The Journal of Immunology

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This information is current as of February 13, 2017.

J Immunol 2016; 197:4696-4703; Prepublished online 7 November 2016;
doi: 10.4049/jimmunol.1600401
http://www.jimmunol.org/content/197/12/4696

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Identification of an Activating Chicken Ig-like Receptor Recognizing Avian Influenza Viruses

Christine A. Jansen,* Daphne A. van Haarlem,* Beatrice Sperling,† Peter J. van Kooten,* Erik de Vries,* Birgit C. Viertlboeck,† Lonneke Vervelde,*,[1] and Thomas W. Göbel†

Chicken Ig-like receptors (CHIRs) represent a multigene family encoded by the leukocyte receptor complex that encodes a variety of receptors that are subdivided into activating CHIR-A, inhibitory CHIR-B, and bifunctional CHIR-AB. Apart from CHIR-AB, which functions as an Fc receptor, CHIR ligands are unknown. In the current study, we used a panel of different BWZ.36 CHIR reporter cells to identify an interaction between specific CHIRs and avian influenza virus (AIV). The specificity of the CHIR–AIV interaction was further demonstrated using CHIR fusion proteins that bound to AIV-coated plates and were able to reduce the interaction of reporter cells with AIV. There was no difference in binding of CHIR to different AIV strains. Furthermore, CHIR fusion proteins reduced AIV-induced in vitro activation of NK cells obtained from lungs of AIV-infected animals, as judged by the lower frequency of $CD107^+$ cells. Because the original CHIR reporter lines were generated based on sequence information about extracellular CHIR domains, we next identified a full-length CHIR that displayed similar binding to AIV. The sequence analysis identified this CHIR as a CHIR-A. Neuraminidase treatment of coated CHIR–human Ig proteins reduced binding of trimeric H5 proteins to CHIR. This suggests that the interaction is dependent on sialic acid moieties on the receptor. In conclusion, this article identifies AIV as a ligand of CHIR-A and describes the functional consequences of this interaction. ***The Journal of Immunology*****, 2016, 197: 4696–4703.**

Natural killer cells play an important role in the immune response against viruses like influenza (1). They express activating and inhibitory receptors, and the balance between these signals determines NK cell activation (2, 3). Inhibitory NK cell receptors are characterized by expression of an ITIM in their cytoplasmic tail. Activating receptors signal via transmembrane-anchored adaptor proteins that contain ITAM (4).

Comparative studies identified several potential NK cell receptor families in the chicken. The syntenic region of the NK gene complex harbors only a few C-type lectins (5). Their role in NK cell function has not been completely resolved. Other C-type lectins encoded by the chicken MHC locus may play a role in NK cell function (6). Members of the Ig superfamily that may be involved in immune responses also were identified in the chicken and include TREM, CD300, and SIRP homologs (7). In addition, the syntenic region of the mammalian leukocyte receptor complex has been intensively studied in the chicken. It is located on microchromosome 31 and encodes the so-called chicken Ig-like receptor (CHIR) family (8, 9). The chicken leukocyte receptor complex has not been entirely sequenced, but studies suggest that it resembles a highly polymorphic locus harboring a variable number of CHIR genes. Within the CHIR family, three major subtypes can be identified based on structural features. CHIR-A contains two C2-type Ig domains, a charged transmembrane residue, and a short cytoplasmic tail, which are features of activating receptors. CHIR-B contains two C2-type Ig domains and a long intracellular tail with ITIM, indicative of inhibitory receptors. The third type, CHIR-AB, combines the charged transmembrane residue with the long ITIM-containing cytoplasmic tail and, therefore, may function as a bifunctional receptor (9, 10). CHIR ligands remain to be identified, with the exception of CHIR-AB1, a high-affinity receptor that binds chicken IgY (11, 12).

One family of activating NK cell receptors in mammalian species is the natural cytotoxicity receptors NKp30, NKp44, and NKp46 (or NCR1 in mice) (13–15). NKp44 and NKp46 bind the viral hemagglutinin (HA) protein of human and swine influenza viruses. Viruses recognize α2,6-linked sialic acid (SA) residues on the receptor, and this induces NK cell activation (16–18).

Interestingly, the crystal structure of CHIR-AB1 shows a structural homology to the D1 domain of the mammalian NKp46 (19).

The present study was initiated to find novel CHIR ligands using a panel of BWZ.36 reporter cell lines stably transfected with prototypic members of the CHIR family. We reasoned that the extraordinary CHIR diversity may be the result of strong selection by a pathogen. Therefore, we tested the reporter cells on AIV-coated plates and found an interaction between AIV and CHIR.

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Received for publication March 8, 2016. Accepted for publication October 12, 2016.

This work was supported by Netherlands Organization for Scientific Research Veni Grant 016.096.049, European Union Sixth Framework Program Flupath Grant 04220, the Programme Impulse Veterinary Avian Influenza Research in The Netherlands of the Dutch Ministry of Agriculture, Nature and Food Quality, and German Research Council Grant DFG GO489/3-7. A travel grant from the Boehringer Ingelheim Fonds enabled the site visit that initiated this project.

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Abbreviations used in this article: AIV, avian influenza virus; CHIR, chicken Ig-like receptor; HA, hemagglutinin; hIg, human Ig; H5N1, A/turkey/turkey/1/05; H5N2, A/chicken/Pennsylvania/21525/83; H7N1, A/chicken/Italy/1067/99; H9N2, A/chicken/Saudi Arabia/SP02525/3AAV/2000; HPAI, highly pathogenic avian influenza; LPAI, low pathogenic avian influenza; SA, sialic acid; VCNA, *Vibrio cholerae* neuraminidase.

