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Vimentin Expressed on *Mycobacterium tuberculosis*-Infected Human Monocytes Is Involved in Binding to the NKp46 Receptor\(^1\)

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We previously showed that human NK cells used the NKp46 receptor to lyse *Mycobacterium tuberculosis* H37Ra-infected monocytes. To identify ligands on H37Ra-infected human mononuclear phagocytes, we used anti-NKp46 to immunoprecipitate NKp46 from NK cells bound to its ligand(s) on H37Ra-infected monocytes. Mass spectrometry analysis identified a 57-kDa molecule, vimentin, as a putative ligand for NKp46. Vimentin expression was significantly up-regulated on the surface of infected monocytes, compared with uninfected cells, and this was confirmed by fluorescence microscopy. Anti-vimentin antiserum inhibited NK cell lysis of infected monocytes, whereas antiserum to actin, another filamentous protein, did not. CHO-K1 cells transfected with a vimentin construct were lysed much more efficiently by NK cells than cells transfected with a control plasmid. This lysis was inhibited by mAb-mediated masking of NKp46 (on NK cells) or vimentin (on infected monocytes). ELISA and Far Western blotting showed that recombinant vimentin bound to a NKp46 fusion protein. These results indicate that vimentin is involved in binding of NKp46 to *M. tuberculosis* H37Ra-infected mononuclear phagocytes.

Previously, we demonstrated that NKp46 and NGK2D play important roles in the lysis of *Mycobacterium tuberculosis*-infected monocytes (13, 14). Of five NGK2D ligands, only UL16-binding protein 1 was involved in lysis of *M. tuberculosis*-infected monocytes and alveolar macrophages. Characterization of the mechanisms by which NKp46 binds to *M. tuberculosis* H37Ra-infected mononuclear phagocytes would be a significant advance in our understanding of innate immunity to intracellular bacteria. In the current study, we found that vimentin contributed to binding of NKp46 to H37Ra-infected monocytes.

Vimentin is a type III cytoskeletal protein that maintains the architecture of cytoplasm. It is also involved in cell adhesion, transcellular migration (15), wound healing (16), and cellular signaling (17). Recent studies suggest that vimentin plays a role during intracellular infection. For example, vimentin interacts with viruses during virion assembly and facilitates virion transport (18, 19). In addition, activated human macrophages secrete vimentin, which contributes to bacterial killing through generation of oxidative metabolites (20).

Immunoprecipitation, followed by mass spectrometry analysis, identified vimentin as a putative ligand for NKp46. Furthermore, flow cytometry and confocal microscopy showed that vimentin was expressed on the surface of monocytes and up-regulated by *M. tuberculosis* H37Ra infection. NK cells lysed CHO-K1 cell transfecants that expressed vimentin, and lysis was inhibited by mAb-mediated masking of NKp46 on NK cells or vimentin on CHO-K1 cells. In addition, binding of vimentin to NKp46 was confirmed by ELISA, radio-iodinated vimentin cell binding assay, and Far Western blotting.

Materials and Methods

**Patient population**

Heparinized venous blood was obtained from 18 tuberculin-negative healthy donors to eliminate effects due to *M. tuberculosis*-reactive T cells.

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3 Abbreviations used in this paper: HSPG, heparan sulfate proteoglycan; MOI, multiplicity of infection; MFI, mean fluorescence intensity.

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Depending on the experiment, 60–140 ml of blood was obtained from each donor. Bronchoalveolar lavage fluid was obtained from six healthy tuberculin-negative donors. All studies were approved by the Institutional Review Board of the University of Texas Health Center at Tyler.

