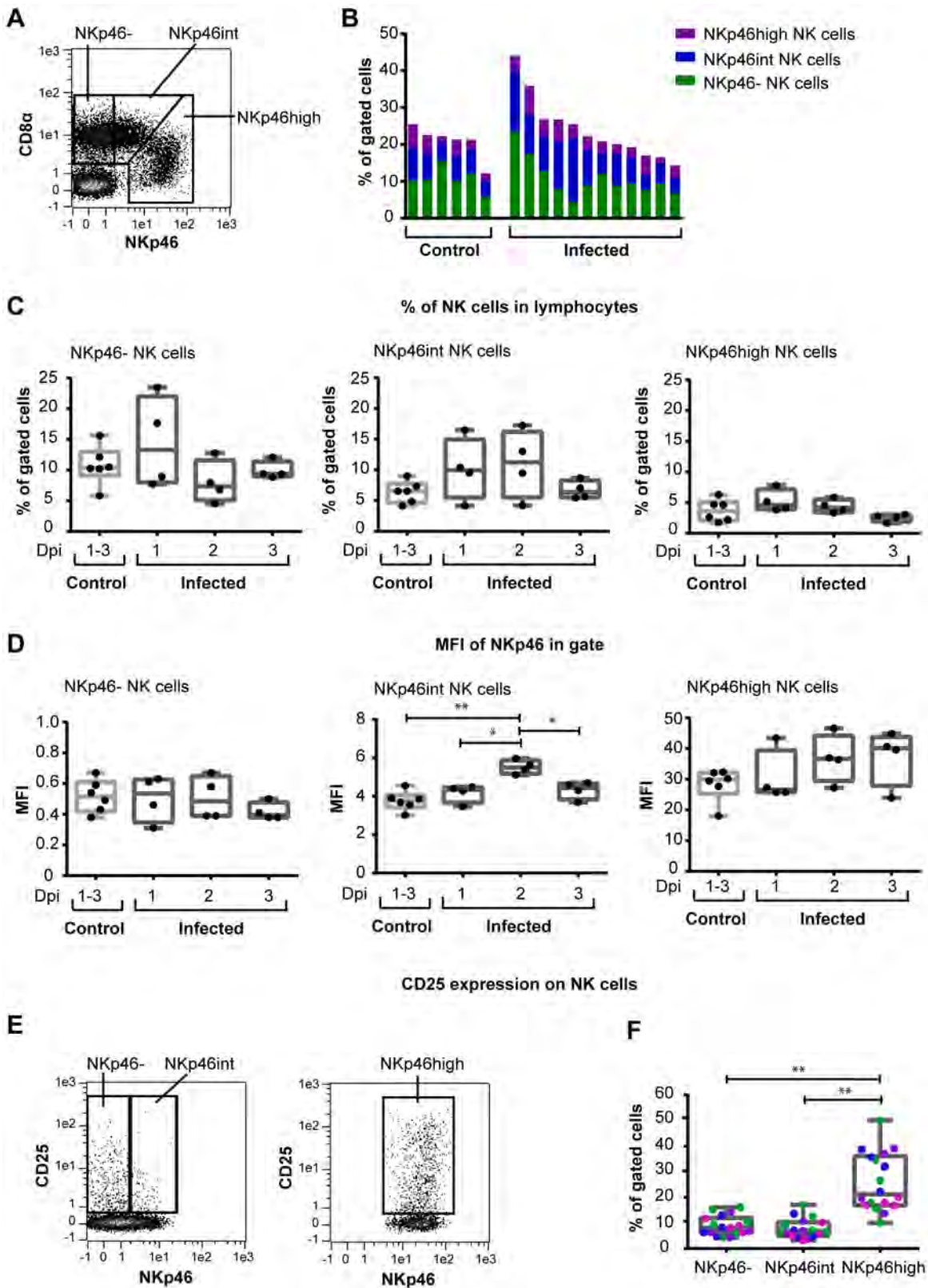
Figure 3. Percentages of NK cells and expression of NKp46 and CD25 in lung tissue. Mononuclear cells were isolated from lung tissue of pigs infected with influenza A virus ($n = 12$) and control animals ($n = 6$) during the first 3 days pi and analysed by flow cytometry. (**A**) Live CD3^{−} lymphocytes were gated as described in Fig 2. NK cells were gated according to CD8α and NKp46 expression and defined as NKp46^{−}, NKp46^{int} or NKp46^{high} cells. Plot shown is from a representative control animal. (**B**) Proportions of NKp46^{−} (green), NKp46^{int} (blue) and NKp46^{high} (purple) NK cells in individual animals, shown as percentages of gated cells in lymphocytes. (**C**) Percentages of NKp46^{−} (left), NKp46^{int} (middle) and NKp46^{high} (right) NK cells among lymphocytes. (**D**) Median fluorescence intensity (MFI) in the NKp46^{−} gate (left), the NKp46^{+} gate (middle) and in the NKp46^{high}

exudate (Fig. 4D). In several areas of the lung tissue, NKp46$^+$ and influenza virus NP$^+$ cells were found in immediate proximity to each other, and in some cases these cells had a direct contact (Fig. 4D).

NKp46$^+$ cells in the lungs are not infected with influenza virus and do not undergo apoptosis

Influenza virus propagation leads to apoptosis of infected epithelial cells through activation of caspase-3 [42], and it has been suggested that induction of apoptosis in NK cells in lung tissue may be an important escape mechanism for influenza A virus [6,8]. Samples from lung tissue were therefore stained for NKp46 (Fig. 5A) and influenza A virus NP (Fig. 5B) in combination with caspase-3 (Fig. 5C). No cells in the lung tissue were stained double positive for influenza A virus NP and NKp46, indicating that virus did not replicate in the NKp46$^+$ cells. Abundant caspase-3$^+$ cells were found in the epithelial lining of bronchi and bronchioles and most of these cells were infected with influenza virus (Fig. 5D).
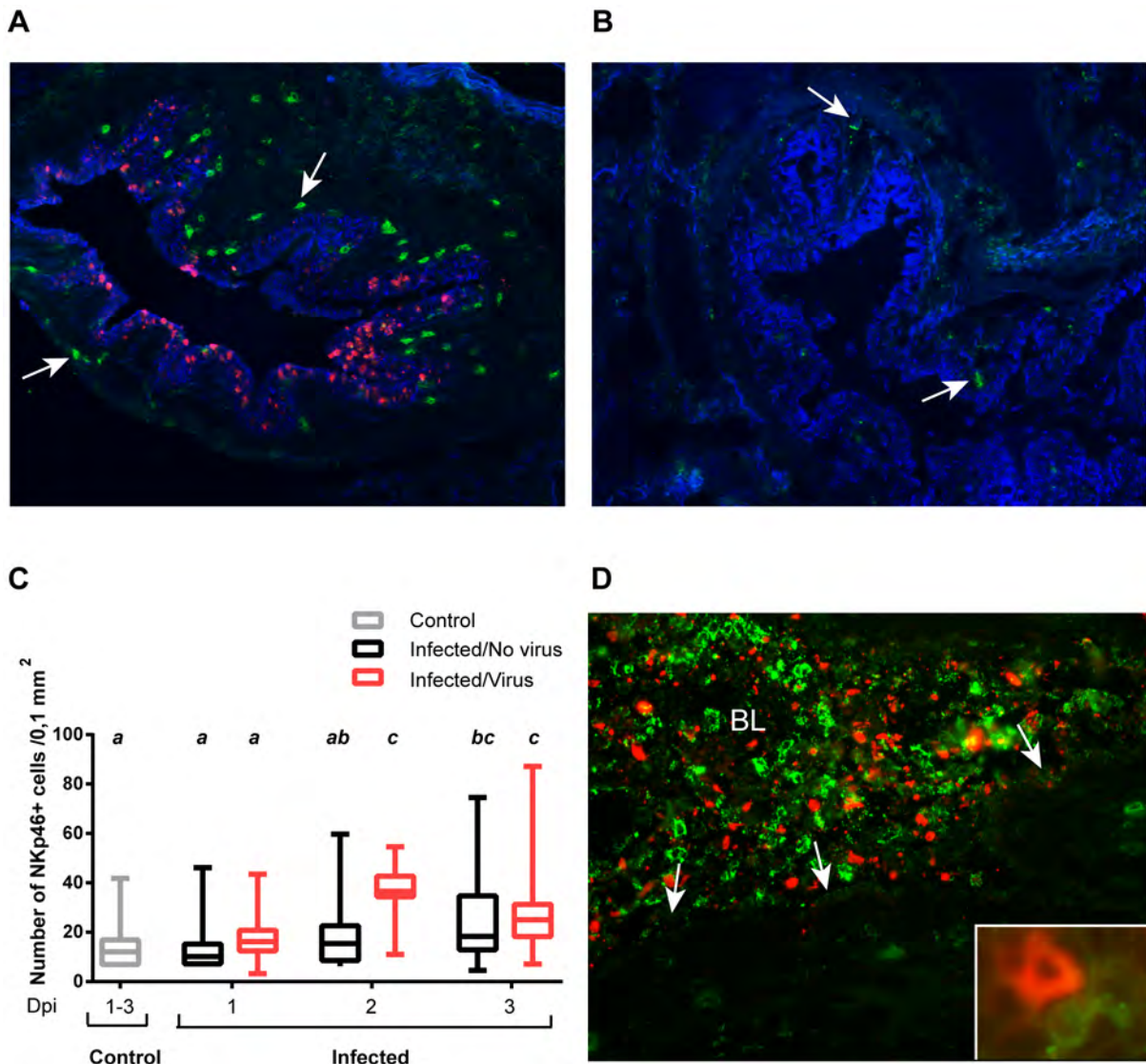
Figure 4. NKp46$^+$ cells in the lungs of influenza virus infected pigs. Lung tissue sections from pigs infected with influenza A virus and control pigs were stained with immunofluorescence markers for cytokeratin (blue), NKp46 (green) and influenza A virus NP (red). NKp46$^+$ cells were counted in areas were influenza A virus NP was (**A**) detected and (**B**) not detected. Representative pictures taken from the same animal on day 1 pi are shown. Arrows point at NKp46$^+$ cells. Immunofluorescence staining, 200x. (**C**) Plot shows number of NKp46$^+$ cells per 0,1 mm^2 in sections ($n = 24$ per animal) from control animals ($n = 6$) and in areas with and without virus in infected animals ($n = 4$ per day) calculated as described in *Material and Methods*. Groups with different letters differ significantly ($p \leq 0.05$). (**D**) NKp46$^+$ cells in the lumen of a bronchus (BL). Arrows point at the epithelial lining. Representative picture of luminal exudate, taken from an infected animal on day 2 pi. Insert shows NKp46$^+$ and influenza A virus NP$^+$ cell in the lung tissue of an infected animal on day 1 pi. Immunofluorescence staining, 400x.
doi:10.1371/journal.pone.0100619.g004

However, no double positive NKp46 and caspase-3 stained cells were found.

Elevated levels of TNF, but not IFN-γ in lung tissue

To investigate the intracellular protein levels of IFN-γ in lung tissue cells, isolated lung mononuclear cells were stained with an antibody against IFN-γ and analysed by flow cytometry (Fig. 6A). The results showed some day to day variation in both control and infected animals and no clear differences in IFN-γ production between the infected and control group could be detected (Fig. 6B). A greater portion of the IFN-γ $^+$ cells were CD3$^+$, but IFN-γ $^+$CD3$^-$ cells were also observed. Some of the IFN-γ $^+$CD3$^-$ cells expressed NKp46, but this population did not differ between control and infected animals (data not shown). IFN-γ production was also investigated on mRNA level by rRT-PCR detection using specific primers for IFN-γ mRNA in isolated lung mononuclear cells from infected animals. Consistent with flow cytometric analysis, the relative expression of IFN-γ mRNA varied between the individual infected animals and no difference between infected and control groups were found (Fig. 6C). Expression of TNF mRNA was also investigated by rRT-PCR and the results showed an increase in TNF levels in all infected animals relative to healthy pigs on day 1 and 2 pi (Fig. 6C).

The NKp46 receptor binds to influenza virus infected cells

The *in vivo* studies indicated a role for NKp46$^+$ cells in the lungs of influenza A virus infected pigs. Therefore, an *in vitro* binding assay was carried out to determine whether porcine NKp46 binds to influenza A virus infected cells as has been shown for human NKp46 [3,4]. Recombinant porcine NKp46-Ig fusion protein showed dose-dependent binding to influenza A virus infected cells (Fig. 7A). No binding of NKp46-Ig on uninfected cells was observed. As expected, a clear H1 expression could be observed on infected cells, whereas no expression was observed on non-infected MDCK cells (Fig. 7B). When using anti-H1 mAbs and NKp46-Ig in combination, a distinct co-staining could be observed on influenza A infected cells (Fig. 7C).

Discussion

Interactions between NK cells and influenza A virus in the lungs during the early phase of infection are still poorly understood. *In vitro* studies have shown that human NK cells can be activated by binding of viral HA to NKp46, leading to direct killing of infected cells [3,4,14,43]. Conversely, blocking of this receptor results in reduced NK cell activation [14,44]. The present study provides evidence that porcine NKp46 also binds to influenza A virus infected cells, suggesting that this receptor has a defined role in influenza virus infections in several species.

A reduction in NKp46$^+$ NK cells was seen in the blood of infected pigs shortly after infection. As demonstrated by complete blood counts, there was a general decrease in lymphocytes. Hence, the reduction of NKp46$^+$ NK cells was not unique, but did differ from NKp46$^-$ NK cells, which increased in numbers. A similar
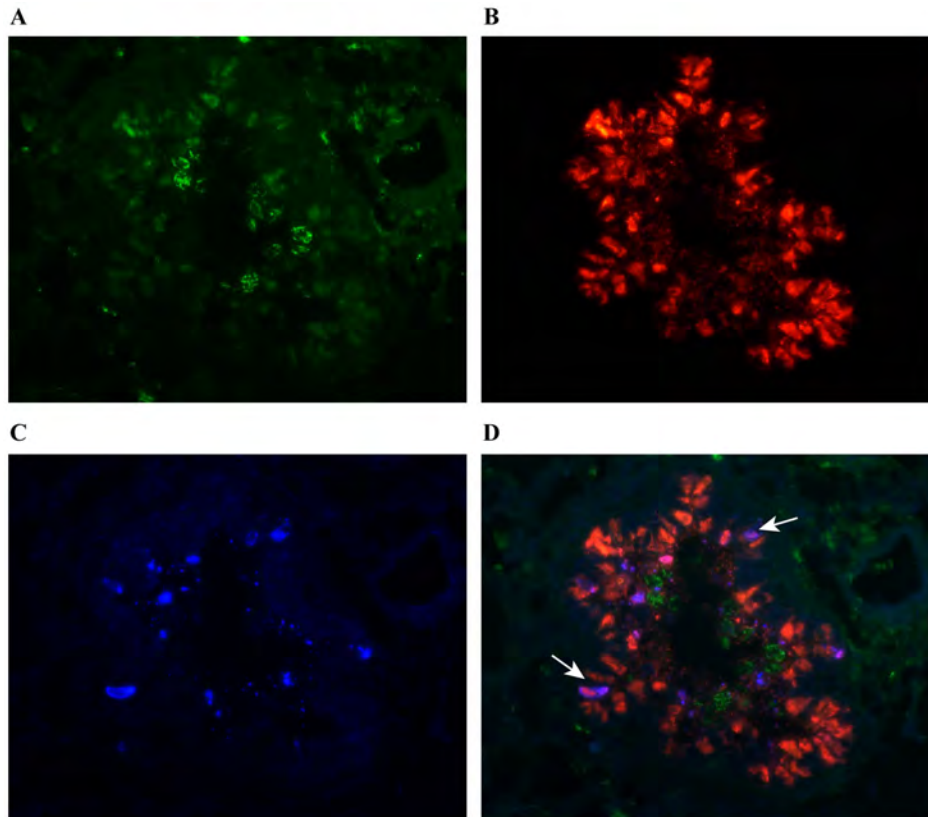
Figure 5. Staining for apoptosis in the lungs. Lung tissue sections from animals infected with influenza A virus (*n* = 12) were stained with immunofluorescence markers against (**A**) NKp46(green), (**B**) influenza A NP(red) and (**C**) the apoptosis marker caspase-3(blue). (**D**) Overlay displaying simultaneously influenza A virus NP$^+$ and caspase-3$^+$ cells as purple (arrows). Representative of virus infected bronchiole at day 1 pi. Immunofluorescence staining, 400x.
doi:10.1371/journal.pone.0100619.g005
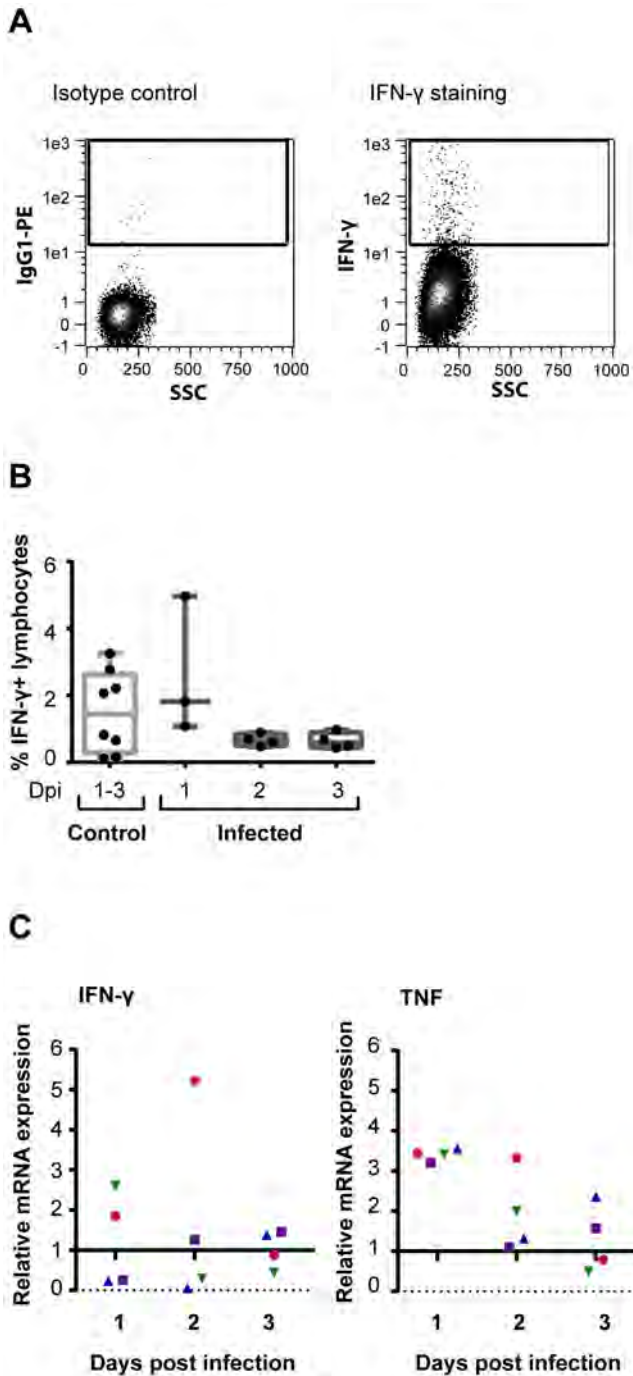
Figure 6. Detection of IFN-γ and TNF in lung tissue. (**A**) Intracellular IFN-γ was analysed in lung mononuclear cells from influenza A virus infected animals and control animals by flow cytometry. IFN-γ [+] cells were gated among live lymphocytes. Plots show representative isotype control (left) and IFN-γ staining (right) from the same infected animal on day 3 pi (**B**) Percentages of IFN-γ [+] cells obtained by flow cytometry in control animals ($n = 8$) and infected animals ($n = 4$ per day). Data from one animal at day 1 is missing due to too few cells isolated. (**C**) Gene expression for IFN-γ and TNF mRNA in infected animals ($n = 4$ per day) was calculated as relative values to the household gene GADPH and to mRNA levels of the target gene in a control group ($n = 4$). Each symbol represents one infected animal, different symbols represents animals sacrificed the same day. Values above 1 indicate an up regulation, whereas values below 1 indicate a down regulation of the target gene.

