CD4 detected from *Lactobacillus* helps understand the interaction between *Lactobacillus* and HIV

Yan Su, Baojiang Zhang, Lingling Su

Department of Microbiology and Immunology, Xinjiang Agricultural University, Xinjiang 830052, China
Xinjiang Academy of Animal Science, Xinjiang 830050, China

**ABSTRACT**

Human immunodeficiency virus (HIV) preferentially infects and destroys CD4+ cells and leads to a gradual decline in the number of CD4 cells. Despite evidence that probiotics increase CD4+ T lymphocytes in patients with HIV/acquired immunodeficiency syndrome (AIDS) and lower the risk of HIV transmission, little is known about the detailed mechanism underlying these effects. In this study, we investigated the cell surface protein of *Lactobacillus* and its role in blocking HIV-1 transmission by lactobacilli. Using reverse transcription–polymerase chain reaction (RT–PCR), immunofluorescence, and flow cytometry (fluorescence-activated cell sorting, FACS), we detected the CD4 receptor on the surface of *Lactobacillus*. Monoclonal antibody (mAb) for the CD4 receptor could partially inhibit HIV-1 binding to *Lactobacillus*. In addition, *Lactobacillus* could decrease HIV-1 pseudovirus infection of TZM-bl cells *in vitro* by 60–70%. Our data suggest that *Lactobacillus* can use this receptor to bind HIV and block HIV infection. This may in turn increase the CD4 T lymphocyte count in patients with HIV. These data provide direct evidence that *Lactobacillus* expresses the CD4 receptor and utilizes it to block HIV transmission.

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1. Introduction

At present, there is an increased interest in using probiotic bacteria for the treatment of human immunodeficiency virus (HIV)-associated diseases and infection. *Lactobacillus* is a fermentative bacterium that resides in the gastrointestinal tract of humans and animals (Casas and Dobrogosz 2000). When utilized as a probiotic, it has many positive effects on health (Weizman et al. 2005). HIV transmits through the mucosal surfaces and causes severe damage to the gut, which has led some scientists to believe that the use of probiotics may help counter its devastating effects and infection. The use of probiotics has been shown to be safe for HIV patients (Wolf et al. 1998; Reid 2002). Clinical studies have demonstrated that probiotics have a beneficial effect on HIV-induced diarrhea (Cunningham et al. 2000). Recent randomized trials in Brazil (Trois et al. 2008) and Nigeria (Anukam et al. 2008) suggested that yogurt supplemented with *Lactobacillus* is significantly associated with an increase in the CD4 count in patients with HIV.

The CD4 receptor is the primary receptor for the entry of T-tropic HIV into its target cells *in vivo* (Planelles and Chen 1993). It has been shown that T-tropic isolates often appear in association with a decline in CD4+ T lymphocytes during disease progression. The CD4 count is therefore an important indicator of disease progression and treatment effect. An increasing number of studies indicate that probiotics can improve the CD4 cell count in HIV-positive individuals and lower the risk of HIV transmission; however, the mechanism by which probiotics increase the mean CD4+ cell count in HIV-positive individuals remains unclear (Irvine et al. 2010). Chang et al. (2009) showed that oral lactobacilli isolated from saliva of healthy humans can bind and capture HIV-1 *in vitro*. The present study was designed to investigate the extracellular proteins of *Lactobacillus* and their interaction with HIV and to test whether cell surface-associated proteins induce an increase in CD4 counts. Our results indicated that the HIV-1 CD4 receptor could be detected on the *Lactobacillus* cell surface. Furthermore, viral binding to *Lactobacillus* appeared to employ the CD4 receptor, and *Lactobacillus* could inhibit infection of cells by HIV-1 pseudovirus *in vitro*. This finding provides clear and direct evidence of the mechanism used by some lactobacilli to increase the CD4 cell count, bind HIV virus, and in turn block HIV transmission.

*Corresponding author. Tel.: +86 01 9918762704.
E-mail address: suyan3399@gmail.com (Y. Su).
2. Experimental procedures

2.1. Materials

*Lactobacillus casei* 393 was obtained from the American Type Culture Collection. *Lactobacillus* species were inoculated at 1% and propagated in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Michigan, MI) at 37 °C for 15 h. CD4 and mAbs to CD4 were obtained from R&D Systems (Minneapolis, MN).