**Antibodies**

**Immunostaining and FACS analysis.** The following mAbs were used: FITC-anti-CD14, FITC-anti-CD3, PE-conjugated anti-CD56 (all from BD Biosciences), and FITC-anti-vimentin (Research Diagnostics). To measure expression of NKp46 ligand(s) on infected monocytes, indirect immunostaining was performed with NKp46F(ab′)2 (R&D Systems) and a FITC goat anti-human secondary Ab (Jackson Immunoresearch Laboratories). Immunostaining was performed by standard methods (21). Based on forward and side light scatter characteristics, we gated on lymphocytes or monocytes, and the percentage of cells labeled with specific surface markers was determined with a FACSCalibur (BD Biosciences).

**Fluorescence microscopy.** To detect vimentin on the surface of monocytes by fluorescence microscopy, we used anti-vimentin mAbs and a mouse isotype control Ab (both from BD Biosciences).

**Neutralization experiments.** The mAbs to NKp46 (BD Biosciences), rabbit antiserum to human vimentin (Biomeda), and control rabbit antiserum (Sigma Genesis) were used for neutralization experiments.

**Culture of alveolar macrophages**

Bronchoalveolar lavage was performed in the right middle lobe and the anterior segment of the right upper lobe of healthy tuberculin-negative donors. For each lobe, 80 ml of saline was instilled, and ~40 ml of fluid return was obtained with a syringe. Bronchoalveolar lavage fluid was centrifuged at 834 × g for 5 min. The cell pellet was resuspended in RPMI 1640 (Invitrogen Life Technologies), supplemented with sodium pyruvate (Invitrogen Life Technologies), penicillin-streptomycin (Invitrogen Life Technologies), and 10% human AB serum (Atlanta Biologicals) (RPMI 1640 complete medium). Approximately 90% of the bronchoalveolar lavage cells were macrophages, as judged by Giemsa staining. Cells (1.2 × 10^6) were adhered to each well of 12-well flat-bottom plates (BD Biosciences Labware) for 2 h. Nonadherent cells were removed by washing each well three times with 4 ml of RPMI 1640, and adherent cells were cultured with RPMI 1640 complete medium. Some adherent cells were infected with frozen aliquoted stocks of H37Ra at a multiplicity of infection (MOI) of 20:1. We used a higher MOI for alveolar macrophages than for monocytes because we previously found that a MOI of 20:1 provided infectivity that was almost 100% in healthy donors.

**Preparation of monocytes for cytotoxicity assays**

PBMC were isolated by differential centrifugation over Ficoll-Paque (Amersham Biosciences). Monocytes were isolated with magnetic beads conjugated to anti-CD14 (Miltenyi Biotech), and positively selected cells were cultured with RPMI 1640 complete medium. Some adherent cells were used for staining with anti-vimentin Abs.

**Infection of cells with Listeria monocytogenes**

Some monocytes, prepared as outlined above for infection with H37Ra, were infected with L. monocytogenes serovar 1/2b strain (ATCC BAA-839) at a MOI of 1:1. After 48 h, uninfected and L. monocytogenes-infected cells were used for staining with anti-vimentin Abs.

**Isolation of NK cells**

CD3^+ CD56^- NK cells were isolated from PBMC. To avoid contamination with CD3^+ CD56^- cells, we depleted CD3^+ cells from PBMC with magnetic beads conjugated to anti-CD3 (Miltenyi Biotec). From the CD3^- cell fraction, CD56^- cells were isolated by positive selection with magnetic beads conjugated to anti-CD56 (Miltenyi Biotec). These cells were 95–100% CD56^- and 95–97% CD3^-, as measured by flow cytometry, and were used as effector cells.

**Cytotoxicity assay**

NK cell-mediated cytotoxicity against infected and uninfected monocytes, alveolar macrophages, and CHO-K1 cells was assayed in a ^51Cr release assay, using standard methods (23). Briefly, target cells were labeled overnight with 100 μCi of sodium chromate. Target cells were washed three times, and triplicate wells of 10^5 cells/well were mixed with effector cells at an E:T ratio of 40:1 in 200 μl of RPMI 1640 with 10% heat-inactivated human serum in 200-μl round-bottom wells. Ten hours after incubation, 100 μl of supernatant was removed from each well, and radioactivity was measured in a gamma counter. When CHO-K1 cells were used as targets, supernatant was removed after 4 h to measure radioactivity. The percentage of lysis was calculated as 100 × (experimental release − spontaneous release)/maximum release − spontaneous release).

