Recognition of Mycobacterial Cell Wall Components by Langerin and the Ligand Recognition Differences between Human and Murine

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1. Abstract

Langerin is a pattern recognition receptor expressed especially on Langerhans cells in the skin but also found in several other organs such as lung and gut epithelium, as well as spleen in mouse and human tonsil. Langerin is a type II transmembrane C-type lectin receptor characterized by the presence of a calcium-containing sugar binding domain. Langerin is known to have a role in degrading viral particles such as HIV, Influenza A, and measles virus. It is also reported to be able to recognize pathogens such as *Mycobacterium leprae* and fungi which are then presented to T helper cells. Molecularly, langerin captures antigens through its carbohydrate recognition domain (CRD). The recognition of ligands by langerin is quite interesting as it is also able to bind ligands independently of its CRD through its neck region.

One of the pathogens known to interact with langerin is *Mycobacterium tuberculosis*. To search for ligands from this micro-organism, we utilized reporter cells expressing langerin-CD3ζ chimeric receptor and report the activation upon ligand binding by NFAT-GFP. We found that human and murine langerin are able to recognize *Mycobacterium tuberculosis* and also *Mycobacterium bovis* BCG by our system. Next, we extracted lipid-soluble components from BCG. This extract was able to activate human and murine langerin reporter cells. Further fractionation allowed us to find that human and murine langerin are activated by different types of ligands. Human langerin was able to recognize mannose-capped lipoarabinomannan and phosphatidylinositol mannosides. Surprisingly, murine langerin did not recognize any glycan ligand in the fraction. Mycolate and cardiolipin were found to be able to activate murine langerin reporter cells. Our data suggest that langerin may recognize other ligands besides glycan and further analysis could help us understand langerin-ligand recognition mode and how to target langerin for several purposes such as antigen delivery.

2. Keywords

Langerin, Pattern Recognition Receptor, Mycobacteria, Lipid, Glycan

3. Introduction

Langerin (CD207) is expressed mainly on Langerhans cells in the skin of mammals. In mouse, langerin can be found in lungs, gut epithelium and spleen1–6. Human is also known to express langerin in tonsils, gut epithelium, and lungs7. Because of this expression pattern, langerin is thought to act primarily by recognizing incoming pathogens from outside the body. some pathogens known to interact with langerin such as HIV, Influenza A virus, measles virus, *Mycobacterium leprae*, and some fungi8–12. Recognized pathogen then internalized, degraded, and loaded to MHCI or MHCII protein13. Pathogen components from langerin internalization known to activate T helper cells after presentation by Langerhans cells or other langerin-expressing dendritic cells (DCs).

One of pathogens that invades the epithelial is *Mycobacterium tuberculosis*. Epithelium is the first contact area with this pathogen, it is possible that Langerhans cells and other langerin-expressing DCs might be the first cells that recognize it using langerin. Some of the molecular ligands from this pathogen has been known as mannose containing substances14. From all of the report about langerin ligand, glycan is the only known ligand. From the structural point of view, langerin has CRD and calcium ion on it CRD. This fact drives the thinking that langerin specifically recognize glycan than any other substances.

In this study, we screened several components from *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. Human langerin was found to be able to recognize water-soluble component, mannose-capped lipoarabinomannan (Man-LAM), as well as lipid-soluble components such as mono-acyl phosphatidyl-myo-inositol dimannosides (AcPIM2) and diacyl phosphatidyl-myo-inositol dimannosides (Ac2PIM2). On the other hand, murine langerin recognized mycolate and cardiolipin. Seeing from its specificity to glycan ligand, finding non-glycan ligands of murine langerin is quite surprising and unexpected. These new ligands might extend our understanding on the langerin-ligand recognition mode and also have some role in receptor-specific antigen targeting for several purposes such as antigen delivery.

4. Experimental procedures

Reagents

PE anti mouse/human langerin (CD207) antibody (4c7) was purchased from Biolegend. Lipoarabinomannan (02449-61) was from Nacalai Tesque. Alpha, methoxy, and keto mycolate (791280, 791281, 791282), 14:0 Cardiolipin (710332), 18:1 Cardiolipin (710335), 18:2 Cardiolipin/heart Cardiolipin (840012), and 16:0-18:1 Cardiolipin (710341) were from Avanti. TDM (T3034) was from Merck.