2.2. Observation of bacteria and HIV interaction by transmission electron microscopy (TEM)

For sample preparation, *L. casei* 393 was grown in MRS medium at 37 °C overnight. *L. casei* 393 was collected, washed three times with PBS to remove lactic acid. Then, a suspension of 1 × 10^9 bacterial cells was incubated with HIV pseudovirus in a total volume of 1 ml at 37 °C for 1 h. After washing three times with PBS and centrifuging at 6000 rpm for 5 min, *L. casei* 393 was fixed with paraformaldehyde (2%, v/v) at 4 °C overnight and diluted with 100 μl PBS. For TEM, copper slot grids (Gröpl, Tulln, Austria) supported with Formvar film and coated with 0.5% bovine serum albumin (BSA; Sigma, Vienna, Austria) were used. Drops of the culture were directly added onto the grids. After 1 min, the drops were carefully drained off with filter paper, and the remaining cells were air-dried and negatively stained with 2% uranyl acetate. Samples were observed using a TEM (JEM-2100EX, JEOL, Japan).

2.3. Confocal microscopic observation of receptor expression on the surface of lactic acid bacteria (LAB)

*L. casei* 393 cultures were collected and washed with PBS with 0.5% BSA, followed by staining with anti-CD4 mAb (R&D Systems) at a 1:500 dilution for 1 h at 4 °C. Alexa Fluor 488-conjugated goat antimouse IgG was used as secondary antibody (Invitrogen) at a 1:50 dilution for 30 min at 4 °C. Anti-GFP mAb was used as a negative control. Cells were visualized using a Leica SP5X confocal fluorescence microscope with a 100× oil immersion objective.

2.4. Flow cytometric analysis of CD4 receptor expression on *Lactobacillus*

For flow cytometry (FACS), *L. casei* 393 cells were washed with PBS with 0.5% BSA and 0.1% NaN₃, incubated with mAb to CD4 (R&D Systems) at a 1:500 dilution for 1 h at 4 °C, and then incubated with FITC-labeled goat antimouse IgG Fc (Sigma) at a 1:100 dilution for 30 min at 4 °C. Samples were fixed with 0.1% paraformaldehyde and analyzed using a FACSCalibur four-laser cytometer (Becton Dickinson). Anti-GFP mAb was used as a negative control. At least 1 × 10⁴ gated events were acquired in each case, and CD4 expression was evaluated by FACS analysis.

2.5. Reverse transcription–polymerase chain reaction (RT–PCR)

RT–PCR for CD4 was performed on cytoplasmic RNA extracted from *L. casei* 393. The RT–PCR reaction was performed in single tubes using the SuperScript III One-Step RT–PCR System according to the manufacturer’s instructions (Invitrogen). In brief, serially diluted RNA for each chemokine receptor was reverse transcribed at 60 °C for 30 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and a final extension at 68 °C for 7 min. Amplified DNA was analyzed on 1% agarose gel. Primers were designed to amplify the coding sequence based on the receptor sequences obtained from the GenBank database. The primers used were 5’-GAAACCTGGTGTGATGAGAG-3’ and 5’-GGGGCTATCATTTCTTTTGAACCCGTT-3’ (438 bp). In addition, control PCR reactions were conducted with primers on samples incubated in the absence of the template. The *Lactobacillus* LDH L gene (ldhL, 972 bp) was used as an internal control. PCR reactions for CD4 were also conducted with other lactobacilli (*Lactobacillus reuteri, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus rhamnosus*). The PCR products were confirmed by direct sequencing using the same primers as for those used the PCR reaction, in an ABI 377 DNA sequencer (PerkinElmer, Hünengen, Switzerland).