**Immunoprecipitation of NKp46 receptor**

We determined the specificity and feasibility of using anti-NKp46 mAb for immunoprecipitation. Approximately 100 ml of blood was obtained from each of 10 healthy donors. CD3^− CD56^− NK cells (5–8 × 10^6) were isolated from PBMC of each donor by magnetic selection, as noted above, and cultured separately with rIL-2 (100 U/ml) and rIL-12 (5 ng/ml) to up-regulate NKp46 expression in methionine-free medium with 100 μCi of [35S]methionine. After 15 h, cells were washed with PBS three times, and membrane extracts were prepared, using Triton X-100 extraction buffer. Extracts from four to five donors were pooled and immunoprecipitated with anti-NKp46 mAb 461-G1-coated protein A-Sepharose beads, using standard methods (10). Immunoprecipitated Ag was resolved by SDS-PAGE and visualized by autoradiography. A single band of 46 kDa, corresponding to the molecular mass of NKp46, was visualized with anti-NKp46 antisera but not with control antisera (data not shown). This demonstrates that the anti-NKp46 mAb 461-G1 can be used for immunoprecipitation.

**Immunoprecipitation of NKp46 ligand(s)**

We next determined whether 461-G1 would immunoprecipitate NKp46 ligand(s) on the surface of *M. tuberculosis*-infected monocytes. Approximately 100 ml of blood was obtained from each of four healthy donors. Approximately 8–10 × 10^6 CD14^- monocytes were isolated from each donor by magnetic selection, as outlined above. The cells were infected with H37Ra and cultured in methionine-free medium with 100 μCi of [35S]methionine. After 48 h, membrane extracts were prepared with Triton X-100 extraction buffer. Membrane extracts from individual donors were pooled. Next, we obtained 100 ml of blood from each of five healthy donors and CD3^- CD56^- NK cells were isolated, as outlined above, and cultured separately with rIL-2 (100 U/ml) and rIL-12 (5 ng/ml) to up-regulate NKp46 expression. After 15 h, cells from each individual donor were washed with PBS three times, and membrane extracts were prepared using Triton X-100 extraction buffer. These unlabeled NK cell membrane extracts were pooled and immunoprecipitated with 461-G1-coated protein A-Sepharose beads. These immunoprecipitated beads were incubated overnight with the [35S]methionine-labeled membrane extracts of infected monocytes. After extensive washing, proteins were eluted with 0.1 M glycine and 150 mM NaCl (pH 2.8), concentrated with Amicon Ultra filters, and analyzed by discontinuous SDS-PAGE under nonreducing conditions. For samples intended for mass spectrometry, an experiment was performed with unlabeled *M. tuberculosis*-infected macrophages, and the gel was stained with Coomassie brilliant blue.

**Fluorescence microscopy**

Expression of vimentin on the surface of infected monocytes was determined by fluorescence microscopy, as described previously (24). Control and infected monocytes and alveolar macrophages grown on Transwell filters were fixed in 2% paraformaldehyde in phosphate buffer (pH 7.2) and washed thoroughly. Nonspecific binding was blocked by incubating cell monolayers in blocking buffer (2% FCS in PBS). Cells were then incubated overnight with mouse anti-vimentin Ab (10 µg/ml) in blocking buffer. As a control, monocytes were incubated with either blocking buffer alone or mouse IgG (10 µg/ml). After washing, cells were incubated with Oregon Green 488-conjugated goat anti-mouse IgG Ab (10 µg/ml) (Molecular Probes) for 60 min. After thorough washing in PBS, Transwell filters were mounted in buffered glycerol. Slides were examined with a Zeiss LSM 510 confocal laser scanning.
microscope using a C-Apochromat ×63, 1.2 water immersion objective (Carl Zeiss), and the 488-nm line of the argon laser for excitation of Oregon Green. The cells were scanned and images saved at 1024 × 1024-pixel/8-bit resolution before importing into Adobe Photoshop (Adobe Systems) for compilation and direct printing.