Cells

Human or murine langerin ECD was cloned from plasmids containing cDNA of each corresponding sequence. The resulting clone was then ligated into CD3ζ containing pMX-P1b/CD3ζ-IRES-hCD8 plasmid vector15. The vectors were then transfected into Phoenix cells in order to infect 2B4-NFAT-GFP reporter cells and induce human or murine langerin expression. Upon ligand recognition by human or murine langerin, activation signal is transduced through phosphorylation of ITAM in CD3ζ.

Ig Fusion Protein production

Extracellular domain of human and murine langerin was amplified by PCR and then ligated to pDisplay plasmid containing Fc portion of human IgG1. The amplified DNA strands was put on the 3’ end of the human IgG1 Fc sequence. Resulting plasmids then transfected into Phoenix cells by PEI (Sigma) for one day. Transfection medium then changed to 10% FBS/DMEM and incubated on 37°C, 5% CO2 incubator for 7 days. Cells culture supernatant was collected and the produced Ig fusion protein concentration was measured.

Ig fusion binding assay

Candidate ligands were coated onto half-area High Bind Plate (Corning) by isopropanol. Block plate with 5% BSA/TSM (20mM Tris HCl, 1mM CaCl2, 150mM NaCl, and 2mM MgCl2) and incubated at RT (room temperature) for 2 hours then Ig fusion protein applied to the well after the plate washed by TSM buffer) and incubated at RT for 2 hours. Anti-human IgG-HRP then applied and incubated at RT for 1 hour. Substrate then applied and incubated for 30 minutes. Color change was measured by plate reader at 450 nm.

Reporter cells stimulation by mycobacteria, its extracts and fractions

For heat-killed mycobacteria, reporter cells were counted and seeded into 96-well plate and the mycobacteria was added in the culture medium. As for the extract, it was coated in the 96-well plate using isopropanol, then counted reporter cells were seeded into the wells. Incubation was done for 18-20 hours in a 5% CO2 incubator at 37°C. After incubation, reporter activity was analyzed by flow cytometry (Calibur-BD bioscience).

Lipid extraction and purification

*Mycobacterium bovis* BCG in dry form was extracted using Chloroform:Methanol = 2:1 (v/v) and distilled water at the same volume for 1 night. The mixture was then centrifuged at 2000 rpm for 5 minutes to separate water-soluble and lipid-soluble layers. Lipid-soluble layer was then evaporated by N2 gas and the exact amount was measured. Fractionation of this extract was done using HPTLC plate and developed by Chloroform:Methanol:Water = 90:30:5 (v/v). Developed plate was then fractionated into 16 fractions for further reporter cells stimulation.

5. Results

**Isolation of Langerin Ligand from *Mycobacterium tuberculosis***

To investigate whether langerin can recognize mycobacteria, we utilized NFAT-GFP reporter cells system which expressed human or murine langerin-CD3ζ chimeric receptor. *Mycobacterium tuberculosis* H37Ra, H37Rv, and *Mycobacterium bovis* BCG heat-killed bacteria were used to stimulate reporter cells. Murine and human langerin-expressing reporter cells were able to recognized heat-killed bacteria (figure 1B). Further, we extracted lipid-soluble components of *M. bovis* BCG and stimulated langerin-expressing reporter cells. Both human and murine langerin-expressing reporter cells were able to be activated by the lipid-soluble extract (figure 1C).

Thin layer chromatography (TLC) was used to separate the lipid-soluble extract into 16 fractions. Reporter readings from human langerin-expressing reporter cells showed that fraction number 2 and 4 of the TLC chromatogram are recognized by the receptor and fraction 4-6 and 16 were recognized by murine langerin-expressing cells. (Figure 1D)

**Phosphatidylinositol Mannoside and Lipoarabinomannan are Human Langerin Ligand**

The major water-soluble component of the mycobacterial cell wall is mannose-capped lipoarabinomannan (man-LAM) which is known to be present ubiquitously among *Mycobacterium* species. As man-LAM contains mannose residues and human langerin has the EPN motif which allows to recognize mannose residue, next we tried to stimulate reporter cells with man-LAM extracted from *Mycobacterium tuberculosis* aoyama-B. Indeed, human langerin-expressing reporter cells were activated by man-LAM but not murine langerin-expressing reporter cells (figure 2A).