doi:10.1371/journal.pone.0100619.g006
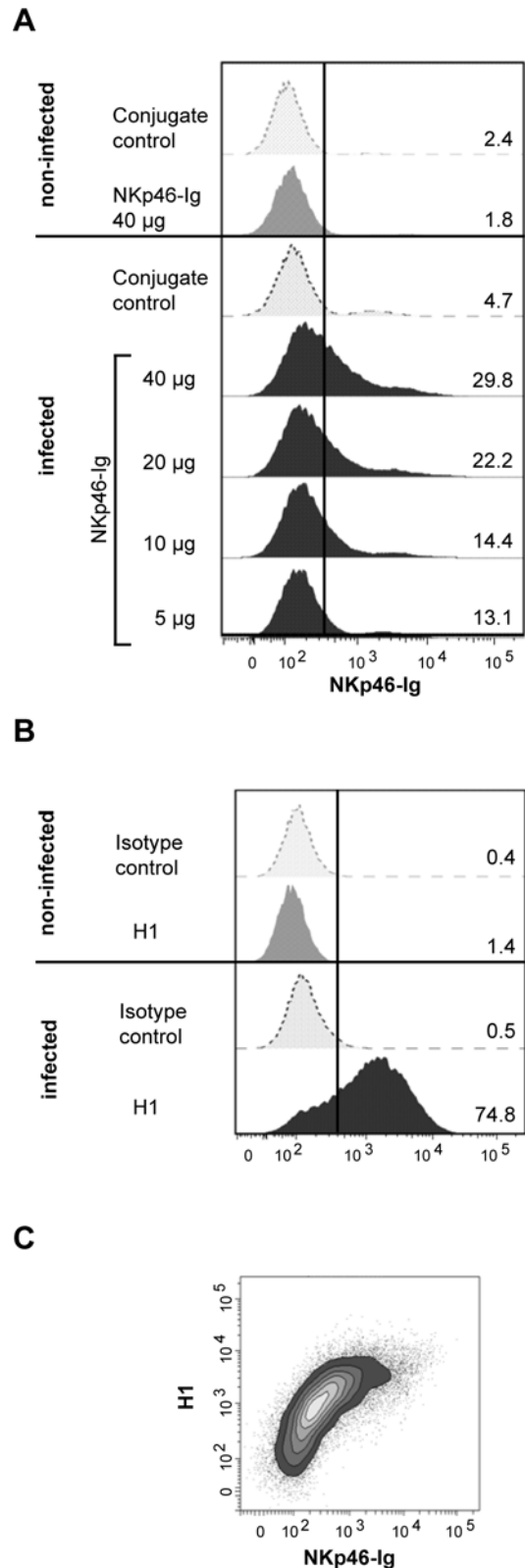
Figure 7. Binding of porcine NKp46 Ig fusion protein to of influenza A virus infected MDCK cells analysed by flow cytometry. Cells were gated according to forward/side scatter characteristics. (**A**) Histograms show binding of different concentrations of NKp46-Ig to infected cells. Percentages of positively stained cells are indicated. (**B**) Infected as well as non-infected MDCK were stained with

anti-H1 mAbs. Corresponding isotype-matched irrelevant mAbs and secondary antibodies only served as controls. (**C**) Double staining for H1 and the highest concentration of NKp46-Ig are shown on influenza infected cells. Results are representative of four independent experiments.

doi:10.1371/journal.pone.0100619.g007

reduction of CD3$^-$CD56$^+$ NK cells has been observed in humans with influenza, leading to the hypothesis that NK cells are recruited to the lungs to participate in the defence against the influenza virus [6,7]. In this study, immunofluorescence staining of lung tissue sections from pigs infected with influenza virus demonstrated a clear difference in the numbers of NKp46$^+$ cells in areas infected with virus, compared to uninfected areas from the same animal. There was also an overall increase in the number of NKp46$^+$ cells in lung sections from the infected animals compared to control animals. It was not possible to separate the NKp46^{int} and the NKp46^{high} NK cell population in the stained tissue sections since no marker has been found to be exclusively expressed by either populations [23] and it is likely that both the NKp46^{int} and the NKp46^{high} NK cells are identified as NKp46$^+$ cells. NKp46$^+$ cells were found to co-localize with influenza virus NP$^+$ cells and in some cases the cells seemed to have contact. This could potentially represent NK cells bound to influenza infected cells as demonstrated by NKp46 binding to infected cells *in vitro*, but other receptors may also account for this contact and need to be further elucidated. These results support the hypothesis that NK cells are recruited to the influenza virus infected parts of the lungs.

Flow cytometric analysis showed only a minor increase in the percentage of NK cells in lung tissue. As other cell types are also recruited to the lungs following influenza virus infection, cell numbers measured as percentages of the mononuclear cell population is likely to underestimate the absolute number of NK cells in lung tissue. Also, since the virus has a multifocal distribution in the lungs, the amount of NK cells will be influenced by the number of microscopic lesions in the sample. This could explain some of the individual differences seen between infected animals in the flow cytometric analysis. The earlier reports of NK cell numbers in lung tissue following virus infection as measured by flow cytometry in swine are diverse. One study found an increase in NK cells in bronchoalveolar lavage fluid from swine starting at 2 days pi [33], while another study found a decrease in NK cells in lung tissue on day 3 pi and in bronchoalveolar lavage fluid on day 6 pi [29].

As *in vitro* studies have shown that influenza virus is able to infect human NK cells, leading to increased detection of apoptotic markers [8] it has been suggested that the reduced number of NK cells in the blood of influenza virus infected humans is caused by apoptosis of infected NK cells in lung tissue causing a drainage of NK cells to the lung [5,6]. In the terminal stages of fatal influenza, humans have diminished numbers of NK cells in lung tissue [5,9]. However, since the lung tissue of human patients was sampled in the terminal stage of the infection, it is impossible to determine whether the NK cells had been present in the lung at earlier time points. In the present study, neither cells that were double positive for NKp46 and the apoptosis marker caspase-3, nor NKp46 and influenza A virus NP, were identified. This indicates that infection and apoptosis of NKp46$^+$ cells is not an important escape mechanism for the influenza virus in the early phase of the infection in swine. NKp46$^+$ cells were observed in the lumen of bronchi and bronchioles of influenza virus infected animals, and the number of NK cells entering the exudate could influence NK cell numbers in the lung tissue and blood.

A population of NKp46^{high} NK cells was found in the lungs of both control and infected animals. This population was also found in liver and spleen in the current study (data not shown) and has been described earlier [22]. Increased expression of NKp46 has been linked to activation of NK cells in both swine and humans [23,45] and in line with this, a higher percentage of the lung NKp46^{high} cells in this study expressed the activation marker CD25 compared to NKp46$^-$ and NKp46^{int} NK cells. A higher expression of CD27 has also been found on this population in spleen [23]. We hypothesise that the NKp46^{high} population in the lungs represents a more mature or activated NK cell population, in line with what has been shown for NKp46^{high} NK cells in spleen [23]. Since NK cells are primarily produced in the bone marrow and released to the blood in steady state [46] and during influenza infections [12], we speculate that the NKp46^{high} NK cell population may have originated from the bone marrow and reached the lungs through the blood, before maturing or being activated in the lungs.

An increased NKp46 expression was also seen in the population of NKp46^{int} cells following influenza A virus infection. It has been shown earlier that porcine NK cells may up-regulate NKp46 in response to *in vitro* cytokine stimulation [22]. This may well be the case *in vivo*, and is also most likely caused by activation of the cells [23,45]. There was a tendency of increased NKp46 expression in the NKp46^{high} NK cells as well, although not significant. This could imply that mainly the cells expressing the NKp46 receptor respond to the influenza A virus infection. Furthermore, these results suggest that the NKp46^{int} cells are the main responders to the infections and therefore might be responsible for the greatest part of the increase in NKp46$^+$ cells seen in the immunofluorescence staining of lung sections.

A decrease in the numbers of NKp46$^+$ NK cells in the blood of infected pigs was observed in both experiments, but a significant reduction was only seen at day 1 pi in the second study. In the first study, the pigs were inoculated with a 1:1 mixture of two different virus variants, namely A (H1N1) pdm09 222D (aspartic acid) and 222G (glycine). These viruses differ only in the amino acid positioned at 222 (H1 numbering) in the viral surface glycoprotein HA. A more severe infection has been associated with the 222G variant, as compared to when only the wild-type 222D variant is detected [47]. In the second experiment, only the 222G virus variant was used. This resulted in a wider distribution of macroscopic changes. The more pronounced NK cell response seen in the second study indicates that the type of virus and severity of the disease may influence the NK cell response in pigs. In humans, a more marked reduction of NK cell numbers in blood has been observed in severe cases of influenza when compared to mild cases [6]. Different influenza viruses have also been shown to activate NK cells to varying extents *in vitro* [43,44].

Elevated levels of IFN-γ have been detected in nasopharyngeal secretions of human patients with influenza [9,19]. Also in swine, elevated levels of IFN-γ in the respiratory tract of influenza infected animals have been reported, but the detection varied over time, indicating the need for more frequent sampling [17,29]. In the present study, no changes in IFN-γ protein levels or mRNA expression in the lungs could be detected. On the other hand, an increased expression of TNF mRNA was found in lung mononuclear cells from all infected animals on day 1 and 2 pi. TNF is an important antiviral cytokine which has been shown to inhibit influenza viral replication in porcine lung epithelial cells *in vitro* and have greater effect against influenza virus replication than IFN-α and IFN-γ [48]. The levels of TNF in the respiratory tract correlates with pulmonary lesion scores and clinical disease in pigs [17,18] and with body temperature in humans [19], indicating an

important link with the induction of clinical signs [17,18]. Unfortunately, TNF was only investigated on mRNA level on the total mononuclear cell population and the relative contribution of NK cells cannot be concluded. Future studies should define the relative contribution of NK cells in cytokine production and further elucidate the role of different cytokines during influenza virus infections. It is also possible that the main function of NK cells in influenza A virus infections is cytotoxic killing of infected cells, rather than cytokine production. NK cells have been shown to kill influenza virus infected cells *in vitro* [3,4,43]. Furthermore, studies in mice point to an important role for NK cell-mediated lysis in clearing influenza A virus from the lungs *in vivo* [4,10], but this needs further investigation.

In summary, a decline in NKp46+ NK cells was demonstrated in the blood of infected pigs; similar to what is seen for CD3−CD56+ NK cells in humans, strengthening the idea of the pig as a model for influenza virus infections in humans. The changes in blood were followed by an increase in NKp46+ NK cells in influenza virus infected areas of the lungs, providing further insight into the dynamics of NK cells during influenza. The specific increase in NKp46 expression on NK cells expressing the NKp46 receptor, cells in the lung tissue of infected pigs indicates a higher activation status of these cells and suggests a functional role for these cells in the lungs of influenza infected swine. Further studies should address the functional aspect of these cells.

Supporting Information

Figure S1 Counting of NKp46+ cells. Numbers of NKp46+ cells per area were counted in lung tissue sections from control animals and influenza A virus infected animals as described in *Material and Methods*. Representative picture of area with virus from infected animal. Immunofluorescence staining, 200x.

(TIF)

Figure S2 Comparison of gross lung lesion score. Lung lesions were compared between the two studies. In the first study, pigs were infected with a mixture of influenza A (H1N1) pdm09 225D and 225G. In the second study, only the 225G variant was used. Macroscopic pathology was evaluated as gross lung lesion score on days 1–5 and 8 pi in the first experiment ($n = 2$ per day) and on days 1–3 in the second experiment ($n = 4$ per day).

(TIF)

Figure S3 Influenza A virus infection causes lymphophenia in pigs. Complete blood counts in influenza A virus infected pigs were determined by CD45 staining and flow cytometric analysis of PBMC. (**A**) Lymphocytes were gated as CD45high cells with a low side scatter (SSC) and granulocytes as CD45dim cells with a high SSC according to CD45 expression and SSC. (**B**) Lymphocyte and granulocyte numbers in infected ($n = 12$) and control animals ($n = 9$) were obtained each day until they were sacrificed. *$p ≤ 0.05$, **$p ≤ 0.01$.

(TIF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: HF AGH FG WG SPG SMB AKS. Performed the experiments: HF AGH MV FG AN WG KHM SPG SMB AKS. Analyzed the data: HF AGH MV AKS. Contributed reagents/materials/analysis tools: WG KHM. Wrote the paper: HF AGH AKS.

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Porcine CD3⁺NKp46⁺ lymphocytes have NK-cell characteristics and are recruited to the lung during early influenza infection

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Abstract

The CD3⁻NKp46⁺ phenotype is frequently used for the identification of NK cells in various mammalian species. Recently, NKp46 expression was analyzed in more detail in swine. It could be shown that besides CD3⁻NKp46⁺ lymphocytes, a small but distinct population of CD3⁺NKp46⁺ cells exists. In this study we report low frequencies of CD3⁺NKp46⁺ lymphocytes in blood, lymph nodes and spleen but increased frequencies in non-lymphatic organs like liver and lung. Phenotypic analyses showed that the majority of CD3⁺NKp46⁺ cells co-expressed the CD8αβ heterodimer, while a minor subset expressed the TCR-γδ which was associated with a CD8αα⁺ phenotype. Despite these T-cell associated receptors, the majority of CD3⁺NKp46⁺ lymphocytes displayed a NK-related phenotype (CD2⁺CD5⁻CD6⁻CD16⁺perforin⁺) and expressed mRNA of NKp30, NKp44 and NKG2D at similar levels as NK cells. Functional tests showed that CD3⁺NKp46⁺ lymphocytes produced IFN-γ and proliferated upon cytokine stimulation to a similar extent as NK cells but did not respond to the T-cell mitogen ConA. Likewise, CD3⁺NKp46⁺ cells killed K562 cells with an efficiency comparable to NK cells. Cross-linking of NKp46 and CD3 led to degranulation of CD3⁺NKp46⁺ cells, indicating functional signaling pathways for both receptors. Additionally, influenza A(H1N1)pdm09 infected pigs had reduced frequencies of CD3⁺NKp46⁺ lymphocytes in blood but increased frequencies in the lung in the early phase of infection. Thus, CD3⁺NKp46⁺ cells appear to be involved in the early phase of influenza infections. In summary, we describe a lymphocyte population in swine with a mixed phenotype of NK and T cells, with results so far indicating that this cell population functionally resembles NK cells.

Introduction

The activating receptor NKp46 (NCR1, CD335) belongs to the family of natural cytotoxicity receptors (NCRs) and was initially described to be specifically expressed on natural killer (NK) cells (1-3). Receptor triggering on NK cells results in the induction of cytokine production like IFN-γ as well as in cytolytic activity (1, 4). The receptor recognizes hemagglutinins (HA) of influenza, parainfluenza and Sendai virus and ligation leads to lysis of infected cells (5-7). Likewise, recognition of currently unknown ligands on transformed cells induces killing of tumor cells (6, 8). Therefore, NKp46 is involved in the defense of viral infections and cancer.

NKp46 is detectable already early in NK-cell development and expression is maintained in later developmental stages (9, 10). Furthermore, NKp46 seems to be evolutionary conserved in mammals and therefore was proposed to be a general marker to define NK cells across mammalian species (9, 11). Hence, NKp46 expression was used to identify and characterize NK cells in humans (1, 2), rodents (3, 9, 12), cattle (13), sheep (14) and pig (15). For swine, three distinct NK-cell subsets could be identified: NKp46^{-}, NKp46^{+} and NKp46^{high} CD3^{-} lymphocytes that display phenotypic as well as functional properties of NK cells (15, 16). It was also shown that porcine NKp46 binds to hemagglutinin of influenza virus and that NKp46^{+} lymphocytes accumulate in lungs of H1N1 influenza virus-infected animals (17).

Moreover, it became evident that NKp46 is also expressed on a minor fraction of different T-cell populations. In mouse, rare subsets of NKp46^{+} cells co-expressing CD3 could be identified (9, 10). Likewise, in humans the existence of CD3^{+}NKp46^{+} cells in lymphatic as well as non-lymphatic tissues could be demonstrated (18). Lymphocytes with this phenotype could be identified within the γδ T-cell population in mice (19) as well as in NKT-cell subsets in mouse and human (20, 21). Although these cells account for only a minute fraction of lymphocytes, it was observed that the NKp46^{+} NKT cells expand during leukemic transformation (20) and viral infection (21). Additionally, it could be shown that NKp46 is induced in cytolytic T cells by chronic activation of cells during autoimmune diseases (22). In cattle it could be shown that γδ T cells from blood and spleen express NKp46 after prolonged *in vitro* stimulation with IL-15 (23). Furthermore, a population of bovine CD3^{+}NKp46^{+} lymphocytes has been

described that represents a non-conventional T-cell subset that is constitutively present in the blood of healthy cattle (24). Likewise, in the dog a CD3$^+$NKp46$^+$ lymphocyte subset could be identified in 79% of animals analyzed (25).

A distinct population of CD3$^+$NKp46$^+$ cells could also be identified in the pig (15). To further investigate this lymphocyte population in more detail, we performed phenotypic and functional studies on porcine CD3$^+$NKp46$^+$ lymphocytes and compared them to NK as well as T cells. We here report that the majority of CD3$^+$NKp46$^+$ cells expresses the CD8αβ heterodimer, comparable to porcine cytolytic T cells while a minor subset belongs to TCR-γδ$^+$ T cells. Nonetheless, CD3$^+$NKp46$^+$ cells express NK-associated molecules like perforin, CD16, NKp30 and NKp44. Functionally, they respond to *in vitro* stimulation in a NK-like manner and have the capacity of spontaneous cytolytic activity. Degranulation could be induced in CD3$^+$NKp46$^+$ lymphocytes by receptor triggering of both, NKp46 as well as CD3. Furthermore, we show that CD3$^+$NKp46$^+$ lymphocytes are recruited from the blood to the lung in the early phase of influenza infection.

Material and Methods

Isolation of porcine lymphocytes

Blood and organs were obtained from healthy 3-7 months old pigs from an abattoir or from animals housed at the University Clinic for Swine at the University of Veterinary Medicine Vienna, Austria. Animals from the slaughterhouse were subjected to electric high-voltage anaesthesia followed by exsanguination, a procedure that is in accordance to the Austrian Animal Welfare Slaughter Regulation. In-house pigs were anaesthetized by intramuscular injection of Ketaminhydrochlorid (Narketan®, Vétoquinol, Vienna, Austria, 10 mg/kg body weight) and Azaperon (Stresnil®, Janssen Pharmaceutica, Beerse, Belgium, 1.3 mg/kg body weight). Subsequently, animals were euthanized via intracardial injection of T61® (MSD Animal Health, Vienna, Austria, 1.0 ml/10 kg body weight). This procedure was approved by the institutional ethics committee and the national authority according to §26 of Law for Animal experiments, Tierversuchsgesetz 2012 - TVG 2012 (reference number bmwf GZ 68.205/0103-II/3b/2013).