**CHO-K1 cell transfection**

CHO-K1 cells were transiently transfected with a plasmid construct encoding vimentin (pCMV.SPORT6) or with the empty plasmid (Invitrogen Life Technologies), using LipofectAMINE 2000 (Invitrogen Life Technologies). Briefly, cells were seeded at 1 × 10^7/well in a 12-well plate. Twenty-four hours later, they were incubated with 1.6 μg of plasmid and 4 μl of LipofectAMINE 2000 in DMEM/10% FCS. After 48 h, transfected cells were used for cytofluorometric analysis. Cell transfectants were stained with FITC anti-vimentin Ab and analyzed by flow cytometry, using a FACSCalibur.

**Far Western blotting**

We used Far Western blotting to measure binding of vimentin to NKp46 fusion protein. We used rCD14 as a control. Recombinant vimentin (Research Diagnostics) and rCD14 (R&D Systems) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA in wash buffer for 1 h at room temperature, followed by overnight hybridization with NKp46:Fc in the same buffer at 4°C. After washing, binding of recombinant vimentin and CD14 with NKp46:Fc were detected by binding to a biotinylated anti-Fc Ab, followed by incubating with streptavidin-HRP (Pierce), and detection by chemiluminescence (Amersham Biosciences).

**125I-vimentin binding**

As an alternative means to evaluate binding of vimentin to NKp46, we performed a traditional binding assay, using recombinant 125I-labeled vimentin, which was prepared by standard methods (25). Recombinant vimentin (100 μg) was radiolabeled with 125I in a glass tube coated with iodogen (Pierce) for 30 min at 0°C, followed by centrifugal desalting to remove free 125I. Specific activities of radiolabeled vimentin ranged from 0.12 to 0.95 μCi/μg. We measured binding of radiolabeled vimentin to freshly isolated NK cells pretreated with mouse anti-NKp46 or mouse isotype control Ab. Bound and free radiolabel were separated as described previously (26). Briefly, 75 μl of the reaction mixture was layered on 250 μg of 20% sucrose, followed by centrifugation for 1 min at 10,000 × g at 22°C. The amount of radiolabeled vimentin bound to cells was determined by cutting the tube and counting the amount of radioactivity in the pellet. The amount of vimentin bound to NK cells in the presence of anti-NKp46 Ab or isotype control Ab was calculated from the radioactivity (counts per minute) bound to NK cells, after subtracting nonspecific binding. To further assess the specificity of vimentin binding, we performed a cold vimentin competition assay. Freshly isolated NK cells were incubated either with recombinant 125I-labeled vimentin (1 μM) alone or with increasing concentrations of cold vimentin (0.0004–1 μM) plus 125I-labeled vimentin. Binding of 125I-labeled vimentin to NK cells was measured by the methods described above.

**Statistical analysis**

Results are shown as the mean ± SE. For data that were normally distributed, comparisons between groups were performed by a paired or unpaired t test, as appropriate. For data that were not normally distributed, the Wilcoxon rank-sum test was used. Values of p < 0.05 were considered statistically significant.

**Results**

**NKp46-fusion Ig binds to M. tuberculosis H37Ra-infected monocytes**

To determine whether putative NKp46 ligands are expressed on infected monocytes, we measured binding of NKp46-fusion Ig to infected and uninfected CD14+ monocytes from three healthy donors by flow cytometry. There was increased binding of NKp46-fusion Ig to infected monocytes, compared with uninfected cells (mean fluorescence intensity (MFI) 107 ± 39 vs 62 ± 14%; Fig. 1), suggesting that H37Ra up-regulates expression of one or more ligands for NKp46 on infected monocytes.