2.6. HIV-1 pseudovirus preparation and antibody inhibition assays

HIV-1 pseudotyped with different envelope proteins was prepared by cotransfecting 293T cells with 10 μg of pNL-LucE-R vector together with 20 μg of env-expressing plasmids (AD8, DH12, and LAI) using a standard calcium phosphate (CaPO₄) precipitation method. As a control, luciferase-expressing reporter viruses without an envelope protein were generated by cotransfecting 10 μg of pNL-LucE-R vector and 20 μg of pCDNA3. Three days after transfection, supernatants of the transfected cultures were clarified by low-speed centrifugation at 3500 rpm for 10 min, followed by filtration of the supernatants through a 0.2-μm Acrodisc 25 filter (Gelman Sciences, Ann Arbor, MI). Pseudotyped virions were quantitated on 1 ml of clarified supernatant using a β-galactosidase (β-gal) staining assay.

For antibody inhibition experiments, *L. casei* 393 was incubated in the presence or absence of anti-CD4 mAb for 1 h at 37 °C. After binding, the lactobacilli were washed three times with 1.5 ml of PBS at 16,000 rpm for 4 min. Viral particles binding to the lactobacilli were lysed in ELISA lysis buffer (ZepetoMetrux) at room temperature for 2 h and then centrifuged at 16,000 rpm for 5 min. The resulting supernatant was used to perform HIV-1 p24 antigen ELISA according to the manufacturer’s protocol.

2.7. In vitro infection inhibition by *Lactobacillus*

*L. casei* 393 was aerobically grown for 16–18 h in MRS broth at 37 °C and then diluted to a concentration of 1 × 10⁶ cells/ml. Aliquots (1 ml) were mixed with HIV-1 AD8, DH12, or LAI. Pseudotyped virus that was not incubated with *Lactobacillus* was used as a control. After incubation, pseudotyped viruses were used to infect Tzmb1 cells (1 × 10⁶ cells per well in 96-well plates). After 2 days, the cells were washed with PBS and β-gal staining was performed to assay pseudovirus infection of the cells. Tzmb1 cells contain the β-gal reporter gene, and the pseudovirus contains the tat gene. When the pseudovirus infects Tzmb1 cells, the tat gene product activates β-gal expression. The β-Gal Assay Kit provides the reagents required to quickly measure the level of active β-gal expressed in the cells. Using β-gal quantitative assays, the number of stained cells was counted under a microscope.

3. Results

3.1. Viral capturing activity observed by TEM

To explore the interaction between *L. casei* 393 and HIV-1 pseudotyped virus (DH12), we used TEM to examine *L. casei* 393 after coinoculation with HIV-1-pseudotyped virus (DH12) and three washes with PBS. Round virus particles approximately 80–100 nm in diameter were found to be distributed very closely around the *L. casei* 393 cells. This suggests there are interactions between the HIV-1-pseudotyped virus and *L. casei* 393 (Fig. 1).
3.2. Confocal microscopy analysis and flow cytometric analysis of CD4 receptor expression on L. casei 393

To show the expression of CD4 receptors on the surface of L. casei 393, we used confocal microscopy and mAbs specific for the CD4 receptor. Green fluorescence could be observed on the surface of the L. casei 393 cells (Fig. 2A). This indicates expression of the CD4 receptor on the cell surface.

Expression of CD4 receptors on L. casei 393 was also examined by flow cytometric analysis using mAbs specific for the CD4 receptor. Representative flow cytometry histograms are presented in Fig. 2B. Compared with the negative control, L. casei 393 exhibited surface fluorescence intensity for CD4. The flow cytometry results showed the presence of a clear signal for CD4.

3.3. Detection of CD4 receptor on Lactobacillus by RT–PCR

Evidence for the presence of LAB chemokine receptors was obtained by PCR of bacterial mRNA with specific primers for CD4. The transcript for CD4 (438 bp) was detected in L. casei 393 and other lactobacilli (L. reuteri, L. plantarum, L. acidophilus, L. rhamnosus) (Fig. 3), and the identity of these transcripts was confirmed by DNA sequencing of the PCR products (data not shown).

3.4. Antibody inhibition assays

We also examined the blocking activity of anti-CD4 mAb on HIV-1 pseudovirus binding to L. casei 393. Fig. 4 shows that anti-CD4 mAb inhibited viral binding by 27%. This demonstrates that virus–LAB binding could be partially blocked by specific antibody and suggests that the CD4 receptor may play an important role in the binding of HIV-1 pseudovirus to L. casei 393.