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Materials and Methods

Ethics statement

Primary lung lymphocytes were obtained from animal experiments involving low pathogenic avian influenza (LPAI) viruses that were performed in strict accordance with the Dutch Animal Experimentation Act and European Union directives 86/609/CEE and 2010/63/EU related to the protection of vertebrate animals used for experimental and other scientific purposes. Animals were hatched from embryonated eggs (Lohmann Tierzucht, Cuxhaven, Germany) and raised in the Central Laboratory Animal Research Facility of Utrecht University. The experimental protocols were approved by the Committee on Animal Experiments of Utrecht University (DEC 2008.II.01.010). The Central Laboratory Animal Research Facility of Utrecht University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Cell lines and primary cells

The mouse thymoma cell line BWZ.36 (kindly provided by W. Yokoyama, Washington University, St. Louis, MO) (20) was cultured in RPMI 1640 with GlutaMAX (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK293 T cells (ATCC CRL-11268) were cultured in RPMI 1640 with GlutaMAX supplemented with 10% ultra-low IgG FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. MDCK cells (a kind gift from prof. Dr. G. Rimmelzwaan, Erasmus Medical Centre, Rotterdam, the Netherlands) were cultured in IMDM (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM GlutaMAX I, and 1% nonessential amino acids. All cells were cultured at 37˚C, 5% CO_2. Primary lung cells were isolated from Lohmann Brown chickens infected with the LPAI virus strain H9N2 isolate A/chicken/United Arab Emirates/99 at 1 d postinfection, as previously described (21), and stored at −140˚C until use. Chickens were housed, handled, and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, and the experiments were performed in accordance with the Dutch regulation on experimental animals.

Viruses

LPAI virus strains H5N2 (A/chicken/Pennsylvania/21525/83; kindly provided by Dr. G. Koch, Central Veterinary Institute, Wageningen Livestock Research, Wageningen University & Research, Wageningen, the Netherlands), H7N1 (A/chicken/Italy/1067/99; kindly provided by Dr. W. Dundon, Istituto Zooprofilattico Sperimentale delle Venezie, Italy), and H9N2 (A/chicken/Saudi Arabia/SP02525/3AAV/2000) were used in this study. The viruses were produced in eggs according to routine procedures. When indicated, viruses were inactivated with β-propriolactone before use (22). Inactivated highly pathogenic avian influenza (HPAI) virus strain H5N1 (A/turkey/turkey/1/05) was kindly provided by Dr. B. Löndt (Animal Health and Veterinary Laboratories Agency, Weybridge, U.K.).

Cloning and transfectants

Reporter gene constructs were cloned from the following CHIR subgroup members (23): CHIR2D-838 (GenBank accession number FM200338, short nomenclature 838), CHIR2D-682 (GenBank accession number FM200233, short nomenclature 682), CHIR2D-692 (GenBank accession number FM200240, short nomenclature 692), and CHIR2D-697 (GenBank accession number FM200243, short nomenclature 697). The short nomenclature is used throughout the figures. The extracellular Ig domains were cloned with an N-terminal FLAG epitope in a modified pcDNA3.1/V5-His expression vector (Invitrogen, Carlsbad, CA), as previously described (8). Briefly, the FLAG-tagged extracellular Ig domains were fused to the transmembrane domain of chicken CD8α (GenBank accession number ABI18344) and the cytoplasmic domain of murine CD3ζ (GenBank accession number NP_001106862). The resulting constructs were electroporated into BWZ.36 cells. Cells were selected using medium supplemented with 800 μg/ml G418 (Biochrom, Darmstadt, Germany), and single clones were screened by staining with an anti-FLAG mAb (clone M2; Sigma-Aldrich, Deisenhofen, Germany).

We generated 10 BWZ.36 reporter cell lines from M11 CHIR sequences that were identified previously. The 10 sequences were selected after constructing a phylogenetic tree from all available CHIR sequences (23). The 10 M11 CHIR sequences used for generating BWZ.36 reporter cell lines each resembled prototypic sequences from one clade, so they represented most diverse CHIR sequences found in a single M11 animal. Because these sequences were derived from analyzing the extracellular domain only, it was not possible to determine to which CHIR subfamily they belong. In addition, we included the previously described reporter cell lines stably transfected with constructs of CHIR-A2, CHIR-AB1, CHIR-AB2, CHIR-B2, and CHIR-B3 to cover all CHIR types.

For the soluble constructs, the extracellular Ig domain was ligated to the hinge region, CH2 and CH3 domains of human Ig (hIg)G1 (as in GenBank accession number AM700586), as described (24). HEK293 T cells were transfected using METAFECTENE reagent (Biontex, Martinsried/Planegg, Germany), and supernatants were harvested at 48 h posttransfection. Soluble dimerized receptors (CHIR–hIg fusion proteins) were affinity purified using a Protein A MIDI Ab purification kit (Serotec), according to the manufacturer's instructions. The CHIR–hIg fusion proteins were quantitated using an ELISA system, as previously described (24).

Full-length CHIRs were amplified from cDNA obtained from M11 PBMC total RNA, as described (23), using the forward primer 5′-CTGCCCCGACCCTCCCTG-3′ and the following reverse primers specific for CHIR-A 5′-AATCCCTTCCCCACCCAGACT-3′, CHIR-B 5′-GCACACCGAGCACACTGGCA-3′, and CHIR-AB 5′-CACGGTAATTCAGTGCTCACTGTGG-3′. The resulting PCR products were cloned into the Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher), and each clone was sequenced following plasmid preparation (GATC Biotech, Konstanz, Germany). Sequence analyses were performed with the Lasergene software package (GATC Biotech). The CHIR used for further experiments in this study was designated CHIR2D-825 (short nomenclature 825).

BWZ.36 reporter assay

Commercially available 96-well plates coated with an inactivated field strain of AIV (IDEXX, Hoofddorp, the Netherlands) and anti-FLAG–coated NUNC MaxiSorp plates were incubated with stably CHIR expressing BWZ.36 reporter cells (3×10^5 cells per well in 200 μl of medium) for 24 h at 37˚C. After cell lysis, β-galactosidase activity was measured using 130 μl per well of chlorophenol red-β-D-galactopyranoside (Sigma-Aldrich) as substrate. Enzyme activity was quantitated by reading at OD 575 nm. Four independent experiments were performed in triplicate, and mean ± SEM were calculated.

In blocking experiments, AIV-coated plates were incubated with CHIR–hIg fusion proteins for 1 h at 37˚C before the BWZ.36 reporter cells were added. Alternatively, NUNC MaxiSorp plates were coated with LPAI virus strains H7N1, H9N2, or H5N2 (1 μg per well in PBS) or the β-propriolactone–inactivated HPAI strain H5N1 (1 μg per well in PBS). All experiments were performed in triplicate, and the results of three independent experiments are shown as mean ± SEM.

