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(54) Title: METHOD FOR MODULATING THE BINDING ACTIVITY OF A NOVEL ICAM-3 BINDING RECEPTOR ON SINUSOIDAL ENDOTHELIAL CELLS IN LIVER AND LYMPH NODES

(57) Abstract: The invention relates to the use of a compound that binds to a C-type lectin on the surface of a sinusoid endothelial layer, in the preparation of a composition for modulating, in particular reducing, the immune response in animal, in particular a human or another mammal. The sinusoid endothelial layer may be either constituted by liver sinusoid endothelial cells (LSEC) or by the lymph node sinusoidal zone.

METHOD FOR MODULATING THE BINDING ACTIVITY OF A NOVEL ICAM-3 BINDING RECEPTOR ON SINUSOIDAL ENDOTHELIAL CELLS IN LIVER AND LYMPH NODES

The present invention relates to the use of a compound binding to a C-type lectin located on the surface of sinusoid endothelial cells in liver and lymph nodes for modulating the immune response in animals.

The molecule DC-SIGN has recently been identified as a DC-specific adhesion receptor that mediates the interaction between DCs and resting T cells through high affinity binding to ICAM-3, thereby facilitating the initiation of primary immune responses. DC-SIGN was shown to be identical to the previously reported type II membrane-associated C-type lectin (Geijtenbeek, T.B. et al., 2000, Cell 100:575-585) that binds HIV-1 envelope glycoprotein gp120 in a CD4-independent manner. The affinity of DC-SIGN exceeds that of CD4 for HIV-1 gp120 (Curtis, B.M. et al., 1992, Proc Natl Acad Sci USA 89:8356-8360), and upon capture of HIV-1, DC-SIGN does not appear to promote viral entry into the DC itself, but rather enhances infection of T cells in trans (Geijtenbeek, T.B. et al., 2000, Cell 100:587-597). DC-SIGN-associated HIV-1 remains infectious over a prolonged period of time, perhaps contributing to the infectious potential of the virus during its transport by DCs from the periphery to lymphoid organs.

A previous search by Yokoyama-Kobayashi et al. (1999, Gene 228:161-167) for cDNA clones encoding type II membrane proteins resulted in the identification of a partial clone that was homologous, but not identical to the cDNA encoding the molecule now known as DC-SIGN. The putative protein product contained a deletion of 28 amino acids in the cytoplasmic domain and was lacking the entire C-type lectin domain relative to the cDNA encoding DC-SIGN.

More recently, Soilleux et al. (2000, J Immunol 165:2937-2942) described the full length cDNA sequence of

the related gene, which they called DC-SIGNR. The genomic organization of DC-SIGN and DC-SIGNR was compared, indicating a high degree of similarity. Concomitant expression of the two genes in placenta, endometrium, and stimulated KG1 cells (a cell line that phenotypically resembles myeloid DCs) was observed, although the expression of DC-SIGNR was very low in both endometrium and stimulated KG1 cells.

In the research that led to the present invention it was now found that the DC-SIGNR gene is expressed at considerably high levels in only two tissues, liver and lymph node, but not in monocyte derived dendritic cells. The receptor was renamed "L-SIGN" because it is a liver/lymph node-specific ICAM-3 grabbing nonintegrin.

The homologous human C-type lectins DC-SIGN and L-SIGN appear to be the products of a recent gene duplication. The corresponding proteins share the same domain organization and overlapping, if not completely identical, ligand specificity. The most diverse region of these molecules occurs in their cytoplasmic tails.

Another obvious difference between the genes for DC-SIGN and L-SIGN is the repeat polymorphism in exon 4 of L-SIGN, which is conserved in DC-SIGN (Table 1). The neck domain of L-SIGN may contain from three to nine repeats while DC-SIGN always consisted of seven repeats among the Caucasians tested. No difference was observed between L-SIGN molecules containing six or L-SIGN molecules containing seven repeats in ligand binding, nor HIV-1 capture and enhancement experiments.

Although the SIGN genes have maintained sequence and functional similarity over their evolutionary history, it was now surprisingly found according to the invention that regulatory elements determining their tissue distribution have evolved along unique paths. Northern analysis of mRNA expression clearly indicated expression of DC-SIGN in monocyte-derived DCs and in tissues where DCs reside, whereas

expression of L-SIGN in DCs was undetectable (Fig. 2). Further, L-SIGN was not detected on monocyte-derived DCs using antibodies specific to L-SIGN (Fig. 3C). Thus, it was found that unique cell types in the lymph node express one, but not both SIGN molecules: L-SIGN is expressed by endothelial cells, as it is in liver, while DC-SIGN is expressed by DCs in T cell area of lymph node. This difference in expression pattern could not be expected based on the sequence homology.