PBMC were isolated from heparinized blood using density gradient centrifugation (Pancoll human, density: 1.077 g/ml, PAN-Biotech, Aidenbach, Germany). Dissected spleens as well as mediastinal lymph nodes were cut into small pieces and mechanically dissociated by a sieve. Obtained spleen cells were applied to density gradient centrifugation. Isolated cells from lymph nodes were applied to cotton-wool filtration to remove dead cells. Lymphocytes from lung tissue were isolated as described elsewhere (17). Briefly, lung tissue was cut in small pieces and incubated for one hour at 37°C in cell culture medium containing 2% FCS (PAA, Pasching, Austria), 20 mM Hepes (Sigma-Aldrich, Vienna, Austria), 25 U/ml DNase I (Life Technologies, Carlsbad, CA) and 300 U/ml Collagenase type I (Life Technologies). The cell suspension was subsequently applied to cotton-wool filtration and density gradient centrifugation.

Isolated cells from PBMC and organs were either immediately used for phenotypic analyses or stored at -150°C. When frozen cells were used for short-term functional assays, PBMC were thawed one day prior to stimulation and rested overnight in culture medium.

Cell culture

The human leukemia cell line K562 (26) and isolated porcine PBMC were propagated in RPMI 1640 with stable glutamine (PAN Biotech) supplemented with 10% (v/v) heat-inactivated FCS (PAA), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (PAA). Cell culture medium for sorted cell subsets was additionally supplemented with 1 mM sodium pyruvate (PAA), non-essential amino acids (PAA) and 50 µM 2-mercaptoethanol (Sigma-Aldrich).

Flow cytometry and antibodies

For flow cytometric (FCM) analyses, cells were either re-suspended in PBS-based buffer containing 10% (v/v) porcine plasma for direct analysis after isolation, or in buffer containing 3% (v/v) FCS for analysis after *in vitro* cultivation. All incubation steps were performed in 96-well round-bottom plates at 4°C for 20 min. The different combinations of primary monoclonal antibodies (mAbs) as well as secondary reagents used for each assay are listed in Table 1. Non-commercial antibodies were produced in-house (27). Where indicated, these antibodies were conjugated either to fluorochromes or Biotin. Alexa Fluor-647 or Alexa Fluor-488 Labeling Kits (Life Technologies) were used according to manufacturer's instructions. FITC conjugation was performed as described elsewhere (28). Sulfo-NHS-LC Biotin (Thermo Scientific, Pierce, Vienna, Austria) was used for the biotinylation reaction according to manufacturer's protocol. If unlabeled and directly conjugated antibodies with the same isotype were used in combination, a sequential staining was performed. After labeling with unconjugated primary mAb and isotype-specific dye-conjugated secondary antibodies, free binding sites were blocked by whole mouse IgG molecules (2 µg per sample, Jackson ImmunoResearch, Suffolk, UK). Thereafter, cells were incubated with directly-labeled primary mAbs. For exclusion of dead cells, Fixable Near-IR Dead Cell Stain Kit (Life Technologies) was used according to manufacturer's protocol with 0.05 µl reactive dye per sample. Appropriate isotype-matched control antibodies were used to assess unspecific bindings. Single-color samples were prepared for automatic compensation.

All samples containing freshly isolated cells were treated with a fixation and permeabilization reagent prior to analyses to lyse remaining erythrocytes (BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit, BD Biosciences, San Jose, CA). Where

applicable, this was followed by an incubation step with directly-labeled mAbs against intracellular antigens. For intracellular staining of perforin, cells were fixed and permeabilized with in-house made saponin-containing reagents as described elsewhere (29).

FCM analyses were performed on a FACSCanto II equipped with a high throughput sampler (BD Biosciences). At least 2×10^5 live lymphocytes were recorded per sample. Data were analyzed with FACSDiva software (Version 6.1.3., BD Biosciences) and FlowJo software (Version 7.6.3., Tree Star, Ashland, OR).

Fluorescence-activated cell sorting (FACS)

PBMC were labeled with primary antibodies against CD3, CD8α and NKp46 as well as corresponding secondary reagents as described in Table 1. PBS containing 5 % FCS (v/v) and 2 mM EDTA was used for all washing steps. PBMC were sorted into CD3$^+$NKp46$^-$ T cells, CD3$^-$CD8α$^{+/dim}$ total NK cells as well as CD3$^+$NKp46$^+$ lymphocytes on a FACSAria (BD Biosciences). All sorted subsets had a purity of 97.0 % or higher. After two washing steps in cell culture medium, sorted cells were cultivated for functional assays. Alternatively, sorted cells were thoroughly washed with PBS and cell pellets were snap-frozen in liquid nitrogen and stored at -80°C for subsequent mRNA analysis.

Intracellular cytokine staining for IFN-γ

Total PBMC were cultivated in 96-well round-bottom plates at 3×10^5 cells per well in a final volume of 200 μl. Cells were incubated in the presence of phorbol-12-myristate-13-acetate (PMA, 50 ng/ml) and Ionomycin (500 ng/ml, both Sigma-Aldrich) for four hours. Alternatively, cells were stimulated with a combination of recombinant porcine (rp) IL-2 (20 ng/ml), rpIL-12 (25 ng/ml) and rpIL-18 (two different concentrations: 5 ng/ml and 25 ng/ml) for 24 hours (all cytokines from R&D Systems, Minneapolis, MN). Cells cultured in medium alone served as negative control. For intracellular cytokine staining, Brefeldin A (GolgiPlug, BD Biosciences) was added at a final concentration of 1 μg/ml per well four hours prior to harvest. Subsequently, cells were subjected to FCM staining as outlined in Table 1.

Proliferation assay

Total PBMC were stained with CellTrace™ Violet Proliferation Kit (Life Technologies) following manufacturer's instructions as described elsewhere (30). Briefly, cells were washed in PBS and adjusted to 5-20 x 10^6 cells per ml, followed by addition of 1 ml of a 5 µM solution of the Violet Cell Trace dye. Labeling was performed for 10 min at 37°C, followed by addition of 2 ml FCS and further incubation for 15 min at room temperature. After three washing steps, cells were cultivated in 96-well round-bottom plates at 3 x 10^5 cells per well in a final volume of 200 µl for four days. Cells were either stimulated with ConA (3 µg/ml, Amersham Biosciences, Uppsala, Sweden), a combination of rpIL-2 (50 ng/ml, R&D Systems) and rpIL-15 (50 ng/ml, Invitrogen, Carlsbad, CA, USA) or left in medium alone as negative control. Following stimulation, cells were harvested and subjected to FCM staining as outlined in Table 1.

Cytotoxicity assay

FACS-sorted T- and NK-cell populations were used in a 4-hour cytotoxic assay as described elsewhere (31). Briefly, sorted cells were plated in 96-well round-bottom plates at the final cell numbers of 20 x 10^4, 10 x 10^4, 5 x 10^4, 2.5 x 10^4 and 1.25 x 10^4 and stimulated with rpIL-2 (20 ng/ml, R&D Systems) and rpIL-15 (20 ng/ml, Biosource) for 36 hours. After pre-activation by cytokines, K562 cells labeled with $DIOC_{18}$ (Sigma-Aldrich) were added to the effector cells with 1 x 10^4 cells per well. After four hours, cells were harvested and 30 µl of a 20 µg/ml propidium iodide solution (Sigma-Aldrich) was added per sample prior to FCM analysis for identification of dead cells. At least 5 x 10^3 DIOC-positive cells were recorded per sample. Percent specific lysis was calculated with the formula: (% of target cell lysis in the test - % of spontaneous cell death) / (% of maximum lysis - % of spontaneous cell death) x 100. For determination of maximum lysis, target cells were fixed and permeabilized with in-house made saponin-containing reagents (29).

Stimulation of lymphocytes by receptor triggering

Receptor triggering was performed by using mAbs against NKp46 (IgG1, clone VIV-KM3) and CD3 (IgG1, clone PPT7). Isotype-matched irrelevant antibodies (IgG1, clone NCG01, Dianova, Hamburg, Germany) served as control. 96-well round-bottom plates were coated with mAbs

at a concentration of 3 μg/ml in PBS (50 μl per well) overnight at 4°C. Plates were washed three times with PBS prior to addition of cells.

Cells used in IFN-γ and CD107a degranulation assays were pre-activated with cytokines prior to functional tests. For IFN-γ production, PBMC were stimulated with rpIL-2 (25 ng/ml) and IL-18 (5 ng/ml, both R&D Systems). For the degranulation assay, cells were pre-incubated with rpIL-2 (10 ng/ml, R&D Systems) and rpIL-15 (10 ng/ml, Biosource) for 24 hours. After pre-activation, cells were transferred at 3 x 10^5 cells in a total volume of 200 μl per well into the mAb-coated plates. Cells were cultured for four hours together with Brefeldin A (GolgiPlug, BD Biosciences; 1 μg/ml) and Monensin (GolgiStop, BD Biosciences; 2 μg/ml). For the degranulation assay, microcultures were additionally supplemented with FITC-conjugated anti-CD107a mAb at a concentration of 4 μg/ml.

For proliferation assays, PBMC were stained with Violet CellTrace™ dye as stated above. Cells were added to mAb-coated plates at 3 x 10^5 cells in a total volume of 200 μl per well and incubated for four days in cell culture medium without further supplements.

After harvesting, cells were forwarded to FCM analysis. Cells were stained with mAb-combinations of respective function assay, as described in Table 1, with the exception of anti-CD8β mAbs that were not included in these analyses. Since NKp46 as well as CD3 were readily internalized after receptor triggering, detection of these two markers by FCM was improved by an additional staining step with CD3 and NKp46-specific mAbs after permeabilization of cells with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit.

Analysis of gene expression by quantitative reverse-transcriptase PCR (RT-qPCR)

Total RNA of FACS-sorted lymphocyte subsets from blood was isolated using TRI Reagent (Sigma-Aldrich) according to manufacturer's protocol. RNA quality control and cDNA synthesis were performed as described elsewhere (32). Expression of the target genes NKp30, NKp44 and NKG2D was determined by real-time PCR. Primers and assay setup for NKG2D was performed as described previously (16). Information on primers is listed in Table 2.

For amplification of target genes, SYBR® green I (0.5x, Sigma-Aldrich) was used as reporter dye. The qPCR reaction-mixes contained iTaq® DNA polymerase (0.3 U/reaction, Bio-Rad, Hercules, CA), gene specific primers (250 nmol/l each), a final concentration of 200 µmol/l dNTP each and 3 mmol/l $MgCl_2$ for NKG2D and 1.5 mmol/l $MgCl_2$ for NKp30 and NKp44 within the reaction buffer (1x, Bio-Rad). PCR conditions as well as optimization and validation of the qPCR assays are described in more detail in the Supplementary Material. The reference genes Cyclophilin A and GAPDH were measured as previously described (PMID: 16223507) and used to normalize each target-gene expression. Corresponding RT-minus controls as well as the no-template controls were included in each assay. All samples were measured in duplicates. Quantitative PCR was performed on a CFX96™ (Bio-Rad) and data analyzed using the CFX manager software (Bio-Rad) in the linear regression mode. Target gene expression was displayed as $2^{-\Delta\Delta Cq}$ values representing the fold changes relative to the mean value of $CD3^+NKp46^+$ cells set to a value of 1.

Influenza infection study

Influenza infection studies were carried out at the Animal Health and Veterinary Laboratories Agency, Addlestone, UK. The project was approved by the Animal Health and Veterinary Laboratories Agency Ethics Committee and all procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under Project License Permit Number 70/7062. Detailed descriptions on infection procedure as well as performed analyses are described elsewhere (17). Briefly, 12 six-week old healthy and influenza-free large white cross-bred pigs were experimentally infected with influenza A/Hamburg/05/2009-e 95 (Ham-e) via the intranasal route with 10^6 $TCID_{50}$ in 2 ml per nostril using a mucosal atomization device (MAD 300, Wolfe Tory Medical). In parallel, nine pigs served as control group, of which six animals were euthanized during the study and three animals were used for blood analysis only. On days 1 to 3 post infection, four infected and two control pigs were euthanized daily.

Heparinized blood was collected from all animals on each sampling day. Samples from lung tissue were taken post mortem from the cranial, middle, caudal and accessory lobes of the right lung. PBMC and lymphocytes from lung tissue were isolated as described above and

analyzed by flow cytometry. The following primary and secondary antibodies were used: CD3-eFluor450 (IgG1, clone PPT3), CD8α (IgG2a, clone 11/295/33, unconjugated or AlexaFluor 647 conjugated), NKp46-biotin (IgG1, clone VIV-KM1), goat anti-mouse IgG2a-AlexaFluor 488 (Life Technologies), Streptavidin-AlexaFluor 647 (Life Technologies) and Streptavidin-PE (eBioscience). For intracellular staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), followed by intracellular staining with Ki-67-FITC mAbs (IgG1, clone B56, BD Biosciences). Appropriate secondary and isotype controls were included. Dead cells were excluded by using Fixable Near-IR Dead Cell Stain Kit (Life Technologies) according to manufacturer's protocol. Acquisition was performed on a MACSQuant Analyser flow cytometer (Miltenyi Biotec) and data were analyzed using MACSQuantify software. A total of 1×10^5 cells were analyzed when possible. Data from one control animal had to be excluded in the Ki-67 staining of lung lymphocytes, due to insufficient cell numbers.

Statistical analysis

Data were analyzed for statistical significance by SPSS® (SPSS Statistics Version 20.0, IBM Corp., Armonk, NY). Obtained values were verified for normal distribution by the Kolmogorov-Smirnov test. Where required, data sets were previously subjected to log-transformation to meet the condition of normality. Data obtained from sets with three or more groups were compared individually by paired two-tailed Student's T-test to the CD3+NKp46+ subset only. No statistical analyses were performed between other cell subsets within these data sets. Box plots were created by SigmaPlot software (Version 11.0, Systat Software Inc., Erkrath, Germany). For data of the influenza infection study, box-plots were created by Graph Pad Prism V6 (GraphPad Software), statistical analyses were performed in JMP V9 (SAS Institute Inc.) by using the Mann-Whitney *U* test as described previously (17). Levels of significance were defined as: *p ≤ 0.05* (indicated by *), *p ≤ 0.01* (indicated by **) and *p ≤ 0.001* (indicated by ***).

Results

Identification of porcine NKp46^{+} lymphocytes co-expressing CD3

Previous studies on NKp46^{+} cells in swine had identified a small but distinct population of lymphocytes that co-expressed NKp46 and CD3 (15). Since these cells phenotypically seem to be positioned at the interface between NK cells and T cells, we aimed to investigate this lymphocyte subset in more detail. Therefore, FCM analyses were performed with lymphocytes isolated from blood as well as lymphatic and non-lymphatic organs. A uniform gating hierarchy was used throughout all experiments (Figure 1A). Doublet cells as well as dead cells were excluded and live lymphocytes were divided upon their CD3 expression. CD3^{-} "non-T cells" were further subgated according to their CD8α/NKp46 expression into the three established porcine NK-cell subsets: CD8α^{+}NKp46^{-}, CD8α^{+}NKp46^{+} and CD8α[dim/-]NKp46^{high} (Figure 1A, bottom row on the left). Additionally, CD3^{+} T cells were analyzed for NKp46.

CD3^{+}NKp46^{+} lymphocytes were analyzed in PBMC of 50 healthy 3-7 months old pigs and compared to the three NKp46-defined NK-cell subsets in regard to their NKp46 and CD8α expression levels (Figure 1B). CD3^{+}NKp46^{+} cells displayed intermediate expression levels of NKp46, with a median fluorescence intensity (MFI) comparable to the CD3^{-}NKp46^{+} NK-cell subset (CD3^{+}: 1429 ± 702, CD3^{-}:1368 ± 1148, Figure 1B, upper graph). However, CD8α expression was significantly higher in the CD3^{+}NKp46^{+} cells (MFI 18015 ± 10129) compared to all three NK-cell subsets (NKp46^{-}: MFI 10195 ± 5063, NKp46^{+}: MFI 9246 ± 4825, NKp46^{high}: MFI 4724 ± 3077, Figure 1B, bottom graph). Therefore, the CD8α expression level of CD3^{+}NKp46^{+} lymphocytes resembles the CD8α^{high} phenotype of CD4^{-}TCR-αβ^{+} cytolytic T cells in swine (33, 34).