ELISA

Commercially available 96-well plates coated with an inactivated field strain of AIV (IDEXX) were incubated with different concentrations of hIg proteins diluted in PBS for 1 h at 37˚C. Plates were washed three times in PBS–0.05% Tween and incubated with goat anti-human IgG-HRP (1:4000; SouthernBiotech) for 1 h at 37˚C. Plates were washed again and developed using TMB (Thermo Fisher). The reaction was stopped with 1 M H_2SO_4, and OD was measured at 450 nm. Goat anti-human IgG-HRP did not react with AIV-coated plates.

Solid-phase binding assay

We investigated the interaction between the HA protein of the H5N1 virus (25) and CHIR 692 using a solid-phase binding assay, as previously described (26). In this assay, 96-well NUNC MaxiSorp plates were coated overnight with 1 μg/ml 692 in PBS. As a positive control, 1 μg/ml fetuin, a blood glycoprotein that contains *N*-linked and *O*-linked sialylated glycan side chains, was used to coat the plates (Sigma-Aldrich). Desialylated fetuin (asialofetuin; Sigma-Aldrich) was included as a negative control. Recombinant HA was precomplexed with HRP-linked anti-Strep–tag Ab at a 2:1 molar ratio for 30 min on ice. Next, 2-fold serial dilutions of the HA precomplexes were added to the coated plate. After a 90-min incubation at room temperature, the plates were washed and developed using TMB. The reaction was stopped with 1 M H_2SO_4, and OD was measured at 450 nm. When indicated, CHIR-coated wells were treated with *Vibrio cholerae* neuraminidase (VCNA; Sigma-Aldrich) for 1.5 h at 37˚C (2 μU/ml) prior to incubation.

MDCK cell infection

MDCK cells were infected with LPAI virus strain H7N1 or with PBS (mock control). MDCK cells were seeded at 5×10^5 cells per milliliter in 24-well plates. The next day, cells were infected with 2×10^6 EID_{50} in 0.2 ml of serum-free medium. After 1 h of incubation at 37˚C, 5% CO_2, cells were washed three times with PBS, and 1 ml of serum-free medium containing

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1 μg/ml trypsin (Worthington) was added. Cells were harvested 24 h postinfection using 5 mM EDTA and analyzed by flow cytometry.

Flow cytometry

Infected MDCK cells were stained with a polyclonal rabbit anti-H7 Ab (Immune Technologies). Next, cells were washed in PBS supplemented with 0.5% BSA and stained with goat anti-rabbit IgG-allophycocyanin. Alternatively, biotinylated CHIR–hIg proteins were coupled to streptavidin beads. Infected cells were incubated with these complexes for 30 min at 4˚C. Cells were washed in PBS supplemented with 0.5% BSA and subsequently stained with goat anti-human IgG-PE (SouthernBiotech).

CD107 expression was analyzed by staining with an allophycocyanin-conjugated mouse anti-CD107 Ab (27). In all experiments, staining with anti-CD107 was combined with mouse anti-chicken CD3-FITC (CT3, IgG1; SouthernBiotech) and mouse anti-chicken CD8α-PE (CT8, IgG1; SouthernBiotech) to exclude T cells from the analysis. $CD3^{-}CD8\alpha^{+}$ cells were gated, and CD107 expression was analyzed in this population. Unless mentioned otherwise, all stainings were performed for 20 min at 4˚C. Prior to flow cytometry, 7-aminoactinomycin D (BD Biosciences) was added to exclude the dead cells. At least 50,000 cells in the live gate were acquired using a FACSCanto flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

CD107 assay

The CD107 assay to study NK cell activation was carried out essentially as described previously (27). Briefly, cells isolated from lungs of AIV-infected chickens were thawed and resuspended in IMDM supplemented with 8% heat-inactivated FCS, 2% heat-inactivated chicken serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM GlutaMAX (NK medium). A total of 200,000 cells was cultured in AIV-coated plates in the presence of 1 μl/ml GolgiStop (BD Biosciences) and an allophycocyanin-conjugated mouse anti-CD107 Ab for 24 h at 37˚C, 5% CO_2. In blocking experiments, AIV-coated plates were incubated with soluble hIg proteins for 1 h at 37˚C before the NK cells were added.

Statistical analyses

Nonparametric statistical tests were used when the assumption of normally distributed data was not met. Differences between the groups were analyzed using Mann–Whitney *U* tests. A p value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 6.05 (GraphPad, La Jolla, CA).

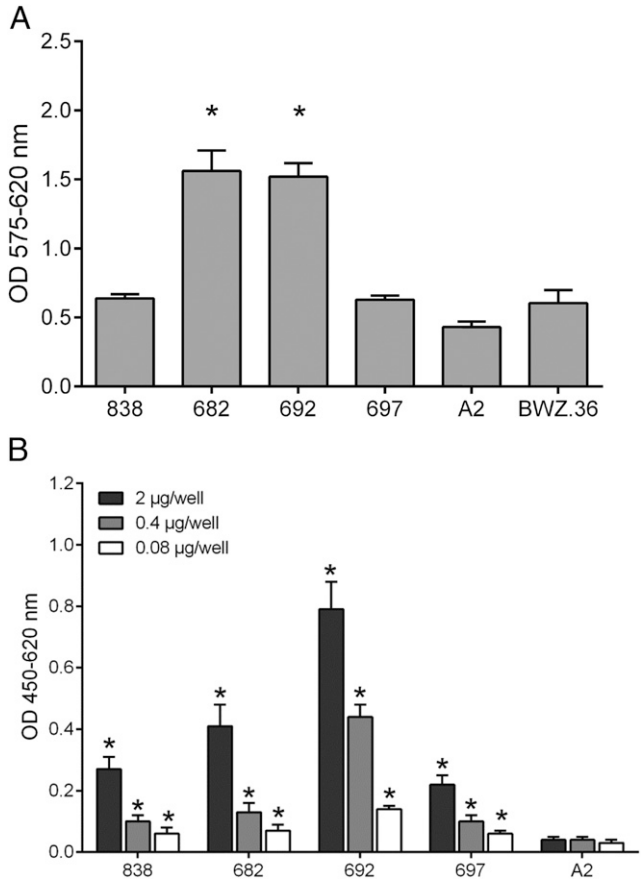

FIGURE 1. AIV binds to CHIR 682 and 692. (**A**) CHIR-expressing reporter cells or untransfected BWZ.36 reporter cells were incubated in AIV-coated plates. (**B**) Soluble CHIR–hIg fusion proteins were serially diluted on AIV-coated plates, and binding was measured by ELISA. All data are mean ± SEM of four independent experiments in triplicate. *$p <$ 0.05 versus negative control A2.