Liver sinusoids are specialized capillary vessels characterized by the presence of resident macrophages adhering to the endothelial lining. The LSEC-leukocyte interactions, which require expression of adhesion molecules on the cell surfaces, constitute a central mechanism of peripheral immune surveillance in the liver. The mannose receptor as well as other costimulatory receptors such as MHC class II, CD80, and CD86 are known to be expressed on LSECs and to mediate the clearance of many potentially antigenic proteins from the circulation in a manner similar to DCs in lymphoid organs.

The inventors established that L-SIGN fits in this category of receptors on LSECs, as its tissue location and ligand binding properties strongly implicate a physiologic role for this receptor in antigen clearance, as well as in LSEC-leukocyte adhesion. The high expression of ICAM-3 on apoptotic cells are the means by which these cells are trapped by L-SIGN-expressing cells in the liver and subsequently cleared.

Like DC-SIGN, L-SIGN is a membrane-associated lectin that enhances HIV-1 infection. The expression of L-SIGN in liver sinusoids indicates that LSECs, which are in continual contact with passing leukocytes, capture HIV-1 from the blood and promote trans-infection of T cells.

In addition, LSECs themselves may be susceptible to HIV-1 infection. Thus, it is possible that L-SIGN promotes infection of these cells thereby

establishing a reservoir for production of new virus to pass on to T lymphocytes trafficking through the liver sinusoid.

Based on the above observations, the present invention relates to the use of a compound that binds to a C-type lectin on the surface of cells of a sinusoid endothelial layer, in the preparation of a composition for modulating, in particular reducing, the immune response in a animal, in particular a human or another mammal. The C-type lectin on the surface of cells of a sinusoid endothelial layer is in particular L-SIGN.

The cells of the sinusoid endothelial layer may either be constituted by liver sinusoid endothelial cells (LSEC) or cells of the lymph node sinusoidal zone.

The composition of the invention may be used for modulating, in particular reducing, one or more interactions between a cell of a sinusoid endothelial layer, in particular a LSEC, and a cell expressing ICAM-2 and/or ICAM-3, in particular a T cell. More in particular, the composition is used for modulating, in particular reducing, the adhesion between a cell of a sinusoid endothelial layer, in particular a LSEC, and a cell expressing ICAM-2 and/or ICAM-3, in particular a T cell, in particular between a C-type lectin on the surface of a LSEC and an ICAM receptor on the surface of a T cell, in particular an ICAM-2 or ICAM-3 receptor on the surface of a T cell.

The composition prepared according to the invention is applied for preventing or inhibiting immune responses to specific antigens, for inducing tolerance, for immunotherapy, for immunosuppression, for the treatment of autoimmune diseases, and/or for the treatment of allergy.

According to a further aspect thereof, the invention relates to the use of a compound that binds or can bind to a C-type lectin on the surface of a cell of the sinusoid endothelial layer, in particular a LSEC, in the preparation of a composition for inhibiting the HIV

infection of cells of a sinusoid endothelial layer, in particular LSECs, in particular for inhibiting the adhesion of HIV surface protein (i.e gp120) to the surface of a cell of a sinusoid endothelial layer, in particular a LSEC and thereby the entry of HIV into said cell.

The invention furthermore relates to the use of a compound that binds or can bind to a C-type lectin on the surface of a cell of a sinusoid endothelial layer, in particular a LSEC, in the preparation of a composition for inhibiting the transfer of HIV from cells of a sinusoid endothelial layer (that may or may not be infected themselves), in particular a LSEC, to non-infected T cells.

Alternatively, the invention provides the use of a combination of: 1) a compound that binds to a C-type lectin on the surface of a cell of a sinusoid endothelial layer, in a particular a LSEC; and attached thereto: 2) an antigen or a fragment or part thereof; in the preparation of a composition for modulating, in particular generating, increasing and/or promoting, an immune response in an animal, in particular a human or other mammal, against said antigen. Preferably, the antigen is covalently bonded to or fused with the compound that can bind to the C-type lectin. The antigen is for example chosen from cancer antigens which can be used to generate an immune response against tumor cells that contain or express said antigen, or antigens as used in vaccines against infectious diseases.

The compound that can bind to a C-type lectin on the surface of a cell of a sinusoid endothelial layer, in particular a LSEC, is preferably chosen from the group consisting of mannose carbohydrates, such as mannan and D-mannose; fucose carbohydrates, such as L-fucose; plant lectins such as concanavalin A; antibiotics, such as pradimicin A; sugars such as N-acetyl-D-glucosamine and galactose; proteins such as gp120 and analogs or fragments thereof; and antibodies directed against a C-

type lectin as expressed on the surface of a cell of a sinusoid endothelial layer, in particular a LSEC, or a part, fragment or epitope thereof.