Although the CD3^{+}NKp46^{+} subset could be identified in the blood of all animals analyzed, these cells represented a small lymphocyte population (0.1 – 3.0%, Figure 1C). This low abundance was comparable to the frequency of the CD8α[dim/-]NKp46^{high} NK-cell subset in blood (0.1 – 1.7%). Since it could be shown that the NKp46-defined NK-cell subsets show different homing preferences in the pig (15, 16), different lymphatic as well as non-lymphatic

organs were analyzed for the frequencies of the CD3$^+$NKp46$^+$ lymphocytes (Figure 1D). In lymphatic organs, the proportion of CD3$^+$NKp46$^+$ lymphocytes was only slightly increased for spleen (0.2 - 3.1%) compared to blood (0.1 – 3.0%), whereas numbers were even reduced in mediastinal lymph nodes (0.1% - 1.4%). However, increased frequencies of CD3$^+$NKp46$^+$ cells were found in non-lymphatic organs like liver (0.6% - 2.8%) and lung (0.9 – 10.6%). This might indicate that this lymphocyte population plays a role in the immune surveillance in these organs.
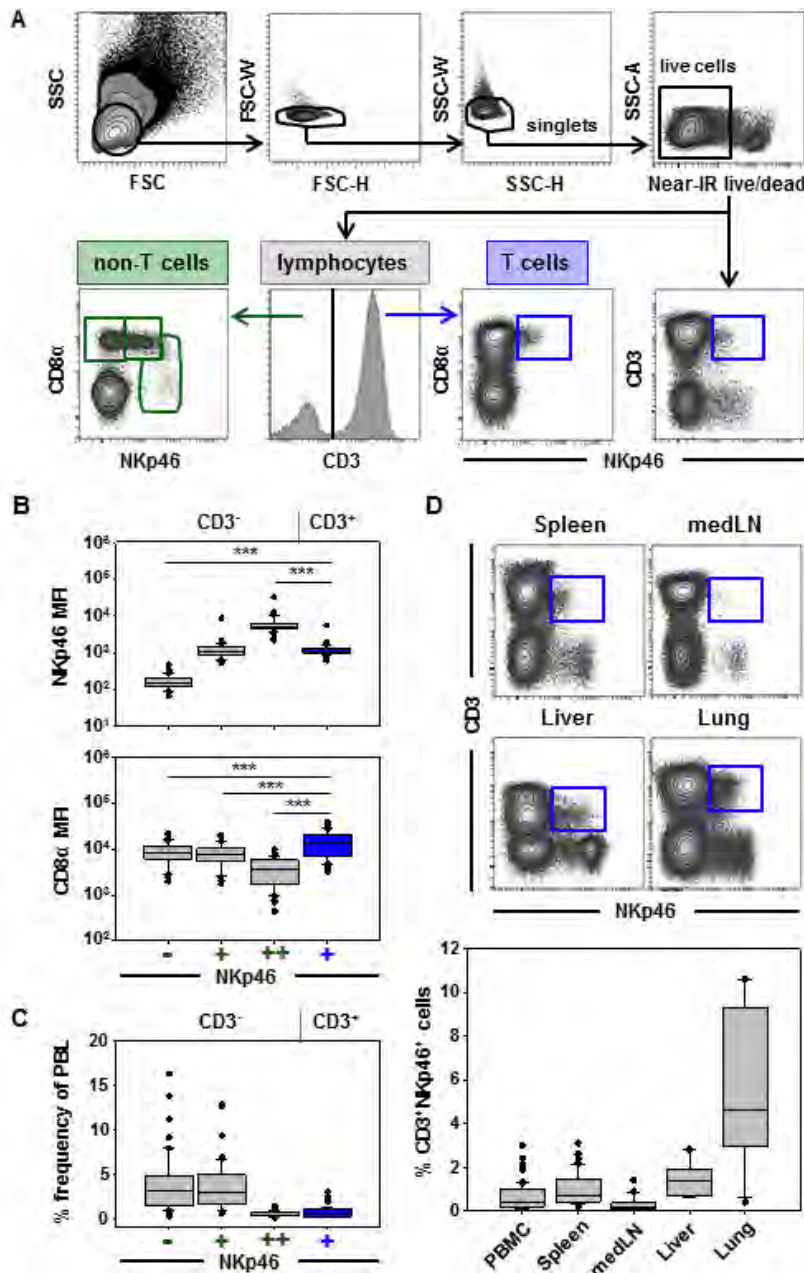
Figure 1: Porcine CD3⁺NKp46⁺ lymphocytes in lymphatic and non-lymphatic organs. (A) Lymphocytes were gated according to their light scatter properties. Potential doublet cells were excluded by consecutive FSC-H/FSC-W and SSC-H/SSC-W gates, followed by gating of Near-IR⁻ cells to exclude dead cells. Live lymphocytes were separated into CD3⁻ non-T cells to analyze CD8α/NKp46-defined NK-cell subsets (CD8α⁺NKp46⁻, CD8α⁺NKp46⁺ and CD8α^(dim/-)NKp46^high, green gates on the left) and CD3⁺ T cells to analyze CD3⁺NKp46⁺ lymphocytes (blue gate on the right). The gating strategy is shown as a representative example for PBMC and was performed correspondingly for all organs analyzed. **(B)** The three CD8α/NKp46-defined NK-cell subsets as well as the CD3⁺NKp46⁺ lymphocytes (shown in blue) in blood of 50 individuals were analyzed for their NKp46 (top) and CD8α (bottom) expression levels. Box-plots show the median fluorescence intensities of both markers within respective subsets. Significant differences between the CD3⁺NKp46⁺ cells and NK-cell subsets are indicated (*** = $p \leq 0.001$). **(C)** Box-plots show the frequencies of the four CD3/CD8α/NKp46-defined lymphocyte subsets in blood ($n = 50$). CD3⁺NKp46⁺ cells are indicated in blue. **(D)** CD3⁺NKp46⁺ cells (blue gates) were analyzed in lymphocytes isolated from blood ($n = 50$), spleen ($n = 40$), mediastinal lymph node (medLN, $n = 20$), liver ($n = 10$) and lung ($n = 10$). Representative examples of one animal are shown in the contour-plots. Box-plots show frequencies of CD3⁺NKp46⁺ lymphocytes within respective organs. All results were obtained from healthy 3-7 month old pigs.

CD3⁺NKp46⁺ lymphocytes show a mixed NK-/T-cell phenotype

For further phenotypic characterization of CD3⁺NKp46⁺ lymphocytes, the expression of T-cell associated markers was analyzed in blood of individual animals by FCM (Figure 2, CD3⁺NKp46⁺ cells shown in blue). In most animals a co-expression of CD8α and CD8β could be observed on the majority of CD3⁺NKp46⁺ cells (Figure 2A+B). Nevertheless, also CD8β⁻ cells could be identified to varying degrees with a frequency of up to 50% of CD3⁺NKp46⁺ lymphocytes in individual animals (Figure 2A, animal Sw#2). Partially, this CD8β⁻ phenotype was associated with co-expression of the TCR-γδ (Figure 2A+B). Overall results from 24 animals (Figure 2B) showed that the vast majority of CD3⁺NKp46⁺ lymphocytes co-expressed the CD8αβ heterodimer, therefore showing phenotypic accordance with porcine cytotoxic T lymphocytes. About 10% of the CD3⁺NKp46⁺ cells expressed the TCR-γδ, which coincided with a CD8αα homodimer expression. No obvious expression of CD4 could be observed within the CD3⁺NKp46⁺ cells in blood of all animals (data not shown).

In a next step, CD3⁺NKp46⁺ lymphocytes were analyzed for further markers associated with either NK cells or T cells (Figure 2C). Therefore, lymphocytes derived from blood (Figure 2C, left side), lymph node and spleen (data not shown) as well as lung (Figure 2C, right side)

were analyzed by FCM. CD2, that is expressed on NK cells as well as T cells was present on all CD3$^+$NKp46$^+$ lymphocytes. The vast majority of CD3$^+$NKp46$^+$ cells was negative for the T-cell related markers CD5 and CD6. However, most cells expressed CD16, that is highly associated with a NK-cell phenotype in the pig (35). CD27 as well as SLA-DR, both expressed on subpopulations of porcine NK and T cells (30, 34), likewise were present to various degrees on CD3$^+$NKp46$^+$ cells. Comparable results for all analyzed surface markers could also be observed for lymph node and spleen (data not shown). Additionally, CD8β was included in the staining panel to investigate potential correlations in regard to the CD8β$^{-/+}$ subsets within CD3$^+$NKp46$^+$ lymphocytes. No obvious differences for most cell surface markers analyzed could be observed within the CD8β$^-$ and CD8β$^+$ subsets. Nevertheless, the majority of CD8β$^-$ CD3$^+$NKp46$^+$ cells expressed CD27, while a more heterogeneous CD27 expression pattern was observed within the CD8β$^+$ subset (Figure 2C, bottom row).
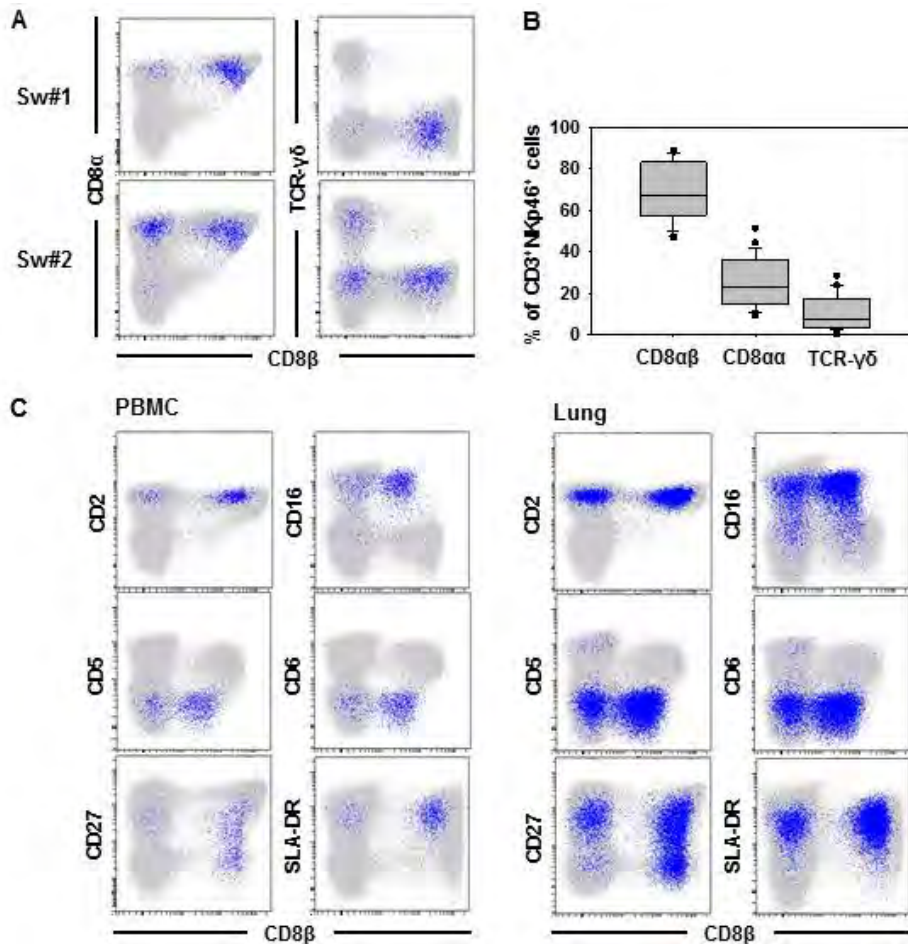
Figure 2: Phenotype of porcine CD3+NKp46+ lymphocytes in blood and lung. CD3+NKp46+ lymphocytes derived from blood and lung were analyzed for their expression of different NK- as well as T-cell associated surface markers by multi-color FCM. CD3+NKp46+ cells are indicated in blue, total live lymphocytes are shown in light gray as background. **(A)** Co-expression of CD8α/CD8β/TCR-γδ on CD3+NKp46+ blood lymphocytes for two representative animals. **(B)** Box-plots show the frequencies of CD8α+/CD8β+/TCR-γδ+ cells within CD3+NKp46+ lymphocytes of 24 animals. **(C)** Expression of CD2/CD5/CD6/CD16/CD27/SLA-DR and CD8β in CD3+NKp46+ cells derived from blood (left) and lung (right) for one individual animal. Data is representative for analyses of six different individuals.

In addition to NKp46, we further wanted to investigate whether other NK-associated activating receptors like NKG2D as well as NKp30 and NKp44 were expressed on the CD3+NKp46+ lymphocytes. Due to the lack of species-specific mAbs for these markers, FACS-sorted lymphocyte subsets were analyzed by quantitative RT-PCR (Figure 3). PBMC of four animals were FACS-sorted in the CD3+NKp46+ subset (Figure 3A, shown in blue) and compared to either CD3+NKp46- T cells (shown in gray) or CD3- total NK cells (shown in

green). For NKp30, NKp44 as well as NKG2D similar mRNA expression levels between CD3$^+$NKp46$^+$ lymphocytes and total NK cells were found, whereas reduced expression levels were observed for T cells (Figure 3B).
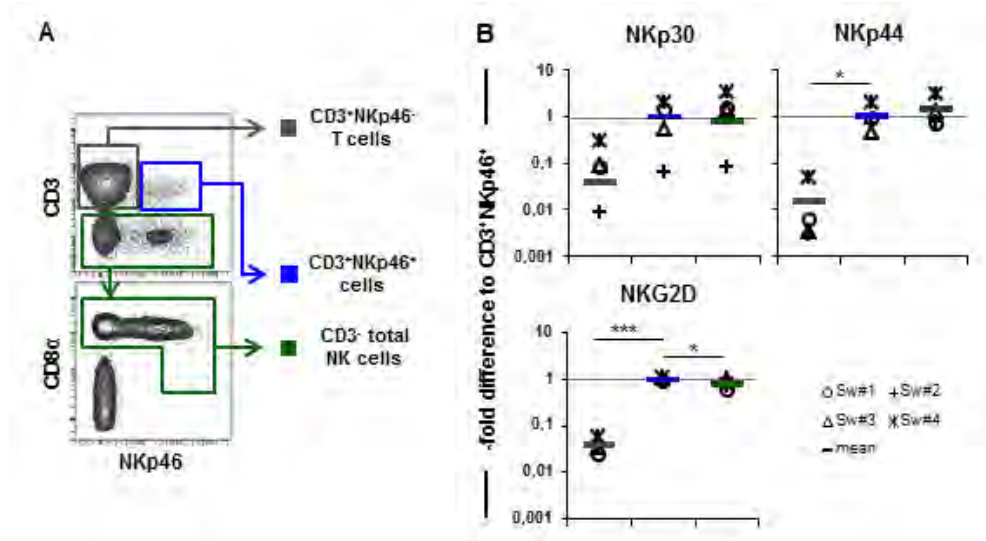

Figure 3: Expression of NK-associated receptor mRNAs in CD3$^+$NKp46$^+$ lymphocytes. (A) PBMC were FACS sorted into CD3$^+$NKp46$^-$ T cells (gray), CD3$^+$NKp46$^+$ cells (blue) and total CD3$^-$ NK cells (green). **(B)** Sorted lymphocyte subsets were analyzed for their mRNA expression levels of NKp30, NKp44 and NKG2D by quantitative RT-PCR. The 2$^{-\Delta\Delta Ct}$ values for the target genes of each individual animal (*n = 4*) are shown as fold differences relative to the mean of CD3$^+$NKp46$^+$ cells from all animals that was set to a value of 1. Colored lines represent mean values of the respective lymphocyte subsets. Significant differences between the CD3$^+$NKp46$^+$ cells and T as well as NK cells are indicated (* = *p ≤ 0.05*, *** = *p ≤ 0.001*).

Therefore, phenotypic analysis indicated a mixed phenotype of CD3$^+$NKp46$^+$ lymphocytes, comprising both, NK- and T-cell associated molecules.

CD3$^+$NKp46$^+$ lymphocytes respond to cytokine stimulation in a NK-like fashion

In a next series of experiments we wanted to address whether CD3$^+$NKp46$^+$ lymphocytes share functional properties with either NK cells or T cells. Therefore, IFN-γ production (Figure 4) as well as proliferative capacity (Figure 5) of blood-derived CD3$^+$NKp46$^+$ cells (shown in blue) were investigated and compared to CD3$^+$NKp46$^-$ T cells (shown in gray) as well as CD3$^-$ total NK cells (shown in green).

For detection of intracellular IFN-γ production (Figure 4) PBMC of six animals were stimulated either for four hours with PMA/Ionomycin or a combination of rpIL-2, rpIL-12 and rpIL-18 for 24 hours. This cytokine combination effectively induces IFN-γ production in porcine NK cells, as shown in previous studies (15, 16, 31). Cells cultured in medium alone did not show any cytokine production (Figure 4A+B, "Medium"). PMA/Ionomycin stimulation induced moderate frequencies of IFN-γ producing cells in all three lymphocyte subsets (Figure 4A+B, "PMA/Ionomycin"). Although the average number of IFN-γ+ cells was lower in T cells (6.2% ± 3.2) compared to CD3+NKp46+ (22.5% ± 11.6) and NK cells (13.6% ± 6.7, Figure 4B), T cells showed at least twice as much IFN-γ production on a per-cell level after PMA/Ionomycin stimulation, indicated by the increased MFI (Figure 4B). As expected, cytokine stimulation induced high frequencies of IFN-γ producing NK cells (42.7% ± 9.1, Figure 4A+B, "IL-2+IL-12+IL-18 low"), which were even more increased with higher levels of rpIL-18 (71.0% ± 5.7, Figure 4A+B, "IL-2+IL-12+IL-18 high"). Comparable frequencies could be observed for CD3+NKp46+ lymphocytes with an average number of 49.8% ± 11.2 (IL-18 low) and 74.8% ± 6.9 (IL-18 high) IFN-γ+ cells. Additionally, IFN-γ production on a per-cell level could be enhanced with higher levels of IL-18 in both subsets (Figure 4B). Although IFN-γ+ T cells also slightly increased with higher concentrations of rpIL-18 (IL-18 low: 11.5% ± 7.0; IL-18 high: 16.1% ± 6.5), the average number of IFN-γ+ T cells was several-fold lower than in the other two lymphocyte subsets. Expression of CD8β was investigated along with IFN-γ production to address potential functional differences in regard to the CD8β-/+ phenotypes of CD3+NKp46+ cells. However, no obvious CD8β-correlated differences for IFN-γ+ subsets could be observed in all animals tested (Figure 4A and data not shown).
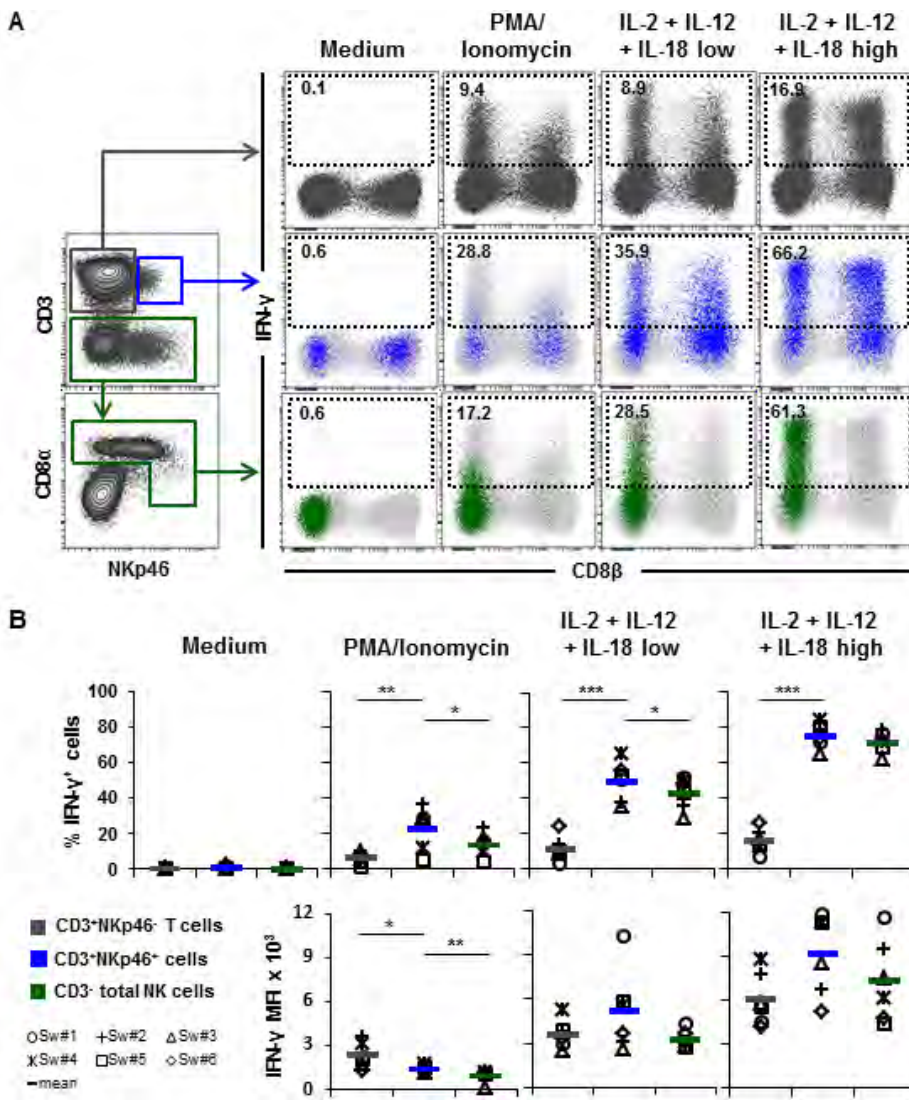
Figure 4: IFN-γ production of CD3+NKp46+ lymphocytes. PBMC were stimulated either with PMA/Ionomycin for four hours or with a combination of rpIL-2 + rpIL-12 + rpIL-18 for 24 hours. IL-18 was used at two different concentrations (low: 5 ng/ml, high: 25 ng/ml). Cells cultured in medium alone served as negative control. Following stimulation, intracellular IFN-γ expression of CD3+NKp46+ lymphocytes (blue) was analyzed and compared to CD3+NKp46- T cells (gray) and total CD3- NK cells (green). **(A)** IFN-γ expression of the respective lymphocyte subsets is shown in the dot-plots on the right in combination with CD8β for one representative animal. Analyzed cell subsets are highlighted in color, total lymphocyte population is shown in light gray in the background. Percentages of IFN-γ+ cells within respective subsets are indicated. **(B)** Frequencies of IFN-γ+ cells (upper row) as well as median fluorescence intensity of IFN-γ+ cells (bottom row) within the three analyzed lymphocyte subsets in blood of six animals are shown. Mean values are represented by colored bars. Significant differences between the CD3+NKp46+ cells and T as well as NK cells are indicated (* = *p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001*).