Results

Identification of CHIRs that recognize AIV

To identify novel CHIR ligands, we generated a panel of BWZ.36 cell lines stably transfected with prototypic CHIRs from different subgroups (23). All CHIRs were tagged with an N-terminal FLAG epitope, and all of the resulting reporter cell lines responded with β-galactosidase production upon cross-linking with an anti-FLAG Ab (data not shown). Using commercially available AIV-coated plates, the BWZ.36 cell lines expressing 682 and 692 responded strongly, whereas minimal signaling was observed for 838 and 697 (Fig. 1A). No binding was observed for BWZ.36 A2, an unrelated negative control cell line, as judged by OD values similar to those for untransfected BWZ.36 cells (Fig. 1A).

To further demonstrate the specificity of binding between certain CHIRs and AIV, we next generated fusion proteins of the extracellular CHIR domain coupled to the CH2 and CH3 domains of hIgG1. Again, AIV binding was observed in a dose-dependent fashion (Fig. 1B), with the strongest binding detected for 692 and 682, followed by weak binding for 838 and 697 and lack of binding for A2.

In a next set of experiments, we investigated whether the fusion proteins were able to block the interaction between AIV and the BWZ.36–CHIR cell lines (Fig. 2). AIV-induced cross-linking of BWZ.36 682 was reduced upon preincubation with 5 μg/ml 682–hIg fusion protein. Preincubation with the nonbinding A2–hIg fusion protein did not inhibit the signal (Fig. 2A). Similar results were observed for 692; AIV cross-linking was inhibited in the presence of 5 μg/ml 692–hIg fusion protein but not in the presence of A2–hIg fusion protein (Fig. 2B). These experiments demonstrated that selected CHIR members can bind to AIV.

AIV proteins expressed on infected cells are recognized by CHIR 692

Next, we investigated whether 692 was able to recognize viral proteins expressed on the cell surface. For this purpose, MDCK cells were infected with LPAI virus strain H7N1. The level of infection was checked by staining the infected MDCK cells with an anti-HA polyclonal antiserum. More than 80% of the cells were found to be infected after 24 h (Fig. 3A). Binding of single 692–hIg fusion proteins to the infected cells could not be detected (data not shown). To amplify the signal, we complexed biotinylated CHIR–hIg fusion proteins to streptavidin beads. In this way, a complex of four CHIR–hIg proteins was formed and was used to stain infected MDCK cells. To determine whether AIV proteins, rather than MDCK cell surface proteins, were recognized by 682–hIg and 692–hIg proteins, infected and mock-infected MDCK cells were stained with CHIR–hIg–strep complexes. Fig. 3B and 3C show a representative example of binding of 682 and 692 to H7N1-infected cells (Fig. 3B, 3C, black line) but not to mock-infected cells (Fig. 3B, 3C, gray line). To compare several experiments, we related the mean fluorescence intensity obtained after staining of infected cells to the staining of mock-transfected cells. In three experiments, staining of H7N1-infected cells with 692–hIg

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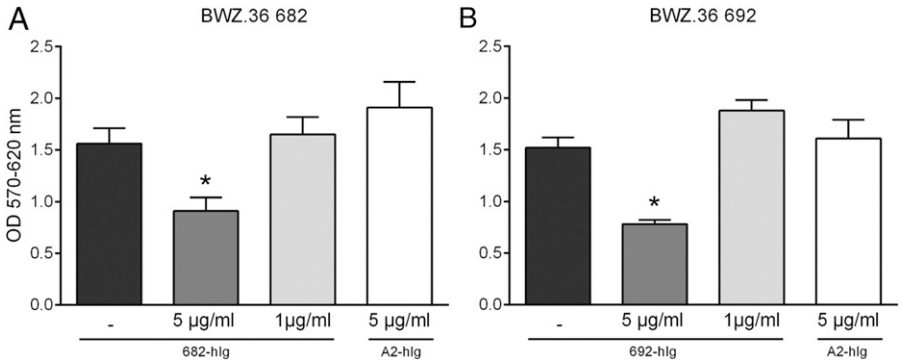

FIGURE 2. The interaction between CHIR and AIV is blocked by CHIR–hIg fusion proteins. BWZ.36 682 (**A**) and BWZ.36 692 (**B**) were incubated in AIV-coated plates that were preincubated with 5 or 1 μg/ml 682–hIg (A) or 692–hIg (B) or with 5 μg/ml A2–hIg as a control, and β-galactosidase expression was measured. Mean ± SEM of three independent experiments in triplicate are shown. *$p < 0.05$ versus the response in the absence of hIg.

proteins resulted in a higher mean fluorescence intensity compared with staining of the mock-transfected cells. In two of three experiments, staining with 682–hIg proteins resulted in a higher mean fluorescence intensity compared with staining of mock-transfected cells. No binding of streptavidin complexes without CHIR–hIg to infected or mock-infected MDCK cells was observed (data not shown). In conclusion, 682 and 692 are able to recognize AIV-infected cells, and the latter shows higher binding.