The C-type lectin on the surface of a cell of a sinusoid endothelial layer, in particular a LSEC, is preferably a protein with the amino acid sequence of Figure 7, or a natural variant or equivalent thereof.

Alternatively, the compound that can bind to a C-type lectin on the surface of a cell of a sinusoid endothelial layer, in particular a LSEC, is a monoclonal antibody, preferably a monoclonal antibody directed against a C-type lectin with the amino acid sequence of Figure 7 or a natural variant or equivalent thereof; and/or a part, fragment or epitope thereof.

According to a further aspect thereof the invention relates to an antibody, preferably monoclonal antibody, directed against a C-type lectin with the amino acid sequence of Figure 7 or a natural variant or equivalent thereof; and/or a part, fragment or epitope thereof. This antibody is preferably AZN-D3, which is obtainable by a method as described in the examples.

The invention further relates to a pharmaceutical composition, containing at least one such antibody and at least one carrier, excipient, adjuvant and/or formulant.

Another aspect of the present invention relates to a combination of: 1) a compound that binds to a C-type lectin on the surface of a cell of a sinusoid endothelial layer, in particular a LSEC; and attached thereto: 2) an antigen or a fragment or part thereof. Preferably, the antigen is covalently bonded to or fused with the compound that can bind to the C-type lectin.

In a combination according to the invention the antigen is for example chosen from cancer antigens which can be used to generate an immune response against tumor cells that contain or express said antigen, or antigens as used in vaccines against infectious diseases.

The compound that can bind to a C-type lectin on the surface of a cell of a sinusoid endothelial layer, in particular a LSEC, is preferably chosen from the group consisting of mannose carbohydrates, such as mannan and D-mannose; fucose carbohydrates, such as L-fucose; plant lectins such as concanavalin A; antibiotics, such as pradimicin A; sugars such as N-acetyl-D-glucosamine and galactose; proteins such as gp120 and analogs or fragments thereof; and antibodies directed against a C-type lectin as expressed on the surface of a cell of a sinusoid endothelial layer, in particular a LSEC, or a part, fragment or epitope thereof.

The antibodies of the invention can furthermore be used in the detection of cells of a sinusoid endothelial layer, in particular LSECs, in a biological sample and in the isolation, preparation and/or purification of cells of a sinusoid endothelial layer, in particular LSECs, from a biological sample or a culture medium.

Alternatively, such antibody can find an application in an assay for determining the presence and/or the expression of C-type lectins, in particular a C-type lectin with the amino acid sequence of Figure 7 or a natural variant or equivalent thereof; and/or a part, fragment or epitope thereof, in a biological sample.

Furthermore, the invention relates to a method for producing, isolating and/or purifying cells of a sinusoid endothelial layer, in particular LSECs, from a biological sample or a culture medium, comprising the steps of:

a) contacting a biological sample or a culture medium that contains said cells with an antibody according to the invention;

b) separating the cells that bind to said antibody from cells that do not bind to said antibody, and optionally from any further constituents of the sample or medium;

and optionally further comprises the step of:

c) separating the cells that bind to the antibody from said antibody.

Preferably, the antibody is attached to a column or matrix, to (para)magnetic beads or to a similar solid support. Biological samples to be tested may be biological fluids such as blood, plasma or lymph fluid.

Finally, the invention provides cells of a sinusoid endothelial layer, in particular LSECs, obtained via the method described above.

The present invention is further illustrated in the example that follows and in which reference is made to the following figures:

Figure 1. Schematic representation of the DC-SIGN / L-SIGN genetic map. Physical distances and gene orientation are based on the sequence provided from BAC clone CTD -2102F19 (GenBank AC008812).

Figure 2. Northern blot analysis of DC-SIGN and L-SIGN. Positions of the 4.3 kb (arrows with solid heads) and 1.9 kb (arrows with open heads) sizes are marked on the left. **(A)** Hybridization with the L-SIGN-specific probe indicating expression of the gene in liver, lymph node, and weakly in thymus. **(B)** Hybridization with the probe recognizing both genes. 4.3 kb bands represent DC-SIGN mRNA. The light upper band (~ 4.2 kb) evident in liver and lymph node using the L-SIGN-specific probe (Fig. 3A) is distinct from DC-SIGN mRNA (4.3 kb) due to the specificity of the probe, intensity patterns, and slight differences in size. **(C)** Reprobing of the blots with the ß-actin cDNA control probe.