To assess proliferation, Violet-labeled PBMC of six animals were stimulated for four days with either ConA or a combination of rpIL-2 and rpIL-15 (Figure 5). Since CD3-downregulation on T cells can be observed after prolonged *in vitro* stimulation (30), the gating strategy for NK cells in this setup was further extended by gating on CD3$^-$CD16$^+$ cells to exclude "CD3 false-negative" cells in the CD3$^-$ gate (Figure 5A). As expected, T cells vigorously proliferated following ConA stimulation (79.2% ± 7.2, Figure 4A+B, "ConA"). In contrast, minor proliferation rates after mitogen stimulation were observed for CD3$^+$NKp46$^+$ cells (5.3% ± 1.8) and NK cells (5.0% ±3.2). After stimulation with rpIL-2 and rpIL-15, CD3$^+$NKp46$^+$ cells showed high proliferation rates (71.4% ± 8.7, Figure 4A+B, "IL-2+IL-15") that slightly exceeded those of NK cells (59.0% ± 6.9), whereas T cells only showed a moderate response to cytokine stimulation (28.9% ±10.0). Unstimulated cells showed minor proliferation rates in all observed lymphocyte populations (Figure 5A+B, "Medium"). Similar to IFN-γ production, we could not observe any CD8β-correlated differences for proliferation within the CD3$^+$NKp46$^+$ population (data not shown).
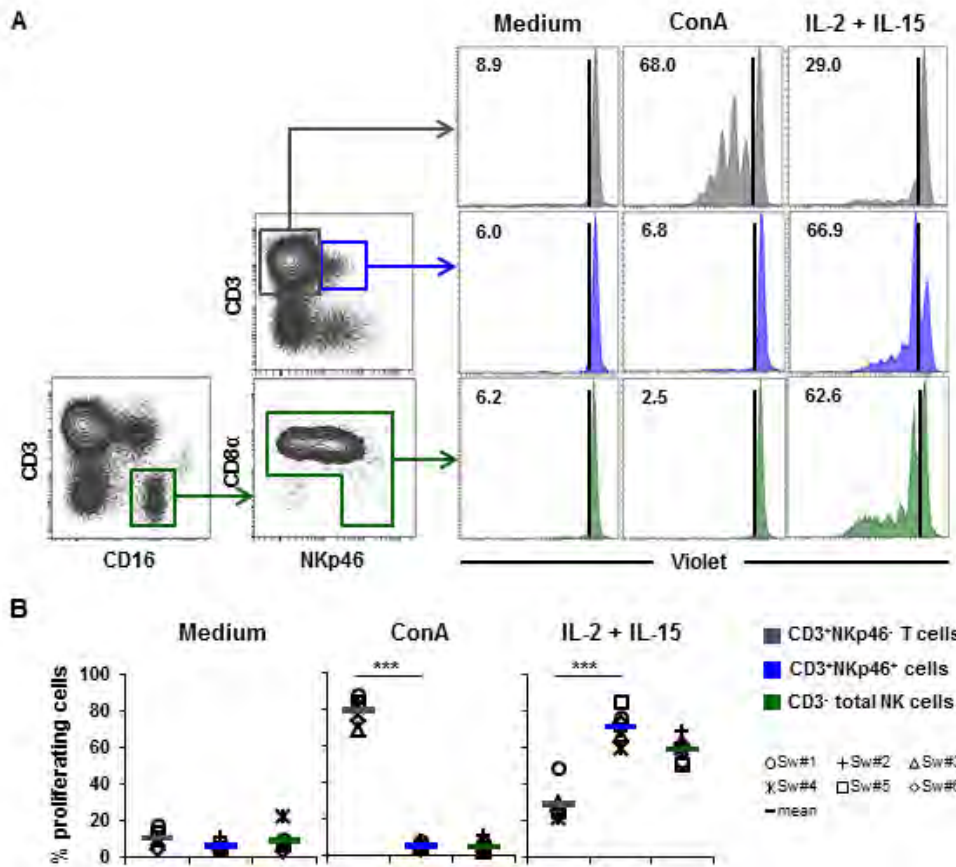
Figure 5: Proliferative capacity of CD3+NKp46+ lymphocytes. PBMC were stained with Violet Cell Trace Dye to analyze proliferating cells following stimulation with either ConA or rpIL-2 and rpIL-15 for four days. Cells cultured in medium alone served as negative control. Proliferation of CD3+NKp46+ lymphocytes (blue) was compared to CD3+NKp46- T cells (gray) and total CD3- NK cells (green). For NK cells an additional pre-gating on CD3-CD16+ was performed. **(A)** Histograms show proliferation of the different lymphocyte subsets following respective stimulation for one representative animal. Percentages of proliferating cells within respective subsets are indicated. **(B)** Frequencies of proliferating cells within the three analyzed lymphocyte subsets in blood of six animals are shown. Mean values are represented by colored bars. Significant differences between the CD3+NKp46+ cells and T as well as NK cells are indicated (*** = $p \leq 0.001$).

Taken together, CD3+NKp46+ lymphocytes showed proliferation rates and IFN-γ production similar to NK cells following cytokine stimulation.

CD3+NKp46+ lymphocytes display cytolytic properties comparable to NK cells

One of the major properties of NK cells is their ability to spontaneously kill susceptible target cells, mediated by effector molecules like perforin. Since the functional properties of CD3+NKp46+ lymphocytes investigated so far were rather comparable to NK cells, we investigated if they likewise show these properties.

Ex vivo perforin expression was analyzed by FCM in lymphocytes isolated from blood and lung of six animals (Figure 6A+B). As negative reference, CD3-CD8α- cells, mainly consisting of B cells, were analyzed in parallel (Figure 6A, shown in light gray). CD3+NKp46+ cells derived from blood and lung showed perforin expression levels (MFI of 652 ± 88 for blood and 667 ± 54 for lung) that were close to expression levels of NK cells (average MFI of 857 ± 118 for blood and 782 ± 117 for lung, respectively). Total T cells on the other hand only showed minor perforin expression in both organs.

Additionally, the ability of CD3+NKp46+ lymphocytes to kill the human leukemia cell line K562 was investigated (Figure 6C). Therefore, PBMC of four animals were FACS-sorted in CD3+NKp46- T cells, CD3- total NK cells as well as the CD3+NKp46+ subset and tested in a 4-hour cytotoxic assay after stimulation with rpIL-2 and rpIL-15 for 36 hours. As expected, T cells showed hardly any cytolytic activity towards the K562 cell line at any E:T ratio. In contrast, NK cells efficiently lysed the target cells, reaching specific lysis of 67.3% ± 6.1 at the highest E:T ratio of 20:1. Likewise, CD3+NKp46+ lymphocytes showed spontaneous lytic activity in an E:T-ratio dependent manner with only slightly reduced levels compared to total NK cells (average specific lysis of 54.5% ± 8.5 at an E:T ratio of 20:1). These data suggest that CD3+NKp46+ lymphocytes are capable of spontaneous lytic activity, comparable to NK cells.
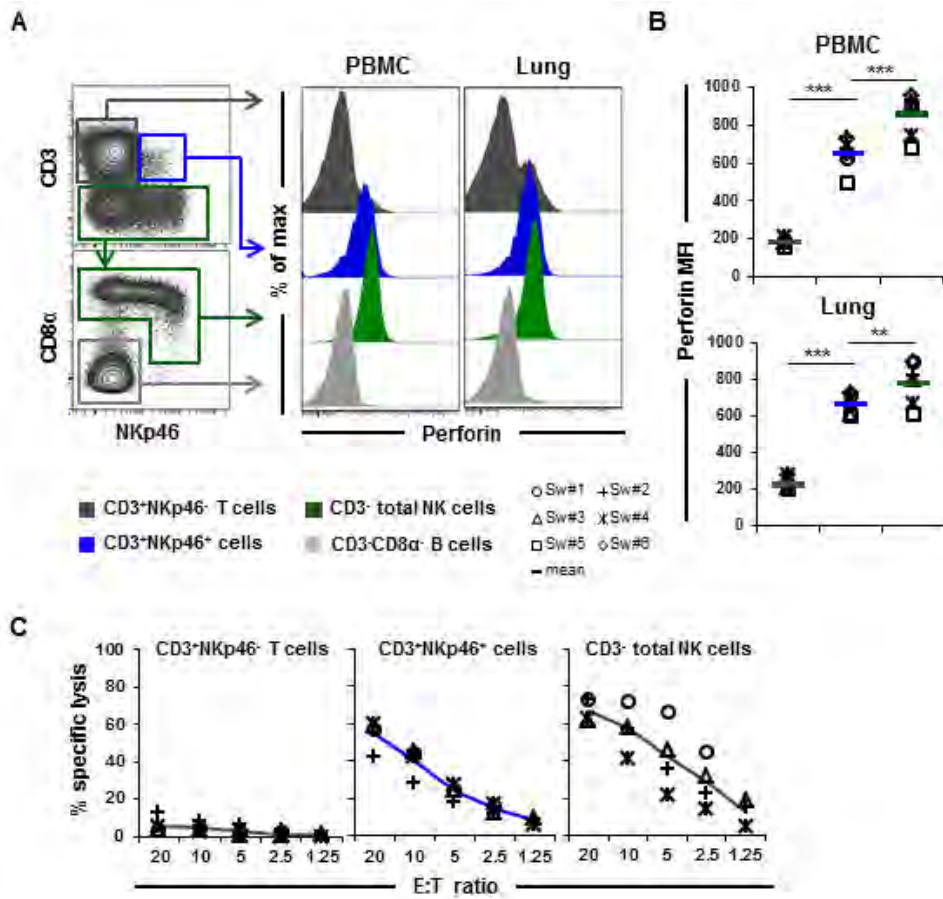
Figure 6: Cytolytic properties of CD3⁺NKp46⁺ lymphocytes. (A) Lymphocytes derived from blood and lung were analyzed for perforin expression. Histograms show the perforin expression levels of CD3⁺NKp46⁺ cells (blue) compared to CD3⁺NKp46⁻ T cells (gray) and total CD3⁻ NK cells (green) for one representative animal. Results for CD3⁻CD8α⁻ cells, mainly consisting of B cells, are shown in light gray and serve as negative reference. **(B)** Median fluorescence intensities of perforin within respective lymphocyte subsets are shown for PBMC and lung for six animals. Mean values are represented by colored bars. Significant differences between the CD3⁺NKp46⁺ cells and T as well as NK cells are indicated (** = $p \leq 0.01$, *** = $p \leq 0.001$). **(C)** FACS-sorted CD3⁺NKp46⁻ T cells (gray), CD3⁺NKp46⁺ cells (blue) and total CD3⁻ NK cells (green) from blood were stimulated with rpIL-2 and rpIL-15 for 36 hours and subsequently used in a 4h-cytotoxic assay with K562 as target cell line. Respective lymphocyte subsets were tested at five different E:T ratios: 20:1, 10:1, 5:1, 2.5:1 and 1.25:1. Results obtained from analyses of four animals are shown. Colored lines represent mean values of the respective lymphocyte subsets.

Cross-linking of NKp46 and CD3 leads to degranulation of CD3+NKp46+ lymphocytes

Since the data so far indicated that CD3$^+$NKp46$^+$ cells functionally resemble NK cells, we further addressed the functionality of NKp46 and CD3 molecules on this lymphocyte subset. For this purpose, PBMC of four animals were stimulated with plate-bound antibodies against either NKp46 or CD3 to analyze activation of the CD3$^+$NKp46$^+$ cells (Figure 7).

Triggering of NKp46 was already shown to induce cytolytic activity in porcine NK cells (15, 16). Therefore in a first attempt, degranulation of CD3$^+$NKp46$^+$ cells (shown in blue) was analyzed in a CD107a assay by FCM (Figure 7A) after NKp46 cross-linking. Total NK cells (shown in green) were analyzed in parallel. Ligation of NKp46 resulted in degranulation of NK cells, shown by a 5-fold increase in CD107a$^+$ cells (36% ± 11) compared to isotype-matched control antibodies. Likewise, triggering of NKp46 on CD3$^+$NKp46$^+$ lymphocytes led to an increase in the frequency of CD107a$^+$ cells (16% ± 3 compared to 3% ± 2). Triggering of CD3 also induced degranulation in CD3$^+$NKp46$^+$ cells (26% ± 7 CD107a$^+$ cells). An increase in CD107a$^+$ cells could also be observed within CD3-stimulated total T cells (shown in gray). These results indicate that NKp46 and the CD3/TCR complex are functional in CD3$^+$NKp46$^+$ lymphocytes.

In addition to cytolytic activity, NKp46 cross-linking is known to induce IFN-γ production in NK cells. Nevertheless, experiments with porcine PBMC showed that although IFN-γ$^+$ cells could be detected in NK cells after cross-linking of NKp46 (14% ± 7, Figure 7B), no obvious IFN-γ production could be observed within CD3$^+$NKp46$^+$ lymphocytes compared to isotype-matched control antibodies (Figure 7B). Similarly, no IFN-γ production could be observed in CD3$^+$NKp46$^+$ lymphocytes as well as CD3$^+$NKp46$^-$ T cells after CD3 cross-linking. Furthermore, cross-linking of CD3 did not induce proliferation in CD3$^+$NKp46$^+$ cells in contrast to T cells where about half of the cells proliferated (60% ± 11, Figure 7C). Interestingly, proliferation could be detected in total NK cells after NKp46 cross-linking (30% ± 12) but no such effect was observed for the CD3$^+$NKp46$^+$ subset.
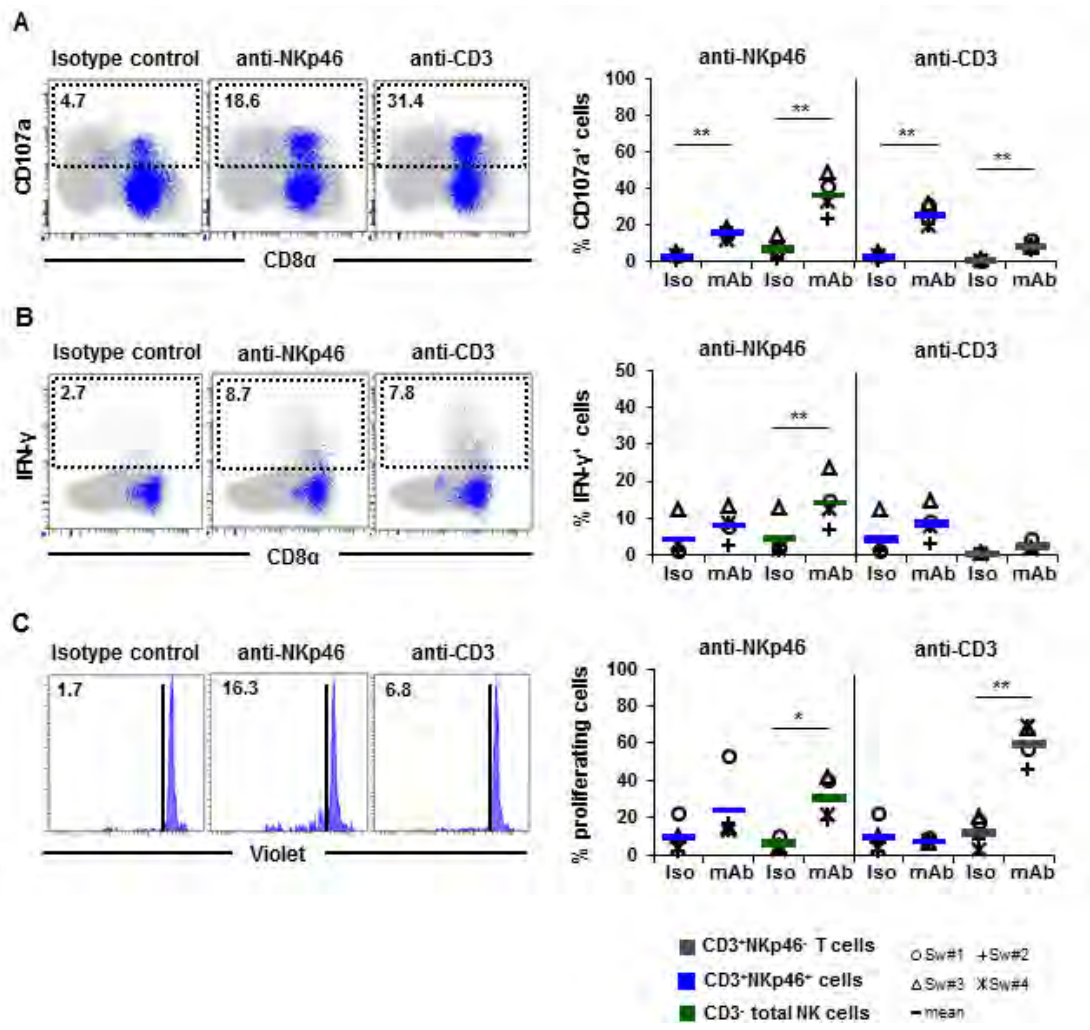
Figure 7: Activation of CD3+NKp46+ lymphocytes by receptor cross-linking. Receptor- mediated degranulation, IFN-γ production and proliferation was assessed by multi-color FCM in response to cross-linking of NKp46 or CD3 by plate-bound mAbs. Isotype-matched irrelevant antibodies served as control. Activation of CD3+NKp46+ lymphocytes (blue) derived from blood of four different individuals was analyzed and compared to either total CD3- NK cells (green) or CD3+NKp46- T cells (gray). Gating for FCM analyses was performed as displayed in the previous figures. **(A)** PBMC were pre-activated with rpIL-2 and rpIL-15 for 24 hours and receptor-mediated degranulation was assessed by CD107a expression on the cell surface after four hours incubation with plate-bound mAbs. **(B)** PBMC were pre-activated with rpIL-2 and rpIL-18 for 24 hours and intracellular IFN-γ production was measured after receptor cross-linking for four hours. **(C)** Total PBMC were stained with Violet Cell Trace Dye and cultured for four days in the presence of plate-bound mAbs. (**A-C**) Dot-plots and histograms show results of CD3+NKp46+ lymphocytes for one representative animal. CD3+NKp46+ cells are shown in blue, total lymphocytes are shown in light gray as background. Frequencies of CD107a+ and IFN-γ+ as well as proliferating cells within lymphocyte subsets are shown for analyses of four animals in the graphs. Mean values are represented by colored bars. Significant differences between stimulated and non-stimulated CD3+NKp46+ cells, T and NK cells are indicated (* = $p \leq 0.05$, ** = $p \leq 0.01$).