**Identification of vimentin on H37Ra-infected monocytes as a putative ligand for NKp46**

The anti-NKp46 Ab 461-G1 was used to immunoprecipitate NKp46 ligand(s) from membrane extracts of M. tuberculosis-infected monocytes, as outlined in Materials and Methods. Proteins were eluted, concentrated with Amicon Ultra filters, and analyzed by discontinuous SDS-PAGE under nonreducing conditions. Coomassie brilliant blue staining showed an intense band of 49 kDa and five other less intense bands of molecular masses 75, 61, 57, 32, and 27 kDa. After staining, the gel was washed three times with water for 60 min. All six bands were excised, and in-gel enzymatic digestion and mass spectrometry analysis were performed by Ampro. Computer analysis and a search of the National Center for Biotechnology Information human protein database showed that peptides in all six bands matched those in vimentin, suggesting that this protein was involved in binding to NKp46.

**Surface expression of vimentin on monocytes infected with M. tuberculosis H37Ra and Listeria**

Although vimentin is generally regarded as a filamentous intracellular protein, the findings above suggest that it may be involved in binding to NKp46. To determine whether vimentin is expressed on the surface of M. tuberculosis-infected monocytes, we isolated CD14+ monocytes from seven healthy donors and infected some monocytes with H37Ra. Vimentin expression by uninfected and infected monocytes was measured by flow cytometry and peaked after 48 h of infection. At that point, the percentage of vimentin-positive cells was increased 2- to 3-fold on infected monocytes, compared with uninfected cells (26 ± 6 vs 10 ± 2%; p = 0.002; Fig. 2). These findings suggest that vimentin is present on the cell surface of infected monocytes and has the potential to interact with NKp46 on NK cells.

**Far Western blotting**

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was present on the surface of both control and infected monocytes (Fig. 3).

To determine whether vimentin expression is increased specifically in monocytes infected with M. tuberculosis H37Ra, we isolated CD14\(^{+}\) monocytes from five healthy donors and infected some monocytes with H37Ra or with another intracellular bacterium, L. monocytogenes. After 48 h, vimentin expression was measured by flow cytometry. Staining with an isotype control mAb showed minimal numbers of positive control or infected monocytes (data not shown).

Effect of anti-vimentin Ab on NK cell-mediated lysis of M. tuberculosis H37Ra-infected mononuclear phagocytes

To determine whether neutralization of vimentin can block NK cell-mediated lysis of infected monocytes, we cultured freshly isolated NK cells with H37Ra-infected monocytes, in the presence of anti-vimentin antiserum or control antiserum (10 \(\mu\)g/ml). Because vimentin is a cytoskeletal protein, we also used antiserum to actin, another cytoskeletal protein, as a control. Anti-vimentin antiserum inhibited NK cell lysis of infected monocytes (mean net specific lysis 13 ± 5 vs 26 ± 6%, \(p = 0.001\); Fig. 4A), whereas anti-actin did not (mean net specific lysis 40 ± 12 vs 26 ± 6%, \(p > 0.05\); Fig. 4A). As shown in Fig. 2, vimentin expression levels on infected monocytes varied in different donors. For five healthy donors, the percentage of vimentin-positive infected monocytes correlated strongly with the net specific lysis of NK cells against autologous infected monocytes (\(r = 0.85\)).

M. tuberculosis is a pulmonary pathogen and it first encounters alveolar macrophages in the lung. To determine whether vimentin mediated NK cell lysis of H37Ra-infected alveolar macrophages, we used NK cells from five donors as effector cells against infected monocytes. As shown in Fig. 4, vimentin expression levels on infected monocytes varied in different donors. For five healthy donors, the percentage of vimentin-positive infected monocytes correlated strongly with the net specific lysis of NK cells against autologous infected monocytes (\(r = 0.85\)).