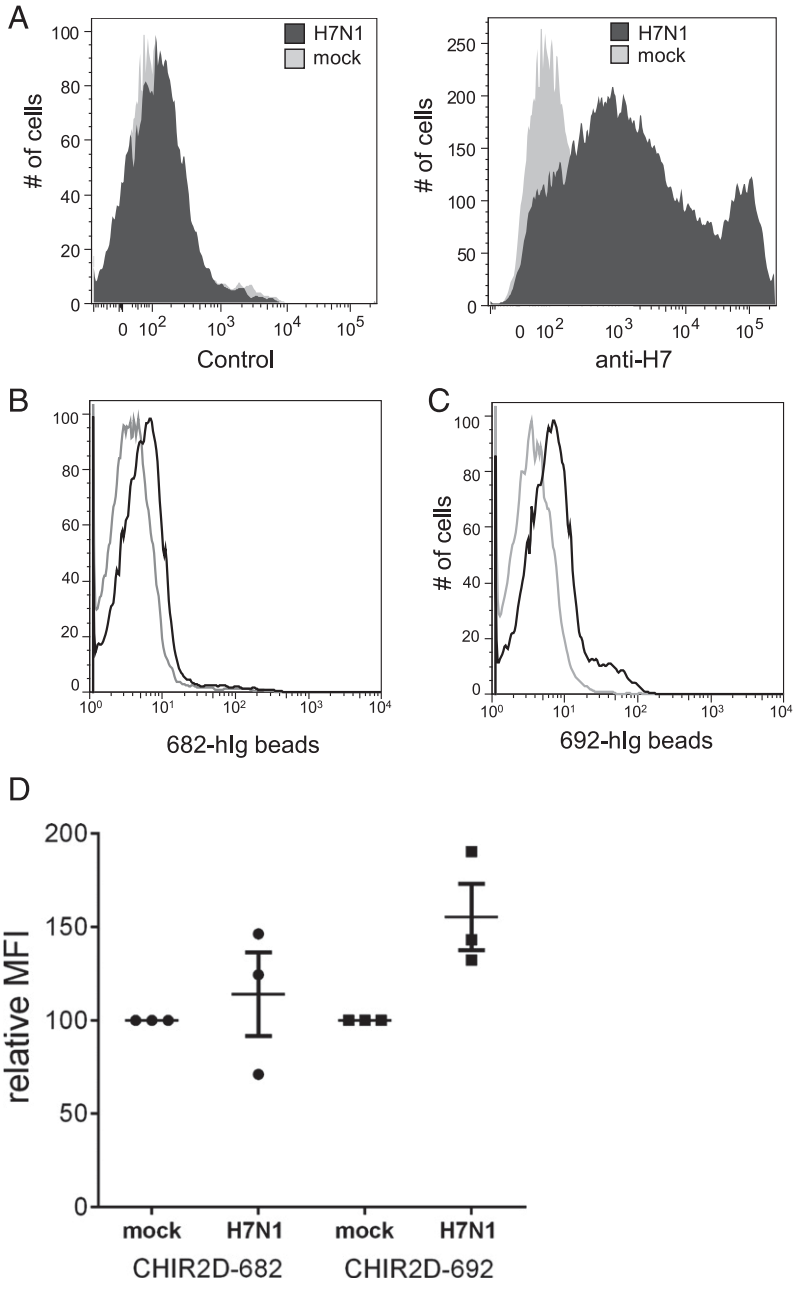

FIGURE 3. AIV-infected cells recognize CHIR–hIg. (**A**) MDCK cells were mock infected or infected with H7N1. Cells were stained with control or anti-H7 mAb 24 h postinfection. An overlay of mock-infected (gray line) and H7N1-infected (black line) MDCK cells stained with 682–hIg complexes (**B**) or 692–hIg complexes (**C**). (**D**) Relative mean fluorescent intensity (MFI) comparing 682–hIg or 692–hIg staining of mock-infected (set to 100) and H7N1-infected MDCK cells. Mean ± SEM of three independent experiments are shown.

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No differences in binding between virus strains

Binding of 682 and 692 to AIV was demonstrated using commercial AIV-coated plates. These plates are coated with a mixture of AIVs; therefore, it is not possible to discriminate between AIV strains. Because we were interested in possible differences in CHIR binding between AIV strains, 96-well plates were coated with various inactivated AIV strains. BWZ.36 682 and BWZ.36 692 cells were incubated with these different viruses, and their activation was analyzed. Cross-linking with plate-bound LPAI virus strains H5N2, H7N1, and H9N2 induced activation of 682 (Fig. 4A) and 692 (Fig. 4B), whereas BWZ.36 A2 did not respond (Fig. 4C). No differences in activation upon cross-linking were observed among the different virus strains; HPAI H5N1 viruses induced similar activation of BWZ.36 682 and BWZ.36 692 compared with LPAI viruses.

Preincubation with soluble CHIR–hIg constructs inhibits AIV-induced activation of primary lung NK cells

Because AIV-induced signaling of BWZ.36 682 and BWZ.36 692 was reduced in the presence of the corresponding soluble CHIR constructs, we next questioned whether the AIV-induced activation of primary NK cells could also be inhibited by these CHIR–hIg fusion proteins. Activation of chicken NK cells can be analyzed by measuring the surface expression of CD107 protein (27), similar to what was shown in mammals (28). Lymphocytes isolated from lungs of AIV-infected chickens were cultured in AIV-coated plates, and CD107 expression was analyzed by flow cytometry on $CD3^{-}CD8^{+}$ NK cells 24 h later. In the absence of hIg fusion proteins, the percentage of activated NK cells was 44.4 ± 2.55% (Fig. 5), which was similar to the percentage observed upon incubation with A2–hIg proteins (45.6 ± 1.9%). Interestingly, in the presence of 682–hIg and 692–hIg fusion proteins, the percentage of $CD107^{+}CD3^{-}$ cells was decreased ~10 and 14%, respectively. In conclusion, specifically blocking the interaction between AIV and primary NK cells inhibits AIV-induced NK cell activation.

CHIR binding to AIV represents activating CHIR-A forms

The prototypic CHIRs that were used to generate BWZ.36 reporter cell lines originated from experiments addressing the complexity of the extracellular CHIR domains. Consequently, only the extracellular portions of CHIR were amplified. Based on their cytoplasmic domains, CHIRs can be divided into activating CHIR-A, inhibitory CHIR-B, and bifunctional CHIR-AB. Therefore, having demonstrated the binding of 692 and 682 to AIV, we next searched for a full-length CHIR with reactivity toward AIV. This search was complicated by the enormous CHIR variability and the fact that 692 and 682 only shared 63% amino acid identity in their extracellular domains. We amplified CHIRs from the identical cDNA source used previously with three sets of primers that amplified full-length CHIRs of each subgroup. We next sequenced a total of 60 clones and performed sequence alignments with 692 and 682, which resulted in amino acid identities between 55 and 86%. CHIR 825 was arbitrarily selected for further studies because it displayed the highest homology of all sequences to 692 (86%), whereas it exhibited only 61 and 71% homology with 682 and A2, respectively (Fig. 6). The charged transmembrane residue and the short cytoplasmic domain identify 825 as a member of the activating CHIR-A subgroup (Fig. 6A).