CD3$^+$NKp46$^+$ lymphocytes are recruited from blood to lung early after influenza infection

Recently, it was demonstrated that NKp46$^+$ lymphocytes accumulate in the vicinity of influenza A-infected cells in the lung of infected pigs (17). To investigate the possibility that besides NKp46$^+$ NK cells also CD3$^+$NKp46$^+$ cells might be involved in this process, this lymphocyte population was analyzed in animals experimentally infected with the 2009 pandemic H1N1 influenza virus strain. Frequencies of CD3$^+$NKp46$^+$ lymphocytes were analyzed in blood and lung at days 1-3 post infection (p.i.) and compared to non-infected control animals. A significant decrease in the total numbers of CD3$^+$NKp46$^+$ lymphocytes in PBMC could be observed in infected animals one day p.i. compared to control animals (Figure 8A+B). Furthermore, a significant increase in the frequency of CD3$^+$NKp46$^+$ lymphocytes could be detected in the lungs of infected animals compared to the control group on day three p.i. (Figure 8C). To investigate proliferative activity of CD3$^+$NKp46$^+$ cells, expression of Ki-67 was analyzed. Compared to analyses from day one p.i., a significant increase in the MFI of Ki-67 could be detected within CD3$^+$NKp46$^+$ cells in the lung on day three p.i. (Figure 8D). The MFI was also significantly higher compared to Ki-67 expression in CD3$^+$NKp46$^+$ cells of the control group.
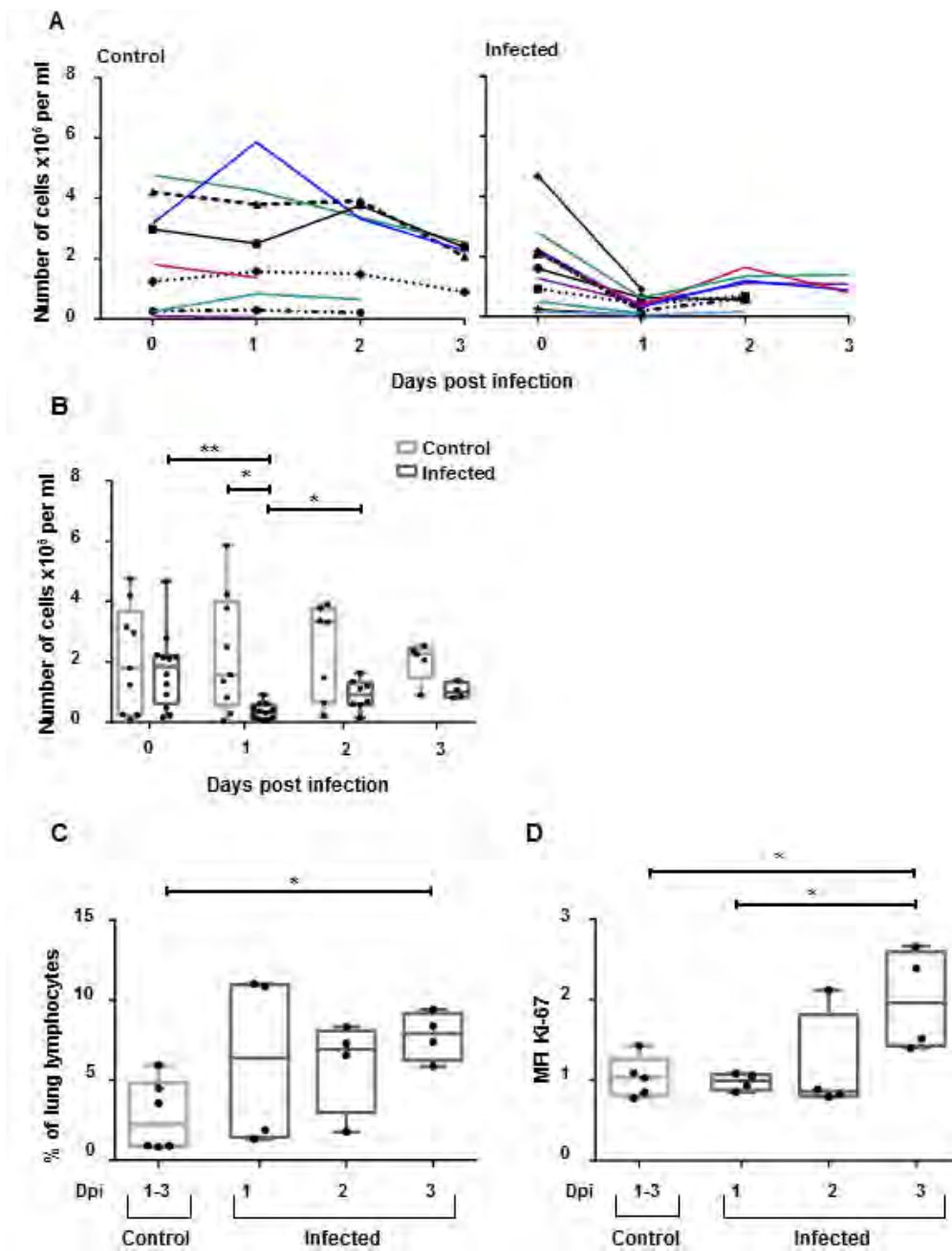
Figure 8: CD3+NKp46+ lymphocytes in influenza A infected piglets. CD3+NKp46+ lymphocytes from blood and lung of influenza A infected piglets and healthy control animals were analyzed by flow cytometry on days 1-3 after infection. **(A)** Absolute numbers of CD3+NKp46+ cells in PBMC of controls (left, n = 9) as well as infected animals (right, n =12) are shown in the course of influenza infection. **(B)** Box-plots show absolute numbers of CD3+NKp46+ cells in PBMC on individual days post infection. **(C)** Frequencies of CD3+NKp46+ cells among lung lymphocytes in control (n = 6) and infected animals (n = 12) in the course of influenza infection. **(D)** Box-plots show median fluorescence intensity of Ki-67 in CD3+NKp46+ lung cells. Significant differences between infected and control animals as well as distinct study days are indicated (* = $p \leq 0.05$, ** = $p \leq 0.01$).

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Discussion

Expression of the activating receptor NKp46 in combination with lack of a TCR/CD3 complex is commonly used to describe NK cells in most mammalian species (9). We could identify a lymphocyte subset in pig with surface expression of both, NKp46 and CD3, thereby displaying a mixed NK-/T-cell phenotype.

Likewise, rare populations of CD3^{+}NKp46^{+} lymphocytes have been identified in other species (9, 18-22, 24, 25, 36) and have been either assigned to TCR-αβ^{+} or TCR-γδ^{+} T cells. These NKp46^{+} T-cell subsets show very heterogeneous phenotypes and seem to be derived from different progenitors rather than representing one distinct lymphocyte population. In most cases they are described as T cells acquiring NK-cell properties upon specific stimuli. This was mainly reported to be a result of chronic activation of the TCR (19), chronic infections and inflammatory conditions (21, 22, 36) as well as prolonged stimulation with IL-15 (23, 37). Additionally, genetic re-programming of T cells into NKp46^{+} cells after transcription factor deletion has been described in mice (38). However, CD3^{+}NKp46^{+} lymphocyte subsets that are present in apparently healthy individuals have been reported in human, mouse, cattle and dog (9, 20, 24, 25), although at very low frequencies. The porcine CD3^{+}NKp46^{+} cells described in this study were also identified in clinically healthy animals but this does not exclude the induction of these cells in certain physiological or pathologic conditions. Indeed, we could show that stimulation with IL-2 and IL-15 led to *in vitro* expansion of this cell population. Also, in influenza-infected animals an increase of Ki-67 expression in lung-derived CD3^{+}NKp46^{+} cells was observed.

Beside the expression of CD3, the majority of porcine CD3^{+}NKp46^{+} lymphocytes expressed the CD8αβ heterodimer and therefore displays phenotypic characteristics of porcine cytolytic T cells (33, 34). Additionally, a minor population of CD8β^{-} CD3^{+}NKp46^{+} cells could be identified that belongs to TCR-γδ lymphocytes. Yet, porcine CD3^{+}NKp46^{+} cells did not express CD5 and CD6, markers that are normally associated with a T-cell phenotype in the pig (34, 39, 40). Instead other NK-associated markers were expressed by CD3^{+}NKp46^{+} lymphocytes at comparable levels to porcine NK cells, like the activating receptors CD16 and NKG2D and the

NCR family members NKp30 and NKp44. Likewise, the effector molecule perforin was found to be expressed in all CD3$^+$NKp46$^+$ cells. Thus, indicating that these cells have more phenotypic similarities with porcine NK cells. Earlier studies in pigs describe a CD3$^+$ lymphocyte population that also expressed CD16 and perforin (35). The majority of these cells was CD5$^-$CD6$^-$ and was suggested to represent porcine NKT cells. The CD3$^+$NKp46$^+$ cells thus might also belong into this category of non-conventional T cells. In a more recent publication a small porcine lymphocyte subset reacting with α-galactosylceramide-loaded CD1d tetramers was described and defined as porcine invariant NKT (iNKT) cells (41). As these cells were described to have a CD5^{high}CD16$^{low/-}$ phenotype, porcine CD3$^+$NKp46$^+$ cells might rather belong to non-CD1d-restricted NKT-like cells.

Despite their mixed NK-/T-cell phenotype, functional properties of the porcine CD3$^+$NKp46$^+$ lymphocytes resemble those of NK cells. Whereas no proliferation could be detected after ConA stimulation, CD3$^+$NKp46$^+$ cells responded in a NK-like manner to cytokine stimulation with proliferation and increased IFN-γ production. Moreover, porcine CD3$^+$NKp46$^+$ lymphocytes showed spontaneous killing activity against the NK-susceptible target cell line K562, comparable to porcine NK cells. As it was already demonstrated that killing of K562 cells by porcine NK cells is NKp46-independent (15), CD3$^+$NKp46$^+$ lymphocytes must harbor other mechanisms for recognition and killing of xenogeneic target cells. Comparable to our results, most of the CD3$^+$NKp46$^+$ subsets identified in mouse (19, 20) and human (20, 22) produced IFN-γ in response to cytokines or triggering of NKp46 receptor. This ability might be closely linked to the expression of the activating receptor itself, as it was shown that induction of NKp46 expression during NK-cell maturation was associated with a higher ability to secrete IFN-γ in response to IL-12 and IL-18 (10). Interestingly, in cattle only an *in vitro* generated TCRγδ$^+$NKp46$^+$ lymphocyte subset was capable of producing IFN-γ in response to cytokines (23), whereas the CD3$^+$NKp46$^+$ lymphocytes found *ex vivo* did not respond to cytokine stimulation or receptor triggering (24). Additionally, we could show that porcine CD3$^+$NKp46$^+$ cells have a functional NKp46 signaling pathway, as triggering of the receptor led to CD107a up-regulation. Likewise, cytolytic function of CD3$^+$NKp46$^+$ cells after receptor activation could be found in human NKp46$^+$ CTL (22) and the bovine CD3$^+$NKp46$^+$ non-conventional T cells (24).

Although functional NK-cell characteristics dominate in porcine CD3+NKp46+ lymphocytes, we could also demonstrate that CD3 stimulation by plate-bound mAbs leads to degranulation of CD3+NKp46+ cells. This indicates a functional TCR-complex. Up to date, analyses on TCR-αβ expression are hampered by the lack of anti-porcine TCR-αβ specific mAbs. Nonetheless, surface expression of the CD3ε chain is strongly connected to TCR co-expression as assembly with the TCR chains is essential for transport of CD3ε to the cell surface (42, 43). Therefore, identification of the CD3+NKp46+ lymphocytes by using mAbs specific for the extracellular part of the porcine CD3ε chain (44, 45) suggests expression of TCR-complexes on these cells. This assumption is further supported by the identification of a small TCR-γδ expressing fraction of porcine CD3+NKp46+ lymphocytes. NK cells on the other hand do not express CD3 proteins with the exception of CD3ζ that serves as a signaling adaptor molecule (46). Rare CD3ε transcripts and proteins could so far only be detected in human fetal NK cells and in *in vitro* generated NK cell clones, but are apparently restricted to the cytoplasm (47, 48).

Nevertheless, analyses of the TCR repertoire would be relevant for a further characterization of porcine CD3+NKp46+ cells, an issue we aim to address in the future. So far, a highly diverse TCR repertoire was found in bovine CD3+NKp46+ cells (24) whereas a rather restricted TCR repertoire was found in the human NKp46+ CTL (22) as it was described for the semi-conserved TCR repertoires in most non-conventional T cells like iNKT and mucosal-associated invariant T (MAIT) cells (49).

At the moment it remains an open question if porcine CD3+NKp46+ lymphocytes can be activated in an antigen-specific manner via their TCR. Nevertheless, due to their NK-like phenotype and function, a potential role in the early defense against infections can be assumed. The role of NKp46-expressing NK cells in the course of influenza infections could already be shown in several studies. NKp46 can bind to hemagglutinin of influenza virus (5, 7) and seems to be actively involved in the defense against infection. Activation of NKp46 leads to killing of infected cells (7, 50) and absence of the receptor in mice results in a lethal outcome of the disease (51). Moreover, NKp46+ NK cells can be found in higher frequencies in lungs of infected mice (52). Likewise, in pig it could recently be shown that NKp46+ cells seem to be involved in the immune response against influenza. NKp46+ lymphocytes

accumulated in influenza-virus infected areas of the lung and it could be demonstrated that influenza HA is also recognized by porcine NKp46 in *in vitro* experiments (17). Comparable to the NKp46^{high} NK-cell subset in the pig (15, 17), also the porcine CD3^{+}NKp46^{+} lymphocytes seem to preferentially reside in non-lymphatic organs like the lung, indicating a role of these cells in the immune surveillance of this organ. Indeed, we could demonstrate a decrease of CD3^{+}NKp46^{+} cells in blood in the early course of influenza infection which is in line with results obtained with CD3^{-}NKp46^{+} NK cells (17). This decrease was accompanied by an increase of CD3^{+}NKp46^{+} cells in lungs of infected animals. Additionally, proliferation of these lung-derived cells could be shown by their elevated levels of Ki-67 expression. Interestingly, this increase in Ki-67 expression could only be detected in the CD3^{+}NKp46^{+} cells and not in CD3^{-}NKp46^{+} NK cells (17). Also in cattle a potential role of the CD3^{+}NKp46^{+} lymphocyte subset as an effector population has been described as they expand during infection with *Theileria parva* and show cytolytic activity towards *Theileria parva*-infected cells (24).

In conclusion, we could identify a CD3^{+}NKp46^{+} lymphocyte subset in swine that shows a mixed NK-/T-cell phenotype with dominating NK-cell properties. Nonetheless, besides NK-signaling pathways our data also suggest a functional CD3/TCR receptor complex, which may allow responsiveness to a broader range of pathogens. From this we propose to position porcine CD3^{+}NKp46^{+} lymphocytes at the interface between the innate and the adaptive immune system.

Conflict of Interest statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

KM, AS and WG were responsible for conception and design of the study. KM performed experiments, analyzed data and wrote the manuscript. MS and ST performed laboratory work and experiments. HF and AKS were responsible for the influenza infection study including experiments and data analyses. AM, CD and SH performed qPCR design, experiments and

data analyses. WG and AS interpreted data and supervised the study. All authors read and approved the final manuscript.

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Table 1 Primary antibodies and secondary reagents used for FCM analyses.

Antigen	Clone	Isotype	Fluorochrome	Labeling Strategy	Source of primary Ab
Phenotyping of isolated cells					
CD2	MSA4	IgG2a	Alexa488	secondary antibody[a]	in-house
CD3	PPT3	IgG1	BV421	Biotin-Streptavidin[b]	Southern Biotech
CD3	BB23-8E6-8C8	IgG2a	PerCP-Cy5.5	directly conjugated	BD Biosciences
CD5	b53b7	IgG1	Alexa488	secondary antibody[c]	in-house
CD6	a38b2	IgG1	Alexa488	secondary antibody[c]	in-house
CD8α	76-2-11	IgG2a	Alexa488 or PE-Cy7	secondary antibody[a] or [d]	in-house
CD8β	PPT23	IgG1	PE	secondary antibody[e]	in-house
CD8β	PG164A	IgG2a	PE	secondary antibody[f]	VMRD
CD8β	PPT23	IgG1	Alexa488	directly conjugated	in-house
CD16	G7	IgG1	Alexa488	secondary antibody[c]	AbD Serotec*
CD27	b30c7	IgG1	BV421	Biotin-Streptavidin[b]	in-house
TCR-γδ	PPT16	IgG2b	BV421	Biotin-Streptavidin[b]	in-house
SLA-DR	MSA3	IgG2a	Alexa488	secondary antibody[a]	in-house
NKp46	VIV-KM1	IgG1	Alexa647	directly conjugated	in-house
Perforin	δG9	IgG2b	PE	directly conjugated	eBioscience**
FACS sort					
CD3	BB23-8E6	IgG2b	Alexa488	secondary antibody[g]	Southern Biotech
CD8α	11/295/33	IgG2a	Alexa647	secondary antibody[h]	in-house
NKp46	VIV-KM1	IgG1	PE	secondary antibody[e]	in-house
Intracellular cytokine staining					
CD3	BB23-8E6-8C8	IgG2a	PerCP-Cy5.5	directly conjugated	BD Biosciences
CD8α	11/295/33	IgG2a	FITC	directly conjugated	in-house
CD8β	PPT23	IgG1	BV605	Biotin-Streptavidin[i] and [j]	in-house
NKp46	VIV-KM1	IgG1	Alexa647	directly conjugated	in-house
IFN-γ	P2G10	IgG1	PE	directly conjugated	BD Biosciences
Violet proliferation assay					
CD3	BB23-8E6-8C8	IgG2a	PerCP-Cy5.5	directly conjugated	BD Biosciences
CD8α	76-2-11	IgG2a	PE	directly conjugated	BD Biosciences
CD8β	PPT23	IgG1	BV605	Biotin-Streptavidin[i] and [j]	in-house
CD16	G7	IgG1	FITC	directly conjugated	AbD Serotec
NKp46	VIV-KM1	IgG1	Alexa647	directly conjugated	in-house
CD107a degranulation assay					
CD3	BB23-8E6-8C8	IgG2a	PerCP-Cy5.5	directly conjugated	BD Biosciences
CD8α	76-2-11	IgG2a	PE	directly conjugated	BD Biosciences
NKp46	VIV-KM1	IgG1	Alexa647	directly conjugated	in-house
CD107a	4E9/11	IgG1	FITC	directly conjugated	AbD Serotec

a) goat anti-mouse anti-IgG2a-Alexa488, Life Technologies

b) Streptavidin-Brilliant Violet 421, BioLegend, San Jose, CA

c) goat anti-mouse anti-IgG1-Alexa488, Life Technologies

d) goat anti-mouse anti-IgG2a-PE-Cy7, Southern Biotech

e) goat anti-mouse anti-IgG1-PE, Southern Biotech, Birmingham, AL

f) goat anti-mouse anti-IgG2a-PE, Southern Biotech

g) goat anti-mouse anti-IgG2b-Alexa488, Life Technologies

h) goat anti-mouse anti-IgG2a-Alexa647, Life Technologies

i) goat anti-mouse anti-IgG1-Biotin, Southern Biotech

j) Streptavidin-Brilliant Violet 605, BioLegend

*AbD Serotec, Raleigh, NC

**eBioscience, San Diego, CA

Table 2: Primers used in the RT-qPCR assays of sorted lymphocyte subsets.