![Figure 2](image2.png)

**FIGURE 2.** Surface expression of vimentin by infected and uninfected monocytes. Monocytes from seven healthy donors were infected with M. tuberculosis H37Ra. After 48 h, CD14\(^{+}\) cells were stained with FITC anti-vimentin mAb, and the percentages of positively stained cells were determined by flow cytometry. Staining with an isotype control mAb showed minimal numbers of positive control or infected monocytes (data not shown).

![Figure 3](image3.png)

**FIGURE 3.** Surface expression of vimentin on M. tuberculosis-infected monocytes by fluorescence microscopy. Monocytes, either uninfected (A) or infected with M. tuberculosis H37Ra (B–D), were fixed and incubated with Abs as follows. A and B, Primary mouse anti-vimentin mAb, followed by Oregon Green 488-conjugated goat anti-mouse IgG secondary Ab, as detailed in Materials and Methods. C, Mouse IgG, followed by Oregon Green 488-conjugated secondary Ab. D, Oregon Green 488-conjugated secondary Ab.

![Figure 4](image4.png)

**FIGURE 4.** Effect of antisera to cytoskeletal proteins on NK cell lysis of infected monocytes (A) and alveolar macrophages (B). A. Freshly isolated NK cells from four healthy donors were cultured with autologous infected monocytes in the presence of 10 \(\mu\)g/ml of the control antiserum shown, and net percent-specific lysis was determined. B. NK cells were incubated with infected autologous or allogeneic alveolar macrophages, in the presence of anti-vimentin antiserum (10 \(\mu\)g/ml), or control antiserum (10 \(\mu\)g/ml), and net specific lysis was determined. Mean values and SEs are shown.
allogeneic alveolar macrophages. Anti-vimentin antiserum reduced net specific lysis of infected alveolar macrophages to 10 ± 4.5% (p < 0.005), compared with 26 ± 4% with control antiserum (Fig. 4B). When NK cells were used as effectors against autologous alveolar macrophages from three additional donors, anti-vimentin antiserum also reduced net specific lysis to 16 ± 1.5% (p < 0.02), compared with 27 ± 3% with control antisera (Fig. 4B).

**NK cell-mediated lysis of transfected cells expressing vimentin**

To more definitely determine whether vimentin is involved in NK cell-mediated lysis of target cells, we assessed the cytolytic capacity of NK cells against CHO-K1 cells that were transiently transfected with different constructs, as detailed in Materials and Methods. As shown in Fig. 5, freshly isolated NK cells from four healthy donors lysed cells transfected with the vimentin construct more efficiently than CHO-K1 cells transfected with an empty plasmid (28 ± 2 vs 16 ± 1%, p = 0.001). NK cell lysis of these transfectants was inhibited by anti-vimentin antiserum (10 ± 5 vs 28 ± 2%, p = 0.001) and anti-NKp46 mAb (10 ± 6 vs 28 ± 2%, p = 0.002). This suggests that interactions between NKp46 and vimentin enhance NK cell-mediated cytotoxicity.

**Direct binding of vimentin to NKp46 by ELISA and Far Western blot**

To determine whether vimentin bound to NKp46, we coated plates with recombinant vimentin, then measured binding of NKp46 and an irrelevant protein by ELISA. Briefly, plates were coated with recombinant vimentin overnight at 4°C. After blocking, plates were washed and incubated overnight at 4°C with NKp46:Fc or B cell maturation fusion protein or BSA as a control. The amount of bound protein was quantified by adding an alkaline phosphatase-conjugated secondary mAb, following a standard ELISA protocol. Vimentin specifically recognized the NKp46 fusion protein, whereas only background binding was observed with the B cell maturation fusion protein (mean OD value 0.42 ± 0.02 vs 0.15 ± 0.01, p = 0.001; Fig. 6A). Far Western blot analysis confirmed that NKp46:Fc bound to recombinant vimentin, a 57-kDa protein, but not to a control protein, CD14 (Fig. 6B).