Figure 3. L-SIGN is expressed on LSECs and not on monocyte-derived DCs. (**A**) The antibody AZN-D1 is DC-SIGN-specific whereas AZN-D3 cross-reacts with L-SIGN. Stable DC-SIGN and L-SIGN K562 transfectants were stained with either AZN-D1 or AZN-D3. (**B**) Immunohistochemical analysis of DC-SIGN and L-SIGN expression in the human liver. Serial sections were stained with either AZN-D1 (DC-SIGN-specific) or with AZN-D3 (detects both DC-SIGN and L-SIGN). AZN-D1 stains infrequent cells that may be

DCs (arrows), whereas AZN-D3 stains cells lining sinusoids. (**C**) Expression of L-SIGN in liver is restricted to LSECs. One day after isolation, primary human liver cells were incubated with fluorochrome labeled ovalbumin. L-SIGN expression was determined by indirect immunofluorescence using an L-SIGN-specific polyclonal antibody. Cells that have taken up ovalbumin (LSECs) and those that did not take up ovalbumin (hepatocytes and other resident hepatic cells) are represented by solid and broken lines, respectively, by gating on the respective cell populations. $2x10^5$ cells were analyzed. (**D**) L-SIGN is not expressed by monocyte-derived DCs. Immature DCs, cultured from monocytes in the presence of GM-CSF and IL-4, do not stain with anti-L-SIGN polyclonal antibody, as determined by FACScan analysis. Solid line indicates staining with anti-L-SIGN polyclonal serum, whereas stippled line (hidden under solid lane) represents staining with rabbit pre-immune serum.

Figure 4. L-SIGN binds ICAM-3 **(A)** and HIV-1 gp120 **(B)**. Adhesion of ICAM-3 and gp120 to the K562-L-SIGN and K562-DC-SIGN cells was measured with the fluorescent bead adhesion assay (Geijtenbeek, T.B. et al., 1999, Blood 94:754-764). The y-axis represents the percent cells binding ligand-coated fluorescent beads. The L-SIGN-cross-reacting mAb AZN-D2 (20 μg/ml) and AZN-D3 (20 μg/ml) inhibit the adhesion of ICAM-3 and gp120 to L-SIGN, in contrast to the DC-SIGN-specific mAb AZN-D1 (20 μg/ml). Adhesion of both ICAM-3 and gp120 to the K562 transfectants is also inhibited by either mannan (20 μg/ml) or EGTA (5mM). Adhesion of both ligands to mock transfectants was less than 5%. One representative experiment out of three is shown (SD<5%).

Figure 5. L-SIGN captures and enhances infection of T cells with HIV-1 <u>in trans</u>. (**A**) L-SIGN captures HIV-1 and transmits it to target cells. Stable DC-SIGN or L-SIGN expressing THP-1 transfectants were pre-incubated with HIV-luc/JRFL pseudovirions to allow

capture of the virus. Cells were washed and THP-1 transfectants were co-cultured with Hut/CCR5 target cells. Cell lysates were obtained after 3 days and analyzed for luciferase activity. For each of the co-culture conditions employed, mock infected controls were uniformly less than 100 counts per second in activity. Each data set represents the mean of four separate wells of infected cells. One representative experiment out of two is shown. (**B**) L-SIGN enhances infection of T cells by pseudotyped HIV-1. HEK293T cells were transiently transfected with cDNA encoding DC-SIGN, L-SIGN or empty vector. Control cells were preincubated with AZN-D2 (20 μg/ml) or mannan (20 μg/ml). Low amounts of pseudotyped HIV-1_{ADA} were added together with activated T cells as described previously (Geijtenbeek, T.B. et al., 2000, Cell 100: 587-597). Infectivity was determined after two days by measuring luciferase activity. One representative experiment of two performed is shown. Each experiment was done in triplicate wells. (**C**) L-SIGN enhances infection of T cells by replication competent HIV-1. Stable K562 transfectants of both L-SIGN and DC-SIGN were incubated with low virus concentrations of replication-competent M-tropic strain HIV-1_{JR-CSF} ($TCID_{50}$ 100/ml). To determine the specificity, cells were preincubated with AZN-D2 (20μg/ml). After two hours, activated T cells were added as described previously (Geijtenbeek, T.B. et al., 2000, Cell 100:575-585). Culture supernatants were collected at day 14 after K562-T cell co-culture and HIV-1 production was measured using ELISA to determine p24 antigen levels. In control experiments, the same amount of virus was added directly to T cells. One representative experiment out of three is shown. Each data set represents the mean of three separate wells of infected cells.

Figure 6. Coding DNA sequence of L-SIGN

Figure 7. Amino acid sequence of L-SIGN.

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摘要(译)

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