Sequences of primers (5´-3´) for target genes, primer positions on (+) strand and length of specific product in base pairs (bp) are indicated.

Target gene Accession No.	Primer sequences forward (F) and reverse (R)	Position on + strand	Product length (bp)
NKp30			
XM_003128312.2	**F:** CGGATGCTGTTGCTCATCTT	7	140
	R: GCCAATCTCCTCTGGCTGG	146	
NKp44			
XM_013977918.1	**F:** TCCGTGAGGTTCCATCTGGCCGTGT	590	140
	R: TGTGAAAGGGCAGCGATGGCAGAGG	729	
NKG2D			
NM_213813.2	**F:** ACAGCAGAGAAGACCAGGATTTCTTCA	408	104
	R: GGAACCATCTTCCCACTGCCAGG	511	

Supplementary Data: Optimisation and validation of qPCR assays for target gene-specific primers in the pig.

Information on Intron-spanning primers.

Primers or products spanning Exon-Exon junctions are indicated in the table including length of intron in base pairs (bp).

Target	Exon junctions in	Intron size (bp)
NKp30	product	1537
NKp44	product	5025

Optimised protocol for the amplification of target sequences by qPCR.

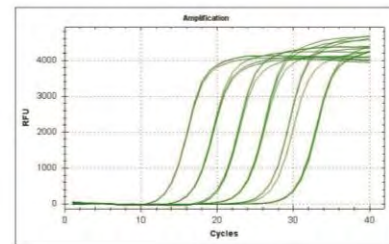| Target	Annealing/Extension temp (°C)/time (sec)	DCt (RT+ to RT-)	Slope	Correlation coefficient (Pearson) R^2	Verified dynamic range	Product melting temperature (°C)
NKp30	66/30	N.D.	-3,387	1,000	10^6	87
NKp44	72/30	N.D.	-3,501	0,998	10^6	90

NKp30: 1:10 serial dilution of PCR product
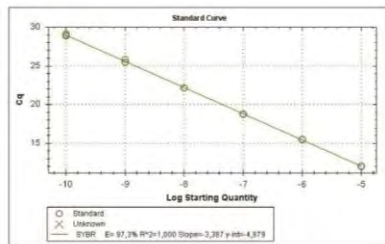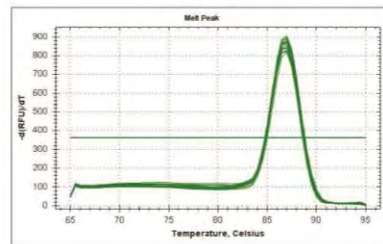
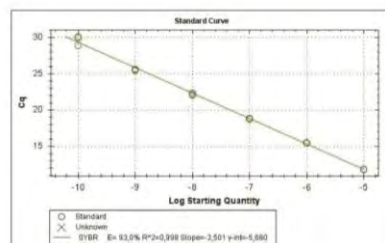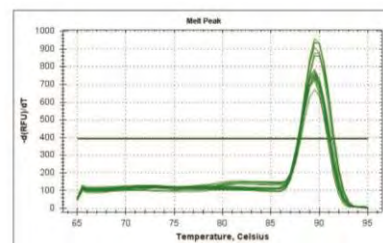calibration curve   melt curve   amplification plot
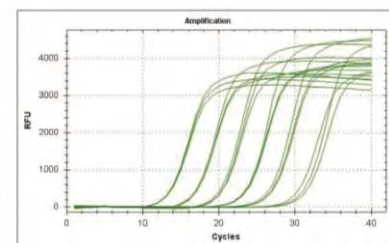
NKp44: 1:10 serial dilution of PCR product

calibration curve   melt curve   amplification plot

4

Influenza A virus leads to degranulation and TNF production in cultured porcine NK cells

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Abstract

Influenza A viruses are important causes of respiratory disease in humans and may lead to hospitalization and death in otherwise healthy individuals. Factors that determine the clinical outcome is poorly understood. NK cells are thought to be important in influenza A virus infections, and swine may represent a good animal model for studying NK cell responses to influenza A virus in humans. Still, little is known about porcine NK cell effector functions during influenza. To study the responses of NK cells to influenza A virus *in vitro*, porcine NKp46^{+} cells were isolated and cultured for 10 days, before phenotyping and stimulation with anti-CD16 and anti-NKp46 monoclonal antibodies. As far as we know, this is the first report on proliferating NK cell cultures from swine. Phenotyping by flow cytometry showed high purity of cells with typical NK cell traits. However, a substantial proportion of the cells did not express NKp46. Cultured NK cells degranulated and produced IFN-γ and TNF in response to ligation of CD16 and NKp46. Cultured NK cells were also stimulated with influenza A virus and were found to degranulate and produce TNF in response to the virus.

Introduction

The last human influenza A pandemic was caused by the influenza A(H1N1)pdm09 virus (H1N1 2009). Since its introduction in 2009, the virus has been circulating endemically in humans, and was the predominant H1N1 virus detected in the 2015/2016 flu season in the Nordic hemisphere [1,2]. The clinical course of H1N1 2009 virus infections in humans varies. Although influenza A virus infections usually causes a self-limiting upper respiratory tract infection, annual influenza A epidemics are estimated to cause between 3 and 5 million cases of severe illness and 250 000 – 500 000 deaths globally each year [3].

Natural Killer (NK) cells are important in the innate immune response against viruses [4,5], and several studies have highlighted their role in the early course of influenza A virus infections [6–10]. The natural killer receptor NKp46 was previously thought to be exclusively expressed on NK cells in humans [11] and several animal species [12–14]. However, the expression of NKp46 on human NK cells has been shown to vary between individuals [15] and between organs within the same individual [16]. Moreover, as different populations of innate lymphocytes are being discovered, NKp46 is found on small, but distinct populations of T cells with innate properties [17–21]. Human NK cells can bind influenza virus hemagglutinin (HA) via NKp46 [10] and directly kill influenza A virus infected cells [9]. A recent study also demonstrated the capacity of human NK cells to bind and respond to infected cells opsonized by influenza A virus specific antibodies through antibody-dependent cellular-mediated cytotoxicity [22]. In addition to their cytotoxic potential, NK cells are important producers of cytokines, including the pro-inflammatory and antiviral cytokines IFN-γ and TNF [23,24]. Elevated levels of TNF and IFN-γ has been found in nasopharyngeal lavage from humans with influenza [25].

Although NK cells are regarded as innate lymphocytes, there is increasing evidence that encounters with microbes generate long lived memory NK cells that are capable of a more efficient secondary response [26]. Influenza A virus vaccination in humans has been shown to elicit potential memory NK cells, with increased IFN-γ production in response to influenza A virus several months following vaccination [27]. Accordingly, NK cells might

also be important in vaccine development. NK cells are likely to be especially important in infections with antigenically new viruses, like pandemic viruses, where the adaptive immune system has no pre-existing memory [7].

In *in vitro* assays, it is of great interest to increase cell numbers. It is well established that NK cells proliferate *in vitro* in response to IL-2 alone [28] and IL-2 activated NK cells are more efficient killers than freshly sorted NK cells [29,30]. To our knowledge, no one has described proliferative cultures from porcine NK cells.

The H1N1 2009 virus originated in swine [31], and is still circulating in swine worldwide [32,33]. Swine are proposed to be an important animal model for studying infection dynamics and immune responses during influenza A virus infections [34], but NK cell responses during influenza in swine have been little studied [35,36]. Porcine NK cells are defined as CD3$^-$CD8α^+ cells that express CD16 and perforin [37]. Swine possess both NKp46$^-$ and NKp46$^+$ NK cells, and it has been shown that NKp46$^-$ cells might up regulate NKp46 in response to cytokines. Moreover, a small population of CD3$^+$NKp46$^+$ cells is found in some pigs [38]. We have previously shown that NKp46$^+$ NK cells may be recruited to the lungs during H1N1 2009 virus infection in pigs and that porcine NKp46 bind influenza virus infected cells *in vitro* [39]. Increased numbers of CD3$^+$NKp46$^+$ cells were also found in the lungs of influenza infected swine, indicating a role of several cell types expressing the NKp46 receptor [Mair et al, submitted 2016]. Elevated levels of mRNA encoding TNF, but not IFN-γ was found in mononuclear cells isolated from lung tissue during influenza infection [39].

To study the responses of porcine NK cells to influenza virus *in vitro*, NKp46$^+$ cells were isolated from blood and spleen from swine and kept in proliferating cultures for 10 days. Subsequently, NK cell responses were studied following stimulation with H1N1 2009 virus.

Material and Methods

Ethical statement

Animals were euthanized in accordance with the Norwegian Animal Welfare Act prior to any subsequent sampling, and the present study is thus not regarded as an animal experiment as stated in The Norwegian regulation on Animal experiments (§ 4).

Influenza virus propagation and sequencing

The virus used in these studies was an influenza A/California/07/2009 H1N1 (Cal09) virus kindly provided by the Norwegian Institute of Public Health. Virus was propagated by passaging in embryonated hen's egg and Madin-Darby Canine Kidney cells. In order to obtain higher virus yields, cell supernatant with virus were precipitated using polyethylene glycol (PEG) 8000 (Amresco) as previously described [41].

Animals and sampling

18 five to nine week-old-pigs were sourced from a Norwegian herd of Landrace/Yorkshire cross breed and euthanized two at the time for nine separate experiments. The Norwegian swine population has a high health status and is documented to be free of respiratory diseases like porcine respiratory corona virus and porcine respiratory and reproductive syndrome [42]. In addition, all pigs were shown to be influenza A virus negative by real-time RT-PCR as described by the Centers for Disease Control and Prevention [43]. All pigs were also shown to be antibody negative to all influenza A virus subtypes by competitive ELISA for detection of nucleoprotein antibodies (ID Vet). Animals used in this study were stunned using captive bolt pistol and euthanized by exsanguination. Blood was collected in containers with EDTA during exsanguination, while samples from spleen were collected in phosphate buffered saline (PBS) with EDTA post-mortem.

Phenotyping of mononuclear cells

Peripheral blood mononuclear cells (PBMC's) and mononuclear cells from spleen were isolated as previously described [39] and finally dissolved in PBS with EDTA and 2 % FCS

at a concentration of 50×10^6 cells/ml. Mononuclear cells were analysed by flow cytometry for the expression of CD3, CD8a, NKp46, CD16 and perforin as previously described [39], using the following monoclonal antibodies (mAbs): Anti-CD3-PerCP-Cy5,5 (Clone PPT3, BD Biosciences), anti-CD8α-biot (clone 76-2-11, AbCam), anti-NKp46-APC (clone VIV-KM1, AbD Serotec)/anti-NKp46-A647 (VIV-KM1, in house production) and anti-CD16-RPE (clone G7, AbD Serotec). Cells stained with CD8α-biot were subsequently incubated with Streptavidin-V450 (BD Biosciences). Cells that were to be analysed for perforin expression were fixated and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences), followed by intracellular staining according to the manufacturer's instructions using anti-perforin (clone δG9, BD Biosciences) and goat anti-mouse IgG2b-A488 (Molecular Probes/Life Technologies). Appropriate secondary and isotype controls were included. Acquisition was performed on a Gallios Flow Cytometer (Beckman Coulter) and data analysed using Kaluza software (Beckman Coulter). Dead cells and duplets were excluded before lymphocytes were gated individually according to forward/side scatter characteristics. Data from at least 50×10^4 cells were recorded when possible. Samples based on total cell numbers below 1000 cells were excluded.

Isolation and culturing of NKp46$^+$ cells

NKp46$^+$ cells were purified from mononuclear cells by incubation with anti-NKp46 mAb (clone VIV-KM1) and positive selection with magnetic beads (Dynal) as previously described [13] with minor modifications. Briefly described, isolated mononuclear cells were incubated with 5 μg/ml NKp46 mAb for 30 min on ice. Subsequently, cells were washed two times in PBS with EDTA and 2 % FCS before incubation with 4×10^6 dynal beads per ml cells for 30 min at 4 °C. Isolated cells were washed four times in PBS with 2 % FCS and dissolved in 4 ml RPMI 1640 supplemented with 10 % FCS, 1mM sodium pyruvate, 60 μg/ml penicillin, 100 μg/ml streptomycin, non-essential amino acids, 50 μM 2-mercaptoethanol (NK cell medium) and 50 ng/ml recombinant porcine IL-2 (RnD) in one well in a six-well tray. After 48-72 hours, magnetic beads were removed and the cells transferred to new wells. Cells were divided and additional medium added when necessary. On day three and 10, cells were counted using an automatic cell counter. Cells were phenotyped and used in assays after 10 days. For phenotyping, cells were stained

with antibodies against CD3, CD8, NKp46, CD16, CD25 and perforin, as described above. For staining of CD25, anti-CD25 (clone 3B2, AbD Serotec) and goat anti-mouse IgG1-A488 (Molecular Probes/Life Technologies) were used. Cells were analysed as described above. NK cells were gated individually according to forward/side scatter characteristics and 1 x 10^4 cells were recorded when possible.

Stimulation of cells

Cultured cells were used in stimulation assays with anti-NKp46, anti-CD16 and Cal09 virus. ELISA plates (Nunc MaxiSorp) were coated overnight at 4 °C with 100 µl of precipitated virus in a 1:10 dilution or 3 µg/ml anti-NKp46 (Clone VIV-KM1) or anti-CD16 (clone G7) mAbs in 0,1 mol/L carbonate buffer (NaHCO$_3$), as indicated. Precipitated cell supernatant from uninfected Madin-Darby Canine Kidney cells, irrelevant mAbs (clone AKS1, in house production) diluted in carbonate buffer at similar dilutions and carbonate buffer alone were used as negative controls. There were no differences between cells incubated with irrelevant mAb or carbonate buffer. To prevent unspecific binding, wells were incubated with 10 % FBS in PBS for two hours at 37 °C and washed three times in PBS before use. Cultivated NK cells were added to coated wells in a concentration of 1-2 x 10^5 cells in 200 µl NK cell media with 10 ng/ml recombinant porcine IL-2, together with 1 µl/ml GolgiPlug containing Brefeldin A and 0,6 µl/ml GolgiStop containing Monensin (both from BD Biosciences).

For assessment of CD107a expression in NK cells, 4 µg/ml anti-CD107a-FITC mAb (clone 4E9/11, AbD Serotec) or isotype IgG1-FITC were added and cells were incubated for 1 hour at 37 °C. Cells were stained with LIVE/DEAD Yellow (Invitrogen) prior to incubation with anti-NKp46-APC and anti-CD16-RPE as described above. To study intracellular amounts of IFN-γ and TNF after stimulation, cells were incubated with GolgiPlug and GolgiStop in coated plates for 4 hours at 37 °C. Cells were then stained with LIVE/DEAD Yellow, fixated and permeabilized prior to incubation with anti-IFN-γ-PE (cloneP2G10, BD Biosciences) and anti-TNF-APC (Mab11, BioLegend) as described above.

Statistical analyses

Statistical analyses were performed using JMP V9 (SAS Institute Inc) and Graph Pad Prism V6 (GraphPad Software). Differences among groups were assessed by the Mann-Whitney test. When three groups or more were present, each pair of groups was compared individually. Box plots show median values, the 25th and 75th percentiles and the lowest and highest values.

Results

Isolation of NKp46+ cells

PBMC and spleen mononuclear cells of swine were isolated before CD3$^+$NKp46$^+$ cells and NK cells defined as CD3$^-$CD8α$^+$ cells were analysed by flow cytometry. NK cell were further classified by their expression of NKp46. In blood, populations of NKp46$^-$ and NKp46$^+$ NK cells were found (Fig. 1A), while a third population of NKp46^{high} NK cells were additionally found in spleen (Fig. 1B). Since all NK cells expressing the NKp46 receptor subsequently were to be isolated without the possibility to separate NKp46^{high} NK cells from those with a dimmer phenotype, NKp46$^+$ and NKp46^{high} NK cells were collectively gated as NKp46$^+$ NK cells also in the initial analyses. Percentages of NKp46$^-$ NK cells, NKp46$^+$ NK cells and CD3$^+$NKp46$^+$ cells in lymphocytes were compared between PBMC and spleen (Fig. 1C). A small population of CD3$^+$NKp46$^+$ cells could be found in PBMC and spleen in some animals. The proportions of NKp46$^-$ cells and NKp46$^+$ NK cells among lymphocytes varied between animals, but there were generally more NKp46$^-$ NK cells in PBMC compared to spleen and more NKp46$^+$ NK cells found in spleen compared to PBMC. NK cells were mainly CD16$^+$ and perforin$^+$ (results not shown).

8
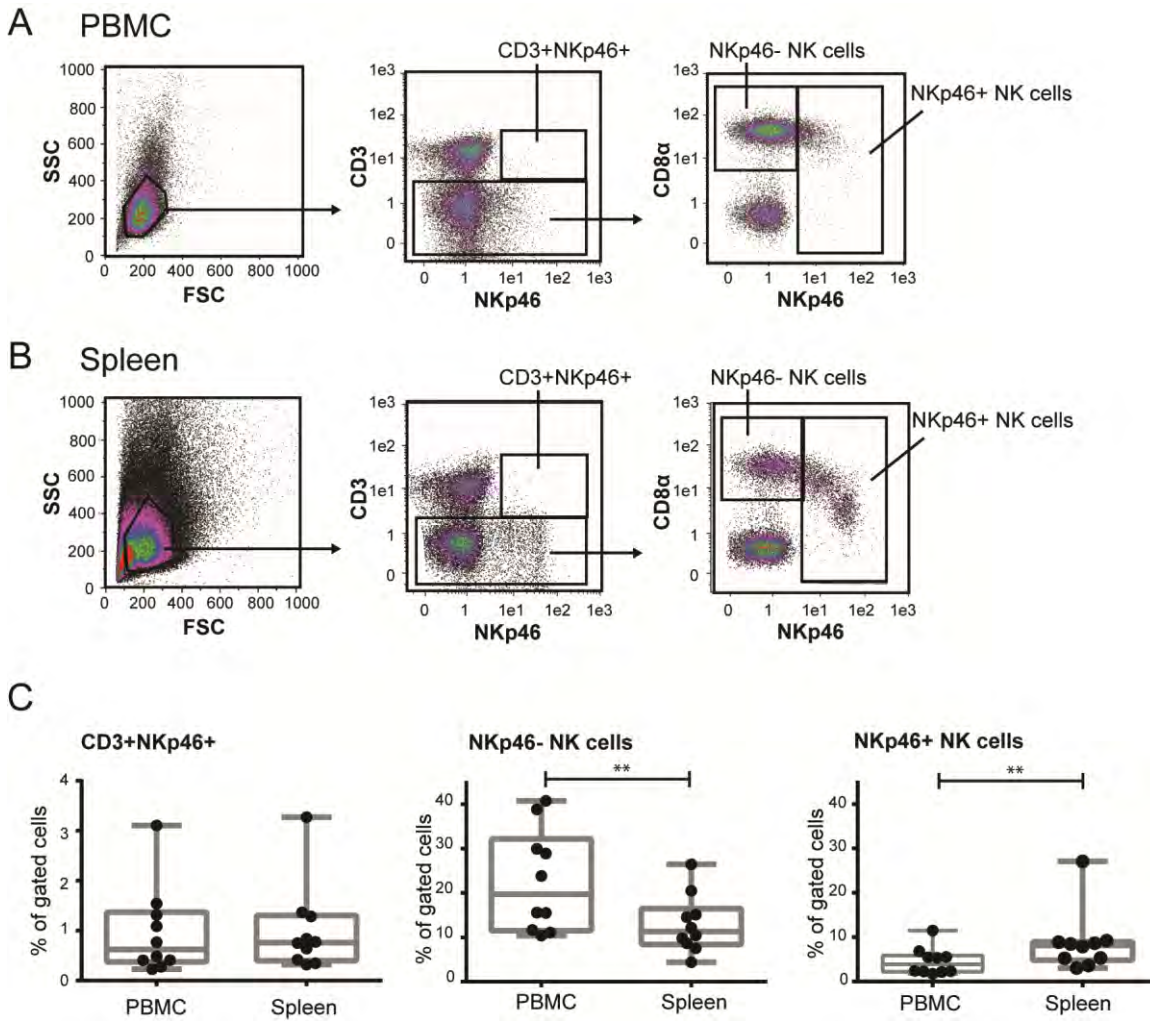
Figure 1 Phenotyping of mononuclear cells from blood and spleen. Pigs were euthanized and blood and spleen taken post mortem. **A)** Isolated PBMCs (n=10) from swine were analyzed by flow cytometry. Cells were gated as live $CD3^+NKp46^+$ cells, $CD3^-CD8\alpha^+NKp46^-$ (NKp46- NK cells) or $CD3^-CD8\alpha^+NKp46^+$ (NKp46+ NK cells). **B)** Mononuclear cells from spleen (n=10) were isolated and gated as described for PBMC. **C)** Percentages of $CD3^+NKp46^+$ (left), NKp46- NK cells (middle) and NKp46+ NK cells (right) in lymphocytes were compared between PBMC and spleen. **$p\leq0,01$.