To confirm whether 825 is also specific for AIV, we produced an 825–hIg fusion protein. The 825–hIg fusion protein bound equally well to the AIV-coated plates as did 692 (Fig. 6B). Therefore, AIV can be specifically bound by a CHIR-A.

CHIR 692 recognizes the HA protein of AIV

We investigated the interaction between the HA protein of H5N1 and 692 using a solid-phase binding assay, as previously described (26). As shown in Fig. 7A, the trimeric H5 protein recognized 692 in a concentration-dependent way. In addition, binding of the HA protein to fetuin was observed, whereas the H5 protein did not interact with desialylated asialofetuin. Interestingly, VCNA treatment of 692 (Fig. 7B) or fetuin (Fig. 7C) decreased the binding of H5 trimeric proteins. Because VCNA treatment of the receptor removes SA residues, these results suggest that the interaction between 692 and trimeric H5 proteins is dependent on SA moieties on CHIR.

Discussion

CHIRs were originally identified by using homology searches with mouse PIR-B probes in the chicken EST database (29). This family

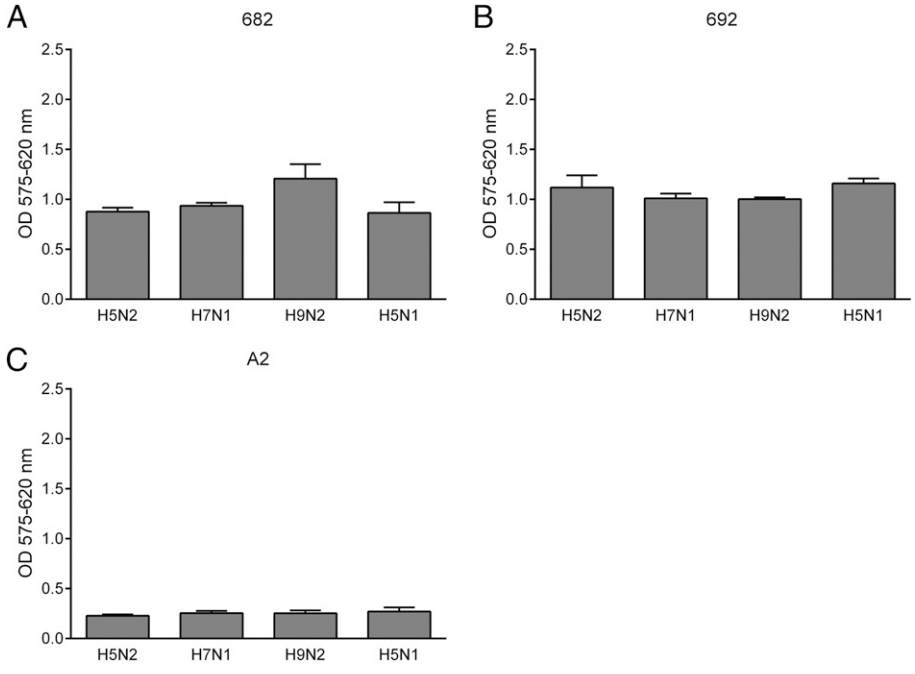

FIGURE 4. No difference in CHIR binding to LPAI and HPAI. 682-expressing BWZ.36 reporter cells (**A**), 692-expressing BWZ.36-reporter cells (**B**), and A2-expressing BWZ.36 reporter cells (**C**) were incubated in wells coated with LPAI virus strains H5N2, H7N1, or H9N2 or with HPAI H5N1 (2 μ per well), and β-galactosidase expression was measured. Mean ± SEM of three independent experiments in triplicate are shown.

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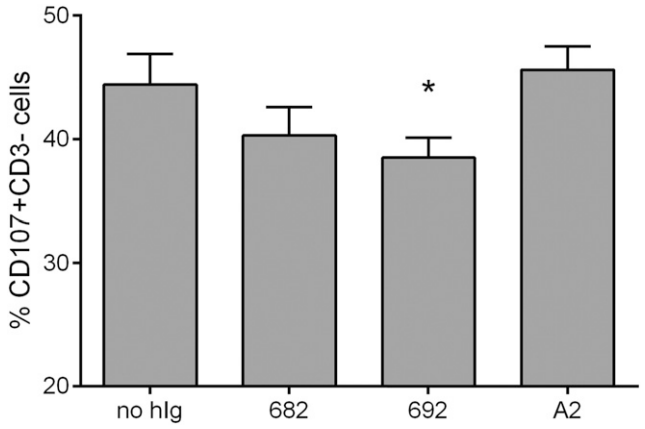

FIGURE 5. CHIR–hIg fusion proteins inhibit AIV-induced activation of lung NK cells. Lung lymphocytes isolated from chickens infected with the LPAI virus strain H9N2 were incubated in AIV-coated plates preincubated with 5 μg/ml 682–hIg, 692–hIg, or A2–hIg or PBS. After 24 h, cell surface expression of CD107 was analyzed on $CD3^{-}CD8\alpha^{+}$ cells by flow cytometry. A representative example of three independent experiments in triplicate is shown. *$p < 0.05$ versus no hIg.

of Ig-like receptors in chickens includes activating, inhibitory, and bifunctional receptors that share homologous extracellular Ig domains but differ in their transmembrane and cytoplasmic domains (30). Only one CHIR ligand has been identified; several CHIR-AB receptors that contain only a single Ig domain and combine features of inhibitory and activating CHIRs bind to chicken IgY with high affinity (10). In contrast to CHIR-AB, CHIR-A and CHIR-B are more numerous, and they generally contain two Ig domains. Therefore, we aimed to identify novel CHIR ligands, with particular emphasis on pathogen-derived molecules, because the high CHIR diversity could have been the result of a selection process driven by a rapidly mutating pathogen.