**125I-vimentin binding**

We evaluated binding of recombinant 125I-labeled vimentin to NKp46 on CD3+CD56+ NK cells from three healthy donors. NK cells were incubated with anti-NKp46 or mouse isotypic control Ab, washed, and incubated with recombinant 125I-labeled vimentin. Anti NKp46 significantly inhibited binding of recombinant 125I-labeled vimentin to NK cells, when compared with isotype control Ab (1326 ± 257 vs 2243 ± 251 cpm, p = 0.01; Fig. 7). As an additional control for specificity, cold vimentin (0.4 μM) significantly inhibited binding of 125I-labeled vimentin to NK cells from two healthy tuberculin reactors (2280 ± 414 vs 4082 ± 1732 cpm).
Discussion

NKp46 is a natural cytotoxicity receptor that is present exclusively on NK cells and is believed to play a central role in NK cell function because it contributes to lysis of a wide variety of tumor cells, as well as cells infected with viruses and bacteria, including *M. tuberculosis* (13, 14). The only definitive ligands that have been identified for NKp46 are the viral hemagglutinins (10), but the mammalian cellular ligands have remained elusive despite intensive investigation. In this report, we demonstrate that vimentin, expressed on the surface of *M. tuberculosis* H37Ra-infected monocytes, participates in the binding of NKp46 to these monocytes and contributes to their lysis. Immunoprecipitation of NKp46-bound ligands on H37Ra-infected monocytes, followed by mass spectrometry analysis, identified vimentin as a putative ligand. Fluorescence microscopy and flow cytometry demonstrated that monocytes expressed vimentin on their surface and that expression was up-regulated by infection with H37Ra. ELISA, Far Western blotting, and radio-labeled NKp46 ligand cell binding assays showed that vimentin binds to NKp46. Furthermore, transfection of CHO-K1 cells with a plasmid that expressed vimentin increased susceptibility to NK cell lysis, which was in turn inhibited by neutralization of vimentin or NKp46, suggesting that interactions between these molecules contribute to NK cell-mediated lysis of infected monocytes. Neutralization of vimentin also reduced the capacity of NK cells to lyse H37Ra-infected alveolar macrophages, which are one of the first cells to encounter intracellular pulmonary pathogens. These results indicate that vimentin may play an important role in the initial stages of the local immune response to *M. tuberculosis* infection. Although we used H37Ra in our studies, our findings are likely to reflect those with virulent *M. tuberculosis* strains, as we previously found no difference in NK cell-mediated lysis of H37Ra- and H37Rv-infected monocytes (14).

Vimentin is generally considered to be an intracellular filamentous cytoskeletal protein (27–29). However, recent studies have shown that vimentin is present in plasma and can be secreted by blood endothelial cells and infected macrophages through the Golgi apparatus (20, 30). Although vimentin lacks a signal sequence, it has a highly positively charged amino-terminal domain that can react with the hydrophobic core of the lipid bilayer and can be directed into cell membranes through the endoplasmic reticulum (31). In addition, the carboxy terminus of vimentin has a di-acidic motif (Asp-X-Glu, where X is any amino acid) preceded by a Tyr-X-X-Y motif (where Y is a bulky hydrophobic residue), which is found in many membrane-associated proteins that are exported from the endoplasmic reticulum (32, 33). The secreted form of vimentin is more highly phosphorylated than the intracellular form (20), has a lower apparent molecular mass, and is recognized by different mAbs (20, 26). In addition, immunofluorescence microscopy showed that vimentin on the surface of EBV-transformed B lymphocytes is organized into diffuse patches, rather than filaments (34). The sum of these findings strongly suggest that secreted vimentin differs in sequence and structure from intracellular vimentin.