Cultivated cells display typical NK cell characteristics

NKp46+ cells were isolated from PBMCs and mononuclear cells from spleen by positively selection and cultured for 10 days. Cell numbers typically increased in culture by a 10-fold in a week (results not shown). Proliferating cells grew as non-adherent cells with
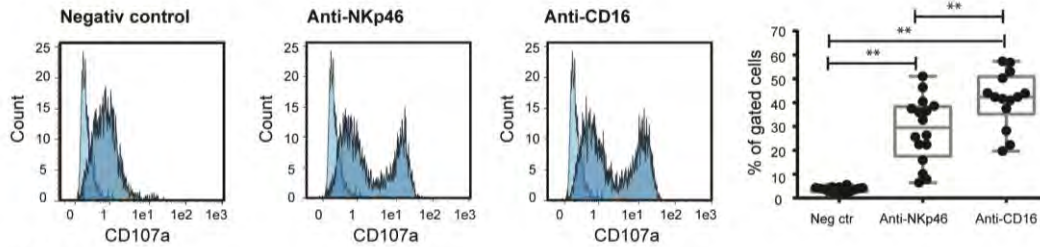
prominent lamellipodia (Fig. 2A). Cells were analysed for their expression of CD3, perforin, CD16 and CD25 (Fig. 2B). Cells were mainly CD3- (median; 99 %, range; 95-100 %), perforin+ (median; 96 %, range; 91-98 %) and CD16+ (median; 96 %, range; 89-98 %), thus displaying a typical porcine NK cell phenotype [37]. Furthermore, the cells had up regulated CD25 (median; 89 %, range; 48-97 %) in response to the IL-2 treatment (Fig. 2B), indicative of increased activation of the cells. Cells were also analysed for NKp46 expression. Although selected for the expression of NKp46, a substantial number of cells were NKp46- (median value; 15 %, range; 9-31 %) (Fig. 2C).
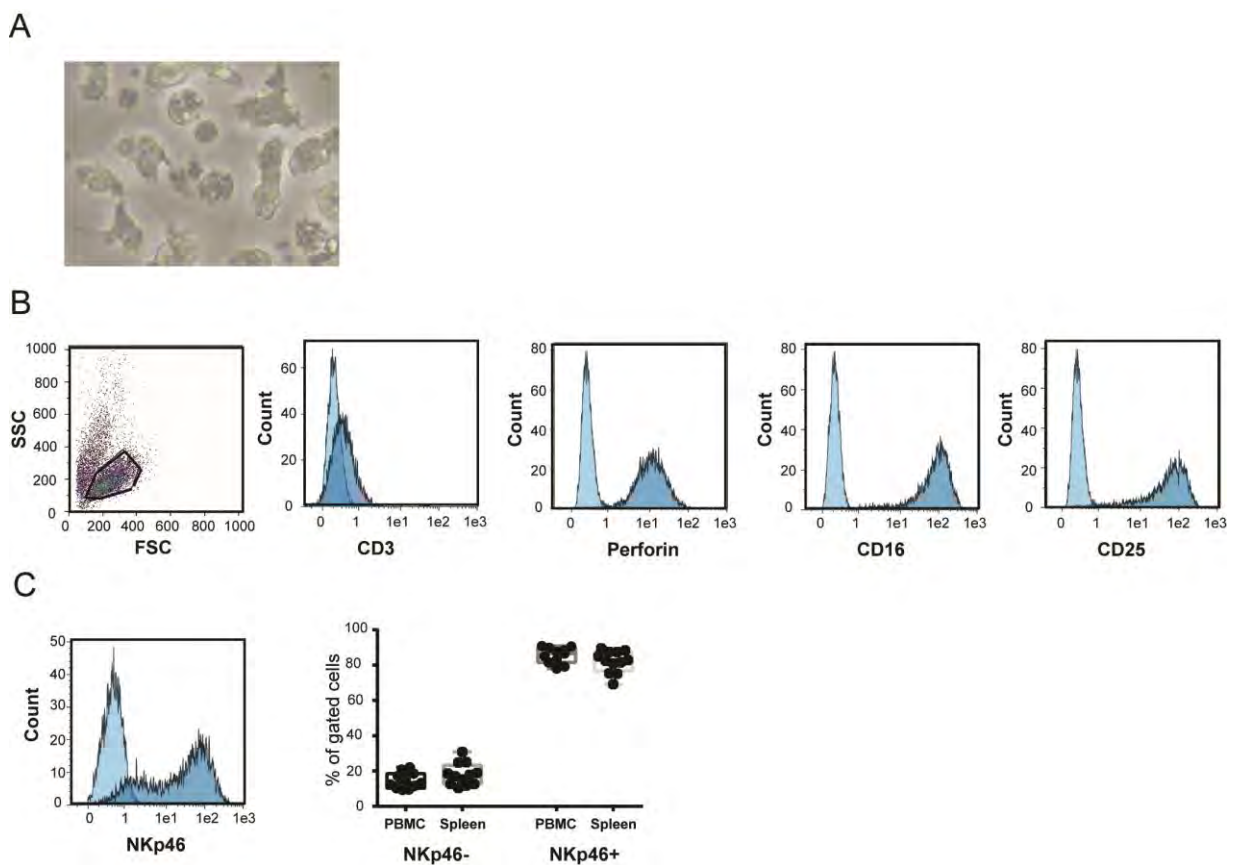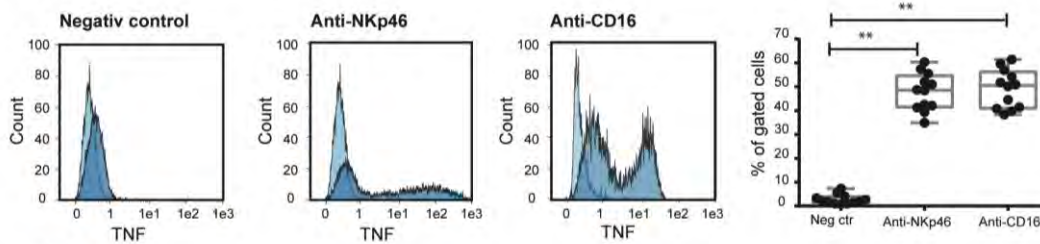

Figure 2. Culturing of NK cells. NKp46+ cells were isolated from blood and spleen mononuclear cells. **A)** Representative picture of cultured cells taken by light microscopy of cells isolated from spleen nine days after isolation. **B)** Cultured live cells were analyzed for their expression of CD3, perforin, CD16 and CD25 by flow cytometry. Light blue histograms show relevant isotype or secondary antibody control. **C)** Percentages of NKp46- and NKp46+ cells in live cultured cells were compared in cell cultures derived from cells isolated from PBMC (n=11) and spleen (n=11).

Cultured cells were incubated in plates coated with mAbs against NKp46 or CD16 and analysed for the expression of the degranulation marker CD107a and intracellular amounts of the two cytokines IFN-γ and TNF. Ligation of NKp46 and CD16 resulted in degranulation in the cultured cells, with ligation of CD16 leading to greater amounts of degranulating cells than NKp46 (Fig. 3A). Cultured cells also produced TNF (Fig. 3B) and IFN-γ (Fig. 3C) following ligation of both NKp46 and CD16. Taken together, these results demonstrate that the cultured cells shared typical features of freshly isolated porcine NK cells [38]. There were no differences between cultures derived from cells isolated from blood and spleen (results not shown).
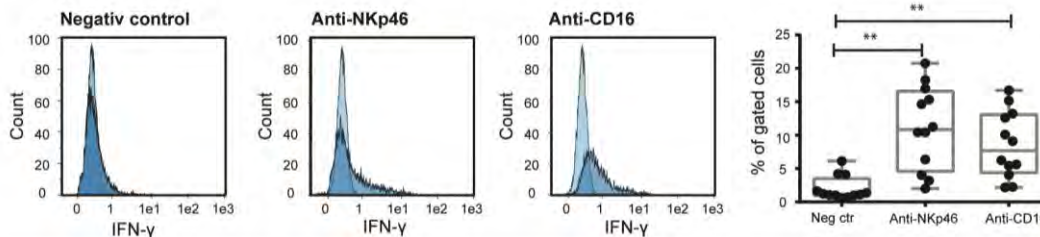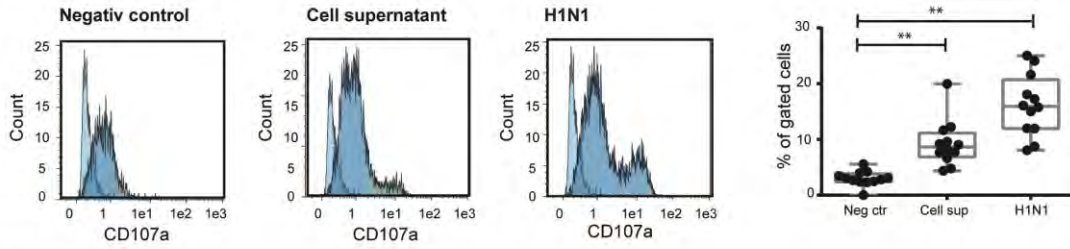

Figure 3. Stimulation of cultured NK cells with anti-NKp46 and anti-CD16 mAbs. A) Cultured cells were incubated in plates coated with irrelevant mAbs (negative control, n=16), anti-NKp46 (n=16) or anti-CD16 (n=14) and analyzed for their expression of the degranulation marker CD107a. Percentages of CD107a+ cells in live cultured cells were compared between the three different mAbs. Stimulated cells (n=12) were also analyzed for intracellular **B)** TNF and **C)** IFN-γ. *******p*≤0,01.
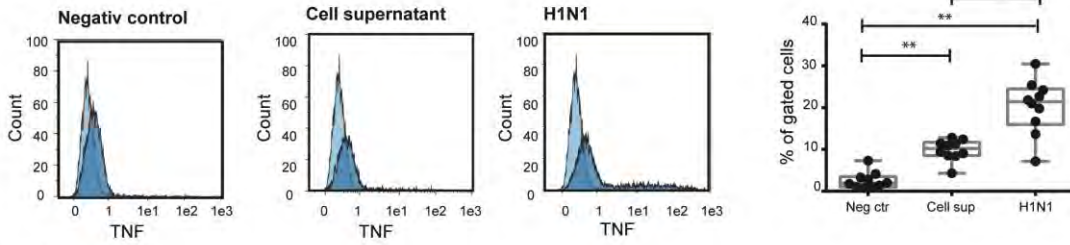
Influenza virus activates NK cells in vitro

We have previously shown that a porcine NKp46 fusion protein bind H1N1 2009 virus infected cells [39]. To see if NK cells were activated to degranulate or produce cytokines in response to H1N1 2009 virus, cultured cells were incubated in wells coated with virus, and compared to NK cells incubated in wells coated with cell supernatant or coating buffer alone (negative control). Although the cell supernatant caused degranulation in a proportion of NK cells, H1N1 2009 virus elicited a significantly greater NK cell degranulation (Fig. 4A) In addition, TNF was produced in greater numbers of cells stimulated with influenza virus compared to both negative controls (Fig. 4B). Production of IFN-γ seemed to be elevated in some cultures stimulated with influenza virus, but the differences between groups were non-significant (Fig. 4C). There were no differences between cultures derived from cells isolated from blood and spleen (results not shown).
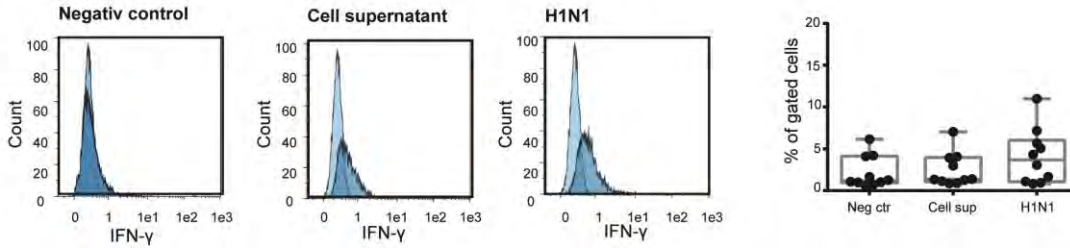
Figure 4. Stimulation of cultured NK cells with influenza H1N1 2009 virus. A) Cultured NK cells (n=12) were incubated in plates coated with coating buffer (negative control), cell supernatant or influenza H1N1 2009 (Cal09) virus and analyzed for their expression of the degranulation marker CD107a. Percentages of CD107a+ cells in live cultured cells were compared between the three different stimulations. NK cells (n=12) were also analyzed for intracellular **B)** TNF and **C)** IFN-γ. *******p*≤0,01

Discussion

In order to see if porcine NK cells can be activated to degranulate or produce cytokines in response to influenza virus, cultured NK cells were incubated in wells coated with precipitated H1N1 2009 virus. As the precipitation process leads to up-concentration of not only viral particles, but also proteins found in the cell media holding the virus, precipitated cell supernatant was included as a negative control in addition to coating buffer alone. As suspected, cell supernatant caused degranulation in a greater proportion of cells than the coating buffer alone. Nonetheless, degranulation of NK cells in response to H1N1 2009 virus exceeded that of the cell supernatant. Incubation of NK cells with H1N1 2009 virus also caused elevated levels of TNF, which correlate with *in vivo* findings of increased TNF production in human cases of influenza [25,44]. The amount of IFN-γ$^+$ cells seemed to be elevated in some cultures, but the increase was not significant between the two groups. This correlates well with our previous findings of increased levels of TNF mRNA, but not IFN-γ mRNA in mononuclear cells of pigs infected with H1N1 2009 [39] and suggests that TNF is more important in H1N1 2009 infections than IFN-γ. To support this, a recent study found elevated levels of TNF only and not IFN-γ in the blood of humans infected with influenza A virus [44]. Moreover, Jost et al [6] suggested that IFN-γ was only produced in severe cases of influenza.

Activation by the H1N1 2009 virus caused less NK cell degranulation and TNF producing cells compared to direct ligation of NKp46 or CD16 in mAb coated plated. This may be due to the short stimulation period of one hour used in the degranulation assay and four hours used in cytokine assay. In support of this notion, an *in vitro* study of human NK cells has shown that NK cell degranulation in response to influenza A virus did not exceed degranulation caused by NKp46 stimulation until 12 hours after viral exposure [45].

The NKp46 receptor has been assigned a special role as an activating NK cell receptor in human NK cells in response to influenza A virus [9]. We have previously shown that porcine NKp46 recombinant protein bind influenza H1N1 2009 infected cells expressing HA on their surface [39]. In order to investigate the involvement of the NKp46 receptor in

activation of porcine NK cells in response to influenza virus, blocking of NKp46 prior to incubation with H1N1 2009 was attempted. Binding was confirmed by the absence of staining by the same mAb afterwards. However, no effect of blocking was seen on either degranulation or cytokine production (results not shown). While NK cells have been shown to bind influenza virus only through specific glycosylation sites in the more proximal of two Ig-like domains [46], the specific binding site of the anti-NKp46 mAb is not known. Thus, the anti-NKp46 mAb and HA may very well bind different epitopes. It has previously been shown that a mAb directed against the distal Ig-like domain does not block activation of human NK cells by influenza A virus infected cells [46]. While ligation of NKp46 caused a loss in NKp46 expression (results not shown) typical for NKp46 receptor engagement [47], stimulation with influenza virus only caused minor changes in NKp46 expression in the present study (results not shown). This might be due to the short stimulation period used in our experiments. Stimulation of human NK cells with influenza infected cells causes an initial upregulation of NKp46 during the first two hours of incubation, followed by a loss of NKp46 expression after six hours [45]. The importance of the NKp46 receptor in response to influenza A virus in porcine NK cells needs to be further investigated.

To our knowledge, this is the first description of proliferating NK cell cultures from swine. NKp46+ cells were isolated by the use of magnetic beads and cultured in the presence of recombinant porcine IL-2. After 10 days in culture, the majority of cells displayed a typical porcine NK cell phenotype [37]. Interestingly, a substantial proportion of NKp46- cells were found in the cultures. After positive selection with magnetic beads, the NKp46 receptor is likely to be internalized as a consequence of receptor engagement [48], and one could hypothesize that on a proportion of the cells, the receptor had not yet been recycled to the surface. However, we have previously shown that 99,5 % of selected bovine NK cells display a NKp46bright phenotype one week after selection by the same method [13]. It is more likely that the NKp46- cells represents contaminating NKp46- NK cells that have proliferated in response to IL-2 or that they were NKp46+ NK cells at the time of selection that have down regulated NKp46. It has been reported that NKp46 can be down regulated during disease on human NK cells [45,49,50].

Ligation of NKp46 and CD16 resulted in degranulation and cytokine production. NKp46 was shown to be a less efficient triggering receptor for NK cell degranulation, than CD16, as previously described by Mair et al [38]. Furthermore, we found that the amount of cells that initiated TNF production was greater than IFN-γ producing cells after ligation of both NKp46 and CD16. This could correspond to the higher amounts of TNF found in cell supernatant by Mair et al [51]. Taken together, these results show that the cultured NK cells displayed typical NK cells behavior.

This study shows that NK cells from swine can be kept in proliferating cultures and that porcine NK cells respond to H1N1 2009 virus by degranulation and cytokine production.

Acknowledgment

Authors contribution

HF, AGH, HS and AKS conceived and designed the study. HF performed experiments, analyzed data and wrote the manuscript. HL participated in the design of the study and performed laboratory work and experiments. CAG performed sampling. PB assisted in developing methods and interpretation of data. AGH, HS and AKS assisted in interpretation of data and supervised the study. All authors critically read and approved the final manuscript.

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