3.5. Inhibition of pseudovirus infection in Tzmbl cells by Lactobacillus

To evaluate the antiviral ability of the L. casei 393, an assay was performed in Tzmbl cells with HIV-1 pseudovirus DH12 (Fig. 5). The results showed that this Lactobacillus strain could reduce the infectivity of different HIV-1 pseudoviruses (AD8, DH12, and LAI) in Tzmbl cells by approximately 60–70% (Fig. 6) after incubation of HIV-1 pseudoviruses with the Lactobacillus strain for 30 min. This indicates that L. casei 393 exhibits inhibitory activity against the HIV-1 strain.

4. Discussion

Increasing numbers of people now believe that probiotics are an exciting prophylactic and therapeutic advance in various infections. There are many potential advantages of probiotics over

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Fig. 1. Electron micrograph of HIV-1 pseudovirus binding to LAB. Binding of HIV-1 pseudovirus to L. casei 393 was studied by TEM. (A) L. casei 393 not coinoculated with HIV-1-pseudotyped virus. (B) L. casei 393 after coinoculation with HIV-1-pseudotyped virus DH12 and three washes with PBS. (C) L. casei 393 after coinoculation with HIV-1-pseudotyped virus AD8 and three washes with PBS.

Fig. 2. (A) Immunofluorescence analysis of CD4 receptor expression on L. casei 393. Samples were prepared as described in Section 2 and observed using a Leica Sp5X confocal fluorescent microscope with a 100× oil immersion objective. (Negative control): HIV mAb b12 was used as a primary antibody control and FITC-conjugated goat antimouse IgG as secondary antibody (CD4). (B) Anti-CD4 mAbs antibody was used as primary antibody and FITC-conjugated goat antimouse IgG as secondary antibody.

Fig. 3. (A) Detection of CD4 receptor-specific mRNA in LAB. The 438-bp CD4 PCR product was shown and amplified DNA was analyzed on 1% agarose gel. RT–PCR results are shown for two strains: (1) L. casei 393, (2) L. reuteri, (3) L. plantarum, (4) L. acidophilus, and (5) L. rhamnosus. To control for possible DNA contamination, the reaction was performed without a template as a negative control. (B) RT–PCR results for internal control (ldhL). The 972-bp ldhL RT–PCR product, analyzed on 1% agarose gel, is shown for five strains: (1) L. casei 393, (2) L. reuteri, (3) L. plantarum, (4) L. acidophilus, and (5) L. rhamnosus. The reaction was performed without a template as a negative control.

Fig. 4. Blocking of HIV-1 binding to L. casei 393 by mAb. The antibody was used at 20 μg/ml. Mean ± SD of triplicate experiments is shown. (Negative control): L. casei 393, (positive control): L. casei 393 + anti-GFP mAb + HIV-1 DH12-pseudotyped virus, (anti-CD4): L. casei 393 + anti-CD4 mAb + HIV-1 DH12-pseudotyped virus. Similar results were obtained in two independent experiments.
conventional therapy, including relatively low cost, the fact that probiotics are unlikely to increase the incidence of antibiotic resistance, and the multiple mechanisms by which probiotics inhibit pathogens, thereby decreasing the chances of infection. At present, the best-studied probiotics are LAB, particularly Lactobacillus sp., and several studies indicate positive effects of Lactobacillus sp. on health (Casas and Dobrogosz 2000; Weizman et al. 2005). Lactobacillus now represents a potentially significant therapeutic advance in HIV clinical treatment. However, there are few data on the mechanism underlying the actions of probiotics, and it will be important to define a clear and scientific rationale for using a selected bacterial strain against a particular pathogen.

There are many possible mechanisms by which probiotics may prevent HIV infection (Lee and Puong 2002; Verdeneili et al. 2009), including stimulation of immunity, competition for limited nutrients, inhibition of epithelial and mucosal adherence, inhibition of epithelial invasion, and production of antimicrobial substances.