Vimentin is an acidic protein (35–37) and phosphorylation further increases its acidity, suggesting that secreted vimentin could interact with a basic protein or with positively charged amino acid residues. The 2.2-Å crystal structure of NKp46 suggests two putative ligand binding sites (38). One resembles the region of FcεRI that binds to Fcε. As with FcεRI, NKp46 contains several basic residues in the N terminus, including Arg39, Lys52, and Arg55 in the C′E loop and Arg80 in the FG loop (38), which have the potential to interact electrostatically with acidic residues of vimentin. The other putative ligand binding site on NKp46 resembles the HLA binding site on a killer Ig-related receptor (39, 40), but has a lower positive charge (38), suggesting that ligand recognition by this region of NKp46 is less influenced by electrostatic interactions than is recognition of MHC by a killer Ig-related receptor.

We found that vimentin is up-regulated on the cell surface of H37Ra-infected mononuclear phagocytes. TNF-α is a potent stimulus for macrophages to secrete vimentin (20), and it is intriguing to speculate that this cytokine, which is produced by *M. tuberculosis*-infected monocytes and is present in high concentrations at the site of disease in human tuberculosis (41, 42), up-regulates vimentin expression, and contributes to recognition of infected monocytes by NK cells. The secreted form of vimentin also enhances the cellular oxidative burst of macrophages (20) and therefore has the potential to mediate antimycobacterial activity. *M. tuberculosis*-infected monocytes also produce the anti-inflammatory cytokine IL-10 (43), which decreases vimentin production by macrophages (20), suggesting that IL-10 may inhibit TNF-α mediated vimentin expression in *M. tuberculosis* infection.

The precise mechanism by which vimentin contributes to binding of NKp46 to H37Ra-infected mononuclear phagocytes remains uncertain. It is possible that vimentin is a primary cellular ligand for NKp46. We attempted to confirm this by using surface plasmon resonance to directly measure binding of NKp46Fc protein to recombinant vimentin, which is only available in the intracellular filamentous form. Although no binding was observed (R. Vankayalapati, unpublished data), this does not exclude the possibility that direct binding occurs to extracellular vimentin. Another possibility is that vimentin is not the primary ligand for NKp46 but acts to facilitate interactions between NKp46 and its primary cellular ligand. This possibility is supported by the finding that neutralization of vimentin inhibited only 50% of NK cell-mediated lysis of infected mononuclear phagocytes (Fig. 4). Actin plays a similar role in stabilizing and enhancing interactions between the TCR, MHC molecules, and the costimulatory molecules CD28 and LFA-1 (44).

HSPG are membrane-associated molecules on the surface of all cells, and heparan sulfate moieties were recently reported to contribute to the lysis of tumor targets by NK cells through NKp30 and NKp46 (11). In contrast, other investigators found that HSPG did not influence the NKp30-mediated binding and lysis of tumor cells by NK cells (12). Because HSPG are tightly bound to cytoskeletal vimentin (45), we studied the potential contribution of HSPG to NKp46-mediated lysis of H37Ra-infected monocytes. Interestingly, flow cytometry showed that surface expression of HSPG on monocytes was significantly reduced by H37Ra infection, and Abs to HSPG only modestly inhibited NK cell lysis of infected monocytes (R. Vankayalapati, unpublished data). These findings suggest that HSPG may not contribute to NK cell interactions with H37Ra-infected cells, similar to the binding of influenza hemagglutinin to NKp46, which is independent of heparan sulfate (46).

In summary, we found that vimentin on the surface of H37Ra-infected monocytes and alveolar macrophages contributed to the capacity of NK cells to lyse infected cells through NKp46. Further studies are needed to determine whether vimentin is a primary cellular ligand for NKp46 or whether vimentin stabilizes and enhances interactions between NKp46 and its other cellular ligand.

Disclosures

The authors have no financial conflict of interest.
References