Human langerin-expressing reporter cells were also able to recognize the lipid-soluble extract of *Mycobacterium bovis* BCG and from the TLC fractionation, we identified the specific fraction that is recognized by human langerin-expressing reporter cells. Previous study by Toyonaga *et al.* has already determined the structure of this fraction as mono-acyl phosphatidyl-myo-inositol dimannosides (AcPIM2) and diacyl phosphatidyl-myo-inositol dimannosides (Ac2PIM2). To ensure that AcPIM2 and Ac2PIM2 are able to activate human langerin-expressing reporter cells, we used AcPIM2 and Ac2PIM2 purified from *Mycobacterium bovis* BCG which had already been analyzed using mass-spectrometry. Human langerin-expressing reporter cells were activated by these glycolipids, and more potently by AcPIM2 than Ac2PIM2 (figure 2B).

From these results, we conclude that human langerin was able to recognize the mycobacterial water-soluble component man-LAM, and also AcPIM2 and Ac2PIM2 from the lipid-soluble extract of the microorganism.

**Cardiolipin and Mycolate are Murine Langerin Ligand**

Murine langerin-expressing reporter cells were activated by two different fractions from the TLC fractionation of the lipid-soluble extract of *Mycobacterium bovis* BCG. These two fractions are already known as cardiolipin and mycolic acid. To confirm the recognition of cardiolipin by murine langerin we utilize extracted cardiolipin from bovine heart, *Mycobacterium* and also synthetic cardiolipin. Cardiolipin 16:0/18:1 extracted from *Mycobacterium* is the most potent activator of murine langerin together with heart cardiolipin 18:2 (figure 3A). Next, we used synthetic mycolic acid to stimulate murine langerin-expressing reporter cells. Indeed, murine langerin was activated by alpha and methoxy mycolate, the latter being the strongest ligand (figure 3B&C). These results suggest that cardiolipin and mycolate could be recognized by murine langerin.

6. Discussion

*Mycobacterium* species infect their hosts through the epithelial route. In the epithelium, langerin-expressing dendritic cells are present and actively engulf pathogen. Although it is known that mycobacteria is recognized by langerin, a report from Kissenpfennig *et al*.16 shows no significant difference between langerin knockout and wild-type mice in survival and bacterial load in the lung after *Mycobacterium tuberculosis* H37Rv infection. Langerin ligand known is limited to glycan or sugar-containing molecule and its roles in immune system is remain elusive. With our reporter cells expressing langerin, we could find several ligands from *Mycobacterium* species. Human langerin recognized man-LAM and PIM2 from Mycobacteria or even the synthetic one. Meanwhile murine langerin recognized ligands that is not a glycan, Mycolate and cardiolipin.

It is reported that C-type lectin receptors can recognize saccharide-free ligands. One example is rat surfactant protein A (rat SP-A) which binds to dipalmitoyl phosphatidylcholine (DPPC), a lipid found in pulmonary surfactant. Each carbohydrate recognition domain (CRD) of rat SP-A is able to bind DPPC17. The recognition involves a three-walled tyrosine cage binding to the choline headgroup, and a positively charged amino acid to a phosphoryl group. Rat SP-A is a homo-trimeric receptor similar to langerin but with just 22% of amino acid sequence identity. Although rat SP-A is not identical to langerin, DPPC binding mode could be implemented into langerin binding to its lipid ligand.

Mutagenesis of some hydrophobic amino acid in murine langerin such as I252 and L319 to alanine (unpublished data) did not abolished cardiolipin recognition or rather slightly increased. Our data in supplementary figure 2C shows that dimeric langerin fusion Ig was not able to recognized methoxy mycolate. Therefore, another possibility of lipid binding to murine langerin is in the interface of trimeric langerin protein. This interface contains a quite big space and mostly hydrophobic so it might fit the lipid.