Now we presume that characterizing the extracellular proteins of lactobacilli, including secreted or cell surface-associated proteins, is crucial for understanding the mechanism underlying their therapeutic functions. At present, 37 extracellular proteins have been detected in the L. reuteri strain DSM 20016 by phage display screening (Wall et al. 2003). By bioinformatic analysis, Bath et al. (2005) showed that 126 genes encoded extracellular proteins with various functions. Numerous extracellular proteins have been identified; however, not all the presumed interactions between Lactobacillus, other organisms, and the environment are understood at present.

The CD4 glycoprotein is a major cell surface receptor for HIV-1 infection. In the present study, surface expression of the CD4 receptor was determined by RT–PCR as well as flow cytometry and immunofluorescence using specific antibodies. In addition, we found that anti-CD4 antibody could partially inhibit the binding activity of HIV-1 pseudovirus. Although anti-CD4 antibody cannot completely inhibit Lactobacillus binding, this may suggest that CD4 is not the only extracellular protein responsible for this binding function.

Our data provide clear and direct evidence that the HIV-1 primary receptor CD4 occurs on the cell surface of Lactobacillus strains and is able to bind HIV-1 pseudovirus and inhibit dual-tropic (DH12) HIV-1 env pseudotypes from entering Tzmbl cells, as determined by enzymatic activity controlled by the β-gal reporter gene. The inhibitory potential ranged from 60% to 70% depending on the test system used. Although in vitro assays may not necessarily correlate with in vivo applications in humans, the results are very encouraging and indicate that in vivo testing is warranted using this bacterial strain L. casei 393 with good potential binding ability.

Valeur et al. (2004) recently found that the administration of L. reuteri induced a significantly higher number of CD4-positive T lymphocytes in the ileal epithelium than no administration of L. reuteri. Another report showed that probiotic yogurt was significantly associated with beneficial effects on the CD4 count (Reid et al. 2003). However, the specific mechanism is not completely understood. There is evidence suggesting that L. reuteri may be able to modulate the immune system in the gastrointestinal tract (Madsen et al. 1999; Tejada-Simon and Pestka 1999; Maasen et al. 2000; Christensen et al. 2002), and a significant increase in CD4+ cells in the ileum was observed after intake of L. reuteri. However, there is no direct evidence to support this. Given that HIV preferentially infects and destroys CD4+ cells, our findings seem to support the idea that Lactobacillus may be involved in the binding and capturing of HIV-1 pseudovirus using the CD4 receptors anchored to its cell wall, thereby reducing viral infection of CD4+ cells and increasing the number of CD4+ cells in the ileum.

Characterizing the mechanisms of HIV-1 binding to Lactobacillus may increase our understanding of the potential of Lactobacillus for preventing HIV-1 infection. This study aimed to investigate and define the role of the CD4 receptor in the binding of HIV-1 to Lactobacillus. To this end, we used HIV-1 pseudovirus for viral binding and p24 antigen ELISA to characterize the binding activity.

CD4 is a transmembrane glycoprotein present on T-helper/inducer cells that participates in the adherence of T cells to target cells and is involved in the transmission of intracellular signals during T-cell activation by class II MHC molecules. Moreover, CD4 has inducer or helper activity for interactions among T cells, B cells, and macrophages. Many clinical trials report beneficial effects of probiotics in diseases in which there are exaggerated Th1 and Th2 immune responses (Gionchetti et al. 2000; Kalliomaki et al. 2003). In addition, it has been recently shown that some probiotic organisms can bind cell receptors specific for DCs (Smits et al. 2004). We therefore surmise that probiotics may have a direct effect on DC function by expressing CD4. Furthermore, probiotics may have some effect on adaptive immunity and autoimmune disease through DC-primed T cells to modulate both Th1 and Th2 immune responses.

A limitation of this study needs to be acknowledged. Because the pseudovirus of each reporter virus was env defective and was competent only for a single cycle of viral replication, the intracellular level of β-gal activity was a reflection of viral entry only. Thus, the behavior of the env pseudotype may not represent the behavior of the intact virus.
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References