To search for influenza-binding NK cell receptor in chickens, we grouped all available CHIR sequences based on sequence comparisons of their extracellular domains, and representative receptors were selected for ligand-binding assays (23). This approach resulted in the identification of AIV as a novel ligand for this family of Ig-like receptors in chickens. AIV was recognized by 682 and 692. The interaction of these CHIRs with AIV was specific, because it was reduced with soluble hIg fusion proteins, and it was confirmed using CHIR–hIg fusion proteins in an ELISA system. Staining of AIV-infected MDCK cells with CHIR–hIg proteins detected only a small fraction of cells, especially compared with the high percentage of infected cells. This may be due to the neuraminidase activity of the influenza virus. Neuraminidase was reported to remove SA residues from NKp46, which results in reduced recognition of the influenza virus (31). Alternatively, this low level of staining

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A

Ig1

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825  LPRPSLSLRPSQGVSLGDPVTLRCHLPHMAAWVWLYHEEGRSYNKGKKKEQDAAAFFFVSTLQEHAGRYWCQYRVSESAELSVESDPVELVLT
682  ........H...E.....T..M.....QP....E..QDGLLRSY.DMD.H.VM.E.SL.CVKL.D.MK.......L.PPGV.EK.V.I...V.
692  ........H..H......N........RQ.......Q.G.WF.S........V.E.S.....R.............W.E.A.EK.........
A2   ........H.........T........RL.......R.GRW..A.YID.K..TTE.S.L..SR....T.S...QL...EDI..M.........
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Ig2

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825  DLRYPPCRISLHPEQHVGTGTNVTISCWNKDYGATFLLHKDGSSDPIQRQESSGGGTATFTLFGVTPADSGIYRCSYRPWHYAFMSSPLGDSVMLE
682  .H..S.PN..IS..KI.EV......Q.C.QG..GIIF.....H.A.L.H.DPR..................T.....H.KASL.V........T..
692  ......S..................R.....................K..N.....................T.....C..R.......P.......
A2   .RSF..PH......ER.........C........A.......R.A....LVPDDVA.VS............T.......RN.S.L......I.T..
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EX, TM, CY

```
825  VTPTP--APPGAELVSCGNLVVAVVRGCAAVLIFGLGVFFVIDARSLWIRRDESLGGEGI
A2   ....AEF.........R..........^....A...A.........................
```

B

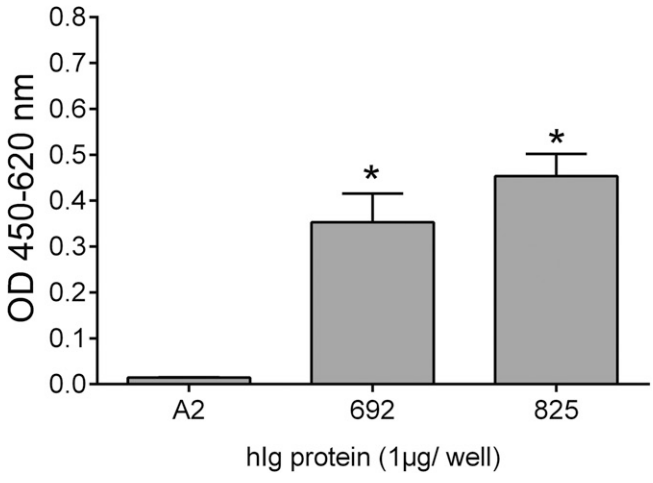

FIGURE 6. AIV-binding CHIRs belong to a family of activating receptors. (**A**) Amino acid alignment of 825 with 682, 692, and A2. The Ig domains (Ig1, Ig2) and the rest of the molecule, including the transmembrane region (line above the sequence), the charged transmembrane residue (arrowhead), and potential *N*-linked glycosylation sites, are indicated. Note that the Ig2 domains of 682 and 692 are not shown in their entirety, because the original amplification strategy used to identify CHIR diversity included the end of the Ig2 domain as primer site. (**B**) hIg proteins of 825, closely related to 692, were generated and binding of the hIg fusion proteins to AIV-coated plates was analyzed by ELISA. Mean ± SEM of three independent experiments in triplicate are shown. *$p < 0.05$ versus A2.

could be explained by a low-affinity interaction between CHIR and AIV. Because we used CHIR–hIg fusion proteins, these may not resemble the native form of the receptor found on the cell surface and, thus, decrease lower the affinity. In fact, many of the interactions described for various Ig-like family receptors are rather weak; this is most likely the reason why a proportion of the Ig-like family members are still classified as orphan receptors, because no potential ligands have been described (32).

Binding of AIVs of various HA types to 692 was analyzed, including the highly pathogenic AIV H5N1, using BWZ.36 reporter cells. All viruses tested resulted in receptor cross-linking and signaling, and no differences between low pathogenicity and highly pathogenic AIV were observed. This is in agreement with the binding of human influenza viruses that vary in HA type to human NKp46 (18).

Our initial findings were limited by the design of the representative BWZ.36 CHIR reporter lines, because they were generated from partial CHIR sequences with no information regarding their transmembrane or cytoplasmic domains. Therefore, it was not possible to further classify the CHIR into one of the three major subtypes. To overcome this limitation, we cloned additional CHIR genes from the same DNA source and isolated a CHIR that was homologous to 692. This 825 bound to AIV with identical characteristics and was further used to explore the CHIR–AIV interaction in more detail. The 825 belongs to the CHIR-A subgroup. This explains the finding that the 692–hIg fusion protein reduced the CD107 activation of lung NK cells, indicating that the CHIR–AIV interaction leads to cellular activation by the association of intracellular adaptor proteins caused by CHIR ligation. The blocking effect observed with the fusion protein was significant, but it did not totally block cellular activation. This is most likely caused by the fact that the lung cells carry many CHIR-A versions. An unknown proportion of these may interact with AIV at different sites and, among those, only receptors with a binding mode similar to 692 can be efficiently blocked. We have no evidence for AIV interaction with CHIRs belonging to the other subgroups, especially CHIR-B; however, it is likely that CHIRs with inhibitory cytoplasmic domains also bind to AIV. So far, we have not found paired receptors within the CHIR family. Extensive genetic analysis showed no receptor pair that was 100% identical in the extracellular region but different in the transmembrane domain or the cytoplasmic tail (33). However, because many CHIR genes are highly homologous (30), we cannot exclude the possibility that two highly similar receptors from different CHIR subgroups that recognize the same ligand may, in fact, be paired receptors. Interestingly, we identified three CHIR sequences derived from a cDNA sample from a single chicken that have the potential to interact with AIV but do not share high homology. Therefore, it can be speculated that individual chickens have several CHIRs that do interact with AIV. It remains to be seen whether all of these CHIRs belong to the activating subgroup. Given that the CHIR family is highly diversified, with multiple receptors in individual animals (30), it is tempting to speculate that it is the interaction between AIV and an individual CHIR haplotype that determines the outcome of the infection.