Mycolate recognition by murine langerin showed an interesting pattern. Murine langerin preferentially recognized methoxy and alpha mycolate than keto mycolate. In alpha mycolate structure, cyclopropane is found in its acyl chain where in methoxy mycolate is a methoxy functional group and in keto mycolate is ketone functional group (supplementary figure 1A). These differences are rather small but affected langerin recognition to mycolate. We suspect that mycolate could make a fold and it is unique to each type of mycolate so langerin recognition also affected by this folding mechanism18. In the case of cardiolipin, cardiolipin that has 14 carbon atoms on its acyl chain and no double bond cannot be recognized by murine langerin but the longer acyl chain and double bond bearing molecule recognized by murine langerin (supplementary figure 1B). Further analysis to determine lipid binding to murine langerin is needed to unravel the binding mode and may also to know the function and interaction of langerin with other protein or receptor.

Upon ligand recognition, langerin is known to phagocytose the ligand and route it to MHCI and MHCII and resulting in ligand presentation. Although this role is well established, intracellular signaling of langerin is not fully understood. It is thought that langerin, upon clathrin binding, could result in phagocytosis of viral particles. It is still needed to be clarified, whether the recognition of mycolate and cardiolipin can activate langerin signaling and induce phagocytosis or other cellular functions.

In conclusion, human and murine langerin are able to recognize different types of ligands. Human langerin recognize mannose-containing compound such as man-LAM and PIM2, whereas murine langerin is able to recognize mycolate and cardiolipin. Further study on how langerin recognizes these ligands is necessary to have a better understanding of langerin-ligand recognition. These results will extend our knowledge on langerin-ligand recognition and targeting langerin for several purposes such as antigen delivery.

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8. Author contributions

HSI perform the experiment, analyze data, and write the manuscript.

10. Conflict of interests

The authors declare that they have no conflicts of interest with the contents of this article.

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**FIGURE LEGEND**

**Figure 1. Recognition of *Mycobacterium* strains by langerin.** (A) Schematic drawings of binding assays using NFAT-GFP reporter cells expressing langerin-CD3ζ chimeric receptor. (B) Heat-killed *Mycobacterium tuberculosis* H37Ra, H37Rv, and vaccine strain BCG were used to stimulate murine or human langerin reporter cells. Expression of NFAT-GFP was analyzed by flow cytometry. (C) Total lipid extracted from vaccine strain BCG by Chloroform:Methanol = 2:1 was coated onto 96-well plate by isopropanol then used for stimulation of murine or human langerin reporter cells. (D) Total lipid of BCG was developed in TLC plate using Chloroform:Methanol:Water = 90:30:5 and fractionated into 16 fraction. Each fraction was coated onto 96-well plate and used for stimulation of murine or human langerin reporter cells. NFAT-GFP expression was then analyzed by flow cytometry.

**Figure 2. Human langerin ligand from *Mycobacterium* strains.** (A) Major component of water-soluble extract of *Mycobacterium*, lipoarabinomannan (LAM), was coated onto 96-well plate and its stimulating effect tested on murine or human langerin reporter cells. (B) AcPIM2 and Ac2PIM2 were coated onto 96-well plate by isopropanol and the ability to stimulate murine or human langerin reporter cells was assessed by analyzing NFAT-GFP expression by flow cytometry.

**Figure 3. Murine langerin ligand from *Mycobacterium* strains.** (A) Murine or human langerin reporter cells were stimulated by various types of Cardiolipin and the expression of NFAT-GFP was checked by flow cytometry. (B) Synthetic Mycolates were used to stimulate murine or human langerin reporter cells. (C) Mycolate extracted from *Mycobacterium tuberculosis* H37Rv and vaccine strain BCG were used to stimulate murine or human langerin reporter cells.

**Supplemental Figure 1.** (A) Schematic representation of Mycolates. (B) Schematic representation of various types of Cardiolipin.

**Supplemental Figure 2.** (A) Schematic representation of pDisplay human IgG1 Fc-Langerin expression vector. (B) Schematic representation of human IgG1 Fc-Langerin Fusion Ig protein. (C) human langerin Ig fusion protein was shown to have binding to Ac2PIM2 while murine langerin Ig fusion failed to bind methoxy mycolate.