The single CHIR crystal structure that is available was reported to display the closest structural homologies to two mammalian proteins. These include the FcαRI receptor and the mammalian NKp46 protein (19). NKp46 was recognized as a pan species NK cell marker (34). Because NKp46 is known to interact with the HA protein of influenza virus, among other ligands (35), we investigated the interaction of 692 with the HA protein of AIV. Solid-phase binding assays using H5 trimers demonstrated an interaction between HA and 692, indicating that the HA protein mediates AIV recognition of this CHIR.

Removal of SA residues on the receptor by VCNA treatment decreased binding of the HA protein, which suggests that binding of HA to the receptor is dependent on SA residues. However, analysis of the sequences did not reveal the presence of *N*-linked glycosylation sites present in binders (Fig. 6). Apart from a single

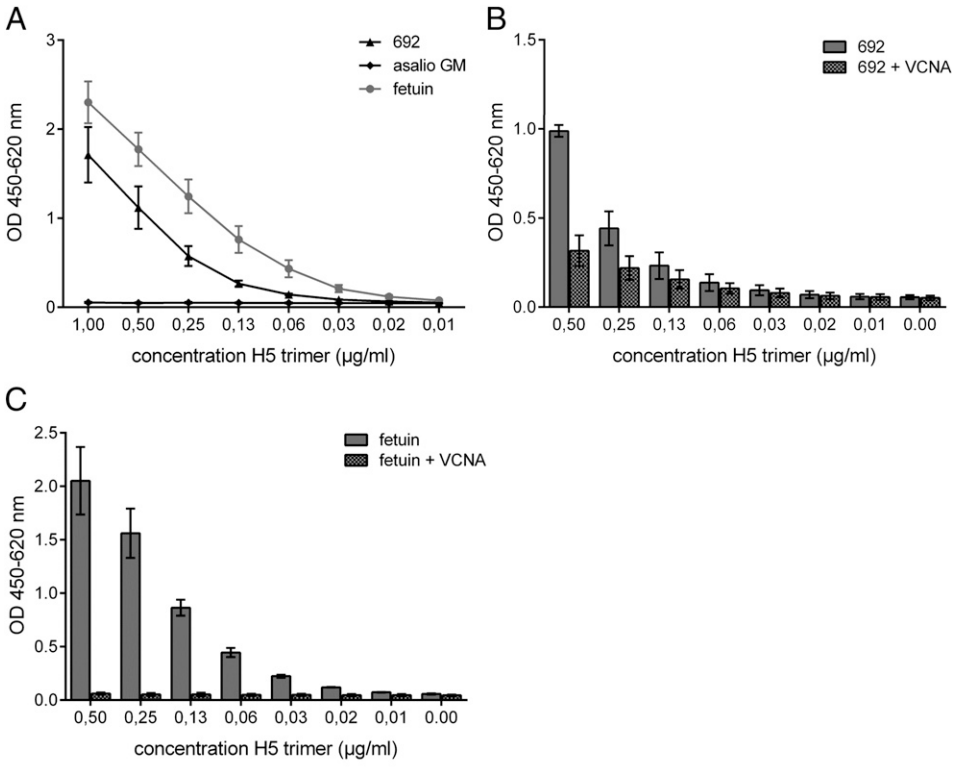

FIGURE 7. Binding of CHIR to trimeric HA proteins is dependent, in part, on SA residues. (**A**) Nunc MaxiSorp plates were coated with 1 µg/ml 692–hIg protein, fetuin, or asialofetuin, and binding of recombinant soluble HA trimers was analyzed. 692–Ig (**B**) or fetuin (**C**) was treated with VCNA prior to analyzing the binding of soluble HA trimers. Mean ± SEM of $n = 5$ is shown in (A); mean ± SEM of $n = 2$ is shown in (B) and (C).

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conserved potential *N*-linked binding site in all sequences, each CHIR analyzed possessed a unique set of potential *N*-linked binding sites. This fact, together with the lack of identical amino acid motifs found only in the binders, may suggest the need for a contribution of both protein binding and glycosylation in ligand binding.

The SA dependence of the HA-CHIR interaction is in agreement with is in agreement with earlier studies describing the interaction between mammalian receptors and HA. Human NKp46 recognizes viral HA in an SA-dependent manner, and *O*-linked glycosylation plays an essential role in this interaction (18). NCR1 also recognizes influenza virus in an SA-dependent manner. In this interaction, *N*-linked glycosylation is important for binding (36), although the exact *N*-linked glycosylation site involved has not been identified.

In vivo studies in mice showed that NCR1 is required for clearance of the influenza virus (37), suggesting an important role for this receptor in influenza-specific immunity. Recently, we (21) reported diminished activation of lung NK cells upon infection with the highly pathogenic H5N1 virus in chickens. Additional studies are warranted to investigate whether the AIV-specific CHIR is involved in this process.

In conclusion, to our knowledge, we identified the first pathogen-derived CHIR ligand. Binding results in activation of NK cells, similar to what was described for mammalian NKp46. The observation that Ig-like receptors that recognize pathogens exist in chickens opens a novel area of avian research in which the outcome of a viral infection is influenced by the binding of virus to immunomodulatory receptors.

Disclosures

The authors have no financial conflicts of interest.

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